UNIVERSITÉ DE MONTRÉAL

THE CALCIUM HOMEOSTASIS OF ENDOTHELIAL CELLS UNDER THE EFFECT OF OXIDATIVE STRESS AND ANTIOXIDATIVE TREATMENT

par

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Thèse présentée à la Faculté des Études Supérieures

En vue d'obtention du grade de

Philosophiae Doctor

(Ph.D.)

En PHYSIOLOGIE

Mai, 2001

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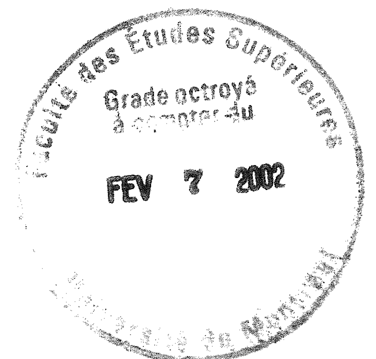

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Cette thèse intitulée :

THE CALCIUM HOMEOSTASIS OF ENDOTHELIAL CELLS UNDER THE EFFECT OF OXIDATIVE STRESS AND ANTIOXIDATIVE TREATMENT

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ACKNOWLEDGEMENTS

In this solemn moment the consciousness, the thoughts and the consideration is turning back in time and is recollecting sequence by sequence an important period of my life, a short episode in which the interlude between two stages of cognitive existentialism has past through important metamorphic changes, not only from the changes in the spectrum of intellectual preoccupation and touching the scientific endeavor, penetrating and filling the accomplishment of the elevated formes of cognitive spheres of biological, physiological and physiopathological understanding, the intimacy of life itself but most important, to lay a hand on and to fill the "*Γν /τηι σεαυτον-Gnôthi seauton*" (Socrate, *Apollon of Delphes*) of personal *alter ego*. This complex metamorphosis was possible only through the joint efforts of the institution and collectivity in which I have past this productive, enriching, illuminating and unforgettable period and which was so supportive. Therefore, I would like to bring all my thanks and the deepest recognition and gratitude to the entire collectivity of the University of Montreal, the Faculté des Études Supérieures, the Department of Physiology and the Membrane Transport Group Research.

My deepest gratitude, recognition, and consideration are graciously offered to prof. Dr. Rémy Sauvé for the entire spectrum of support offered during this period. If Victor Hugo was right when he said "*La conscience de l'homme c'est la pensée de Dieu*" (V. Hugo, *Les Châtiments*) then your personal and professional example and attitude is coming from the most elevated and superiorly organized forms of the human spirit. If more than 2000 years ago was sad "*Macte nova virtute, puer; sic itur ad astra*" (Virgil, *Eneida*, IX, 641) (deploy your young courage child, like this we are rising up to the stars), it became again a reality and a topicality under your guidance, supervision, training and forge of my thinking, interpretation and working style.

Beside the technical skills, creativity, thinking, and rationalizing your personal qualities have transferred to me the goodness ("*Véritablement bon est l'homme rare qui jamais ne blâme les gens des maux qui leur arrivent*", Paul Valéry, *Choses tues*), character ("*On peut tout acquérir dans la solitude, hormis du caractère*" Henry Beyle Stendhal, *De l'amour*), devotion and the certainty in a world of incertitude, because "*l'honneur de l'homme est d'atteindre à ce centre où la certitude se fait vertige et le vertige certitude*", (Emmanuel, Noël Mathieu, *Versant de l'âge*). Neither me nor somebody else can compensate the teaching of such valuable standards, moral, and ethics but only by insuring their further horizontal and vertical transmission. Therefore, the single manner and possibility to honor and compensate your efforts is to insure you that I will do all my best to transmit everything further because "*Tristo è quel discepolo che non avanza il suo maestro*" (Léonardo da Vinci, *Carnets*) and, even more, because "*Les disciples de la lumière n'ont jamais inventé que des ténèbres peu opaques*" (Robert Desnos, *Corps et biens*).

My deepest gratitude, thanks, and consideration are graciously offered also to prof. Dr. Guy Roy for his contribution and input to the correction of the text, the valuable suggestions, and guidance to the stylizing the form and the content of this work. Forging the thinking is an art ("*it is the thinking who gives the beauty of the style, but to the pseudo-thinker is the style who's decorating the thinking*" ("*Der Stil erhält die Schönheit vom Gedanken, statt daß bei den Scheindenkern durch den Stil schön werden sollen*", Artur Schopenhauer, *Parerga und Paralipomena*) but we have to avoid the trap on which are involuntarily falling because "*L'instinct, c'est l'âme à quatre pattes; la pensée c'est l'esprit debout*" (Victor Hugo, *Tas de pierres*) and we are pushed to think instinctively, whereas the first thinking has to be avoid ("*Je ne me fie quasi jamais aux premières pensées qui me viennent*", René Descartes, *Discours de la méthode*). Your contribution and guidance has brought to my thinking, the so necessary, final finishing touch.

I am sincerely grateful to Mrs. Line Garneau for her helpful advices, technical assistance and solutions to many problems we have encountered during the time. "*L'amitié est un contrat par lequel nous nous engageons à rendre de petits services afin qu'on nous en rende de grands*" said Montesquieu (*Mes penssées*), thus your kindness, helpful and advice can be considered as proof for a friendship developed over the time for which I am so thankful. I am grateful to all my collegues from Dr. Sauvé's laboratory, Dr. Helen Klein, Fadi Hobeila and Manuel Simoes, for your kindness, friendship, and support. *"Les âmes grandes sont toujours disposées à faire une vertu d'un malheur"*, (Honoré de Balzac, *Illusions perdues*).

I am sincerely thankful and grateful to Mrs. Dr. Lingyum Wu and to Dr. Pierre Bissonnette for their support, help, and collaboration to the development of the project and for their scientific contribution to the results and publications. Your contribution is a clear example of what Louis Pasteur said: "*La science n'a pas de patrie*", 1888, 14 novembre, *Discours d'inauguration de l'Institut Pasteur*, because where ever we are coming from, "*le commencement de toutes les sciences, c'est l'étonnement de ce que les choses sont ce qu'elles sont*" (Aristote, *Métaphysique,* I, 2.), and where ever we are from, "*On ne connaît pas complètement une science tant qu'on n'en sait pas l'histoire*", (Auguste Comte, *Cours de philosophie positive*).

My deepest gratitude and thanks are directed towards Mrs. Dr. Lucie Parent and Dr. Jaques De Champlain for their help, advice, patience, and collaboration, which have conducted me to the accomplishment of this project and completion of publications. Your positive attitude, encouragement and help was more then dignifying, because "*Nous ne savons rien. Le seul espoir de savoir, c'est de savoir tous ensemble, c'est de fondre toutes les classes dans le savoir et la science*" said Lev Nikolaïevitch Tolstoï, *Carnet,* 28 mars 1861 and, moreover, "*Ah! not in*

knowledge is happiness but in the acquisition of knowledge" said Edgar Allan Poe, in *Tales of the grotesque and arabesque,* from The Power of Words.

My deep recognition and gratitude go also to all my professors who teach me during the courses and which were always helpful with precious suggestions and advices: Prof Dr. Guy Roy, Prof Dr. Raynald Laprade, Prof. Dr. Alfred Bertelot, Prof. Dr. Cristiane Malo, Dr, Josette Noël, Prof Dr. Pierre Haddad, Prof. Dr. Madhu Anand-Srivastava, Prof. Dr. Réjan Couture et Prof. Dr. Pierre Gautier.

Special thanks and gratitude are directed towards the technical personnel of GRTM, Mrs. Joane Vallé, Mrs. Julie Verner and Mr. Michel Brunette, which were all the time very kind, helpful and they always came out with the best technical solutions during so many years.

I want to address my thanks and gratitude to all the laboratories and the technical personnel for their help and assistance, which have been helpful and supportive, especially the laboratory of Prof Dr. Jaques De Champlain and to the colleagues Ms. Hélène Girouard and Ms. Chantal Chulak.

Finaly, I am thankful and profoundly grateful to my dear Adela for her patience, understanding, and such a tremendous moral support for all these long years. For your love and support, I will always be grateful.

DEDICATION

This work represents a moral dedication to the memory of all the past generation of my family which transcendentally have fought vigorously the vicissitudes of life for a better human existence and to my children for which here is only one impetus and motivation: "*Generosos animos labor nutrit*", (Lucius Annaeus Seneca, *Epistulae Morales Ad Lucilium,* XXXI), because "*Labor optimos citat*" (Lucius Annaeus Seneca, *De Providentia, 5*), and never forget that only "*Labor omnia vincit improbus*", (Publius Vergilius Maro, *The Georgics*).

The following poem is a dedication to all readers. It represents the soul of Romanian people.

A DACIAN'S PRAYER
Mihai Eminescu, 1879.

When death did not exist, nor yet eternity,
Before the seed of life had first set living free,
When yesterday was nothing, and time had not begun,
And one included all things, and all was less than one,
When sun and moon and sky, the stars, the spinning earth
Were still part of the things that had not come to birth,
And You quite lonely stood... I ask myself with awe,
Who is this mighty God we bow ourselves before.

Ere yet the Gods existed already He was God
And out of endless water with fire the lightning shed;
He gave the Gods their reason, and joy to earth did bring,
He brought to man forgiveness, and set salvation's spring
Lift up your hearts in worship, a song of praise enfreeing,
He is the death of dying, the primal birth of being.
To him I owe my eyes that I can see the dawn,
To him I owe my heart wherein is pity born;
When e'er I hear the tempest, I hear him pass along
Midst multitude of voices raised in a holy song;
And yet of his great mercy I beg still one behest:

That I at last be taken to his eternal rest.

Be curses on the fellow who would my praise acclaim,
But blessings upon him who does my soul defame;
Believe no matter whom who slanders my renown,
Give power to the arm that lifts to strike me down;
Let him upon the earth above all others loom
Who steals away the stone that lies upon my tomb.

Hunted by humanity, let me my whole life fly
Until I feel from weeping my very eyes are dry;
Let everyone detest me no matter where I go,
Until from persecution, myself I do not know;
Let misery and horror my heart transform to stone,
That I may hate my mother, in whose love I have grown;
Till hating and deceiving for me with love will vie,
And I forget my suffering, and learn at last to die.

Dishonored let me perish, an outcast among men;
My body less than worthy to block the gutter then,
And may, o God of mercy, a crown of diamonds wear
The one who gives my heart the hungry dogs to tear,
While for the one who in my face does callous fling a clod
In your eternal kingdom, reserve a place, o God.

Thus only, gracious Father, can I requitance give
That you from your great bounty vouched me the joy to live;
To gain eternal blessings my head I do not bow,
But, rather ask that you in hating compassion show.
Till comes at last the evening, your breath will mine efface,
And into endless nothing I go, and leave no trace.

LIST OF ABBREVIATIONS

aa:	Amino acid;
AC:	Adenylyl cyclase;
ACE:	Angiotensin converting enzyme;
ADP:	Adenosine diphosphate;
AHR:	Aldosterone-salt hypertensive rats;
AMI:	Acute myocardial infarction;
ATP:	Adenosine triphosphate;
BAECs:	Bovine aorta endothelial cells;
BHQ:	2,5-di-(t-butyl)-1,4-benzohydroquinone;
$[Ca^{2+}]_i$:	Intracellular calcium concentration;
$[Ca^{2+}]_o$:	External calcium concentration;
$[Ca^{2+}]_l$:	Iintra-luminal Ca^{2+} concentration;
$^b[Ca^{2+}]_i$:	Basal (resting) intracellular calcium concentration;
CaM:	Calmodulin;
cAMP:	Adenosine-3',5'-cyclic monophosphate;
CAT:	Catalase;
CCE:	Capacitative Ca^{2+} entry;
cGMP:	Guanosine 3',5'-cyclic monophosphate;
CHF:	congestive heart failure;
CICR	Ca^{2+}-induced Ca^{2+} release;
CPA:	Cyclopiazonic acid;
Cu^{2+}:	Cupric ion;
Cu^{+}:	Cuprous ion;
Cyt P_{450}:	Cytochrome P-450;
CRAC:	Ca^{2+} release-activated Ca^{2+} channels;
CVD:	Cardiovascular diseases;
$\Delta[Ca^{2+}]_i$:	Mean increase in $[Ca^{2+}]_i$ during stimulation;
DAG:	*sn*-1,2-Diacylglycerol;
DEF:	Deferoxamine;
DMEM:	Dulbecco's modified Eagle medium;

DOCA:	Deoxycorticosterone acetate;
DTT:	Dithiothreitol;
ECs:	Endothelial cells;
ECGF:	Endothelial cell growth factor;
EDHF:	Endothelium-derived hyperpolarising factor;
EETs:	11,12 and 5,6-epoxyeicosatrienoic acids;
ER:	Endoplasmic reticulum;
Fe^{2+}:	Ferrous iron;
Fe^{3+}:	Ferric iron;
G protein:	GTP-binding protein;
GC:	Guanylyl cyclase;
GDP:	Guanosine diphosphate;
GTP:	Guanosine triphosphate;
$GS^{\bullet}$:	Sulphur-centred thiyl radical;
GSH:	Reduced glutathione;
GSH-Px:	Glutathione peroxidase;
GSSG:	Oxidized glutathione;
$(GSSG)^{\bullet-}$:	Glutathione disulphide radical anion;
H^+:	Proton;
4-HNE:	4-hydroxy-2-nonenal;
$HO_2^{\bullet}$:	Hydroperoxyl radical;
H_2O_2:	Hydrogen peroxide;
HOCl:	Hypochlorous acid (undissociated);
OCl^-:	Dissociated hypochlorous acid;
HOONO:	Peroxynitrous acid;
HUVEC	Human umbilical vein endothelial cells;
HX:	Hypoxanthine;
I_{CRAC}:	Ca^{2+} release-activated Ca^{2+} channels current;
IHD;	Ischemic heart disease
IP:	Iinositol phosphate;
IP_1:	Inositol 1-monophosphate;
IP_2:	Inositol 1,4,-biphosphate;

IP_3	Inositol 1,4,5-trisphosphate;
IP_3R	Inositol 1,4,5-trisphosphate receptor;
IP_3R_1	Type-1 IP3 receptor;
IP_3R_2	Type-2 IP3 receptor;
IP_3R_3	Type-3 IP3 receptor;
IP_3R_4	Type-4 IP3 receptor;
K:	Equilibrium constant;
KO:	Knocked out;
LDLs:	Low-density lipoproteins;
MDA:	Malondialdehyde;
MEL:	Melatonin;
MPO:	Myeloperoxidase;
NAC:	N-acetylcysteine;
NAD^+:	Nicotinamide adenine dinucleotide, oxidized form;
NADH:	Nicotinamide adenine dinucleotide, reduced form;
$NADP^+$:	Nicotinamide adenine dinucleotide phosphate, oxidized form;
NADPH:	Nicotinamide adenine dinucleotide phosphate, reduced form;
NE:	Norepinephrine;
$NO^\bullet$:	Nitric oxide;
NO_2:	Nitrogen dioxide;
$NO_3^\bullet$:	Nitrate;
NOS:	Nitric oxide synthase;
NSC:	Non-selective cation channel;
$O_2^{\bullet-}$:	Superoxide anion;
OFR:	Oxygen-derived free radicals;
OH^-:	Hydroxide ion;
$OH^\bullet$:	Hydroxyl radical;
$ONO^\bullet$:	Peroxynitrous acid;
$ONOO^-$:	Peroxynitrite;
PDE:	Phosphodiesterase;
PGI_2:	Prostacyclin;
PIP_2:	Phosphatidylinositol 4,5-biphosphate;

pK:	Negative logarithm of the ionization constant (K);
PKA:	cAMP-dependent protein kinase;
PKC:	Protein kinase C;
PKG:	cGMP-dependent protein kinase;
PLA:	Phospholipase A;
PLB	phospholamban;
PLC:	Phospholipase C;
PLD:	Phospholipase D;
RAMEC	Rat adrenal medulla microvascular endothelial cells;
ROI:	Reactive oxygen intermediates;
ROS:	Reactive oxygen species;
RyR:	Ryanodine receptor;
SERCA	sarco/endoplasmic reticulum Ca(2+)-ATPase;
SD:	Sprague-Dawley rats;
SDECs:	Sprague Dawley rat aorta endothelial cells;
SHR:	Spontaneously hypertensive rats;
SHRECs:	Spontaneously hypertensive rat aorta endothelial cells;
SMCs:	Smooth muscle cells;
SOD:	Superoxide dismutase;
SR:	Sarcoplasmic reticulum;
t-BuOOH:	t-butyl hydroperoxide;
TG:	Thapsigargin;
trp:	Transient receptor protein;
*trp*L:	Transient receptor protein like;
Vitamin C:	Ascorbic acid;
Vitamin E:	α-Tocopherol;
WKY:	Wistar-Kyoto rats;
WT:	wild-type;
XO:	Xanthine oxidase.

SOMMAIRE

Les cellules endothéliales font partie de la régulation tissulaire et jouent un rôle capital dans le contrôle et la modulation du tonus vasculaire. Les fonctions des cellules endothéliales sont fondamentalement compromises en état physiopathologique associé avec le stress oxydant. Le mécanisme moléculaire associé aux dommages des cellules endothéliales induits par le stress oxydant demeure en grande partie inconnue. L'objectif principal de cette étude est de caractériser l'homéostasie du Ca^{2+} dans les cellules endothéliales d'aorte de bovin, de rats normotendus et hypertendus, sous l'impact des radicaux libres et des antioxydants. Des expériences de spectrophotométrie fluorescente du Ca^{2+} ont été effectuées, ainsi que des mesures sur la production d'$InsP_3$ et des analyses de l'expression et de l'activité des enzymes. L'analyse des données démontre que le Ca^{2+} largué par des agonistes dépendants et indépendants de la production d'$InsP_3$ évoquent des réponses calciques 55% plus petites dans les cultures primaires des cellules de rats normotendus comparativement aux cellules BAE. La quantité de Ca^{2+} largable des réservoirs intracytosoliques dans les cultures primaires des cellules endothéliales est nettement inférieure au Ca^{2+} largable par des agonistes dans les cellules endothéliales clones. L'application du traitement antioxydant spécifique (catalase et SOD) et non spécifique (vitamine E et C, desferioxamine, mélatonine) récupère les réponses calciques induites par des agonistes dans les cellules endothéliales soumissent au stress oxydant dans une proportion de 50-100% et abaisse de manière importante le Ca^{2+} intracytosolique de repos. L'application du traitement antioxydant aux cellules endothéliales de rats SHR jeunes et adultes augmente les réponses calciques induites par des agonistes de 60-100%, établissant les réponses calciques des cellules endothéliales de rats hypertendus statistiquement égaux aux cellules endothéliales rats normotendus. L'effet bénéfique des antioxydants sur les réponses

calciques de cellules endothéliales de SHR, en comparaison avec les cellules endothéliales de rats normotendus, n'est pas associé à une modification dans la production d'$InsP_3$. En dépit d'un effet inhibiteur de la mélatonine sur le largage du Ca^{2+} induit par des hormones et des agonistes dans différents types de cellules, dans la présente étude, la mélatonine démontre une capacité d'augmenter de 30% le largage du Ca^{2+} induit par des agonistes dépendants d'$InsP_3$. Après le traitement simultané des cellules endothéliales de rats avec la mélatonine et le stress oxydant, la mélatonine améliore de 60% le largage du Ca^{2+} induit par des agonistes. Les cellules endothéliales de SHR ont eu une augmentation de 55% du largage du Ca^{2+} induit par des agonistes suivant un pré-conditionnement de 20 minutes avec la mélatonine. Dans les protocoles expérimentaux d'autocontrôle, les effets de stimulation sur l'augmentation du largage du Ca^{2+} induit par des agonistes ont été démontrés clairement et irréfutablement, ainsi qu'un puissant effet de stimulation sur l'entrée capacitative du Ca^{2+} dans des cellules endothéliales BAE et des cultures primaires de cellules endothéliales de rat. En conclusion, cette étude met en évidence une image en perspective sur l'homéostasie du Ca^{2+} dans les cultures primaires de cellules endothéliales des rats normotendus et hypertendus et indique de puissants effets des antioxydants spécifiques, non-spécifiques et de la mélatonine en condition de stress oxydant. C'est la première évidence convaincante au sujet de l'effet préjudiciable du peroxyde d'hydrogène sur l'homéostasie du Ca^{2+} pendant le stress oxydant dans les cellules endothéliales des rats. Elle représente également la première démonstration sur l'expression et l'activité des enzymes antioxydants des cellules endothéliales de rats normotendus et hypertendus en cultures primaires.

RÉSUMÉ

La régulation du tonus vasculaire est une conjonction d'activités de contrôle à multiniveaux en provenance du système nerveux central, du système nerveux autonome, du système endocrine et du contrôle local ou tissulaire. Dans la décennie passée, il est devenu évident que les cellules endothéliales vasculaires sont de petites usines pour la production d'une variété de substances paracrines. L'endothélium produit des facteurs biologiquement-actifs impliqués dans l'agrégation de plaquettes, la thrombose et la fibrinolyse, l'inflammation (facteurs de perméabilité vasculaire), l'immunité, l'angiogenèse (facteurs de croissance vasculaire d'origine endothéliale), la mécano-réception, les hormones (les endothélines et probablement la mélatonine) et les facteurs endothéliaux de croissance (facteur de croissance de cellules endothéliales). Par conséquent, l'endothélium devrait être élevé au statut d'organe. Les cellules endothéliales font partie de la régulation tissulaire et jouent un rôle capital dans le contrôle et la modulation du tonus vasculaire. Le tonus des cellules musculaires lisses vasculaires sousjacentes est entretenu par la sécrétion, dans un équilibre dynamique, de deux types des facteurs vasoactifs, des facteurs de vasodilatation dérivés de l'endothélium (EDRF) et des facteurs de contraction dérivés de l'endothélium (EDCF). L'hypertension essentielle de l'humain et plusieurs modèles d'hypertension animale sont associés à une augmentation importante de la résistance vasculaire périphérique. Les fonctions des cellules endothéliales sont fondamentalement compromises en état physiopathologique associé avec le stress oxydant. Les changements induits par le stress oxydant sur l'homéostasie du Ca^{2+} et les blessures infligées dans l'endothélium pourraient être responsables et expliquer une grande partie de la physiopathologie cardiovasculaire observée et associés aux maladies cardiovasculaires. La dysfonction endothéliale s'est avérée posséder une importante contribution à la pathogénèse de

l'hypertension en raison de la destruction des capacités paracrines des cellules endothéliales. L'événement précoce induit par le stress oxydant dans les cellules endothéliales est caractérisé par le dysfonctionnement du mécanisme de signalisation transmembranaire et en particulier l'homéostasie cellulaire du Ca^{2+}. D'un point de vue physiologique, l'augmentation de $[Ca^{2+}]_i$ est une nécessité absolue pour que la cellule endothéliale puisse déclencher la sécrétion des facteurs paracrines et vasomoteurs. Toutefois, d'un point de vue physiopathologique, l'augmentation prolongée de $[Ca^{2+}]_i$ s'avère nocive aux paramètres physiologiques de la cellule. Le changement dans l'homéostasie du Ca^{2+} et l'augmentation du Ca^{2+} libre dans le cytosol suivant les dommages produits par le stress oxydant génèrent des modifications fondamentales dans la réponse physiologique de l'endothélium. Le mécanisme moléculaire associé aux dommages des cellules endothéliales induits par le stress oxydant demeure en grande partie inconnu. Le point de départ et la plus importante source des radicaux libres dans le système biologique est la réaction en chaîne de réduction de l'oxygène. Cette réaction génère l'anion superoxide ($^{\bullet}O_2^-$), lequel possède une activité intrinsèque vasoconstrictrice *per se,* et constitue le point de départ pour la production de tous les radicaux libres dérivés de l'oxygène. En parallèle avec le lancement de la réduction univalente de l'oxygène, évolue la réaction en chaîne de la boucle d'azote, dans laquelle se produit la formation de l'acide peroxynitreux et ses produits dérivés. De même, la réaction de l'ion Cl^- avec l'anion superoxide génère une autre boucle parallèle, responsable de la formation de l'acide hypochloreux induite de manière spontanée et/ou de façon prépondérante par la réaction catalysée par la myeloperoxidase. La formation des espèces réactives de l'oxygène est augmentée dans les cellules endothéliales suite au stress oxydant. La participation des radicaux libres dans la pathogénèse de la cardiomyopathie humaine et animale et de l'hypertension essentielle a été démontrée par la présence des concentrations élevées de $^{\bullet}O_2^-$ et de H_2O_2. La présomption de l'implication de

radicaux libres dans la physiopathologie d'hypertension a été définitivement démontrée par l'effet induit par l'application d'injections intraveineuses de superoxide dismutase (SOD) liée à des protéines de fusion, lesquelles injections ont abaissé de manière significative la pression artérielle dans les rats spontanément hypertendues (SHR). On a observé la même manifestation de redressement de l'hypertension artérielle chez les rats hypertendus induits par l'angiotensine-II, où la pression artérielle élevée et la réactivité vasculaire ont été restaurées suite à l'administration exogène de la SOD encapsulée dans des liposomes.

L'objectif principal de cette étude est de caractériser l'homéostasie du Ca^{2+} dans les cellules endothéliales d'aorte de bovin, de rats normotendus et hypertendus, sous l'impact des radicaux libres et des antioxydants. Dans la première partie de l'étude, des cellules endothéliales provenant de l'aorte de bovin (BAE) ainsi que de l'aorte de rats normotendus Sprague Dawley (SD), en conditions de contrôle et en conditions de "stress" oxydant, seront comparées aux cellules endothéliales de rats SHR hypertendus, relativement aux dommages infligés par les radicaux libres ainsi qu'à la capacité protectrice des antioxydants spécifiques et nonspécifiques. Dans la deuxième partie de l'étude, sera étudié l'impact de la mélatonine sur l'homéostasie de Ca^{2+}, dans des conditions normales et dans des conditions de stress oxydant. À cet égard, des expériences de spectrophotométrie fluorescente du Ca^{2+} ont été effectuées, ainsi que des mesures sur la production d'$InsP_3$ et des analyses de l'expression et de l'activité des enzymes. L'étude suit des protocoles expérimentaux dans des conditions normales, de stress oxydant imposé, et de traitement avec des antioxydants. Les comparaisons sont basées sur l'évaluation du largage intracytoplasmique du Ca^{2+} après la stimulation de la production d'$InsP_3$ induite par des agonistes comme l'ATP et la bradykinine. Dans un ensemble parallèle de protocoles expérimentaux, les effets complexes de la mélatonine sur l'homéostasie du Ca^{2+} des cellules endothéliales ont été évalués pour la première fois. L'évaluation est basée sur le

largage du Ca^{2+} induit par la mélatonine, les effets de la mélatonine sur les réservoirs internes du Ca^{2+} et sur l'entrée capacitative du Ca^{2+}, ainsi que sur l'effet antioxydant de la mélatonine sur l'homéostasie du Ca^{2+} dans toutes les cellules endothéliales sous étude.

L'analyse des données et des protocoles expérimentaux démontre que le Ca^{2+} largué par des agonistes dépendants et indépendants de la production d'$InsP_3$ évoquent des réponses calciques 55% plus petites dans les cultures primaires des cellules de rats normotendus comparativement aux cellules BAE. Encore que les réponses calciques dans les cellules endothéliales de SHR sont 60% plus petites que celles des cellules endothéliales de rats normotendus. La quantité de Ca^{2+} largable des réservoirs intracytosoliques dans les cultures primaires des cellules endothéliales est nettement inférieure au Ca^{2+} largable par des agonistes dans les cellules endothéliales clones. Le stress oxydant provoqué par le système générateur des radicaux libres HX/XO induit une inhibition dépendante du temps et de la dose sur les réponses calciques induites par des agonistes, parallèlement à une augmentation du Ca^{2+} libre intracytosolique.

Les réponses calciques induites par des agonistes dans les cellules endothéliales de rats normotendus ne sont pas sensibles au traitement antioxydant. L'application du traitement antioxydant spécifique (catalase et SOD) et non spécifique (vitamine E et C, desferioxamine, mélatonine) récupère les réponses calciques induites par des agonistes dans les cellules endothéliales soumisent au stress oxydant dans une proportion de 50-100% et abaisse de manière importante le Ca^{2+} intracytosolique de repos. L'application du traitement antioxydant spécifique et non spécifique aux cellules endothéliales de rats SHR jeunes et adultes augmente les réponses calciques induites par des agonistes de 60-100%, établissant les réponses calciques des cellules endothéliales de rats hypertendus statistiquement égaux aux cellules endothéliales

de rats normotendus. Cependant, dans toutes les préparations cellulaires sous étude, les traitements antioxydants n'affectent pas les réservoirs thapsigargine-sensibles des cellules endothéliales. Dans toutes les expériences, la catalase s'est avérée être l'antioxydant le plus efficace, indiquant indirectement que le plus délétère des sous-produits de la réduction de l'oxygène est le peroxyde d'hydrogène. L'effet bénéfique des antioxydants sur les réponses calciques induites par des agonistes dans les cellules endothéliales de SHR, en comparaison avec les cellules endothéliales des rats normotendus, pourrait être associé à une modification importante dans la production d'$InsP_3$. Les mesures d'$InsP_3$ prouvent une augmentation de 15 fois la quantité d'$InsP_3$ basal dans les cellules endothéliales soumises pendant 90 minutes au stress oxydant avec le système générateur de radicaux libres HX/XO. Cependant, l'augmentation d'$InsP_3$ induite par la stimulation avec l'ATP n'est que de 2 fois. Le traitement de cellules endothéliales de SHR avec des antioxydants n'a pas augmenté de manière significative la quantité d'$InsP_3$ produite, démontrant que l'effet bénéfique des antioxydants n'est pas associé avec l'augmentation de la production d'$InsP_3$. L'effet bénéfique des enzymes antioxydants pourrait être dû à une expression et/ou à une activité déficiente des enzymes antioxydants spécifiques endogènes. L'immunobuvardage démontre que l'expression de la catalase et celle de la SOD sont équivalentes dans l'endothélium des rats normotendus et hypertendus. La détermination de l'activité enzymatique pour la catalase et la SOD démontre des activités presque identiques des deux enzymes antioxydants dans les deux types de cellules endothéliales de rat. L'entrée capacitative du Ca^{2+} dans les cultures primaires des ECs est un ordre de grandeur plus petit que l'entrée capacitative du Ca^{2+} dans les cellules endothéliales clones, étant situé à la limite de détection. L'augmentation de l'entrée capacitative du Ca^{2+} est dans une corrélation positive avec la durée et la dose de stress oxydant appliqué. Cependant,

l'entrée capacitative du Ca^{2+} n'est pas affectée par le traitement avec les antioxydants, exception faite de la mélatonine qui augmente l'entrée capacitative du Ca^{2+} jusqu'à huit fois.

Les résultats des effets de la mélatonine sur les cellules endothéliales indiquent beaucoup de mécanismes inconnus. Les résultats démontrent que la mélatonine ne possède pas de récepteurs membranaires spécifiques associés avec la production d'$InsP_3$ et le largage du Ca^{2+} induit par la mélatonine des réservoirs intracytosoliques. Cependant, les études de liaison spécifiques effectuées dans ce projet n'excluent pas la présence des récepteurs membranaires, cytoplasmiques et/ou nucléaires pour la mélatonine, couplés à des voies de signalisation transmembranaire indépendante du Ca^{2+}. Toutefois, d'autres études de liaison spécifiques soutiennent une liaison agoniste-antagoniste spécifique avec la calmoduline. En dépit d'un effet inhibiteur de la mélatonine sur le largage du Ca^{2+} induit par des hormones et des agonistes dans différents types de cellules, dans la présente étude, la mélatonine démontre une capacité d'augmenter de 30% le largage du Ca^{2+} induit par des agonistes dépendants d'$InsP_3$. Après le traitement simultané des cellules endothéliales de rats avec la mélatonine et le système produisant des radicaux libres HX/XO, la mélatonine améliore de 60% le largage du Ca^{2+} induit par des agonistes. Les cellules endothéliales de SHR ont eu une augmentation de 55% du largage du Ca^{2+} induit par des agonistes suivant un préconditionnement de 20 minutes avec la mélatonine. Néanmoins, la mélatonine, comme d'autres antioxydants, n'a pas amélioré la disponibilité des réservoirs TG sensibles. Par un effet d'anticalmoduline, la mélatonine montre un effet de puissante stimulation sur l'entrée capacitative du Ca^{2+} dans toutes les souches de cellules endothéliales. Dans les protocoles expérimentaux d'autocontrôle, les effets de stimulation sur l'augmentation du largage du Ca^{2+} induit par des agonistes ont été démontrés clairement et irréfutablement, ainsi qu'un puissant effet de stimulation sur l'entrée capacitative

du Ca^{2+} dans des cellules endothéliales BAE et des cultures primaires de cellules endothéliales de rat.

En conclusion, cette étude met en évidence une image en perspective sur l'homéostasie du Ca^{2+} dans les cultures primaires de cellules endothéliales des rats normotendus et hypertendus et indique de puissants effets des antioxydants spécifiques, nonspécifiques et de la mélatonine en condition de stress oxydant. C'est la première évidence convaincante au sujet de l'effet préjudiciable du peroxyde d'hydrogène sur l'homéostasie du Ca^{2+} pendant le stress oxydant dans les cellules endothéliales des rats. Elle représente également la première démonstration sur l'expression et l'activité des enzymes antioxydants des cellules endothéliales de rats normotendus et hypertendus en culture primaire. Cette étude constitue la première évidence scientifique démontrant l'altération de l'homéostasie du Ca^{2+} par la surproduction des radicaux libres dans les cellules endothéliales de SHR, laquelle surproduction est due à l'accumulation du peroxyde d'hydrogène. Les effets de la mélatonine comme agoniste et antioxydant n'ont jamais été étudiés dans les cellules endothéliales jusqu'ici. Les résultats au sujet de l'effet de la mélatonine sur l'homéostasie du Ca^{2+} dans les cellules endothéliales présentées dans cette étude peuvent expliquer, en majeure partie, l'effet bénéfique de la réduction de la pression artérielle par la mélatonine dans l'hypertension essentielle humaine et dans les modèles d'animaux hypertendus.

CHAPTER I. INTRODUCTION

I.1. VASOACTIVE FUNCTION AND PHYSIOPATHOLOGY OF ENDOTHELIUM

Up to the last two decades, it was considered that the vascular endothelium forms an inert and nonthrombogenic surface that facilitated blood flow through the body. More recently, it has been demonstrated that the endothelial cells form a selective diffusion barrier between the vascular and interstitial space, not only in the brain, but also in the entire organism and they play a capital role in both, the short- and long-term regulation of vascular resistance. The endothelium is highly differentiated to mediate an extensive bi-directional exchange of small molecules and to limit the passage of large molecules.

I. 1.1. Vasoactive Function of Endothelium

At the cellular level of the microvessel, there is a triple control system made of endothelial cells, primary effector cells of the specific organ and the local autonomic nerve (Figure 1). This cell system is also implicated in the metabolism of blood-born substances such as bradykinin and angiotensin, which are metabolized by angiotensin-converting enzyme, and the adenine nucleotides, which are metabolized by ecto-adenosine triphosphatases, present on the surface membrane of the endothelial cells. Intra- and extracellular oxidases and oxidoreductases are implicated in the metabolism and/or degradation of endogenous amines, and in the biotransformation of many pharmacological agents.

It has become increasingly evident that endothelial vascular cells are small factories for the production of a variety of paracrine substances. The endothelium produces biological active

factors implicated in platelet aggregation, thrombosis and fibrinolysis, inflammation, immunity, and angiogenesis.

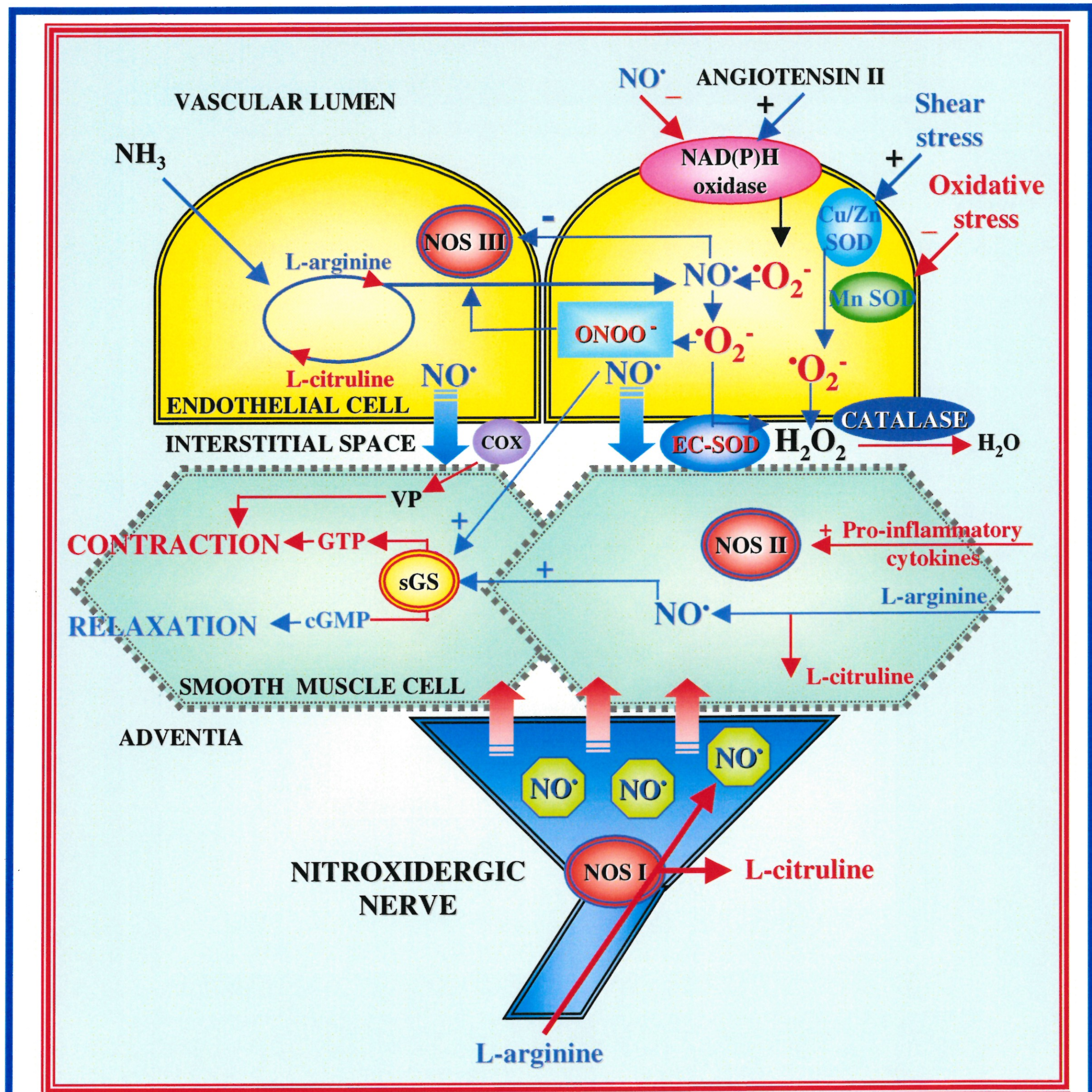


FIGURE 1.

Some of the complex interaction involved in regulating the balance of nitric oxide (NO•) and superoxide anion (•O_2^-) within the vasculature.

NOS I indicates neuronal NOS; NOS II indicates inducible NOS;
NOS III indicates endothelial NOS;
Mn SOD manganese SOD; Cu/Zn SOD copper/zinc SOD;
sGC soluble guanylate cyclase;
H_2O_2 hydrogen peroxide; $ONOO^-$ peroxynitrate;
GTP guanosin 5'-triphosphate; COX cyclooxygenase;
VP vasoconstrictor prostanoids;

Endothelial cells play an important role in the regulation of vascular tone by secreting a variety of vasoactive factors in response to physical, humoral, and/or hormonal stimuli. The control of the tonus of subjacent vascular smooth muscle cells is realized by secreting two types of vasoactive factors, the **endothelium derived relaxing factors** (EDRF) and the **endothelium derived contracting factors** (EDCF).

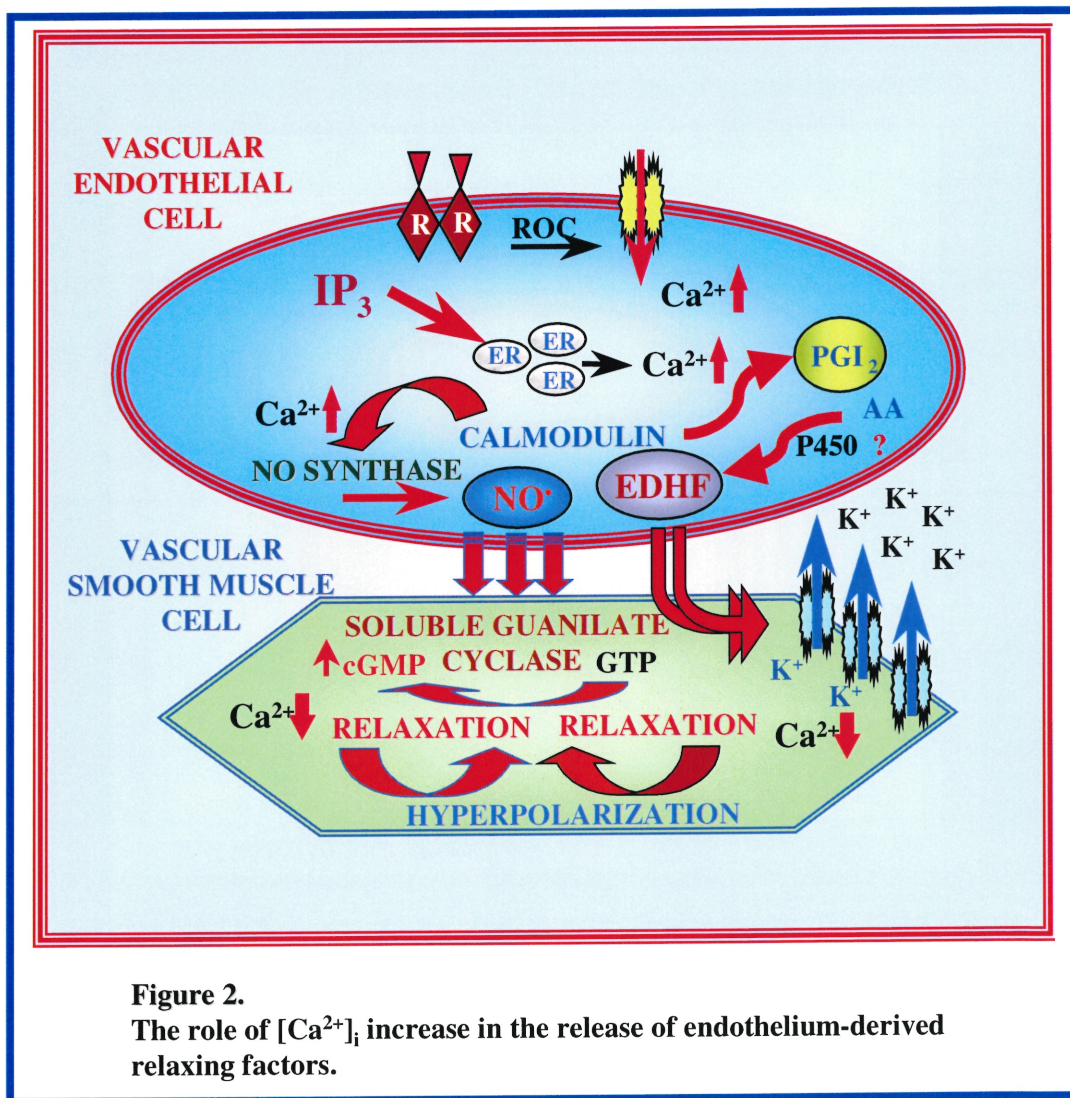


Figure 2.
The role of $[Ca^{2+}]_i$ increase in the release of endothelium-derived relaxing factors.

The identified ***vasorelaxing agents*** (EDRF) (Figure 2) are: **endothelium-derived hyperpolarizing factor** (EDHF), **prostacyclin** (PGI_2), and **nitric oxide** ($NO^{\bullet}$).

In fact, from a chemical and structural point of view, $NO^{\bullet}$ can be considered as a free radical [1]. In 1980, Furchgott and Zawadzchi [2] observed that acetylcholine from endothelium was able to relax precontracted aorta rings. The diffusible factor was generically called EDRF. In 1987, Palmer, Ferrige and Moncada, [3] identify EDRF as being a simple diatomic molecule, ***nitric oxide***, synthesized from L-arginine by the nitric oxide-synthase [4-6]. The molecular identity of EDHF has not yet been identified but its effect has been clearly demonstrated. The common feature of EDHF is the activation of calcium-dependent potassium channels inhibitable by charybdotoxin and/or apamin [7]. In the coronary circulation of many species, the EDHF seems to be a cytochrome P450-dependent non-prostanoid metabolite of arachidonic acid, activated by a number of chemical and physical stimuli and similar to those that are known to activate the endothelial nitric oxide synthase [8].

The ***vasoconstrictor factors*** known until now are: **superoxide anion** ($^{\bullet}O_2^-$), **endothelin-1** (and perhaps other endothelins), **endoperoxides** (PGH_2), and **thromboxane A_2**.

The superoxide anion acts primarily by an elimination of $NO^{\bullet}$ following a spontaneous radical-radical reaction between $^{\bullet}O_2^-$ and $NO^{\bullet}$ and by inflicting oxidative lesions to functional structures implicated in vasodilatation through its oxidative potential and through its capability to generate in a step wise manner, the oxygen-derived free radicals.

Endothelin-1 appears to be the predominant member of the endothelium-derived peptide family generated by vascular ECs that possess powerful vasoconstrictor properties. In addition to its direct vascular effects, endothelin-1 has inotropic and mitogenic properties [9], influences homeostasis of salt and water, alters central and peripheral sympathetic activity and stimulates the renin-angiotensin-aldosterone system [10]. Endogenous generation of endothelin-1 appears

to contribute to the maintenance of basal vascular tone and blood pressure through activation of vascular smooth muscle ET_A receptors. At the same time, the endogenous endothelin-1 acts through endothelial cell ET_B receptors to stimulate the tonic formation of nitric oxide and to oppose vasoconstriction. It also appears likely that endothelin participates in the adverse cardiac and vascular remodeling of hypertension, as well as to the renal damage associated with hypertension [11].

Arachidonic acid metabolic pathway is a complex chemical chain reaction (Figure 3). The prostaglandins, prostacyclin, and thromboxanes are produced via the cyclooxygenase pathway, where prostaglandin endoperoxide synthase (PES) route transforms arachidonic acid into the cyclic endoperoxides. The arachidonic acid metabolites, the prostaglandins, possess a variety of complex effects (e.g. PGI_2 vasodilatation and PGH_2, TXA_2 vasoconstriction) whereas their specific enzymes produce a remarkable amount of superoxide radical. Enzymes that transform arachidonic acid into eicosanoids are ubiquitous within animal cells. The majority of cells present specific membrane receptors for PGH_2/TXA_2. The initial rate-limiting step in the arachidonic acid metabolic cascade is the enzymatic release of the fatty acid from membranes; this is accomplished by acyl hydrolases, such as phospholipases A_2 or C. Phospholipase A_2 releases arachidonate from the second glycerol carbon of phospholipids. Phospholipase C specifically cleaves phosphatidylinositol diphosphate (PIP_2) into inositol phosphate and diacylglycerol (DAG), whereas a diglyceride lipase liberates arachidonate from diacylglycerol. Only free arachidonate can be metabolized. Two single-protein isoenzymes of PES exhibit both cyclooxygenase and peroxidase activities and they are located within endoplasmic and nuclear membranes.

Vasoactive Function of Endothelium

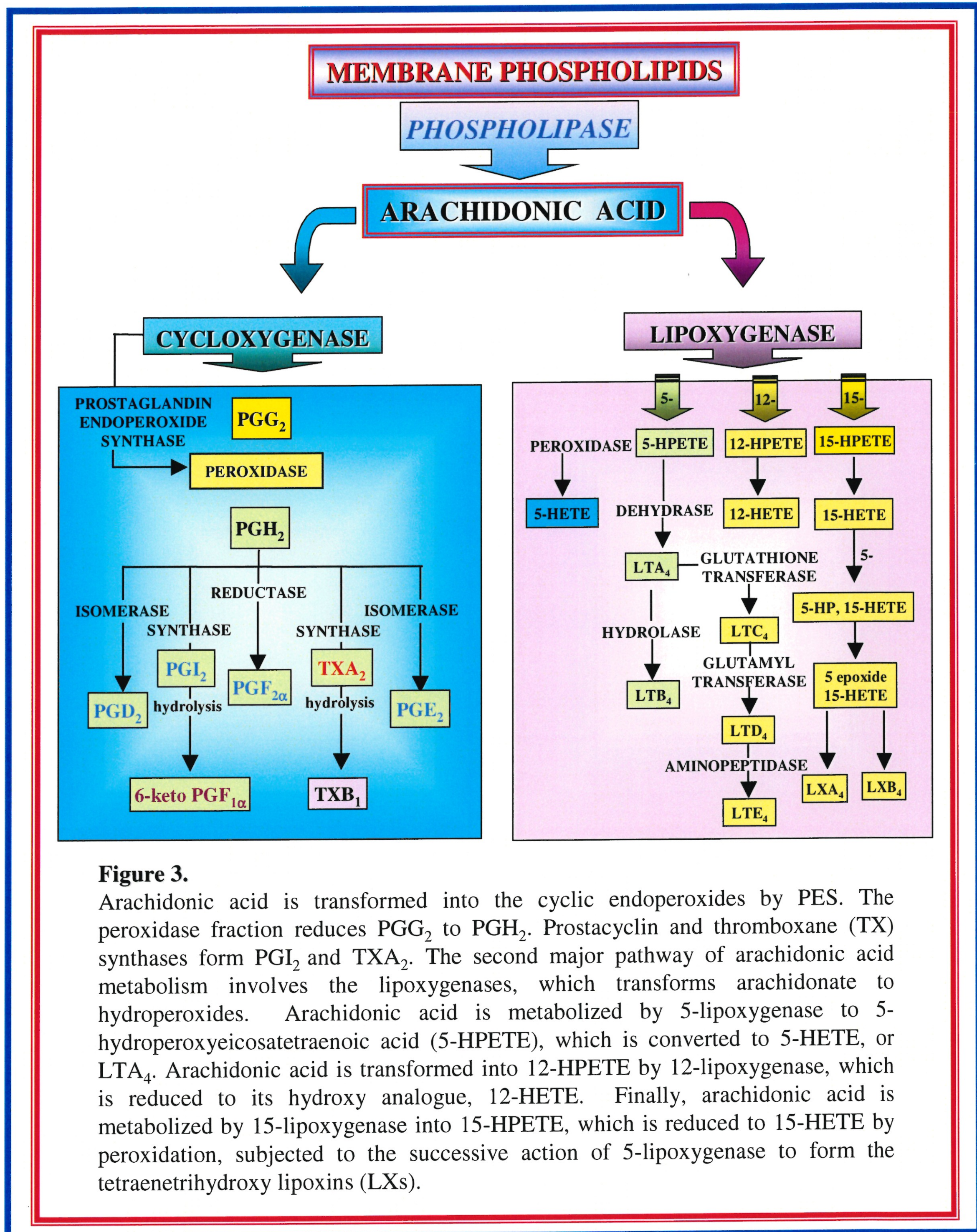


Figure 3.
Arachidonic acid is transformed into the cyclic endoperoxides by PES. The peroxidase fraction reduces PGG_2 to PGH_2. Prostacyclin and thromboxane (TX) synthases form PGI_2 and TXA_2. The second major pathway of arachidonic acid metabolism involves the lipoxygenases, which transforms arachidonate to hydroperoxides. Arachidonic acid is metabolized by 5-lipoxygenase to 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is converted to 5-HETE, or LTA_4. Arachidonic acid is transformed into 12-HPETE by 12-lipoxygenase, which is reduced to its hydroxy analogue, 12-HETE. Finally, arachidonic acid is metabolized by 15-lipoxygenase into 15-HPETE, which is reduced to 15-HETE by peroxidation, subjected to the successive action of 5-lipoxygenase to form the tetraenetrihydroxy lipoxins (LXs).

The cyclooxygenase introduces two molecules of oxygen into arachidonate to yield PGG_2 and the peroxidase reduces PGG_2 to PGH_2.

A variety of tissue-specific enzymes compete for the same unstable substrate, PGH_2, dictating the relative amounts of prostaglandins, prostacyclin (PGI_2), or thromboxanes synthesized.

The second major pathway of arachidonic acid metabolism involves a class of enzymes known as the lipoxygenases; these cytosolic dioxygenases transform arachidonate to hydroperoxides. The cellular distribution of lipoxygenases is more restricted than that of PES, but they are actively present in endothelial cells [7,12].

I. 1.2. Physiopathology of Endothelium

Cardiovascular diseases (CVD) are highly recognized as the main cause of death in developed countries, with an incidence of approximately 50%. CVD is a general expression used to designate a wide range of diseases. According to the World Health Organization (1999), the CVD classification is divided into 69 groups, including ischemic heart disease (IHD), transient cerebral ischemic attack and ischemic stroke, occlusive arterial disease of the lower extremities, and other related diseases such as acute myocardial infarction (AMI).

The dynamic equilibrium between EDRF and EDCF plays a capital role in the physiopathology of the vascular reactivity. The slightest alteration of ECs homeostasis predisposes the endothelial function to a disease state, which more likely implies the initiation and the evolution of the oxidative stress and the disruption of the dynamic equilibrium between EDRF and EDCF. This unbalanced endothelial state, which may qualify for an idiopathic oxidative syndrome, can settle into the primordial etiology of different cardiovascular diseases (Figure 4). Therefore, it has been suggested that the endothelium has a crucial role in the

initiation and in the progression of several cardiovascular diseases, including atherosclerosis, hypertension, and ischemic injury.

The microvessels and the endothelial cells have the disadvantage of being exposed to two major sources of oxidants at the same time. Firstly, the transport and transfer function of blood (gases, nutrients and cellular excreta) involves mostly oxido-reductive reactions, in which oxygen's reactions occupy an important part of the transport's function chemical spectrum. In plus, in the circulatory as well as in the interstitial space are present important oxidative reactions derived from humoral defensive system, from which almost all the byproducts have a free radical character. Both conditions bring exogenous oxidant sources to the endothelium. Secondly, from the intrinsic endothelial cells respiration and metabolism result a large variety of free radicals (regularly derived from oxygen, nitrogen and chloride), which act as a potential endogenous source of oxidative stress. Therefore, the oxidative stress may arise as an intrinsic source, starting with the univalent reduction of O_2, or extrinsically induced by the activation of the oxidative burst in polymorphonuclear leukocytes, by a secondary effect of drug metabolism and by the uptake of oxidized low-density lipoproteins.

In all aerobic organisms and cells, as well as in ECs, the first and most widely produced free radical is the superoxide anion ($^{\bullet}O_2^-$). In the vascular system, independent of its source, $^{\bullet}O_2^-$ possesses an intrinsic vasoconstrictor activity *per se* [13]. Despite the *"ab ovo"* significance of oxygen in aerobic organisms for oxidation reactions, which provide energy and kill invaders, unwanted reactions are inescapable. For example, the univalent reduction by cytochrome oxidase of molecular O_2 to water, which produces $^{\bullet}O_2^-$ and H_2O_2 in consecutive steps, has induced the genetic expression of specific enzymes, such as superoxide dismutases (SOD), catalases, and glutathione peroxidases (GSH-Px) to remove the intermediate reactive

oxygen species. This genetic adaptation suggests that a significant amount of O_2 is reduced via this route and that far from negligible quantity of intermediate O_2 species (OIS) and/or oxygen derived free radicals (OFR) are produced via this pathway. It was estimated that a typical human cell metabolizes $\approx 10^{12}$ molecules of O_2 per day and generates some 3×10^9 molecules of H_2O_2 per hour [14]. In a collateral reaction loop, $^{\bullet}O_2^-$ reacts spontaneously with $NO^{\bullet}$ producing the peroxynitrate ($ONOO^-$), successfully reducing the bioavailability of endothelium derived $NO^{\bullet}$, inducing automatically less vasodilatation and the harmful effect of $ONOO^-$, which is further promoted to peroxynitrous acid. The cleavage products of peroxynitrous acid are among the most reactive oxygen derived species in the biological system [15]. The oxidant stress induced by different mechanisms appears to influence vascular tone and reactivity through alterations in the production, release, or interaction with endothelium-derived paracrine products. The molecular mechanism associated with oxidant-induced cell injury remains largely unknown. However, recent results seem to favor the hypothesis of a primordial role for the superoxide anion in the oxidant-induced cell damage theory.

Oxidant state such as hyperoxia, ischemia and/or reperfusion oxygenation in conjunction with organic peroxides and O_2, N_2 and/or carbon centered free radicals may cause cell injury by oxidation of sulfhydryl groups [16], induction of lipid peroxidation [16,17], DNA strand breaks and injuries [18], and activation of ADP-ribose polymerase [19], altered protein synthesis [20], rearrangements of microfilaments [21] and reduction of membrane fluidity [22]. An early event associated with endothelial cells dysfunction in oxidative stress implies alteration of transmembrane signaling mechanism, in particular the cellular Ca^{2+} signaling mechanism [23-25].

From a physiological point of view, the increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) is an unconditional necessity for endothelial cell to be able to trigger the release of paracrine factors (Figure 2). This is a *sine qua non* condition for the control of vascular reactivity and permeability of ECs. It is now accepted that the oxidative stress under physiopathological conditions is the outcome of the activation of both the sympathetic nervous system and the renin-angiotensin system, associated into a tight and dynamic partnership with the activation of lymphocytes and endothelial system [10,26]. The physiopathological mechanisms implicated in atherosclerosis and hypertension, as well as contractile dysfunction and arrhythmia, are associated with oxidative stress. Calcium entry into myocytes, promoted by oxidative stress, stimulates neointimal hyperplasia, promoting the occurrence of atherosclerosis as well as vasoconstriction for the progress of hypertension. The Ca^{2+} overload of myocytes produces cell damages, as seen in cardiomyopathic or ischemic reperfusion lesions of the heart, and promote the transition of cardiac hypertrophy to heart failure [27].

In recent years, the implication of "oxidative stress syndrome" has been demonstrated in a wide variety of degenerative processes, diseases and syndromes, including the following: mutagenesis, cell transformation and cancer; atherosclerosis, arteriosclerosis, heart attacks, strokes and ischemia/reperfusion injury; chronic inflammatory diseases, such as rheumatoid arthritis, lupus erythematosus and psoriatic arthritis; acute inflammatory processes, such as wound healing; photo-oxidative stresses of the eye such as cataract; central-nervous-system disorders, such as certain forms of familial amyotrophic lateral sclerosis, certain glutathione peroxidase-linked adolescent seizures, Parkinson's disease and Alzheimer's dementia; and a wide variety of age-related disorders, perhaps even including factors underlying the aging process itself. Some of these oxidation-linked diseases or disorders can be exacerbated, perhaps even initiated, by numerous environmental pro-oxidants and/or pro-oxidant drugs and foods.

Physiopathology of Endothelium

I. 1.2.1. Hypertension

Essential hypertension is a classic example of complex, multifactorial, and polygenic disease. The endothelial dysfunction has been revealed as an important contributor to the pathogenesis of hypertension because of altered paracrine capabilities of vascular endothelial cells. Human essential hypertension and several animal models of hypertension are associated with an important increase in the peripheral vascular resistance. Since EDRF is inactivated by $^{\bullet}O_2^-$ *in vitro*, oxidative stress in and around vascular ECs may affect dramatically the vascular resistance. $NO^{\bullet}$ is one of the most rapid and potent endogenous vasodilator and its bioavailability is, in all likelihood, dependent of the ECs' production potential of highly active free radicals and the amount of substrate for $NO^{\bullet}$ production. Its reduced bioavailability would lead to vasoconstriction and hence, increased peripheral vascular resistance.

Recent works have revealed that angiotensin-II stimulates the production of $^{\bullet}O_2^-$ by increasing the activity of NAD(P)H cytochrome oxidoreductase [28,29]. Blood pressure and vascular reactivity could be restored by exogenous liposome-encapsulated SOD in the angiotensin-II hypertensive rats, but not the noradrenalin hypertensive rats, which further implicate the role of $^{\bullet}O_2^-$ in hypertension, associated with high angiotensin-II level [26]. It has been reported also that purified rat brain $NO^{\bullet}$-synthase (NOS) produces $^{\bullet}O_2^-$ in a reaction that is inhibited by N^{ω}-nitro-L-arginine methyl ester (L-NAME) but not by N^{ω}-monomethyl-L-arginine [30]. The purified porcine NOS-I can produce H_2O_2 under the conditions of low L-arginine concentration [31]. It is suggested that NOS-III from human umbilical vein ECs is a source of $^{\bullet}O_2^-$ after stimulation with native low-density lipoproteins, as it can be inhibited by L-NAME [32]. The perfusion of isolated rabbit lungs with a medium containing the oxidant-producing system, purine/xanthine oxidase or glucose/glucose oxidase, stimulates a vasopressor response.

This may occur via a direct effect of oxidative stress on smooth muscle cells or the vasopressor response may reflect either an increase of EDCF or a decreased release of EDRF from endothelium [33]. Gurtner et al. [34] demonstrated that the perfusion of rabbit lungs with the membrane-permeant oxidant *tert*-butyl hydroperoxide (*t*-BOOH) results in a vasoconstrictive response, the magnitude of which positively correlates with the ratio of thromboxane A_2 (a powerful vasoconstrictor) to prostaglandin I_2 (a potent vasodilator) in the effluent. In isolated rat heart, ischemia followed by reperfusion results in a diminished vasorelaxing response to acetylcholine but does not affect the action of the direct muscle relaxant, nitroglycerin, suggesting the implication of an endothelium-based mechanism [35]. A diminished acetylcholine relaxant effect has also been demonstrated on precontracted pulmonary arteries rings after exposure of rats for 7 days to oxygen rich atmosphere (85%). Because the muscle does not lose its sensitivity to relaxants agents, such as nitroprusside and papaverine, it was suggested that the oxidant stress decreased the release and/or bioavailability of EDRF from endothelial cells [36].

Using a reversed therapeutic strategy, Nakazono et al. were able to lower blood pressure in SHR, but not in WKY rats, by intravenous administration of a fusion protein HB-SOD, a protein that has a high affinity for heparin-link proteoglycans on vascular ECs (Figure 4) and an important capacity to scavenge the $^{\bullet}O_2^-$ [37]. Recently it has been demonstrated that in SHR stroke-prone (SHRSP) rat strain, $NO^{\bullet}$ production was higher than in WKY strain [38]. Despite this increased production, the $NO^{\bullet}$ bioavailability was reduced in the hypertensive strain, suggesting that $NO^{\bullet}$ may be scavenged by an overproduction of $^{\bullet}O_2^-$.

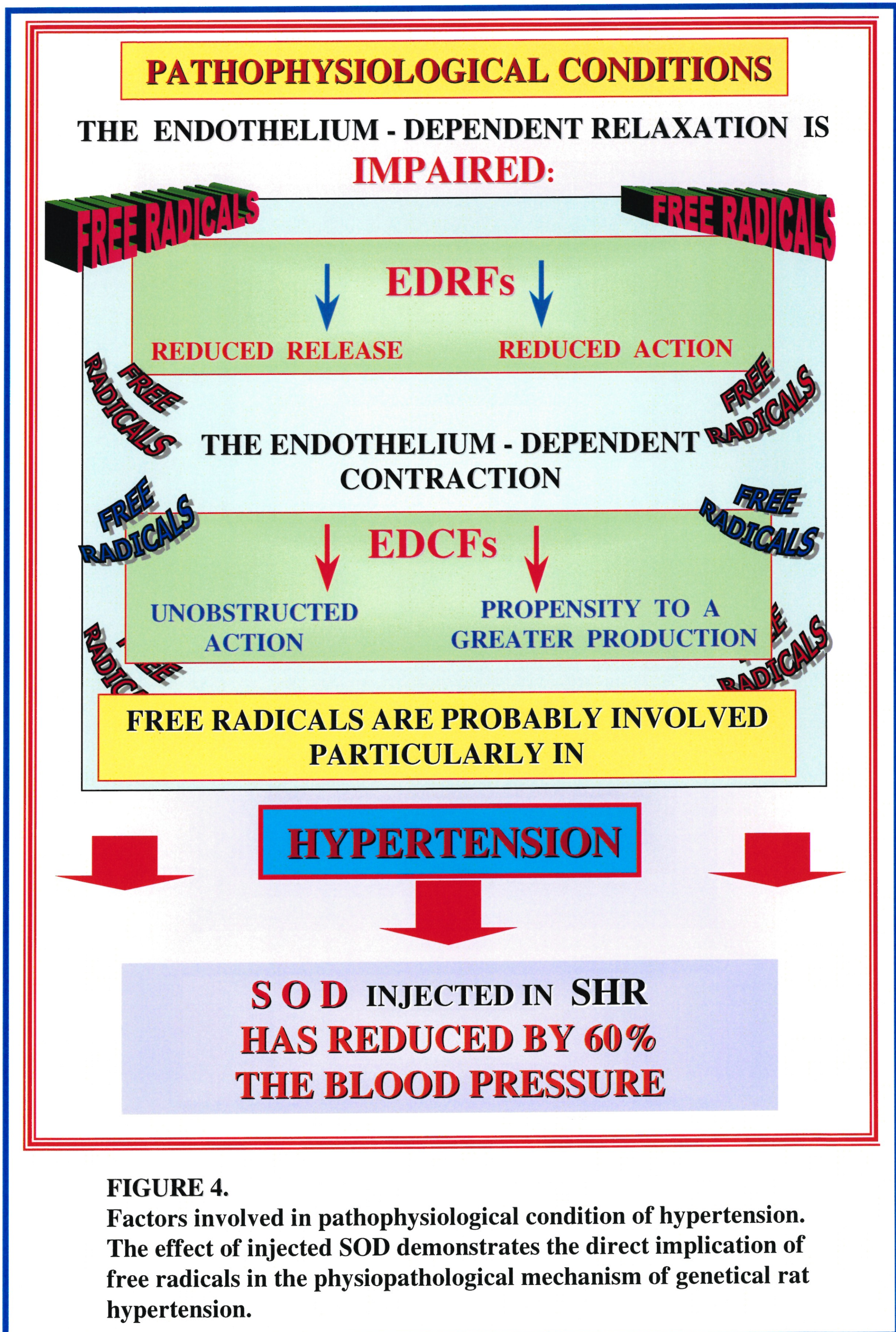


FIGURE 4.
Factors involved in pathophysiological condition of hypertension. The effect of injected SOD demonstrates the direct implication of free radicals in the physiopathological mechanism of genetical rat hypertension.

It was also demonstrated that the generation of $^{\bullet}O_2^-$ was greater in aortae from SHRSP. The source of $^{\bullet}O_2^-$ was the endothelium and it could be inhibited by N^{ω}-nitro-L-arginine methyl ester, suggesting that endothelial NOS-III is the enzyme responsible of $^{\bullet}O_2^-$ overproduction [39].

The involvement of free radicals in the pathogenesis of human essential hypertension has been demonstrated by the presence of elevated concentrations of $^{\bullet}O_2^-$ and H_2O_2 (Figure 1) [40] Similar effects were observed in artificially induced hypertension; administration of high salt diet to Dahl rats and the initiation of a hypertensive state has been consequently associated with the accumulation of high levels of H_2O_2 [41,42].

Antioxidant treatment with vitamin E, GSH and SOD has had a suppressive effect on high blood pressure of human hypertensive subjects and was without effect on control normotensive subjects, demonstrating that free radicals are involved in the hypertensive state [43,44]. Acute hypertension induced in normotensive rats via the administration of angiotensin-II, showed severe and extensive endothelial and smooth muscle injuries that were inhibited upon antioxidant treatment.

It is not possible yet to establish if oxidative stress is the cause or the effect of hypertension. There is confirmation however, that the relaxation capability to acetylcholine is reduced in proportion with the increase in blood pressure and, implicitly, with age in SHR. Moreover, increase in hypertension with aging decrease the inhibitory effects of the endothelium against norepinephrine-induced contractions whereas the intraluminal, but not extraluminal, activation of endothelium-derived relaxing factor release become impaired [45].

Abnormalities in Ca^{2+} handling by smooth muscle cell have been postulated to play an important role in the pathogenesis of hypertension. Elevated resting cytosolic free Ca^{2+}

concentrations in smooth muscle cells from SHR have been confirmed in the hypertensive and prehypertensive stage [46].

In ECs from SHR, bradykinin (BK) and thapsigargin (TG) have induced significantly smaller Ca^{2+} responses than in ECs from WKY, despite equivalent responses to angiotensin-II and endothelin-I, in both strains. These results presume that ECs from SHR might have an alteration in bradykinin receptor or modified bradykinin degradation. It is also possible that the intracellular calcium stores that are sensitive to thapsigargin and bradykinin may differ from those that are activated by angiotensin-II and endothelin-I [47]. Liu Y et al. [48], have demonstrated in aorta isolated from aldosterone-salt hypertensive rats (AHR), that the acetylcholine-induced endothelium-dependent relaxation and ${}^{b}[Ca^{2+}]_i$ in endothelial cells did not differ from matched control-salt rats (CSR). The maximum relaxation was significantly reduced for AHR. However, the $[Ca^{2+}]_i$ induced by acetylcholine was significantly smaller in AHR than in CSR.

I.2. FREE RADICALS, MECHANISM OF DEFENSE AND EFFECT OF OXIDATIVE STRESS IN BIOLOGICAL SYSTEM

Free radical reactions are ubiquitous in living organisms. Under physiological conditions, homeostasis is incessantly challenged by stressors arising from both internal and external media [49]. From the physiological reactions and metabolic processes in aerobic organism, a wide succession of byproducts are generated from oxygen metabolism called reactive oxygen intermediates (ROI) and free radicals (FR) [49,50]. The reductive environment of the cellular milieu provides abundant opportunities for oxygen to undergo consecutive univalent reductions. Thus, the superoxide anion radical, hydrogen peroxide, and the extremely reactive hydroxyl radical are common byproducts of life in an aerobic environment, and these molecular structures appear to be responsible for oxygen toxicity. In response to univalent oxygen reduction, the cellular defense reaction involves mobilization of various cellular constituents and the functional integration of specific defense systems, including membrane-associated and cytosolic soluble free radical-neutralizing scavenging enzymes [51]. The evolutionary survival process has provided aerobic organisms with an "antioxidant defense system", some precisely designed devices and mechanisms proficient to neutralize the oxidative effects of oxygen and its reactive metabolites [52,53].

I. 2.1. Chemistry of Free Radicals

The univalent reduction of oxygen generates reactive intermediate structures due to its propensity to take part in single electron transfer. Oxygen's electronic structure is more stable if electrons are paired, with antiparallel spin. In terms of electron orbital configuration, a free radical is an atom or a molecule with an unpaired electron on its outer orbital.

Defense Systems Against Oxidative Stress

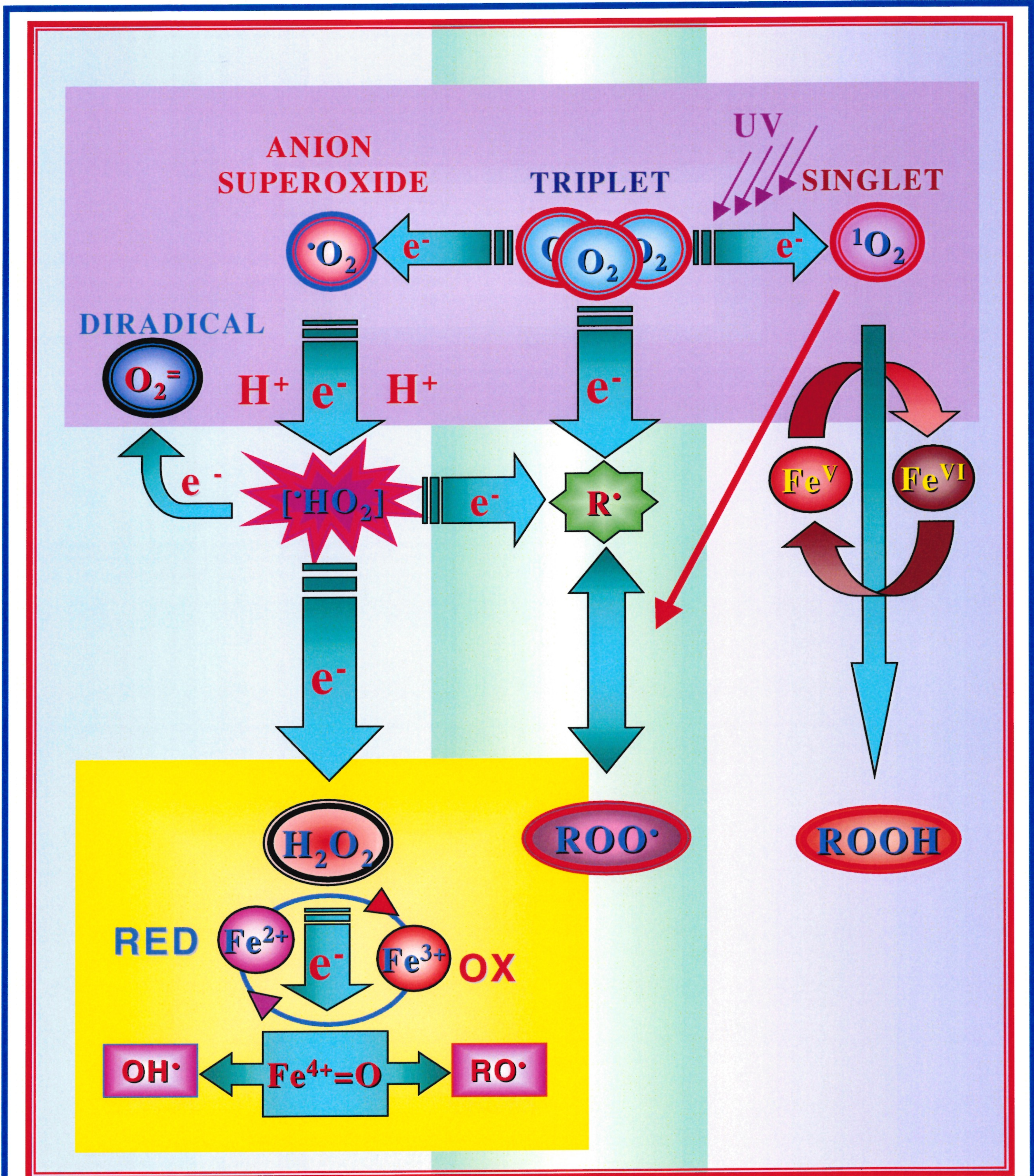


Figure 5.
The activation and the natural forms of activated O_2 present in biological systems.

In reality, the atmospheric O_2 is a "biradical", since it possesses two unpaired electrons, each situated on a different π orbital, both possessing the same quantum numbers and a parallel

spin configuration. This electronic configuration is giving special and unique properties to oxygen (Figure 5). This spin restriction seems advantageous to aerobic organisms because it slows oxygen reactions [54], but it creates the unwanted situation favorable to one electron transfer, allowing the formation of a molecule or an atom with an unpaired electron, a free radical [55]. As seen in Figure 6, the standard reduction potential of O_2 is variable, depending on the proton concentration of the medium in which the reaction is taking place.

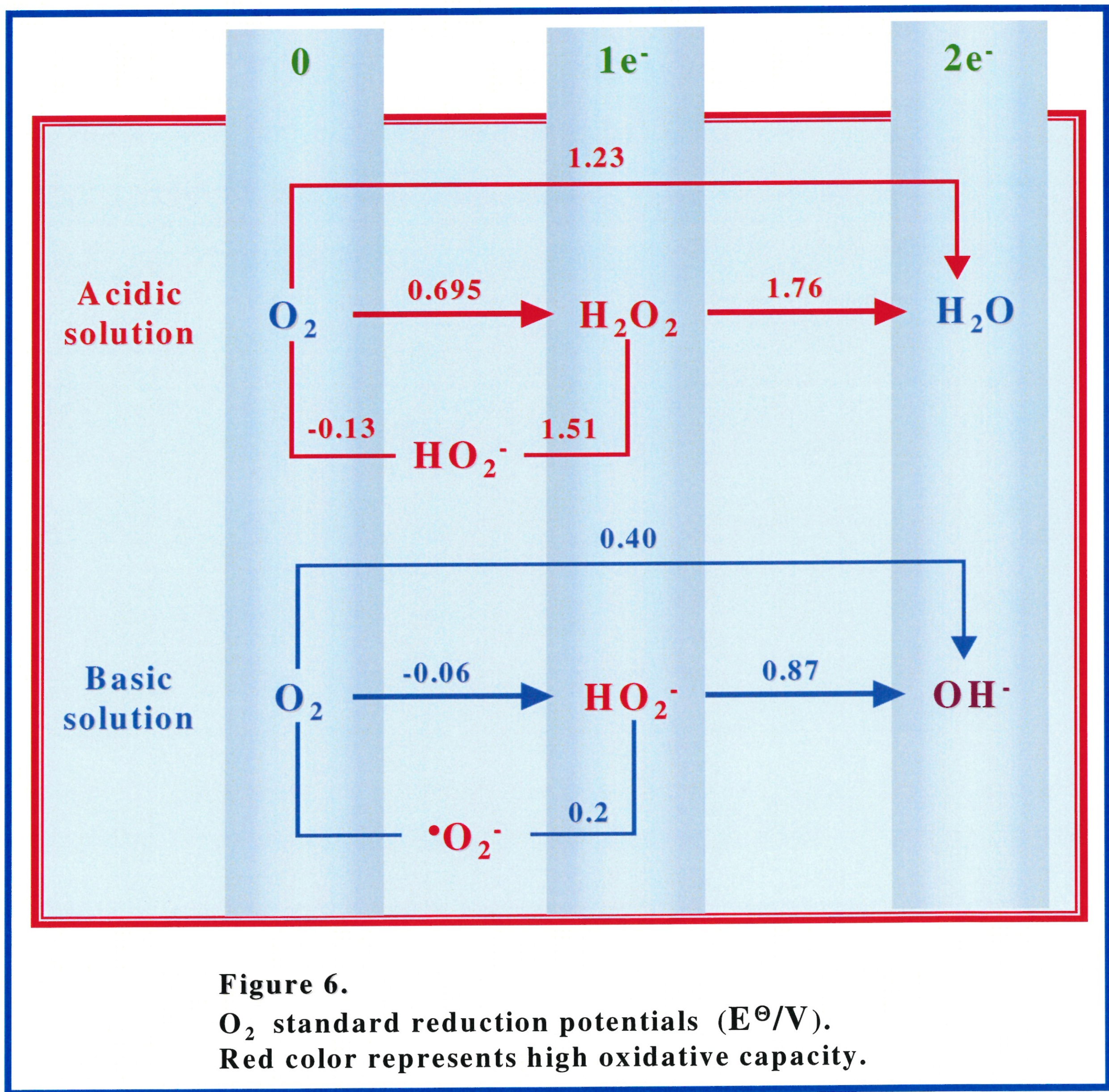


Figure 6.
O_2 standard reduction potentials ($E^{\ominus}/V$).
Red color represents high oxidative capacity.

In biological systems, these excited forms of oxygen can arise from the activity of a variety of peroxidases (prostaglandin hydroperoxidase) and the singlet oxygen can be the promoter of oxygen radical chain reactions (Figure 5) [56].

The major route for the metabolism of molecular oxygen involves its complete reduction to H_2O by accepting four electrons:

Biological systems contain a wide variety of organic free radical species, but the number of oxygen-derived free radicals is limited [57].

I.2.1.1. The superoxide anion (${}^\bullet O_2^-$)

It is the first intermediate in the sequential univalent reduction of O_2 (Figure 7). Superoxide anion is the dissociated form of a weak acid, the hydroperoxyl radical, $HO_2^\bullet$ (Table 1), which dissociates into water by protonation according to reaction (1), in a manner similar to acetic acid (reaction 2). The most prominent feature of ${}^\bullet O_2^-$ is its ability to act as a Brönsted base. In an acidic environment, ${}^\bullet O_2^-$ will spontaneously generate H_2O_2, whereas at physiological pH values, the spontaneous dismutation will occur, but mostly catalyzed by SOD. The $HO_2^\bullet$ is a much stronger oxidative species than ${}^\bullet O_2^-$ itself [53]. The distinction between $HO_2^\bullet$ and ${}^\bullet O_2^-$ is important for two reasons. Firstly, the negative charge on ${}^\bullet O_2^-$ will inhibit its diffusion across lipid membrane, since charged molecules generally have much lower solubility in lipids than uncharged molecules. Secondly, the equilibrium of reaction (1) controls the natural lifetime of superoxide radicals in the absence of any other reactant or catalyzing enzyme [53].

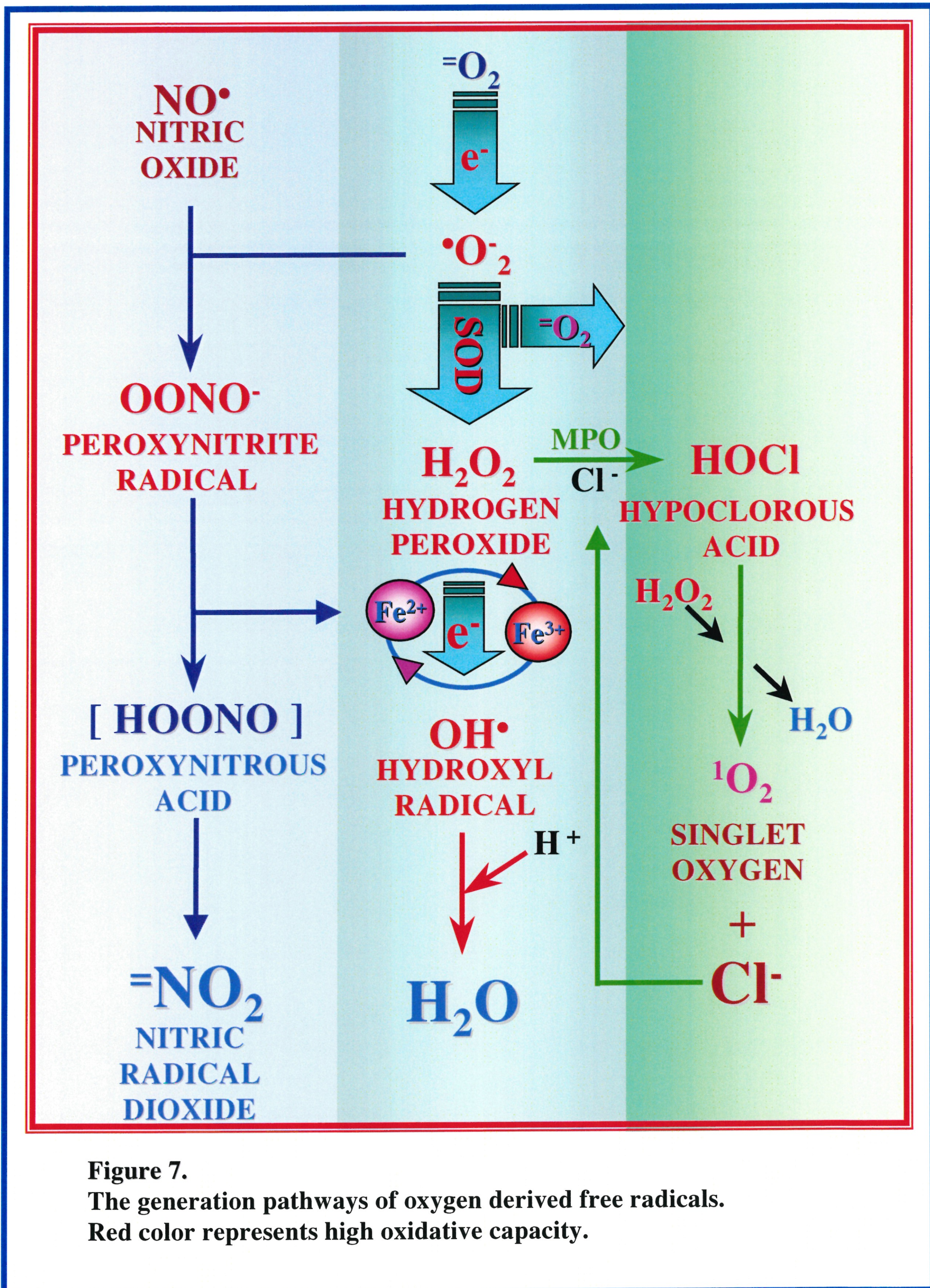


Figure 7.
The generation pathways of oxygen derived free radicals.
Red color represents high oxidative capacity.

I.2.1.2. The hydroxyl radical ($^{\bullet}OH$).

The hydroxyl radical is a three-electron reduction byproduct of oxygen (Figure 7). While H_2O_2 is an oxidizing agent, it is the combination with $^{\bullet}O_2^-$ which yield the most potent oxidant encountered in biological systems, the $^{\bullet}OH$; it has a short half-life ($1*10^{-9}$ s, at 37°C) and a diffusion capability restricted to only about two molecular diameters before reacting. Reaction (4) (see Table 1), known also as Haber-Weiss reaction [58], was suspected as a potentially important way in which the cell could generate highly reactive and damaging hydroxyl radicals, in the absence of ionizing radiation or free transitory metals.

TABLE 1

SPECIFIC REACTIONS IMPLICATED IN UNIVALENT REDUCTION OF O_2

Reaction	No.
$HO_2^{\bullet} = {}^{\bullet}O_2^- + H^+$	(1).
$CH_3CO_2H = CH3CO_2^- + H^+$	(2).
$2\,{}^{\bullet}O_2^- + 2\,H^+ = H_2O_2 + O_2$	(3).
$2\,HO_2^{\bullet} \rightarrow H_2O_2 + O_2$	(3a).
$HO_2^{\bullet} + {}^{\bullet}O_2^- + 2\,H^+ = H_2O_2 + O_2$	(3b).
$2\,{}^{\bullet}O_2^- + 2\,H^+ = H_2O_2 + O_2$	(3c).
${}^{\bullet}O_2^- + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$	(4)
${}^{\bullet}O_2^- + H_2O_2 = {}^1O_2\uparrow\uparrow + OH^-$	(5)
Haber-Weiss reaction:	
${}^{\bullet}O_2^- + H_2O_2 = {}^{\bullet}OH + H_2O + O_2$	(6)

The chemical rates of reaction (3) and (4) from Table 1 were measured and compared, to determine their possible incidence in biological systems. It was concluded that the rate of reaction (4) from Table 1 is too slow for biological system and it cannot be considered as an

important source for the production of $^{\bullet}OH$ [53]. However, the acceleration realized by the specific enzyme SOD, in reaction (3) from Table 1, represents the most pertinent source of $^{\bullet}O_2^-$ in living system. A second quantitatively important source of $^{\bullet}OH$ is the decomposition of hydrogen peroxide via the Fenton reaction (1) in Table 2

TABLE 2

$^{\bullet}O_2^-$ REACTIONS WITH TRANSITION METALS IONS

Reaction	No.
Fenton reaction:	
$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$	(1)
$^{\bullet}O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$	(2)
$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + {}^{\bullet}OH + OH^-$	(3)
$^{\bullet}O_2^- + Cu^{2+} \rightarrow Cu^+ + O_2$	(4)
$Cu^+ + {}^{\bullet}O_2^- \rightarrow Cu^{2+} + O_2^{2-}$	(5)
$^{\bullet}O_2^- + \text{Ferritin-}Fe^{3+} \rightarrow O_2 + \text{Ferritin} + Fe^{2+}$	(6)
Initiation of lipid peroxidation	
$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\bullet} + OH$	(7)
Propagation of lipid peroxidation	
$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet}$	(8)
$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$	(9)
$LOO^{\bullet} + LH \rightarrow LOOH + LO^{\bullet}$	(10)

More than 100 years ago, Henry Fenton had observed that the reducing agent, ferrous iron, (Fe^{2+}) in the presence of H_2O_2 was able to oxidize some organic compounds [59].

The hydroxyl radical is also generated in the presence of a reducible form of copper in reaction (3) Table 2. $^{\bullet}O_2^-$ acts firstly to reduce Cu^{2+} (cupric ion) in reaction (4) from Table 2, and then to oxidize Cu^+ (cuprous ion) in reaction (5). Copper is present in the redox center of SOD. The

highly instable byproduct, O_2^{2-} from reaction (5) in Table 2, represents the H_2O_2 since it interacts immediately with water/proton.

I.2.1.3. The hydrogen peroxide (H_2O_2).

Hydrogen peroxide, a two-electron oxygen reduction byproduct, represents a source of free radicals and at the same time, an important oxidizing agent. Its definition is complicated because, technically, H_2O_2 has all its electrons in pairs and is not a free radical; on the other hand, it is the second byproduct of one of the consecutive steps in the reduction of O_2 (reaction (3) in Table 1), which means it is a ROI [60].

Concerning in reaction (3) from Table 1, the spontaneous dismutation of $^{\bullet}O_2^-$ occurs in biotic medium at a rate of $2*10^5$ $M^{-1}*s^{-1}$, while the catalytic rate induced by SOD is $2*10^9$ $M^{-1}*s^{-1}$ (a factor 10^4) and represents the major source of H_2O_2 in biological systems [61]. In aqueous solutions, H_2O_2 is not reactive enough to oxidize many organic molecules, but in the presence of free transition metals (Fe^{2+}, Cu^+), it generates the highly oxidant hydroxyl radical (Figure 8) [62].

The most important characteristic of H_2O_2 is its capacity to diffuse through the nearest hydrophobic lipid membrane with relative ease because of its nonionized state. Therefore, it modifies molecules close to its site of generation, mainly by hydrogen abstraction from unsaturated bonds of polyunsaturated fatty acids. Finally, the addition of the fourth electron to the $^{\bullet}OH$ in the forth step of the univalent reduction of O_2 ultimately results in the formation of H_2O.

I.2.1.4. Free radicals derived from $NO^{\bullet}$.

After the demonstration in 1980 that EDRF was the simple diatomic free radical molecule $NO^{\bullet}$, an additional step was the demonstration that superoxide anion inactivates EDRF through reaction (1) in Table 3, whereas SOD enhances its biological activity. This chain reaction occurs in endothelial cells where $NO^{\bullet}$ will promote the initiation of two reaction pathways. Firstly, the $NO^{\bullet}$ production of ECs will be buffered before being able to diffuse in adjacent smooth muscle cells. Secondly, the intermediary products generated during the radical-radical reactions, notably peroxynitrous acid (HOONO) and peroxynitrate ($ONOO^-$), will initiate the process of membrane lipid peroxidation and, depending on the duration and intensity, the setting of oxidative stress in ECs (Figure 7).

TABLE 3

REACTION OF $^{\bullet}O_2^-$ WITH $NO^{\bullet}$

Reaction	No.
$^{\bullet}O_2^- + NO^{\bullet} = ONOO^-$ PEROXYNITRATE	(1)
$HOONO = H^+ + ONOO^-$	(2)
$HOONO \rightarrow H^+ + NO_3^-$	(3)
$HOONO = HO^- + ONO^{\bullet}$ $(=NO_2^-)$ PEROXYNITROUS ACID	(4)
$2NO^{\bullet} + {}^{\bullet}O_2^- \rightarrow 2NO_2^{\bullet}$	(5)

In aqueous medium, the peroxynitrate is rapidly transformed into nitrate (NO_3^-) (reaction (3), Table 3), in competition with a reaction that is believed to involve the decomposition of peroxynitrous acid to the hydroxyl radical and nitrogen dioxide (reaction (4), Table 3). It is still debatable, however, if this reaction occurs in biological systems and if the free $^{\bullet}OH$ is released. What is known with certainty is that HOONO is a powerful oxidant with cytotoxic properties, implicated in the initiation of lipid peroxidation and thiole oxidation. Immune-stimulated macrophages produce abundant amounts of $NO^{\bullet}$, and inhibition of its production reduces the microbiocidal and tumouricidal activities of macrophages. Reaction (5) (Table 3) occurs in atmosphere, related with urban smog, chemical waste stacks, and cigarette smoke, proceeding through intermediate steps involving molecules such as N_2O_4 and N_2O_3 and all being recognized as potent carcinogens.

The intermediate reduction byproducts of $NO^{\bullet}$ are implicated in numerous collateral reactions with most of the fundamental components of living organism. For example, nitros(yl)ation is a widespread protein modification reaction that occurs during many physiological and pathological processes. S-nitrosothiols may cause many of the biological effects of $NO^{\bullet}$, and cellular effects have been attributed to S-nitrosylation of reactive protein sulfhydryls. It can alter both the structure and function of protein. Nitric oxide has been implicated in this process, but its mechanism remains vague and mostly unknown.

I.2.1.5. Free radicals derived from $^{\bullet}O_2^-$ and Cl^-.

The immune-activated macrophages and neutrophils utilize reaction (1) (Table 4) to combine H_2O_2 with Cl^- through a specific enzyme, myeloperoxidase, to produce the protonated

hypochlorous acid (HOOCl) (bleach), a strong weapon against microorganisms, parasites, and all xenobiotic-recognized invaders.

Since the pK of reaction (3) is 7.45, the HOOCl exists in roughly equal amounts in the undissociated (HOCl) and dissociated (OCl^-) forms at pH between 7-8 (Figure 8), which is at physiological pH. Highly reactive hydroxyl radicals can be formed from $HOCl/OCl^-$ into reactions with reductants that are one-electron donors, as in reaction (4) and (5) (Table 4). HOOCl is chemically reactive in its own and if we compare the reaction (5) from Table 4 with Fenton reaction (1) from Table 2, we see that these reactions are analogous with peroxide in reaction (1) being replaced by HOCl. In *vitro*, reaction $HOCl + Fe^{2+} \rightarrow {}^{\bullet}OH + Cl^- + Fe^{2+}$ has been shown to be about a thousand times faster than Fenton reaction (1) in Table 2, suggesting that spontaneous and/or myeloperoxidase-induced $^{\bullet}OH$ can be produced in sufficient amount to cause considerable harm to a biological system.

TABLE 4

REACTIONS OF $^{\bullet}O_2^-$ WITH Cl^-

Reaction	No.
$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$	(1)
$Cl_2 + 2OH^- = OCl^- + Cl^- + H_2O$	(2)
$HOCl = H^+ + OCl^-$	(3)
$HOCl + {}^{\bullet}O_2^- \rightarrow {}^{\bullet}OH + Cl^- + O_2$	(4)
$HOCl + Fe^{2+} \rightarrow {}^{\bullet}OH + Cl^- + Fe^{2+}$	(5)

I.2.1.6. Free radicals derived from $^{\bullet}O_2^-$ reactions with transition metal ions.

Under normal physiological conditions, $^{\bullet}O_2^-$ does not oxidize all organic substances, and H_2O_2 interacts with organic substances only sluggishly. Free radicals attack and inflict putative cellular damage on the biochemical constituents and cellular structures, partially through reactions supported by transition metals, such as iron and copper. The antioxidant property of blood plasma is due to metal-binding serum proteins that chelate the metals necessary for peroxidation. As seen in Table 2, reactions (2) and (4), $^{\bullet}O_2^-$ interacts with oxidized forms of free iron (Fe^{3+}) and copper (Cu^{2+}), inducing the formation of a redox cycle. Both, Fenton (1) (Table 2) and Haber-Weiss reactions (6) (Table 1), require the participation of a reducible state of metal catalysts. Oxygen complexes with iron in different oxidative states (perferryl ion complex = Fe^{2+}-O_2, oxiferryl ion complex = Fe^{3+}-O_2 and probably Fe^{IV}, Fe^{V} and even Fe^{VI}), which may serve as the primary active-initiation reactants of NADPH-dependent lipid peroxidation (Figure 6, 8) [63]. Healthy organism is transporting iron in a chelated ferric state, bound to transferrin in a complex that is especially difficult to reduce. Superoxide anion is capable of reducing the ferritin-bound iron to the ferrous state, whereupon it is released freely (reaction (6) in Table 2). The subsequent propagation process also requires oxidant metal ion complexes for the enhancement of the decomposition of lipid hydroperoxides, which are converted to other organic free radicals such as alkyl or alkoxyl-peroxyl radicals (LOOH $\rightarrow Fe^{3+} \rightarrow LOO^{\bullet} + Fe^{2+}$ or LOOH $\rightarrow Fe^{2+} \rightarrow LO^{\bullet} + Fe^{3+}$) and which are finally responsible for membrane lipid peroxidation. Liberated iron causes the reductive lysis of the oxygen-oxygen bound in a preexisting hydroperoxide molecule, therefore serving as an initiator radical for the propagation of lipid peroxidation (Table 2, reactions (8)-(10)). Metal catalysis was shown to be essential likewise with peroxidation induced by hydroxyl radicals [64].

Defense Systems Against Oxidative Stress

I.2.2. Biological Sources of Free Radicals

The reductive conversion of O_2 to H_2O by oxidative process requires a sequential transfer of electrons, which is accompanied by free radical (FR) formation. Some of the most important FR properties, sites of production, and the most effective FR scavengers are presented in Table 5. High rates of $^{\bullet}O_2^-$ production occur in **mitochondria**, when the inner membrane-associated respiratory chain carriers are in the reduced state (when ADP is low) and when the concentration of oxygen is increased. The in progress understanding suggests that the first site of mitochondrial $^{\bullet}O_2^-$ production is the ubiquinone to cytochrome c_1 step, which passes through the intermediate ubisemiquinone.

The second site for $^{\bullet}O_2^-$ formation is the **NADH-dehydrogenase** and the oxidation of ubisemiquinone in the autoxidizable electron carrier pathway [65]. The intramitochondrial steady-state concentration of superoxide has been estimated at $8*10^{-12}$ M [66]. For example, heart mitochondrion becomes uncoupled upon exposure to ischemia and the electron transport chain is specifically inhibited at a step between NADH dehydrogenase and ubiquinone [67]. This inhibition is associated with an increased rate of H_2O_2 formation by NADH dehydrogenase and can be caused by a mitochondrial Ca^{2+} overload, which activates phospholipases or proteases, causing uncoupling and inhibition of respiration. Mitochondria accumulate Ca^{2+} electrogenically, using the H^+ gradient for this purpose instead of producing ATP, even in the presence of a high level of ADP [65]. The microsomal and nuclear membranes also contain the electron transport system, cytochrome P-450 and cytochrome b_5, and their activation and $^{\bullet}O_2^-$ production is function of the changes in isoenzymes status [64].

Isoenzymes of cytochrome P-450 are balanced by their ratio of low- and high-spin energy state. A shift to the high-spin state would result in increased $^{\bullet}O_2^-$ and H_2O_2 production, due to increased cytochrome *P*-450 reduction [68]. Thus, free radical production is influenced by the extent of uncoupling of the respiratory chain, which is modulated by cytochrome *P*-450 spin state on the electron transport pathway. During NADPH oxidation, liver microsomes generate $^{\bullet}O_2^-$ and H_2O_2, both in the absence and in the presence of mixed oxidase substrates. The nonmitochondrial respiratory burst, (because it is cyanide-insensitive) during phagocytic activity generates reactive oxygen radicals essential for the defense against invading xenobiotics [68].

Endothelial cells are in direct contact with the white blood cell lines. During phagocytosis, the leukocyte and **neutrophil membrane-bound oxidase** is activated, leading to an increase in O_2 uptake and ensuing production of $^{\bullet}O_2^-$, H_2O_2, and HOCl. The genetic inability to produce superoxide dismutase causes the life-threatening condition known as chronic granulomatous disease. Infected tissues display the cardinal signs of inflammation (redness, heat, swelling, pain, and loss of function) not necessarily because of the invading microbe, but largely due to the attack of neutrophils and the unavoidable damages induced by $^{\bullet}O_2^-$ overproduction in the host tissue. In autoimmune diseases and allergies, after the formation of the immune-complex, the “marked” components become the target of the neutrophils, generalized, or localized, resulting in the attraction of neutrophils and the installation of localized or generalized symptoms and lesions [69].

Xanthine oxidase is another source of $^{\bullet}O_2^-$ generation, as indicated by its ability to reduce cytochrome *c*. It uses NAD^+ as an electron acceptor during the oxidation of xanthine and hypoxanthine.

Defense Systems Against Oxidative Stress

Table 5
SOME CHARACTERISTICS OF REACTIVE OXYGEN SPECIES

SPECIES	SYMBOL	ENDOGENOUS PRODUCTION	EXOGENOUS PRODUCTION	ANTIOXIDANT	PROPERTIES
Superoxide anion	$^{\bullet}O_2^-$	Leakage from mitochondria; Xanthine oxidase; NO-synthase; Cyclooxygenase; Lipoxygenase; NADH/NADPH oxidase;	Auto-oxidation of: catecholamines, ascorbate, GSH, hexoses; Redox cycling (ascorbate) Free transition metals;	SOD; Vitamin E; Vitamin C; Uric acid;	Good reductant; Poor oxidant; Initiate lipid peroxidation; Poor diffusion capability;
Hydroxyl radical	$^{\bullet}OH$	Leakage from mitochondria; Cyclooxygenase; Lipoxygenase;	Fenton (like) reaction Auto-oxidation of: catecholamines, ascorbate,	Vitamin E; Vitamin C; Glucose;	Low diffusion distance; Highly reactive; (addition, abstraction, electron transfer)
Perhydroxyl radical	$HO_2^{\bullet}$	Leakage from mitochondria; Xanthine oxidase; Cyclooxygenase; Lipoxygenase; NADH/NADPH oxidase; NO synthase; Cytosol reactions;	Auto-oxidation of: catecholamines, ascorbate, GSH, hexoses; Redox cycling (ascorbate) Free transition metals;	Catalase; GSH Redox cycle; GSH;	Strong oxidant; Lipid soluble; Poor diffusion capability; Initiate lipid peroxidation;
Peroxyl radical	$ROO^{\bullet}$	Lipoxygenase; Lipid peroxidation reaction Cytosol reactions;	Auto-oxidation of: catecholamines, ascorbate, GSH, hexoses; Redox cycling (ascorbate) Free transition metals; Oxidative burst;	Catalase; GSH Redox cycle; GSH;	Highly lipid soluble; Relative low oxidant; Disseminate lipid peroxidation; Lipid soluble; High diffusion capability; Sluggish reactions with organic substrate
Alkoxyl radical	$RO^{\bullet}$	Lipoxygenase; Lipid peroxidation reaction Cytosol reactions;	Auto-oxidation of: catecholamines, ascorbate, GSH, hexoses; Redox cycling (ascorbate) Free transition metals;	Vitamin E; Vitamin C; Glucose;	Strong oxidant; Initiate lipid peroxidation; Lipid soluble;
Hydrogen peroxide	H_2O_2	Leakage from mitochondria; Xanthine oxidase; Monoamine oxidase; Cytosol reactions;	Oxidative burst; Auto-oxidation of:;, GSH, hexoses; Redox cycling (ascorbate) Free transition metals;	Catalase; GSH Redox cycle; GSH;	Oxidant; High diffusion capability; Sluggish reactions with organic substrate;
Singlet oxygen	$^1O_2$	Leakage from mitochondria	Oxidative burst;	Vitamin E; Vitamin C; Glucose;	Highly oxidant; High diffusion capability; Initiate lipid peroxidation;
Nitric oxide	$NO^{\bullet}$	NO synthase			Good diffusion capability; Highly oxidant;
Peroxynitros acid	HOONO	NO synthase; Cytosol reactions;	Cytosol reactions;	GSH Redox cycle; Vitamin E; Vitamin C;	Highly oxidant; High diffusion capability; Initiate lipid peroxidation;

In tissues containing abundant xanthine dehydrogenase (heart, intestine), ischemia is inducing the conversion of dehydrogenase into oxidase by proteolysis and oxidation. After the brake-down of the adenine nucleotide pool, the xanthine oxidase becomes an important source of $^{\bullet}O_2^-$ upon tissue reoxygenation after ischemia or after hypotension [70].

I.2.3. Defense Systems Against Oxidative Stress

To provide maximum protection against deleterious effects of free radicals, ECs contain, from internal synthesis or as external intake, a variety of substances capable to scavenge different species of free radicals, including lipid peroxides and organic-centered free radicals (Figure 8). All the substances that neutralize the potential destructive effect of free radicals are grouped in the **antioxidant defense system**. They are generically called **antioxidants, free radical scavengers, chain reaction terminators, or reductants**, and they are part of wide panoply of substances having many different properties. Living organisms have found a fine tuned strategy for the compartmentalization of all internally generated antioxidants. Davies classified scavenging enzymes and antioxidants as **"primary"** and **"secondary"** [71]. **Primary defense system** includes: 1) antioxidant scavenging enzymes such as SOD, catalase and peroxidase; 2) antioxidant compounds such as vitamin E, A, and C, glutathione, and uric acid. The **secondary defense systems** include lipolytic enzymes, phospholipases, proteolytic enzymes, proteases, peptidases, DNA repair enzymes, endonucleases, and ligases.

I.2.3.1. Antioxidant Scavenging Enzymes

I.2.3.1.1. Superoxide Dismutase (SOD)

It is a metal protein discovered by McCord and Fridovich in 1969 [72], and represents the starting point of the primary defense system because this enzyme catalyzes specificlly the dismutation of $^{\bullet}O_2^-$ into H_2O_2 and it prevents further accumulation of $^{\bullet}O_2^-$ (Figure 8):

$$\mathbf{SOD}\ ({}^{\bullet}O_2^- + {}^{\bullet}O_2^-) \rightarrow 2H^+ \rightarrow H_2O_2 + O_2.$$

SOD exists in virtually all O_2-respiring organisms. Although its activity was identified in the extracellular space, the bulk of its activity takes place intracellularly, at the $^{\bullet}O_2^-$ generation site itself, divided between mitochondrial and cytosolic compartments. SODs are classified according to the metal found in their enzyme active center, Cu/Zn-SOD, Mn-SOD, or Fe-SOD.

H_2O + O_2
GSSG
GPx
CATALASE
MELATONIN
GSH
$\cdot O^-_2$
SOD
H_2O_2
OH·
LIPID PEROXIDATION
Fe^{2+}
Fe^{3+}
LACTOFERRITIN
DEFFRIOXAMINE
CERULOPLASMINE
RADICAL SCAVENGERS
VITAMIN E
VITAMIN C
β CAROTEN

Figure 8.
Defense mechanisms against damages inflicted by oxygen reactive species.

The most common is Cu/Zn-SOD, which is present in the cytosol and is functionally active as a dimer of two subunits of equal size with an overall MW ≈32.000 Da. The Mn-SOD is present in mitochondria and it is a tetramer with a MW of ≈80.000 Da.

The tissue distribution is widespread and the induction of protein expression is function of the abundance of the specific substrate. The rate of enzymatic dismutation is $\approx 10^4$ times greater than that of chemical dismutation.

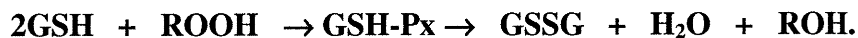
I.2.3.1.2. Catalase

Catalase is the major primary antioxidant enzyme responsible for catalyzing the decomposition of H_2O_2 to H_2O (Figure 8). It is not a specific enzyme since catalase is implicated in the catalytic transformation of organic peroxides. This cytoplasmic heme enzyme is a tetrameric protein of MW ≈240.000 Da and shares with glutathione peroxidase GSH-Px an important detoxification capacity against peroxides. Catalase has a wide range of activity against all peroxides. In the presence of low doses of H_2O_2, organic peroxides are catalyzed by peroxidases, whereas at high concentrations of H_2O_2, the organic peroxides are catalyzed by catalase [73]. The peroxides are already present in the biotic environment and their presence is important for many physiological processes, but the concentration at which catalase starts its activation and how its activity is regulated is still unknown.

I.2.3.1.3. Glutathione Peroxidase

Glutathione peroxidase also catalyzes the reduction of H_2O_2 (Figure 8) and organic peroxides as follows:

$$\mathbf{2GSH + H_2O_2 \rightarrow GSH\text{-}Px \rightarrow GSSG + H_2O} \quad \textbf{or}$$



$$\mathbf{2GSH + ROOH \rightarrow GSH\text{-}Px \rightarrow GSSG + H_2O + ROH.}$$

It is located in the interior of the cytosol and in the matrix of mitochondria and is divided in two categories: the selenium-dependent GSH-Px, which exhibits a low capacity for reduction of H_2O_2 and the selenium-independent GSH-Px, which utilizes organic hydroperoxides as a preferred substrate over H_2O_2. Both types are involved in the inhibition of lipid peroxidation [74].

I.2.3.2 Nonspecific Antioxidants

I.2.3.2.1. Vitamin E (α-tocopherol)

As a generic term, *Vitamin E* represents a group of eight structural isoforms of tocopherol. The α-tocopherol is the best known and possesses the most powerful antioxidative activity and it is characterized by an important lipophilic property [75]. Firstly, it is synthesized as a highly liposoluble structure and integrated in the membrane structure of all cells, in both the vegetal and animal kingdom. Secondly, in lipophilic environment, it is the major free radical chain terminator. α-Tocopherol is uniformly distributed through the entire organism, with an accumulation propensity for the organs with high metabolic rate. Intracellularly, vitamin E is associated with all lipid-rich membranes such as mitochondria and endoplasmic reticulum. It reacts with free radicals yielding a stable form of tocopherol dimers or quinones (lipid hydroperoxides) that can be easily removed by the activity of phospholipase-GSH-Px system. This reaction is believed to interrupt the radical chain reaction process that propagates peroxidation of membranes (Figure 8). Thus, the antioxidant action of tocopherol is highly effective in protecting against membrane lipid peroxidation by reacting with lipid peroxyl and alkoxyl radicals. Vitamin E functions synergistically with ascorbic acid (Vitamin C) to terminate free radical reactions. In *vitro* and in *vivo* experiments demonstrate the important role of α-tocopherol in antioxidant therapy and its effect in the protection against ischemia-

reperfusion injury [76]. When oxidative stress is sufficiently increased to disrupt the antioxidant resources and balance of intracellular antioxidants, it is imperiously necessary to ensure an external input of vitamin E, even though the level of physiological protective concentrations is not yet established.

I.2.3.2.2. Vitamin C (ascorbic acid)

Vitamin C has a hydrophilic character and functions better in aqueous environment. If compared with other water-soluble antioxidants, vitamin C offers the most effective protection against plasma lipid peroxidation [77]. It is found in blood serum and in the cytosol in micromolar concentrations [78]. Because the pK_a of ascorbic acid is 4.25, ascorbate anion (AH^-) is the predominant form existing at physiological pH. Ascorbate (AH^-) is produced from oxidative forms of dehydroascorbic acid (DHA) through a reversible oxidation, with an intermediate formation of ascorbyl radical (A^-). Vitamin C serves as both an antioxidant and a prooxidant. As antioxidant and reducing agent, it directly reacts with $^{\bullet}O_2^-$ and $^{\bullet}OH$, and with different lipid hydroperoxides producing dehydroascorbate; it exerts a sparing effect on the antioxidant actions of vitamin E and selenium (Figure 8). Ascorbic acid is capable of restoring the antioxidant properties of oxidized vitamin E, suggesting that the role of vitamin C would be to recycle the pools of vitamin E by reducing the vitamin E radical [79]. As an oxidant, in serum concentrations over 1 mM and in the presence of free metal ions, vitamin C is able to inflict lipid peroxidation because of its ability to reduce Fe^{3+} to Fe^{2+}, which represents the catalytic factor for many oxygen free radicals [80,81]. The lipid peroxidation in microsomes is enhanced by ascorbate in vitamin E-deficient animals but it is suppressed by ascorbate under normal conditions. In contrast, Aruoma et al, demonstrated enhanced oxidative modifications inflicted to DNA bases in the presence of ascorbate [82]. Because the ascorbic acid complex

chemistry is associated to all the pathophysiological implications and interrelationships generated by the production of free radicals, it is very difficult to find the boundary between its antioxidant and prooxidant properties.

I.2.3.2.3. The carotenoids

Carotenoids protect lipids against peroxidation by quenching free radicals and other reactive species, notably singlet oxygen (Figure 9). These reactions operate maximally at low-oxygen tension (<150 Torr) and may provide some synergism to vitamin E, which reacts most efficiently at higher oxygen concentrations. At higher O_2 partial pressure, its free radical-trapping capacity shows autocatalytic effects with concomitant loss of antioxidant activity [83]. The structural arrangement of β-carotenes with their long chain of conjugated double bonds make excellent scavengers of free radicals. β-Carotene, like vitamin C, appears to function as both, an antioxidant and a prooxidant [84].

I.2.3.2.4. Glutathione (GSH)

Reduced glutathione (GSH) is a small tripeptide, γ-glutamyl-cysteine-glycine, which is present in high concentrations in all eukaryotic cells. The intracellular concentration in normal conditions is generally ≈ 0.5 mM, but in pathological state (oxidative stress), it can rise to 10 mM. Biosynthesis of GSH from its constituent amino acids is catalyzed by glutamylcysteine synthase and GSH synthase. Its reactive thiol group and its γ-glutamyl bond, is giving it resistance to peptidase attack. It reduces $^{\bullet}O_2^-$, H_2O_2, and $RO^{\bullet}$ by a radical transfer process yielding the thiol radical ($GS^{\bullet}$) and GSSG (oxidized glutathione) (Figure 9). It interacts with $^{\bullet}OH$ and carbon radicals by donating a hydrogen atom. As an effective reductant, GSH plays an important role in a variety of detoxification processes, including the anihilation of peroxide damages, as evidenced by GSH depletion [85]. The decrease of GSH level with age may be

related either to the age-related increase in the oxidation rate or to a decrease in overall GSH turnover resulting from an increased degradation and/or reduced biosynthesis of total GSH. Accumulation of toxic substances during senescence elevate the activities of enzymes such as GSH peroxidase and transferase, resulting in the intracellular GSH depletion [86,87].

I.2.3.2.5. Uric acid

Uric acid is the end product of purine metabolism in humans and higher apes. Ames suggested a possible physiological antioxidant potential role of uric acid [88]. Davis et al. (84) suggests that urate represents a major extracellular and intracellular substrate for the defense mechanism against free radicals through its metal chelation activity. The mechanism is not yet understood, but it is proposed that uric acid may act by preserving the plasma ascorbate, probably by complexing transition metals such as iron and copper [89], or its action might be related to the activation of prostaglandin synthesis initiated by arachidonate [90].

I.2.3.2.6. Melatonin

In the past 15 years, melatonin was in the highlight of research and it revealed many of its properties. Besides its effects in the regulation of circadian rhythm and reproduction, regulation of behavior and hormone secretion, which are mediated through high affinity specific receptors on target cells, melatonin performs functions within cells, which do not require an interaction with a receptor located on cell membranes. Melatonin was shown to be a highly efficient scavenger of both hydroxyl and peroxyl radicals. When compared with GSH, melatonin proved to be five times more effective in neutralizing $^{\bullet}OH$, and compared to vitamin E, melatonin was twice as effective in inactivating $ROO^{\bullet}$ (Figure 8) [91]. Melatonin has some indirect effects on the regulation of oxido-reductive status of the organism by stimulating glutathione peroxidase activity [92] and inhibiting $NO^{\bullet}$-synthase capacity to produce $^{\bullet}NO$. In

endothelial cells, melatonin suppresses the H_2O_2-induced inhibition on $^{\bullet}NO$ production, mostly through its ability to scavenge the $^{\bullet}OH$ [93] and by an important effect against membrane lipid peroxidation [94]. Melatonin is able to prevent both cell proliferation and apoptosis by an important antioxidant activity, regulating the cell cycle through an up regulation of mRNA for oxidant enzymes [95]. The administration of melatonin to hypertensive patients [96] and hypertensive rats [97] induced an important lowering of blood pressure despite evidence for an important vasoconstrictor effect on cerebral arteries [98]. Melatonin was also shown to protect against rat ischemia-reperfusion injury [99]. The effects of melatonin are very complex and are tissue-specific but its antioxidant properties are such that melatonin constitutes the most powerful natural antioxidant.

I.2.3.3. Secondary Antioxidant Defense System

Cells are equipped with various enzymatic systems responsible for the reconstruction of the damaged or altered membrane constituents. The secondary antioxidant defense system can also play a role by either eliminating damaged molecules or restoring them through a repair process. Because the first line of defense does not completely protect against free radical attack, some damage occurs that must be dealt with by the second line of defense. The phospholipase-mediated removal of damaged fatty acids moieties from membrane lipids could be an effective way to maintain membrane integrity and at the same time provide a means to regulate membrane lipid turnover [100]. Peroxidation of membrane lipids can stimulate the lipolytic action of phospholipase A_2 and the peroxidized form of phospholipid has been shown to be the preferred substrate for phospholipase A_2 [73].

Substantial amount of altered proteins are produced by metal-catalyzed free radical reactions [101]. Observations suggest that the **proteasome**, a multicatalytic proteinase complex,

may be part of the major defense system that degrades and eliminates oxidative-damaged proteins. The exact role of proteasomes and their mode of action are not understood, although it is believed that they are related with another proteolytic complex termed macroxyproteinase, which appears to be mainly responsible for degrading oxidative-modified proteins [102]. Biological systems possess a variety of other mechanisms that minimize the levels of free metal ions such as Fe^{2+} and Cu^{2+}, therefore slowing the production of free radicals. Free ion concentrations are kept low by binding to such storage proteins as ferritin, transferrin, and lactoferrin. **Deferrioxamine** is a high affinity iron chelator that inhibits lipid peroxidation and reduces the injury associated with ischemia and reperfusion (Figure 8) [103]. Copper concentrations may be modulated by ceruloplasmin, a copper binding protein that chelates specifically copper and has antioxidant properties [104]. In addition to the conventional antioxidants, many drugs and chemicals have antioxidant properties and are capable of lowering free radical production. The **β-blockers** have been shown to inhibit peroxidation in a concentration dependent manner, with the following order of potency: propranolol > pindolol > metoprolol > atenolol > sotalol [105]. **Calcium antagonists** have been shown to possess significant antioxidant activity. **Nifedipine**, the most effective blocker of calcium channels, was found to be the most active antioxidant and to inhibit the production of superoxide anion. Cardiomyopathic protection induced by **verapamil** was accompanied by a significant reduction of oxygen free radicals and lipid peroxides [106]. **ACE inhibitors** seem to have good efficiency against free radical generation beside their specific pharmacological effects. **Captopril** scavenges the $^{\bullet}O_2^-$ produced either by xanthine/xanthine oxidase system or by activated neutrophils [107]. **Probucol** has been shown to inhibit lipid peroxidation by a chain-braking mechanism and to protect against adriamycin-induced cardiomyopathy [108].

I.2.4. The Effect Of Oxidative Stress In Biological System

Advances in free radical chemistry indicate that no biological substance is impervious to free radical attack. It is very evident that a series of important structural modifications occur during oxidative stress, involving the oxidation of proteins, lipids and carbohydrates, as well as genomic and DNA structural damages.

I.2.4.1. Damages Induced by Oxidative Stress to Proteins

Protein molecules are undergoing substantial modifications through oxidative reactions. Studies on oxidative-damaged amino acids indicate that the building blocks of peptides and proteins are targets of free radical attacks resulting in oxidative-induced changes in the conformational structure of proteins. The oxidative-induced changes consist of oxidation of sulfhydryl groups, oxidative adduction of amino acid residues close to metal-catalyzed oxidation, reactions with aldehydes, protein-protein cross-linking, and peptide fragmentation. Hence, these changes are classified in three groups: aggregation, fragmentation, and susceptibility to proteolytic digestion. Protein oxidation has been demonstrated at the level of the peptide backbone and of amino acid structure.

At physiological and pathological level, there has been little scrutiny of the role and differences regarding protein activity during the passage between reduced and oxidized state. A quantitative comparison of bovine serum albumin and glutamine synthase has shown susceptible residues of the former (at the level of methionine and the aromatic amino acid residues) to be oxidized about twice as fast as those of the latter [109].

The oxidation of amino acids by Fenton reagent (H_2O_2 + Fe^{2+}) leads mainly to the formation of NH_4^+, alpha-ketoacids, CO_2, oximes, and aldehydes or carboxylic acids containing one less carbon atom. Oxidation is almost completely dependent on the presence of bicarbonate ions and it is stimulated by iron chelators [110]. Cysteine and methionine are the two sulfur-containing residues normally found in proteins. Cysteine residues are functionally implicated in the catalytic cycle activity of many enzymes and they can form disulfide bonds that contribute to protein structure and spatial conformation changes. In contrast, the specific functions of methionine residues are not known. A variety of oxidants reacts readily with methionine to form methionine sulfoxide, and surface exposed methionine residues represent an extremely high concentration of reactants available as efficient oxidant scavengers. Methionine residues would thus constitute an important antioxidant defense mechanism [111]. Moreover, cysteine residues of channel proteins are affected by oxidant stress. For instance, a discriminatory study on the oxidation sensitivities of various cloned K^+ channels from T lymphocytes, cardiac cells and neurons revealed that five of the cloned channels were highly sensitive to oxidation; but an equal number were resistant and the ratio HX/XO free radical generating system had no effect on all channels studied [112]. Observations on intermediate conductance $IK_{(Ca)}$ channels from bovine aorta ECs are indicating that the application of oxidative stress induces a membrane depolarization consequent to the inhibition of $IK_{(Ca)}$ via the oxidation of critical cysteines' SH groups of the intimate structure of the channel protein [113]. Cytosolic application of hydrophilic oxidative reagent, DTNB and/or thimerosal, reduces gradually the $IK_{(Ca)}$ channel activity, with no modification of the channel unitary conductance. The inhibitory effect of oxidants was not changed by the withdrawal of the oxidant agents but channel activity was partially recovered by the addition of the SH group reducing agents dithiothreitol (DTT) or reduced glutathione (GSH) [113].

The Effect of Oxidative Stress in ECs

A particularly intriguing observation is that a number of enzymes possessing active-site iron-sulfur clusters are hypersensitive to inactivation by $^{\bullet}O_2^-$. Mammalian mitochondrial aconitase is inactivated *in vitro* and *in vivo* by treatments that increase mitochondrial $^{\bullet}O_2^-$ generation, such as growth under hyperbaric conditions. The mechanism of aconitase inhibition by $^{\bullet}O_2^-$ has been demonstrated to involve the release of free iron from the enzyme; that free iron can exacerbate oxidative stress [114]. It has been also demonstrated that the fragmentation of albumin, collagen, and α-globulins occurring during the oxidative damage is selective for proline residues because of a higher susceptibility of proline to $^{\bullet}OH$ attack. Selective attacks of $^{\bullet}OH$ occur against histidine and arginine residues as well, since those amino acids are often in close association with transition metals that serve as catalysts in the production of $^{\bullet}OH$ [115]. Oxidation of amino acids in proteins leads invariably to physical changes characterized by alteration in fluorescence and in the conformation of their tertiary and quaternary structure, inducing potential functional modifications.

I.2.4.2. Damages Induced by Oxidative Stress to Lipids

One of the most important features of free radical related cellular injuries is concerned with the lipid peroxidation arising from the reaction of lipids with free radicals. Although the peroxidation of lipids results from free radical reactions, peroxidation of lipid molecules under in *vivo* conditions has not been fully elucidated. Up to date, in *vitro* experiments are demonstrating that the peroxidation of polyunsaturated fatty acids usually involves three specifically defined steps: *initiation*, *propagation,* and *termination* [116].

The initiation phase of peroxidation usually proceeds with the formation of conjugated diene bonds generated by the elimination of a hydrogen atom. Although that reaction occurs

with relative ease, it is important to mention that the initial interaction of O_2 with unsaturated bonds of fatty acids is unfavorable because of a "spin-forbidden reaction". To overcome this energetically imposed barrier, it is strictly necessary that the activated oxygen be present in the form of reactive singlet oxygen or some free radical species already produced by the biotic system. The initiation process can be substantially facilitated by the presence of redox-active metals such as iron and copper [63]. The major fatty acids that undergo lipid peroxidation in the cell membrane are linoleic acid, arachidonic acid, decosahexaenoic acid, and other polyunsaturated fatty acids, resulting in different hydroperoxide and aldehyde species. Peroxidation of lipid molecules invariably changes or damages the lipid molecular structure of the membrane [64,117]. Beside the self-destructive nature of lipid peroxidation induced by the generation of free radicals, lipid peroxidation is a major source of other cytotoxic products, such as aldehydes, produced from the decomposition of lipid hydroperoxides [118]. The 4-hydroxynonenal, a major byproduct of 1,6-arachidonic acid peroxidation is both cytotoxic and mutagenic [119]. These aldehydes are able to form protein cross-linkages that inactivate many cellular constituents, including membranes and enzymes. The mitochondrial oxidation of malondialdehyde, with the aid of aldehyde dehydrogenase, represents a mechanism of aldehyde detoxification process, which has been developed by the cell to eliminate the toxic effect of aldehydes. The mitochondrial oxidation capacity is substantially lost with age, providing a cellular explanation for the accumulation of malondialdehyde and lipofuscin-like substances in aged organisms.

I.2.4.3. Damages Induced by Oxidative Stress to Carbohydrates

Glucose and other monosacharides undergo oxidation when the conditions are appropriate and if the oxidation of glucose can serve as a scavenger of $^{\bullet}OH$, glucose, in turn,

becomes oxidized, creating a vicious circle. Carbohydrate compounds in reaction with an α-hydroxyaldehyde structure can enolize in the presence of a transition metal ion to become ketoaldehydes. In physiological conditions, simple monosaccharides readily undergo autoxidation to form dicarbonil compounds and H_2O_2 [120]. The autoxidation is a process resulting in a large number of activated molecules and compounds capable of interacting with new non-oxidized molecules, continuing the propagation and the progress of oxidative stress.

I.2.4.4. Genomic and DNA Structural Damages

Many processes, including extrinsic factors such as radiation and chemicals, and intrinsic factors, such as oxygen-derived free radicals, can inflict damages to DNA. Much of our understanding of free radical DNA damages is derived from radiation chemistry. At present, no method is available to quantitatively assess the relative contribution of these factors to the DNA damages. A sensitive analytical method utilizing 8-hydroxydeoxyguanosine to detect oxidized DNA molecules will be hopefully useful for explorations into DNA damages and DNA oxidation reactions occurring in living cells. The $^{\bullet}OH$ radical inflicts both base alteration and strand breaks to DNA. The $^{\bullet}OH$ sources are xanthine/xanthine oxidase and H_2O_2 through the Fenton reaction. The DNA breakdown and the strand breaks can be prevented by antioxidants, demonstrating that damages are inflicted by the deleterious activity of free radicals [14,18,121]. The DNA double helix is much less susceptible to oxidative injury than is isolated DNA. Of the five components, thymine and cytosine are the most susceptible to $^{\bullet}OH$ damage, followed by adenine, guanine, and the deoxyribose sugar moiety. Thymine looks very sensitive to different aggressive factors because the treatment of mammalian cells with irradiation produced increased amounts of thymine glycol. Mitochondrial DNA damage has attracted substantial interest because mitochondria are the major sources of oxygen-derived free radicals. They are a

preferential target for many xenobiotic chemical carcinogens and are poorly endowed with DNA repair enzymes. It has been also reported that a large deletion of mitochondrial genome accumulates exponentially with age in humans and animals, mainly due to the deleterious impact of oxidants generated in mitochondria [73].

I.3. Ca^{2+} SIGNAL TRANSDUCTION IN ENDOTHELIAL CELLS

The disruption of Ca^{2+} homeostasis has been proposed to play a crucial role in the development and evolution of endothelial dysfunction under pathological conditions such as atherosclerosis, hypertension, hypercholesterolemia, reoxygenation-reperfusion injury, diabetes mellitus, and septic shock. Hormones, agonists, and autacoids induce in ECs a biphasic increase of $[Ca^{2+}]_i$. The stimuli-induced increase in $[Ca^{2+}]_i$ in vascular endothelial cells is both necessary and sufficient for the release of EDRF or/and EDCF [122]. The first component of the biphasic increase in $[Ca^{2+}]_i$ is an inositol 1,4,5-triphosphate ($InsP_3$) mediated release from intracellular stores, directly coupled with the second component made of a capacitative Ca^{2+} entry from the external medium [123]. Evidence is indicating that agonist-stimulated Ca^{2+} influx is dependent on membrane potential and hyperpolarization of the membrane is responsible for an increase of Ca^{2+} entry [124-126]. This process is mediated in part by the activation of various types of ECs' Ca^{2+}-dependent K^+ channels [126-128]. In contrast, the Ca^{2+} entry in excitable cells is stimulated following a depolarization of their membrane.

I.3.1. The Heterogeneity of Signaling Pathway in ECs

In the sequence of events occurring after the stimulation of ECs' membrane receptors, the production of inositol triphosphate ($InsP_3$) and diacylglycerol (DAG) through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and the related increase of intracellular Ca^{2+}, constitute the key events underlying the regulation of a large number of cellular processes. The formation of $InsP_3$ is the focal point for two major signaling pathways: one is initiated by a family of G protein-linked receptors and the other by receptors linked to tyrosine kinases (Figure 10). Both classes of receptors are coupled to a transducing mechanism, which activate

phospholipase C (PLC) to hydrolyze the membrane lipid precursor PIP_2 and to produce both $InsP_3$ and DAG.

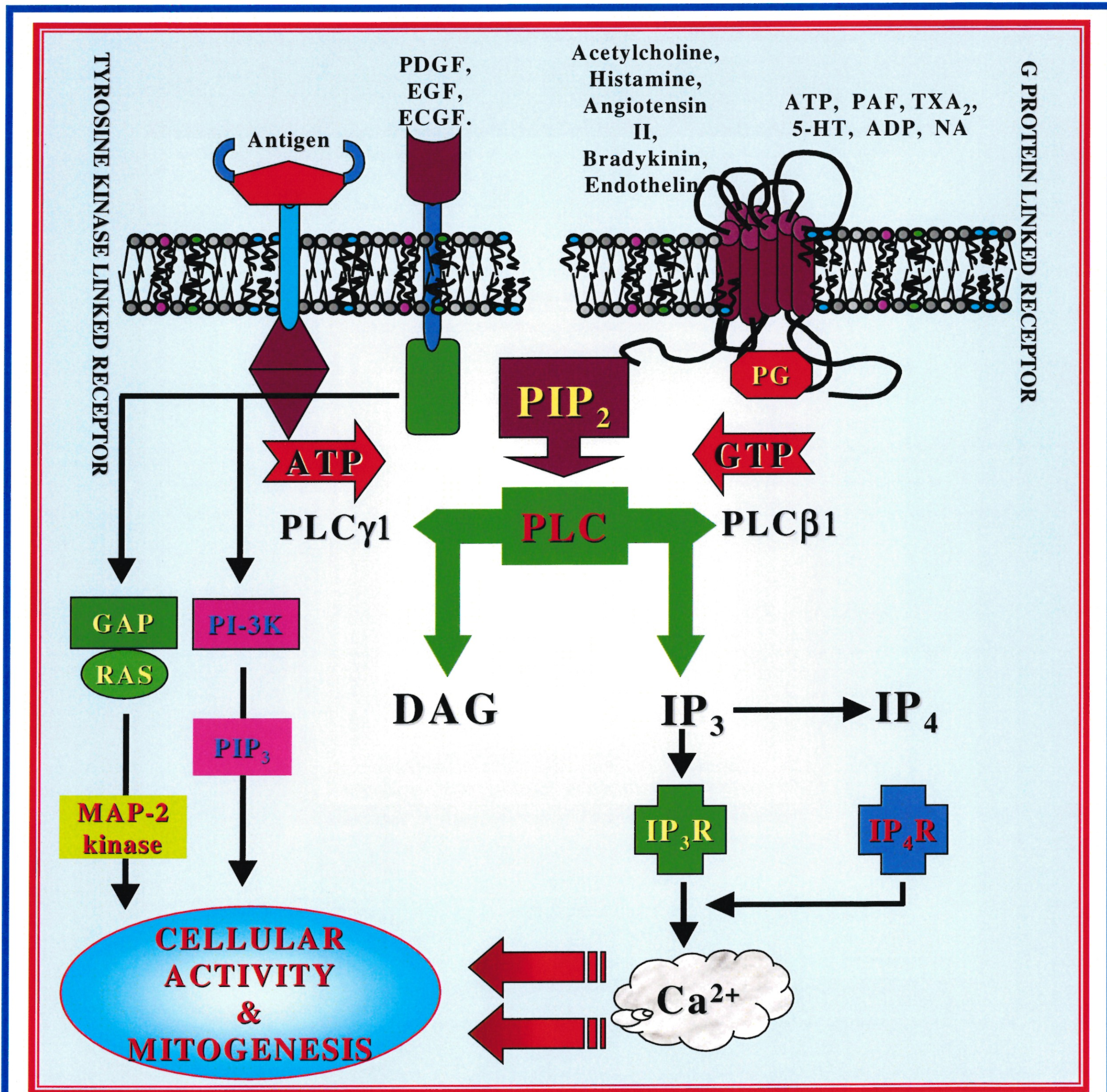


FIGURE 9.
Summary of the two major receptor-mediated pathways for stimulating the formation of Inositol triphosphate (IP_3) and diacylglicerol (DAG). Numerous agonists bind to 7-membrane-spaning receptors (R), which use a GTP binding protein (PG) to activate phospholipase C (PLC-β1), whereas PLC-γ1 is stimulated by the tyrosine kinase-linked receptors. The tyrosine kinase receptor activates other effectors such as the phosphatidylinositol (3,4,5)-triphosphate (PIP_3)and the GTPase-activating protein (GAP), that regulates RAS.

$InsP_3$ binds to a specific receptor embedded in the endoplasmic reticulum membrane, causing the release the Ca^{2+} from intracytosolic pools. Vasoactive agents such as bradykinin, histamine, ATP, and ADP that stimulate cultured ECs, induce a several fold increase in $[Ca^{2+}]_i$ to a peak, which subsequently declines to a sustained elevated level. The transient response is observed in the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o$) or in the presence of La^{3+} [129]. This demonstrates that the transient component reflects a release of Ca^{2+} from internal stores, whereas the sustained component reflects an influx of Ca^{2+} from extracellular space. Despite the molecular heterogeneity of the two signaling receptor pathways, the effect of an external signal is the triggering of a cascade of events, ending with the utilization of $InsP_3/Ca^{2+}$ and DAG/PKC as ultimate effectors for the regulation of a wide range of cellular activities. The membrane-signaling pathway controlled by receptors linked to G-protein has three sequential steps: the ligand-activated receptor induces through a G protein transducer an activation of phospholipase C (PLC). Most of the G protein-linked receptors possess seven membrane-spanning domains connected by extracellular and intracellular loops. The second and third intracellular loops of the receptor have an essential role in the activation of the heterotrimeric G protein, which in turn is responsible for the stimulation of the β_1 isoform of the PLC family [130]. The G protein family can be divided in two classes (stimulatory and inhibitory), depending on whether or not they are sensitive to pertussis toxin.

$InsP_3$ production is also stimulated in ECs through the activation of tyrosine kinase-receptors, which relay the agonist information through a direct interaction between the receptor tyrosine residues and the γ1-form of PLC (Figure 9). This pathway is an energy requiring process because ATP is consumed not only as the two receptors interact (autophosphorylation), but also during the subsequent phosphorylation of $PLC_{\gamma 1}$.

Ca^{2+} Signal Transduction in Endothelial Cells

In general, growth factors such as platelet-derived growth factor (PDGF) and endothelial cell growth factor (ECGF) operate by gathering two receptors together which enables their cytoplasmic kinase domains to phosphorylate each other on the tyrosine residues and to create the docking sites to $PLC_{\gamma 1}$ [131]. This association has two essential consequences for the activation of $PLC_{\gamma 1}$, firstly, it is phosphorylated by the receptor on specific tyrosine residues, and secondly, its membrane translocation brings the $PLC_{\gamma 1}$ into direct contact with its substrate, PIP_2. The kinetics of $InsP_3$ production and Ca^{2+} liberation by the two signaling pathways are different, with smaller amplitude but longer lasting of Ca^{2+} responses for tyrosine kinase-receptor pathways [132].

The free basal $[Ca^{2+}]_i$ ($^b[Ca^{2+}]_i$) is maintained between 0.05-0.2 μM [133]. The huge difference (4 orders of magnitude) between free $[Ca^{2+}]_i$ and the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) generates a large electrochemical driving force in favor of a net intracytosolic Ca^{2+} entry. A long lasting elevated free $[Ca^{2+}]_i$ is not suitable for cells because of the rapid cytotoxic effects induced by free elevated $[Ca^{2+}]_i$. To prevent a long lasting $[Ca^{2+}]_i$ increase, cells use two mechanisms, an extrusion system located on the plasma membrane and a coordinated Ca^{2+} sequestration mechanism, situated at the level of endoplasmic reticulum, mitochondrial and nuclear membranes. The sequestration of Ca^{2+} in intracytosolic organelles, mostly in the endoplasmic reticulum, gives rise to the notion of intracellular Ca^{2+} stores (pools).

I.3.2. Characteristics of Intracellular Calcium Stores in ECs

The store membranes from which Ca^{2+} is released contain three major components: 1) a pump for sequestration and extrusion of Ca^{2+}; 2) binding proteins for an efficient and substantial

sequestration (calsequestrin and calreticulin); and 3) specific receptors $InsP_3R$ and ryanodine receptor (RyR) to release Ca^{2+} back to the cytosol in responses to receptor stimulation.

I.3.2.1. Ca^{2+}-ATPases

In the organization of exchanging intracellular Ca^{2+} stores, an essential role is played by Ca^{2+} pumps, the molecular devices devoted to the accumulation of the cation against its electrochemical gradient. The vectorial movement of Ca^{2+} from cytosol to the lumen of endoplasmic reticulum (ER) is provided by **sarco-endoplasmic calcium ATPase** (SERCA).

Evidence for the existence of Ca^{2+} pumps has been available for over three decades, but important developments in the study of Ca^{2+} uptake in ER were made after the discovery of SERCA inhibitors and the development of molecular biology tools. Before the molecular structure became available, the general organization of SERCAs in the membrane had been deduced and inferred from structure-function relationship data. It was proposed that the pump was an asymmetrical transmembrane protein, with a small luminal domain, a membrane-spanning portion, and a bulky cytosolic head [134]. An analysis of hydropathy profile of the amino acid sequence revealed that the protein was composed of 10 putative α-helices membrane-spanning domains. The two termini were located on the cytoplasmic side along with a large hydrophilic region, responsible for the binding of ATP and a phosphorylation site represented by an aspartate residue. The high-affinity Ca^{2+} binding site and the ion permeation pathway were generated by helices M_4 M_5, $M_{6,}$ and M_9. The most prominent features of SERCA were obtained following the cloning of skeletal SERCA cDNA and the utilization of pump inhibitors, both specific (thapsigargin and cyclopiazonic acid) and non-specific (vanadate, fluoride).

Thapsigargin is a tumor promoting sesquiterpene lactone, which binds stoichiometrically to all SERCAs and causes an essentially irreversible inhibition of their activity by blocking the enzymes in the Ca^{2+} free E_2 state in almost all eucaryotic cells [135]. The 2,5-di(tert-butyl)-1,4-benzohydroquinone has the same action but with lower affinity (K_d= 0.4 μM) whereas the mycotoxin cyclopiazonic acid, an indole metabolite produced by *Aspergillus* and *Penicillium*, is highly specific for SERCA inhibition. Despite the powerful inhibitory capability and specificity of TG, in the past years several results have demonstrated the presence of TG-resistant pools in many cellular types [136-138]. The thapsigargin-resistant Ca^{2+} pools are capable of generating rapid cytosolic Ca^{2+} signals in response to the $InsP_3$-generating agonists. They could maintain a physiological $^{b}[Ca^{2+}]_i$ level and mediate the accumulation of Ca^{2+} within the $InsP_3$-sensitive pools in the presence of TG, and most likely they are responsible for the ability of cells endowed with TG-resistant Ca^{2+} pools to function and grow in the presence of thapsigargin [139]. The existence of TG-resistant SERCA was further demonstrated by mutation of the wild type phenylalanine at position 256 with leucine or serine, which induced a 4-5-fold increase of the SERCA resistance to thapsigargin [137].

The isolation of cDNA and the genes of SERCA have revealed that the SERCAs are encoded by a family of homologous and alternatively spliced genes that show a high degree of changeable phenotypic expression. The expression of isoforms is of great importance for the fine modulation and properties of Ca^{2+} stores in different tissues and cells because of the critical role of Ca^{2+} stores in Ca^{2+} signaling and Ca^{2+} homeostasis in all excitable and nonexcitable cells, and particularly in different developmental stages or states of activity. Three genes and five isoforms have been identified so far. $SERCA_3$ is expressed selectively in cells in which Ca^{2+} signaling plays a critical and sensitive role in the regulation of physiological processes, like

ECs [140]. Expression patterns of SERCA isoforms and $InsP_3$ receptor isoforms were studied in endothelial cells at the mRNA level by the ratio RT-PCR technique in three types of ECs: rat adrenal medulla microvascular endothelial cells (RAMEC), rat aortic endothelial cells (RAEC), and human umbilical vein endothelial cells (HUVEC). Wu et al. demonstrated [141] the presence of a dynamic expression of multiple SERCA isoforms in each type of endothelial cells. $SERCA_3$ is one of two Ca^{2+} pumps serving intracellular Ca^{2+} signaling pools in non-muscle tissues, unlike the ubiquitous $SERCA_{2b}$. However, freshly isolated HUVEC were an exception in this respect, since they contained only $SERCA_3$, without $SERCA_{2b}$ messengers. The expression patterns changed upon cell proliferation: $SERCA_3$ messengers decreased whereas $SERCA_{2b}$ messengers increased. In HUVEC, the expression of $SERCA_3$ only decreased and returned, upon differentiation, to the levels observed in the freshly isolated cells. Therefore, the plasticity of various SERCA isoforms expression shows that, in all probability, different Ca^{2+} pools may play distinct roles during cell proliferation and differentiation [141].

Another interesting property of the various SERCAs concerns the interaction with phospholamban. Phospholamban (PLB) is a 24- to 27-kDa phosphoprotein that modulates activity of SERCA after cAMP mediated phosphorylation. In its unphosphorylated form, PLB inhibits $SERCA_1$ and $SERCA_2$ but not $SERCA_3$ by reducing their Ca^{2+} affinity. Expression of PLB is limited to cardiac, slow-twitch skeletal and smooth muscle in which PLB is an important regulator of $[Ca^{2+}]_i$ and contractility. However, evidence demonstrates the existence of PLB in the vascular endothelium, a nonmuscle tissue, and provides functional data on PLB regulation of vascular contractility through its action on endothelium. Endothelium-dependent relaxation to acetylcholine was attenuated in aorta of PLB-deficient (PLB-KO) mice compared with wild-type (WT) controls. This effect was not due to actions of nitric oxide on the smooth muscle, because sodium nitroprusside-mediated relaxation in either denuded or endothelium-intact

aortas was unaffected by PLB gene ablation. In the WT mouse aorta ECs, both RT-PCR and Western blot analyses revealed the presence of PLB in endothelial cells. These data convincingly indicate that even though PLB expression was thought to be limited only to cardiac, slow skeletal and smooth muscle, it is present and modulates vascular function because of its interactions with SERCAs in endothelial cells as well [142]. Therefore, the presence of PLB in endothelial cells points to an unrecognized pathway for modulation of endothelial cell $[Ca^{2+}]_i$ and of vascular contractility, demonstrated by a decreased endothelium-dependent relaxation in the PLB-deficient aorta ECs [143].

I.3.2.2. Endoplasmic reticulum and the fast exchanging Ca^{2+} pools

The endoplasmic reticulum is a complex interconnected network of tubular and flat vesicular structures, delimited by a highly proteinic lipid bilayer membrane. The surface of the endoplasmic reticulum can exceed the surface of the cell by a factor of 30-40. Metabolic synthesis begins in the ER and can be transferred to the Golgi apparatus and other organelles. The granular (rough) ER is characterized by a large number of ribosomes attached to the outer surface of the reticulum membrane and the complex is actively implicated in protein synthesis, glycosylation, and accumulation of glycoproteins. The smooth endoplasmic reticulum is responsible for phospholipid and cholesterol synthesis, which is incorporated in the ER membrane, thus causing the ER membrane to grow continuously. This growth is kept under control by a steady process of vesicle formation, which are continuously breaking away and migrating to the Golgi apparatus. There is also an association of smooth ER subdomains with mitochondria. This association would be implicated in phospholipid transfer, and the sequestration and modulation of cellular and mitochondrial free Ca^{2+}. Consequently, ER-mediated Ca^{2+} uptake by mitochondria may activate mitochondrial metabolic activity by

stimulating Ca^{2+}-dependent mitochondrial dehydrogenase, as well as regulate the subcellular pattern of Ca^{2+} oscillations generated by receptor activation [144]. The association/dissociation process between ER subdomains and mitochondria, particularly under conditions of high local free cytosolic Ca^{2+}, are therefore of functional importance. It was demonstrated that low $[Ca^{2+}]_i$ (<100 nM) would favor a dissociation of the ER-mitochondria complex whereas a high $[Ca^{2+}]_i$ (>1 μM) would induce the formation of the complex, most likely to favor the mitochondrial Ca^{2+} uptake [145]. The ER provides all the enzymatic components for the detoxification of cells through oxidation, hydrolysis, coagulation, conjugation, and other chemical processes. The ER represents the main buffer capacity for the accumulation and storage of Ca^{2+}. Its inner endoplasmic matrix is filled with many Ca^{2+} binding proteins, the most important being **calsequestrin** and **calreticulin**. These storage proteins have two important basic characteristics expected for a dynamic Ca^{2+} storage function: high storage capacity (25-50 mol:mol) and low affinity, with a K_d in the (1-4 mM) range [146]. The ER intraluminal Ca^{2+} concentration ($[Ca^{2+}]_l$) is in the millimolar range under physiological conditions, and it is not expected to vary dramatically when Ca^{2+} is released from endoplasmic reticulum.

I.3.2.3. $InsP_3$ and Ry receptors

I.3.2.3.1. Distribution of $InsP_3$ and Ry receptors

Inositol 1,4,5-triphosphate Receptor ($InsP_3R$) and **Ryanodine Receptor** (RyR) are embedded in the ER membrane and form the channels responsible for the release of Ca^{2+} from the rapidly exchanging Ca^{2+} stores. The $InsP_3R$ and RyR are tetramers composed of large subunits (300 and 550 kDa, respectively) with six spanning transmembrane segments to form the channels. During evolution, both receptors have been conserved in the animal kingdom and

have a geometrical resemblance to a cauliflower or rosette. They are located on modified portions of the endoplasmic reticulum and are forming the so-called "calciosome". The calciosome was thought to be a new cytosolic organelle, specialized in Ca^{2+} accumulation and strictly representing the $InsP_3$ sensitive store [147], but it was later demonstrated to be heterogeneous and a part of it has revealed sensitivity to ryanodine. During the early stage of ontogenesis, a dominant presence of $InsP_3$-mediated intracellular Ca^{2+} release has been identified for most of the embryonic cell Ca^{2+} signaling pathways. As development proceeds, virtually all cell types continue to express $InsP_3R$ channels, but in excitable cells, the major Ca^{2+} release channels became RyRs. As organogenesis proceeds, more specialized RyR channels are expressed in many cell types and the triggering mechanisms for intracellular Ca^{2+} release became more diverse, to include $InsP_3$-dependent and voltage-dependent Ca^{2+} release and the Ca^{2+}-induced Ca^{2+} release mechanism. This developmental switch from predominantly $InsP_3$-mediated to both $InsP_3$-mediated and $InsP_3$-independent pathways for intracellular Ca^{2+} release is consistent with data showing that $InsP_3R$ plays an important regulatory role in cellular proliferation and apoptosis, whereas RyR is required for other cellular functions including muscle contraction [148].

I.3.2.3.2. The $InsP_3R$ of ECs

Calcium contained within intracellular stores is released in the cytosol when $InsP_3$ binds to its receptor [149]. A family of $InsP_3$ receptors ($InsP_3Rs$) has been identified with a molecular diversity arising from both alternative splicing and the existence of separate genes. Expression patterns of $InsP_3R$ isoforms were studied in ECs at the mRNA level by the RT-PCR technique and subsequent restriction-enzyme analysis. So far, in RAMEC, RAEC, and HUVEC, $InsP_3R_1$ and $InsP_3R_3$ were identified; they change their expression depending on the cellular proliferation

state. Therefore, in freshly isolated ECs, the presence of both $InsP_3R$ mRNA was identified. However, the expression patterns changed upon cell proliferation: $InsP_3R_1$ messengers decreased, while $InsP_3R_3$ increased with culturing. Upon induced cell-culturing differentiation, the expression pattern demonstrates that $InsP_3R_1$ was reduced in all three cellular strains, while $InsP_3R_3$ rose significantly [140]. Data demonstrate that the $InsP_3R_3$, in contrast to the $InsP_3R_1$, is not stimulated by sulfhydryl oxidation and is less sensitive to ATP, and that $InsP_3R_1$ and $InsP_3R_3$ differ markedly in their response to protons [150]. The $InsP_3R_1$ and $InsP_3R_2$-type have a homology of ≈70%, whereas the $InsP_3R_3$ and $InsP_3R_4$ (which was not identified in ECs so far) have only a partial homology related only to their C-terminal domains. The N-terminal domain is lying free in the cytoplasm with the $InsP_3$ binding sites located at its end, far-off from the channel-forming C-terminal. The functional $InsP_3$ receptor exists as a tetramer, with the C-terminal regions co-operating to form the Ca^{2+} channel and the N-terminal regions forming bulbous heads that project into the cytosol. The $InsP_3R$ is sensitive to $InsP_3$ and adenine nucleotides and partially sensitive to cytosolic Ca^{2+}. The $InsP_3R$ is insensitive to ryanodine, whereas heparin and caffeine are recognized as potent but non-specific inhibitors. The $InsP_3R$ sensitivity may change depending on Ca^{2+} content of ER and the available evidence suggests that its sensitivity increases as the stores charge up with Ca^{2+}. The $InsP_3R$ sensitivity may vary also depending on the receptor heterogeneity (different gene products, alternative splicing and/or post-translational modifications). There are neither specific agonists nor antagonists for $InsP_3R$ [149]. The functional properties of $InsP_3R$ have been extensively studied after the isolation and cloning of $InsP_3R$, revealing its physical, chemical, and biological characteristics. The binding of $InsP_3$ to its receptor induces a large conformational change leading to channel opening. Biophysical analyzes of a single $InsP_3R$ channel reveal unambiguous channel opening and closing events with increase in open probability in response to $InsP_3$. The mean open time

is <10 ms, with evidence showing a four-conductance state of ≈20-25 pS for each conductance state [151]. Evidence demonstrates that channel opening depends upon sequential $InsP_3$ binding to the four putative binding sites of the tetrameric protein. Each binding step could cause a partial opening of the channel in order to account for the different conducting states observed in patch-clamp recordings of purified $InsP_3R$ [151]. The $InsP_3R$ sensitivity is positively correlated with the loading of Ca^{2+} content in the endoplasmic reticulum. Considerable variability has been reported regarding the sensitivity of $InsP_3$-induced Ca^{2+} release, attributed to a quantal Ca^{2+} release. As the level of $InsP_3$ rises, a fixed proportion of the stored Ca^{2+} is released, leaving Ca^{2+} available for higher doses of $InsP_3$ [152].

I.3.2.3.3. The RyR of ECs

The RyR can be found essentially in excitable cells such as muscle cells, cardiomyocytes and smooth muscle cells, but its presence in conjunction with $InsP_3R$ has been described in a broad spectrum of non-excitable cells. The type-1 RyR channel (characteristic of skeletal muscle) couples structurally and functionally with L-type voltage-gated Ca^{2+} channels inserted in the T tubules of the sarcolemma. Because of this coupling, activation of RyR is thought to be triggered directly by plasma membrane depolarization, with the L-type channels working to sense the voltage and transfer the physical signal intracellularly. Activation by voltage sensor is, however, not the only mechanism proposed for RyR. When type-1 Ry-channels are isolated, they resemble cardiac type 2 receptors in their responsiveness to rapid and large $[Ca^{2+}]_i$ increase (in the order of μM), and known as Ca^{2+}-induced Ca^{2+} release process (CICR). The type-2 RyR is the receptor of cardiomyocyte sarcoplasmic reticulum, which is activated through so-called CICR mechanism (the extracellular Ca^{2+} is allowed to enter the cardiac myocyte through L-type voltage-gated Ca^{2+} channels). RyR is stimulated by ryanodine, caffeine, and likewise by $InsP_3$

and PIP_2. The presence of RyR and ryanodine sensitive Ca^{2+} pools was not extensively studied in endothelial cells and it remains highly controversial. Evidence demonstrates the existence of ryanodine receptors in bovine aorta ECs [153] and caffeine sensitive stores have been identified in rabbit aorta ECs that are able to initiate some Ca^{2+}-induced Ca^{2+} release events [154]. In disagreement with these observations, Schilling has demonstrated that neither ryanodine nor caffeine were capable to affect the $^{b}[Ca^{2+}]_i$ and BAECs do not exhibit specific high- or low-affinity [^{3}H]ryanodine binding [155]. However, in many EC preparations, such as HUVEC line [156], freshly isolated rabbit aortic ECs [157], BAECs [153,158], human pulmonary artery ECs, and freshly isolated EC preparation from rabbit aorta [159], it was demonstrated that both types of receptor are expressed and functional. It is believed that the two receptors are operating in separate stores, even though the two stores may interact cooperatively with each other to generate the Ca^{2+} response. The nucleotide derivative cyclic ADP-ribose (cADPR) is believed to play a regulatory role in the receptor activity. An additional similarity with $InsP_3R$ is the adaptation process that appears to correspond to the quantal or incremental Ca^{2+} release property of the $InsP_3R$ [160,161].

I.3.2.4. CICR and Ca^{2+} waves

Calcium-induced calcium release is a common characteristic for both RyR and $InsP_3R$. The small hot spots of Ca^{2+} resulting from the quantal localized Ca^{2+} release are capable to suddenly induce the release of a huge local amount of Ca^{2+}, similar to an **"all or none"** response, which appears as expanding circular or spiral waves. This "all or none" response seems to arise through a positive feedback mechanism, whereby Ca^{2+} stimulates its own release. In the absence of Ca^{2+}, the $InsP_3R$ is less active even in the presence of increased amount of $InsP_3$. This Ca^{2+}-dependent sensitivity modulation of the $InsP_3$ receptor may contribute to the

oscillatory Ca^{2+} release in endothelial cells [162]. An interesting phenomenon is intercellular Ca^{2+} waves, expressed by an increase of $[Ca^{2+}]_i$ from one cell to another and which occurs in many cell types through two separate mechanisms. In cells lacking gap junctions, waves are spread by means of a secreted intermediate ATP. In cells with gap junctions (glial and neuronal cells), the transmission of the wave is probably realized through the diffusion of either Ca^{2+} or $InsP_3$. As the wave reaches the cell periphery, enough calcium may diffuse across to activate neighboring cell. The cross-coupling model would predict a localized Ca^{2+}-activated elevation of $InsP_3$, which can diffuse across the gap junction.

Ca^{2+} Signal Transduction in Endothelial Cells

CHAPTER I.4. THE MODULATION OF CALCIUM INFLUX IN ENDOTHELIAL CELLS

The Ca^{2+} entry from external space is represented by the second component of the Ca^{2+} response triggered in ECs by hormones and autacoids. This portion of the agonist induced $[Ca^{2+}]_i$ increase is responsible for the sustained elevated phase of $[Ca^{2+}]_i$. The release of Ca^{2+} from intracytoplasmic pools and its entry from external space usually occur either simultaneously or sequentially.

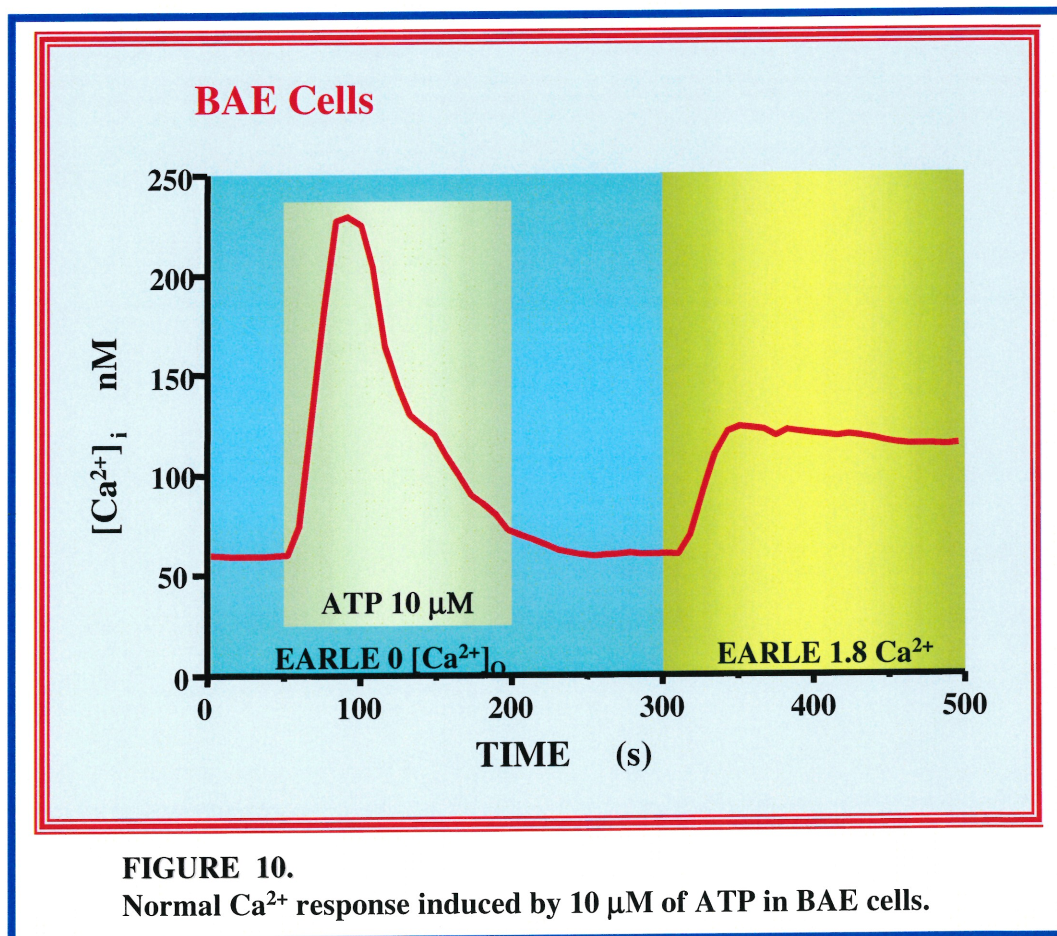


FIGURE 10.
Normal Ca^{2+} response induced by 10 µM of ATP in BAE cells.

A Ca^{2+} emptying of internal pools induces the opening of capacitative Ca^{2+} channels. The capacitative Ca^{2+} entry (CCE) channels are different from the voltage-dependent and the ligand-gated Ca^{2+} channels, which are present only in specialized cells, whereas the CCE channels are

The active extrusion of Ca^{2+} from the cytosol against a concentration gradient and the recapture in the intracytoplasmic stores is realized by two structures, the plasmalemma Ca^{2+}-ATPases and the SERCAs, respectively. Figure 10 represents a typical Ca^{2+} response of endothelial cells from bovine aorta (BAE), following stimulation by ATP. The experiment was carried out in an external free Ca^{2+} solution with the physiological Ca^{2+} concentration added subsequently near the end of the experimental protocol to illustrate the activation of CCE pathway. In the absence of $[Ca^{2+}]_o$, the initial increase in $[Ca^{2+}]_i$ is followed by a rapid return to a basal $[Ca^{2+}]_i$ resting level, without a sustained phase of increased $[Ca^{2+}]_i$. The addition of Ca^{2+} to the bathing solution leads to a substantial increase of $[Ca^{2+}]_i$ due to the activation of CCE pathway. In 1986, Putney [164] proposed the term **"capacitative Ca^{2+} entry"** to define the Ca^{2+} influx which appears after emptying of $InsP_3$-sensitive Ca^{2+} stores (Figure 12) [165,166].

I.4.1. Models of Capacitative Ca^{2+} Entry

Numerous studies have documented the existence of more than one type of Ca^{2+} entry in response to stimulation of cells by PLC-activating agonists [163]. The mechanism of CCE is very complex and it implies many unknown messengers and communication pathways. In ECs and some other cells, thapsigargin, cyclopiazonic acid, 2,5-di-*tert*-butylhydroquinone or the calcium ionophore ionomycin, can artificially induce CCE.

There are many signaling mechanisms and pathways proposed for the mediation of CCE activation: 1. - direct physical interaction of the CCE channel with an element of the storage compartment, possibly the $InsP_3$ receptor itself; 2. - formation of a diffusible messenger of unknown identity; 3. - physical translocation of CCE channel containing intracellular vesicles to the plasma membrane; 4. - the participation of a transient receptor potential (*Trp*) and transient receptor potential like (*Trp*L) proteins as components of CCE channels; 5. - slowly

transient receptor potential like (*Trp*L) proteins as components of CCE channels; 5. - slowly activating pertussis toxin-sensitive G proteins; 6. - a small GTP-binding proteins; 7. - cGMP; 8.- $InsP_3$ and/or $InsP_4$ cellular phosphates; 9. - tyrosine phosphorylation; 10. - activation of cellular phosphatases; 11. - arachidonic acid.

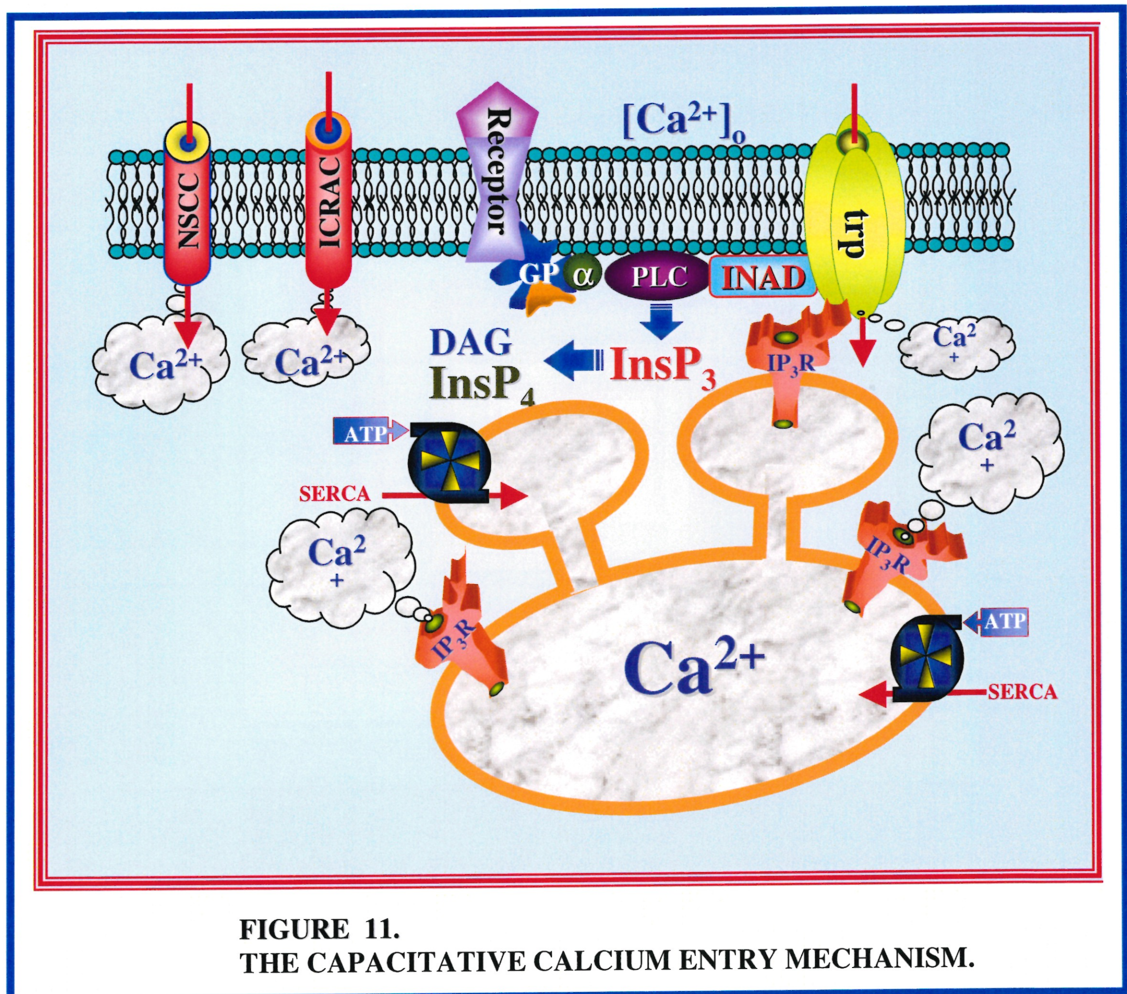


FIGURE 11.
THE CAPACITATIVE CALCIUM ENTRY MECHANISM.

By analogy with the excitation-contraction coupling mechanism, several studies have proposed a direct coupling between $InsP_3R$ and plasma membrane. The large head of $InsP_3R$ may convey the information between the filling up state of ER and the plasmalemma Ca^{2+} channel [167,168]. In the conformational coupling hypothesis proposed by Irvine (1990), $InsP_3$ binding would induce a change that opens the ER channel. As the pool loses its calcium, $InsP_3R$

protein-protein interaction [167]. The channel opening would also require the participation of $InsP_4$ [169].

Inspired from the mechanism of insulin receptors translocation, it was proposed that vesicles filled with CCE channels could be translocated to the surface of ECs, after emptying Ca^{2+} pools [163].

A **passive permeability pathway** for CCE entry has also been proposed, because of the magnitude of its flux, its voltage sensitivity, and its ability to conduct Ba^{3+}. The conductance of this passive pathway should be very small because a membrane current of merely 4 pA/cell is observed [126].

Numerous observations support the implication of a stimulation-induced second messenger for opening CCE. For instance, indomethacin, an inhibitor of cyclooxygenase, prolongs, whereas nordihydroguaiaretic acid, an inhibitor of lipoxygenase, abolished the sustained phase of the $[Ca^{2+}]_i$ response in cultured bovine pulmonary artery ECs. However, these observations have been contradicted by the observation that the metabolic products of cyclooxygenase or lipoxygenase are not implicated in bradykinin-stimulated Ca^{2+} influx [155].

Despite an important amount of evidence for the implication of cGMP (guanosine 3'5'-cyclic monophosphate) in the activation of Ca^{2+} influx [170], the exogenous administration of dibutyryl-cGMP, or activators (nitroprusside) and inhibitors (methylene blue and LY 83583) of soluble guanylyl cyclase, suggests that cGMP plays no significant role in either the release of Ca^{2+} from internal stores or the influx of Ca^{2+} from extracellular space [155,171].

Evidence suggests that some metabolites of PLC may be involved in both, the release of Ca^{2+} and the stimulation of Ca^{2+} influx. U731229 inhibits the agonist-stimulated $InsP_3$ and

DAG production as well as the associated increase in $[Ca^{2+}]_i$ in neutrophils, without direct effect on protein kinase C or PLA_2, and on the basal or stimulated cAMP levels [172]. Therefore, if the agonist-stimulated Ca^{2+} influx occurs via a direct receptor-operated channel or via a G protein-activated channel, the inhibition of PLC should prevent the release of Ca^{2+} from internal stores but have no effect on the Ca^{2+} influx component of this response. Actually, in ECs, U73122 blocked bradykinin-stimulated phosphoinositide accumulation and caused a time and dose-dependent inhibition of both the release of Ca^{2+} from internal stores and the influx of Ca^{2+} from the extracellular space, suggesting that activation of the influx requires some metabolite of phosphoinositide hydrolysis. These may represent a direct interaction of $InsP_3$ and/or $InsP_4$ with plasmalemmal channels [169] or the influx may be linked to a depletion of $InsP_3$-sensitive pools, as suggested by the CCE model of Putney [173]. In non-excitable cells, the capacitative Ca^{2+} influx is strictly correlated with the level of Ca^{2+} in the intracytosolic pools. After the depletion of stores, the Ca^{2+} influx pathway remains activated following removal of the agonist until the internal stores are filled again. Thus, sustained phosphoinositide hydrolysis is not required to maintain the Ca^{2+} entry activated [174]. Furthermore, the Ca^{2+} release-activated Ca^{2+} channel current (I_{CRAC}), a putative channel gated by store depletion that was called CRAC (calcium release activated Ca^{2+} channel) or SOC (store-operated Ca^{2+} channel) [175], is fully activated by simply lowering $[Ca^{2+}]_i$ even though the I_{CRAC} is likewise activated by the administration of TG, which actually increased $[Ca^{2+}]_i$; both conditions do not involve phosphoinositide breakdown.

Evidence for a capacitative Ca^{2+} entry has come from experiments carried out in Ca^{2+}-free solutions, where SERCA pump inhibitors were used to empty the intracellular Ca^{2+} pools by a slow Ca^{2+} leakage, generating a "signal" of Ca^{2+} pool emptying. This "signal" is transmitted to the plasmalemma without any production of phosphoinositide second messenger.

As a result, the addition of Ca^{2+} in the external bathing medium will increase $[Ca^{2+}]_i$ by generating the CCE (Figure 11). Thus, it appears that inhibition of Ca^{2+} uptake via SERCAs causes an increase of $[Ca^{2+}]_i$ and a concomitant stimulation of Ca^{2+} influx, the whole process being associated with the depletion of $InsP_3$-sensitive and insensitive Ca^{2+}-pools. The Ca^{2+} influx pathway activated by the depletion of internal stores is identical with that activated by agonists, because both activated pathways are inhibitable by La^{3+} and cell-depolarization (with high $[K^+]_o$ buffers), along with a total insensitivity to the inhibitors and/or activators of voltage-gated Ca^{2+} channels [176].

Strong evidence suggests that the channels encoded by *trp* and *trp*L genes would form CCE. Therefore, the mammalian homologues of Drosophila photoreceptor constituent proteins *trp* and *trp*L have been proposed as candidates for CCE components. Niemeyer et al. [177] have demonstrated that the double mutant *trp* and *trp*L in Drosophila induced not only an absence of persistent phase in the electroretinogram but failed to exhibit any electrical response. Schilling et al. have demonstrated that the expression of Drosophila *trp* gene in insects SF9 cells induced the appearance of a store depletion activated Ca^{2+}-selective conductance, while *Trp*L induces the expression of a Ca^{2+}-permeable but store depletion-insensitive channel, with a nonselective cation conductance that tended to activate spontaneously and appeared to respond to $InsP_3$ [178,179]. The specific **over expression** of *trp* has been shown to substantially increase the CCE [180,181], even though in many instances these findings have been difficult to reproduce in other laboratories [182,183]. However, the most difficult task is to reconcile the evidence for so many CCE models. At this moment, it appears that there are multiple mechanisms for regulating the CCE. The most valid body of evidence is coming from ECs with $InsP_3$ receptor mediated conformational coupling to endogenous store-operated channels [184], and from the co-immunoprecipitation amino acid sequences of a *trp* and of $InsP_3R$ that interact

directly and form stable complexes [185]. On the other hand, there is also a convincing body of evidence indicating that such a mechanism cannot account for the activation of all store-operated channels.

I.5. THE CHANNELS IMPLICATED IN Ca^{2+} HOMEOSTASIS IN ECs

Channels implicated in ion transport in endothelial cells constitute key determinants for Ca^{2+} signaling, mechano-sensor functions, cell-to-cell communication, vessel permeability, pH, volume regulation, and vasogenesis. An important function of ion channels in ECs is the modulation of cell membrane potential. In these cells, the driving force for Ca^{2+} influx increases at hyperpolarizing potentials. This hyperpolarization is produced by K^+ and Cl^- channel activities that maintain the membrane potential sufficiently negative to insure the necessary driving force for Ca^{2+} entry. The initial increase of $[Ca^{2+}]_i$ induced by hormones, agonists and autacoids activates many Ca^{2+} dependent K^+ currents, which modulate the driving force for Ca^{2+} influx.

With respect to the membrane potential, ECs can be divided in two classes. One has a membrane potential mainly controlled by a K^+ conductance through inwardly rectifying K^+ channels, with a potential between –70 and –60 mV. The second class has a potential maintained by a Cl^- conductance and the vectorial movement of Cl^-, with a resting potential between –40 and –10 mV (a value close to E_{Cl}) [124]. Receptor mediation or mechanical stimulation induces a hyperpolarization through the activation of Ca^{2+} dependent K^+ current ($IK_{(Ca)}$), particularly in cells with a resting potential not dominated by inwardly rectifying K^+ currents. In ECs, the Na^+/K^+-ATPase contributes about -8 mV to the resting potential. Evidence for the existence and activity of a Na^+/Ca^{2+} exchanger has been documented in ECs

currents. In ECs, the Na^+/K^+-ATPase contributes about -8 mV to the resting potential. Evidence for the existence and activity of a Na^+/Ca^{2+} exchanger has been documented in ECs from pulmonary artery but its electrogenic participation to the resting membrane potential was not extensively studied [186].

I.5.1. Ca^{2+} Permeable Channels

In non-excitable cells, the release of Ca^{2+} from the $InsP_3$-sensitive store induces the activation of Ca^{2+} entry. Investigating the nature of this influx pathway with intracytosolic $InsP_3$ injection has demonstrated that Ca^{2+} entry was activated by $InsP_3$-induced pool depletion. This depletion current implicated both a phosphatase and an unidentified diffusible messenger, which might be responsible for Ca^{2+} entry activation [187]. Two low molecular weight tyrosine kinases, the 42 and 44 Kd MAP-kinases [188], and another MAP-kinase, (integrin-associated Ca^{2+} entry kinase) [189], as well as PKA and PKC [190] were implicated in the modulation of Ca^{2+} influx. Cations like Ni^{2+}, La^{3+}, natural organic molecules like heparin, synthetic molecules like SK&F 96365, the tyrosine kinase inhibitor genistein, cytochrome P-450 inhibitors and a Cl^- channel blocker (5-nitro-2-3-phenylpropylamino- benzoic acid, NPPB) all inhibit the Ca^{2+} influx [176]. The contribution of the Ca^{2+} influx to the whole-cell membrane current has been estimated from the Ca^{2+} signals induced by reapplication of high $[Ca^{2+}]_o$ ($\cong$10 mM) to thapsigargin-depleted Ca^{2+} stores in free $[Ca^{2+}]_o$. However, the CRAC current amplitude was merely about 1 pA/cell at a potential of –100 mV [191]. ATP and bradykinin activated a cation channel, selective for Ca^{2+} and Mn^{2+} in BAECs, that has an open probability increased by $InsP_4$ but not $InsP_3$. The channel open probability is about 50% lower at 1 μM $[Ca^{2+}]_i$ than at 1 mM $[Ca^{2+}]_i$. A Ca^{2+} permeable channel has been identified in numerous types of ECs and it was activated by agonists (ATP, BK) and by SERCA inhibitors (thapsigargin, BHQ). The channel

was 10 times more permeable to Ca^{2+} than Na^+, was characterized by a strong inward rectification and could be activated by $InsP_3$ but not by $InsP_4$ in excised patches [184]. The current decreased in elevated $[Ca^{2+}]_o$ while La^{3+} and heparin completely blocked it [184]. An $InsP_3$ receptor-like protein channel has been identified in the plasma membrane of endothelial caveolae, and it was implicated in the modulation of Ca^{2+} entry [192].

I.5.2. Nonselective Cation Channels in ECs

Many of the agonists linked to the Ca^{2+} signaling pathway in endothelial cells are activating nonselective cation channels (NSCCs) [193]. NSCCs open after agonist stimulation and the release of Ca^{2+} from internal pools. The single channel conductance for monovalent cations in physiological solutions range between 20 and 30 pS, whereas that for bivalent cations (Ca^{2+}, Ba^{2+}), it is between 4 and 12 pS [193]. So far, four different transmembrane pathways that are all nonselective for cations have been described in HUVECs. The first is a nonselective cation channel more permeable to Na^+ and K^+ than to Ca^{2+}, and agonists such as histamine can gate it. This channel, which provides an agonist-gated entry route for Ca^{2+}, has a single-channel conductance of 25 pS for Na^+ and K^+, and approximately 4 pS for Ca^{2+}. The second pathway corresponds to an ionic current activated by the selective SERCA blocker thapsigargin, BHQ and CPA, as well as by a direct intracytosolic application of $InsP_3$; it seems to be related to a Ca^{2+}-release from ER Ca^{2+}-stores. In ECs, this Ca^{2+}-entry route is cation selective, but cannot differentiate between Na^+ and K^+. Activation of this nonselective current is associated with an increase in intracellular Ca^{2+} and it is assumed that it corresponds to the Ca^{2+}-entry activated by store depletion in micro-vessels as well as macro-vessels [194]. A third Ca^{2+}-permeable pathway can be activated by shear stress, whereas the fourth pathway is a nickel-blockable, Ca^{2+}-permeable nonselective leak, which is described in nonstimulated ECs [195]. In general,

these channels do not discriminate between monovalent cations, and the permeability ratio of Ca^{2+} over monovalent cations ($P_{Ca}/P_{Na,K}$) is between 0.2 and 0.6 [196]. The antihypertensive drug LP805 activates a NSCC but independently of a $[Ca^{2+}]_i$ increase and a depletion of Ca^{2+} pools [194]. Another 30 pS NSCC channel, equally permeable to Na^+ and K^+, is also permeable to Ca^{2+} and it is activated by oxidative stress. This channel opens in two gating modes that do not depend on $[Ca^{2+}]_i$ and the depletion of Ca^{2+} stores. For that reason, the activation of this channel and the concomitant membrane depolarization can substantially limit the entry of Ca^{2+} in ECs submitted to oxidative stress [194]. This channel activity was weakly voltage dependent at negative membrane potentials and its activity did not depend on $[Ca^{2+}]_o$. Ca^{2+} permeated this channel and the calculated permeability ratios was: $P_K:P_{Na}:P_{Ca}$ = 1:1:16. Histamine, when applied inside the pipette, transiently activated the channel. Neither acetylcholine nor GTPγS applied to the intracellular surface of the patch could mimic the effect of histamine and it was concluded that the channel is not Ca^{2+} dependent and histamine, but not acetylcholine, activates this cation channel to elevate endothelial $[Ca^{2+}]_i$ [197].

I.5.3. The I_{CRAC} Channel

In many electrically non-excitable cells, the store-operated Ca^{2+} influx pathway mediates predominantly the entry of Ca^{2+}. The best-characterized store-operated Ca^{2+} current is the Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}). It is present in many non-excitable cells, like endothelial cells, CPAE, HEK, HEK-293 (an embryonic kidney cell line) and Jurkat cells (the reference cell model for I_{CRAC}). It is generally believed that high concentrations of intracellular Ca^{2+} buffer are required to measure I_{CRAC}, due to Ca^{2+}-dependent inactivation of these channels. In bovine pulmonary artery endothelial cells (BPAE), depletion of intracellular Ca^{2+} stores by $InsP_3$, Ca^{2+} ionophores and Ca^{2+} pump inhibitors, activates a Ca^{2+}-selective conductance in the presence of

the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N'N'-tetra acetic acid (BAPTA). The current shows inward rectification, a highly positive reversal potential and it is blocked by micromolar concentrations of La^{3+}. As shown in Jurkat cells, larger Na^{+} currents are detectable in BPAE cells following store depletion in Ca^{2+}- and Mg^{2+}-free medium [198].

I.5.4. The trp and trpL Channels

A family of proteins related to *trp* genes is conserved from *Caenorhabditis elegans* to humans, and recent evidence indicates that at least some of these proteins are components of SOCs. In *Drosophila melanogaster* eye, the photoactivation of rhodopsin, through a phospholipase C-dependent mechanism, leads to the opening of light-sensitive cation channels, called *trp* (**t**ransient **r**eceptor **p**otential) and *trp*L (**t**ransient **r**eceptor **p**otential-**L**ike). *Drosophila*'s visual transduction channels, associated with the receptor *trp* and *trp*L, were the first members of this category of channels to be identified at the molecular level.

A steadily growing number of genes coding for proteins that are structurally related to the *trp* ion channels have been cloned in recent years. The expression of these genes in antisense orientation in L cells suppressed the agonist-induced CCE. On the contrary, the expression of two full-length cDNAs encoding human *trp* homologs, H*trp*1 and H*trp*3 in COS cells, significantly increased the agonist-induced CCE [182]. All these proteins display a topology of six transmembrane segments shared with some voltage-gated Ca^{2+} channels and cyclic-nucleotide-gated channels.

Based on their homology, the proteins encoded by *trp* genes can be divided into three *trp* protein-channel (*trp*C) subfamilies: *short* (S), *long* (L) and *osm* (O), (from osmolarity). The subdivisions were made according to channel function; therefore the "S*trp*C" family includes

Drosophila *trp* and *trp*L and the mammalian homologues, *trp*C from 1 to 7. Accordingly, *trp*C is a family of Ca^{2+}-permeable cation channels activated after receptor-mediated stimulation of different isoforms of phospholipase C and for emptying Ca^{2+} pools. Members of the "O*trp*C" family are Ca^{2+}-permeable channels involved in pain transduction (vanilloid and vanilloid-like receptors), epithelial Ca^{2+} transport and, at least in *Caenorhabditis elegans*, in chemo-, mechano- and osmoregulation. The human *trp*-related proteins may mediate many of the store-operated conductances that have been identified previously in a plethora of human cells.

There are 7 mammalian *trp*s designated either *trp*1 through *trp*7 or TRPC1 through TRPC7. In ECs, Vaca and Kunze have demonstrated the presence of a channel, which was activated by the depletion of Ca^{2+} pools with the SERCA inhibitor TG. This small channel, when analysed in excised patch, ran down quickly but its activity was restored after the addition of $InsP_3$ [184,199]. Later, Kiselyov et al observed the same behaviour in stably transfected HEK-293 cells with the gene for *trp*3 channel. Moreover, after prolonged perfusion in excised patch the run-down occurred even in the presence of $InsP_3$ and recovery required the addition of $InsP_3R$, or more specifically, the N-terminal $InsP_3$ binding domain of $InsP_3R$ [200]. Furthermore, amino acid sequences of a *trp* and of $InsP_3R$ that interact directly and form stable complexes were evidenced by co-immunoprecipitation. These sequences generate *in vivo* interacting complexes that modulate the evolution of CCE. The finding that a *trp* and $InsP_3R$ interacting sequence interferes with natural CCE leads to the conclusion that the *trp* proteins are structural members of CCE channels. The direct coupling of $InsP_3R$ to *trp* is a physiological mechanism by which cells trigger CCE in response to signals that stimulates phosphoinositide hydrolysis and $InsP_3$ formation [185,201]. It was concluded that CCE have *trp* as one of their normal structural components, and that these channels are directly activated by $InsP_3Rs$.

$InsP_3Rs$ have the dual role of releasing Ca^{2+} from stores and activating Ca^{2+} influx in response to either increasing $InsP_3$ or decreasing luminal Ca^{2+} [185].

I.5.4.1. *Trp*

Trp appears to be a store-operated channel (SOC) when expressed in mammalian cells. Expression of *trp* in SF9 insect cells induces an increase in $^b[Ca^{2+}]_i$, with a basal Ba^{2+} influx and an increase of plasmalemmal Ca^{2+} permeability index. SKF-96365 and La^{3+} block all these Ca^{2+} manifestations. However, the thapsigargin-induced change in $[Ca^{2+}]_i$ are greater in *trp*-expressing cells than in controls. Whole-cell membrane currents recorded in *trp*-expressing cells increased as a function of post-infection time and were inwardly rectifying in symmetrical sodium gluconate solutions. The currents were not selective with respect to Na^+, Ca^{2+} and Ba^{2+} and blocked by La^{3+}; their conductance was not voltage dependent. Additionally, *trp* currents were increased by thapsigargin whereas the dialysis of the cell with $InsP_3$ or with solutions containing high concentrations of the Ca^{2+} chelator, EGTA were without effect. The m*trp*6 is activated by G_q-dependent receptor stimulation but its activation is insensitive to TG-induced pool emptying. Kiselyov et al. have demonstrated that *trp*3 is activated by a direct interaction with $InsP_3R$ [181], whereas Hofmann et al. sustain that *trp*3 and *trp*6, more likely have a DAG-dependent activation, independent of $InsP_3$-production and/or PKC activation [202]. The relative permeability of Ca^{2+} is higher than the permeability of Na^+ (25-40:1) whereas the Mg^{2+} permeability is smaller in comparison with the Ca^{2+} permeability. Despite the higher permeability for Ca^{2+} than for Na^+, the *trp*-dependent currents are larger when Na^+ rather than Ca^{2+} is used as permeant ion, most likely due to the Ca^{2+}-mediated inactivation of *trp*, a feature that may be similar with the Ca^{2+}-dependent inactivation of I_{CRAC}. Thus, as a general consensus, *trp* forms non-selective cation channels that are constitutively active when expressed in Sf9

and/or mammalian cells; but they are sensitive to depletion of internal Ca^{2+} stores, suggesting that *trp* may be a functional subunit of a SOC, which alone can form a functional channel. However, more likely, these channels require additional subunits or cytoplasmic factors, present in mammalian cells, to display SOC activity [203].

Despite these data, the co-expression of *trp* and *trp*L *in vitro* (insect cells, *Xenopus levis* oocytes) and the expression of functional heteromultimer proteins lead to the appearance of store-operated currents distinct from currents produced by expression of the individual proteins. In addition, the transient transfection experiments with H*trp*C1 antisense construct in cells, already stably expressing H*trp*C3 construct, induced an important level of inhibition (55%) of store-operated Ca^{2+} entry [204]. An examination of tissue expression of *trp* and *trp*L in five mammalian *trp* genes shows tissue-specific and cell-specific co-expression of multiple *trp* forms. This implies that the subunit composition of a particular CCE channel may vary in structure and function depending on the cell type [203].

There is a striking difference in ion selectivity and conductance between I_{CRAC} channels, which are highly Ca^{2+} selective but with a very small conductance (≈20 fS) and the trp and trpL channels characterized by a relative low Ca^{2+} selectivity and a larger Ca^{2+} conductance (≈3 pS). These facts indicate that the trp and trpL channels are a family of store-operated channels different from the highly selective CRAC channels.

I.5.4.2. *Trp*L

*Trp*L channels were studied in cell-attached, inside out, and outside-out membrane patches from Sf9 insect cells transfected with *trp*L cDNA. The single-channel current-voltage relationship was linear from -100 to +80 mV with a slope conductance of 89-110 pS. It was associated with the appearance of an outwardly rectifying nonselective cation current, related to a Ca^{2+} entry

and activated without external stimulation. The opening probability was voltage sensitive, increasing at positive potentials and contributing to the outwardly rectifying properties of the whole cell currents. These channels are nonselective with respect to Na^+, Ca^{2+}, and Ba^{2+}, and are blocked by Gd^{3+} as well as by 4-8 mM Mg^{2+}. Therefore, *trp*L differs essentially from *trp* in its lack of ion selectivity, with respect to Na^+, Ba^{2+} and Ca^{2+} and relative insensitivity to inhibition by La^{3+}. However, the rank selectivity in the presence of extracellular bivalent cations was Ca^{2+}>Sr^{2+}>> Ba^{2+}. The single *trp*L channel activity increases spontaneously through time after patch formation and its activity is further increased by application of bradykinin to cells expressing both the B2-bradykinin receptor and the *trp*L protein, suggesting that activation occurs via a diffusible cytoplasmic messenger, possibly $InsP_3$ or some other downstream effector such as Ca^{2+} [205]. Birnbaumer et al. conclude that these *trp*L channels are not activated by depletion of internal Ca^{2+} stores; they are most likely activated by $InsP_3$ and/or DAG and possess two putative calmodulin (CaM)-binding domains, which favour a *trans*-stimulation by Ca^{2+} entry via CCE [201].

I.6. EFFECT OF OXIDATIVE STRESS ON CALCIUM SIGNALING

Endothelial cells are remarkable examples of a multifunctional cell type. These multiple functions are mediated by the production and the release of a variety of vasoactive agents that affect the cells in the vessel wall or in its immediate vicinity, including ECs. Our current knowledge of the mechanisms that regulate the production of these substances is very limited. However, it is well documented that production and release of most of paracrine agents is initiated by Ca^{2+}-dependent mechanisms. Solid evidence demonstrate that $NO^{\bullet}$ production by endothelial isoforms of nitric oxide synthase (cNOS or eNOS) is regulated continuously by calmodulin binding, triggered by transient elevations in $[Ca^{2+}]_i$ levels. Additional modes of cNOS regulation are implicated in the effects of some stimuli such are shear-stress, estrogens, insulin or insulin-like growth factors, that elicit a sustained increase in cNOS activity despite undetectable or transient increases in intracellular Ca^{2+} in endothelial cells [206]. However, consistent with its present classification as a Ca^{2+}-calmodulin-dependent enzyme, eNOS can be activated by receptor-dependent and/or -independent agonists as a consequence of an increase in $[Ca^{2+}]_i$ and the association of the Ca^{2+}-calmodulin complex with eNOS. Therefore, it was hypothesized that the oxidative stress is responsible for the alteration of Ca^{2+} homeostasis in ECs, associated with a subsequent reduction of $NO^{\bullet}$ availability. The entire cascade of this physiopathological process is positively correlated with human essential hypertension and some animal models of experimental hypertension [207]. The intracellular redox steady state is one of the most important characteristics of the chemical environment in all cells. The uncontrolled production of oxygen derived free radicals or the oxidative stress will perturb irreversibly the intracellular redox state (Figure 12).

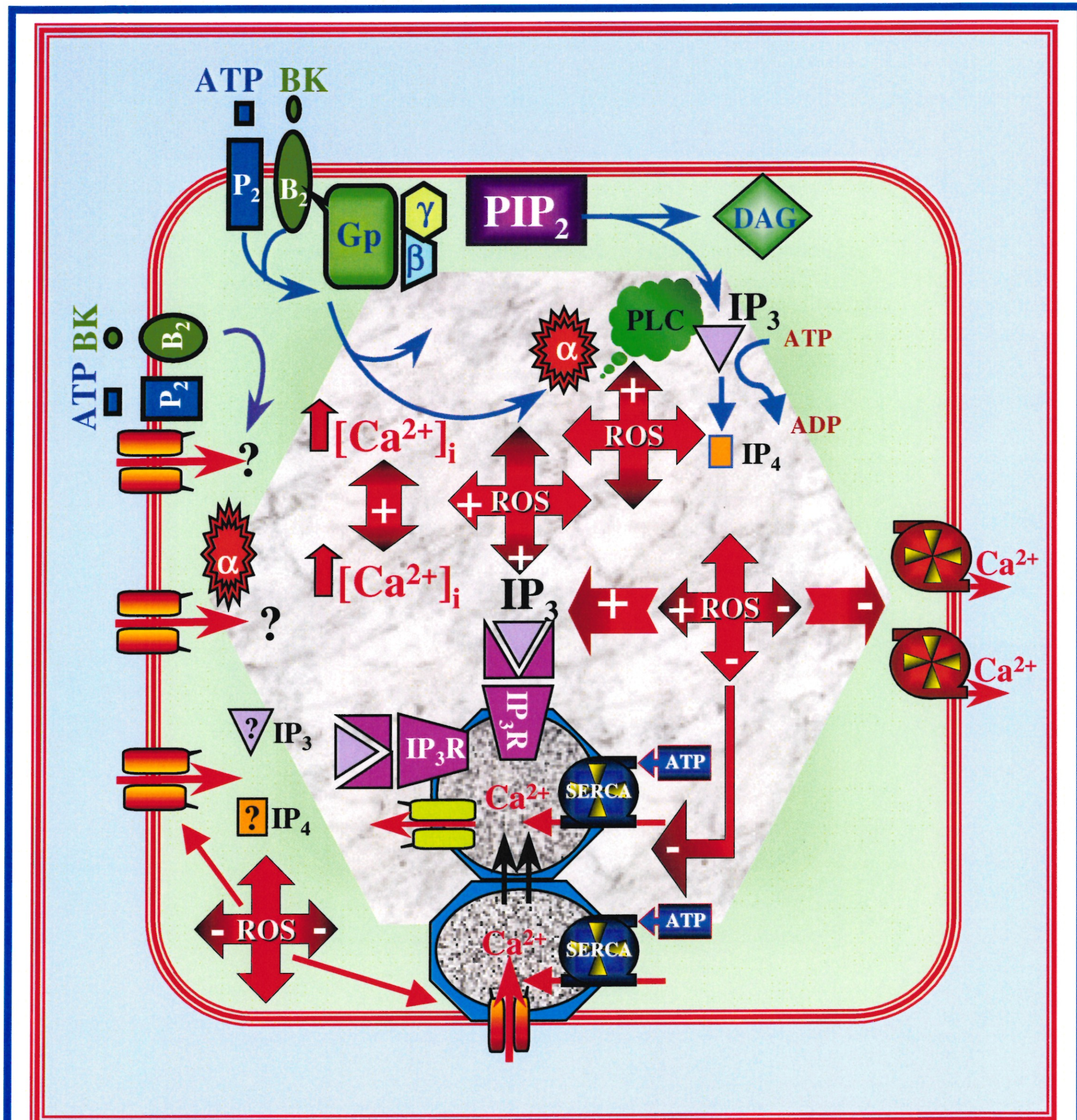


Figure 12.
Pathways sensitive to ROS in agonist - induced changes in [Ca^{2+}]$_i$.
ROS induce stimulatory effects on the activity of PLC and increase the Open probability of IP_3R, both effects inducing an increase of [Ca^{2+}]$_i$.
ROS have inhibitory effect on the activity of SERCA and the plasmalemmal Ca-ATPase with the same outcome on the increase of [Ca^{2+}]$_i$.
Thus, the outcome of ROS effect is an inevitable increase of [Ca^{2+}]$_i$.

The endothelium regulates vascular reactivity through its paracrine capability, a function that rigorously depends on Ca^{2+} homeostasis. Oxidative stress alters the control mechanisms of Ca^{2+} homeostasis of endothelial cells. Consequently, the oxidative stress ought to affect

vascular reactivity and permeability via alteration in the production, the release, and/or the effect of endothelium-derived paracrine substances.

Schilling and Elliott [122] describe the establishment phases of artificially induced oxidative stress through *tert*-butyl hydroperoxide (t-BOOH) in BAECs. t-BOOH is a liposoluble hydroperoxide with a high penetration capacity and not destroyed by catalase. Actually, it is such a strong oxidant that, in the micromolar ($3\text{-}5*10^{-4}$M) range, it is able to overcome the buffering capacity of GSH and inhibit γ-glutamyl synthase, breaking the regenerative capacity of GSH. The classical effect of t-BOOH in inducing oxidative stress is characterized by an alteration of agonist-stimulated Ca^{2+} signaling in three phases.

I.6.1. The initial phase of oxidative stress

In the **initial phase of oxidative stress** induced by t-BOOH, the agonist-induced Ca^{2+} influx is diminished, but the basal level of $[Ca^{2+}]_i$ remains under control, at a low level. If the t-BOOH-induced oxidative stress is prolonged, the receptor-activated release of Ca^{2+} from intracellular stores is further inhibited and the $^b[Ca^{2+}]_i$ progressively begins to rise. At this stage, if the aggressor continues to be present and active, the $^b[Ca^{2+}]_i$ progressively rise and the Ca^{2+} responsiveness diminishes until complete annihilation. The rise of $^b[Ca^{2+}]_i$ activates the Ca^{2+}-dependent K^+ channels, which leads to a depletion of cellular K^+ and Cl^-. The mechanism by which oxidative stress inhibits the agonist-stimulated Ca^{2+} entry pathway during the initial phase remains obscure.

Free radicals may directly modify channel protein properties responsible for the agonist-induced $[Ca^{2+}]_i$ release from intracytosolic pools. Electrophysiological studies on frog cardiac myocyte have shown that t-BOOH induces selective inhibition of voltage-activated Na^+ current,

without alteration of K^+ and Ca^{2+} currents [208]. Moreover, a loss of membrane potential has been induced by dihydroxyfumaric acid in isolated canine myocytes [209] and the depolarization induced by H_2O_2 (from –60 mV to –27 mV) in endothelial cells may be responsible for the reduction of CCE [210].

The first phase of oxidant stress would appear through an alteration of agonist-induced Ca^{2+} response.

I.6.2. The intermediate phase of oxidative stress

In the **intermediate phase** of oxidative stress induced by t-BOOH, there appears an inhibition of receptor-activated Ca^{2+} release from intracytoplasmic stores and a substantial increase of the $^{b}[Ca^{2+}]_i$. A prolonged incubation (up to 2 h) of ECs in t-BOOH will substantially decrease the agonist induced Ca^{2+} response.

This phenomenon is also observed when ECs are stimulated in Ca^{2+} free solutions, indicating that besides the inhibition of Ca^{2+} influx, the oxidative stress inhibits the receptor-activated release of Ca^{2+} from intracytoplasmic pools. This process occurs due either to an alteration of the signaling cascade responsible for Ca^{2+} release or to a slight depletion of Ca^{2+} from intracellular stores. After stimulation, Ca^{2+} is released into the cytosol and it is extruded in extracellular space by the membrane Ca^{2+}-ATPases. In experiences with calf pulmonary artery ECs, bradykinin induced a 12-fold increase in unidirectional efflux of $^{45}Ca^{2+}$, whereas in oxidative stress with ECs treated during the intermediary phase, the $^{45}Ca^{2+}$ efflux was reduced by 50% compared with control conditions [23]. This inhibition by t-BOOH may reflect important damages inflicted to the endoplasmic reticulum SERCAs and/or plasmalemma Ca^{2+}-ATPases of ECs, which will lead to depletion of Ca^{2+} internal stores. In fact the t-BOOH-

induced oxidative stress was reported to cause an inhibition in the breakdown of inositol polyphosphates [211], a decreased microsomal Ca^{2+} sequestration due to the inhibition of Ca^{2+}-ATPase activity [212] and an inhibition of ATP-dependent Ca^{2+} sequestration in inside-out plasmalemmal vesicles [213]. The mechanism responsible for oxidant inhibition of Ca^{2+}-ATPase activity seems to be the oxidation of critical sulfhydryl groups [213,214]. The inhibition of bradykinin-stimulated $^{45}Ca^{2+}$ efflux argues for an oxidant-induced inhibition of the plasmalemmal Ca^{2+}-ATPase. Alternatively, $^{45}Ca^{2+}$ may actually bypass the cytoplasm and exit the cell via specialized junctions between the ER and the plasmalemma. Therefore, an alteration in the coupling between the internal store and the plasmalemma may contribute to oxidant-induced reduction in agonist-stimulated $^{45}Ca^{2+}$ efflux [23].

I.6.3. The final phase of oxidative stress

The **final phase** of oxidative stress induced by t-BOOH is characterized by a loss of responsiveness to agonist and a sustained elevation of $^{b}[Ca^{2+}]_i$. Following incubation with t-BOOH for 3h, ECs seem to loose their ability to maintain their normal $^{b}[Ca^{2+}]_i$. It increases to ≈200 nM, which is more than twice the normal value and a large inward Ca^{2+} gradient is maintained. This final phase is characterized by failure of $[Ca^{2+}]_i$ to increase following bradykinin stimulation, by the annihilation of intracytosolic $^{45}Ca^{2+}$ uptake and extracellular efflux [215].

Unidirectional ouabain-sensitive $^{86}Rb^{+}$ influx is stimulated by the application of oxidative stress to ECs. This is suggesting that the Na^{+}-K^{+}-ATPase pump activity is actually stimulated by oxidative stress [216]. Therefore, the pool of ATP might be sufficient to sustain an increased activity of Na^{+}-K^{+}-ATPase under these conditions, suggesting that intracellular ATP depletion is not the major cause for changes in Ca^{2+} signaling.

The oxidative stress activated Na^+-K^+-ATPase pump is associated also with an increase in $[Na^+]_i$ and a consecutive decrease of $[K^+]_i$. The elevation of $[Ca^{2+}]_i$ activates the $K^+_{(Ca)}$ channels, favouring an increase in K^+ efflux. The cumulative effect of these events will induce important changes in the ionic composition of the intracellular environment. The measurements of whole cell currents obtained from oxidant-treated cells in confluent monolayers reveal a current with a reversal potential under normal ionic conditions ($[K^+]_{pipette}$=140 mM and $[Na^+]_{bath}$=140 mM) of -28 mV and, at the single channel level, a calculated conductance of 25 pS. This suggests that oxidative stress may be associated with the activation of a nonselective cation channel, which in association with an increased permeability of the membrane to Na^+, will depolarize the cell and will contribute to the sustained increase of $[Ca^{2+}]_i$ [217].

I.7. THE PHYSIOLOGY OF MELATONIN

Melatonin is the main hormonal hormone of the pineal gland and among the hormones secreted by the diffuse neuroendocrine system. Melatonin is involved in photic regulation, adaptation to circadian and seasonal light photoperiods, and seasonal cycles of reproduction, fasting, thermoregulation, and hibernation. Melatonin controls, in response to light, the circadian rhythms driven by endogenous oscillators. The duration of a pineal melatonin pulse positively correlates with the length of photoperiods, with short pulses in summer and long pulses in winter. In all species, the rhythmic production of melatonin during the night makes it the nocturnal endocrine marker in the entire animal kingdom [218]. In the last decade, melatonin was intensively promoted as an anti-aging and a life prolonging agent [219], as well as in insomnia [220], "jet lag" [221] and as an antioxidant treatment. Melatonin decrease DNA damage in rats treated with the carcinogen safrole [222] and limits paraquat damage to rat lung [223]. It suppresses the development of cataracts in newborn rats treated with the glutathione depleting agent buthionine sulfoximine [224], decreases lipid peroxidation in the liver of rats exposed to carbon tetrachloride [225] and limits alloxan toxicity to pancreatic cells in mice [226].

I.7.1. Melatonin Synthesis and Metabolism

Melatonin synthesis occurs mainly in pinealocytes but also in plenty of melatonin-producing cells of the diffuse neuroendocrine system. The mechanism triggering the synthesis of melatonin by pinealocytes and the diffuse neuroendocrine system is still unknown. Melatonin is synthesized from serotonin in two steps (Figure 13). The first step is controlled by light since the enzyme arylalkylamine N-acetyltransferase, implicated in the first step of

melatonin synthesis, is light sensitive (light inhibits enzyme's activity). Thus, in the first step, in the absence of light, serotonin's *N*-acetylation is catalyzed by the enzyme arylalkylamine N-acetyltransferase from which are derived one or more intermediary by-products. The second step is an *O*-methylation of the unidentified *N*-acetylated by-product from the first step, catalyzed by hydroxyindole-O-methyltransferase [227]. Apart from the pineal gland, melatonin synthesis has been identified, with the same rhythmical daily base, in the retina, Harderian gland, gut mucosa, cerebellum, airway epithelium, liver, kidney, bone marrow, adrenals, thymus, thyroid, pancreas, ovary, carotid body, placenta and endometrium, mast cells, natural killer cells, eosinophilic leukocytes, platelets and endothelial cells [227].

I.7.2. Melatonin activity and signal transduction

McCord and Allan demonstrated the first biological activity of melatonin in 1917 with bovine pineal gland extracts, which induced the bleaching of tadpole skin from *Rana Pipiens*. After exposure to light, the melatonin concentration declines, resulting in dispersion of the pigment granules and darkening of the cell. Melatonin, synthesized in the retina, participates in the modulation of day and night cyclic variations in photoreceptor and retinal pigment epithelium function. It does so by regulating the amount of light reaching the photoreceptor through the control of the melanosome's granule movement within the retinal pigment epithelium. During dark adaptation, rod input is favoured, whereas light adaptation leads to the suppression of rod input and enhancement of cone input [228]. Specific high-affinity receptor proteins mediate the effects of melatonin. These receptors belong to the subfamily of seven transmembrane receptors present in plasma membrane of targeted cells. Their structure shows multiple consensus sites for protein kinase C phosphorylation and they are coupled with pertussis toxin and cholera toxin sensitive G-proteins.

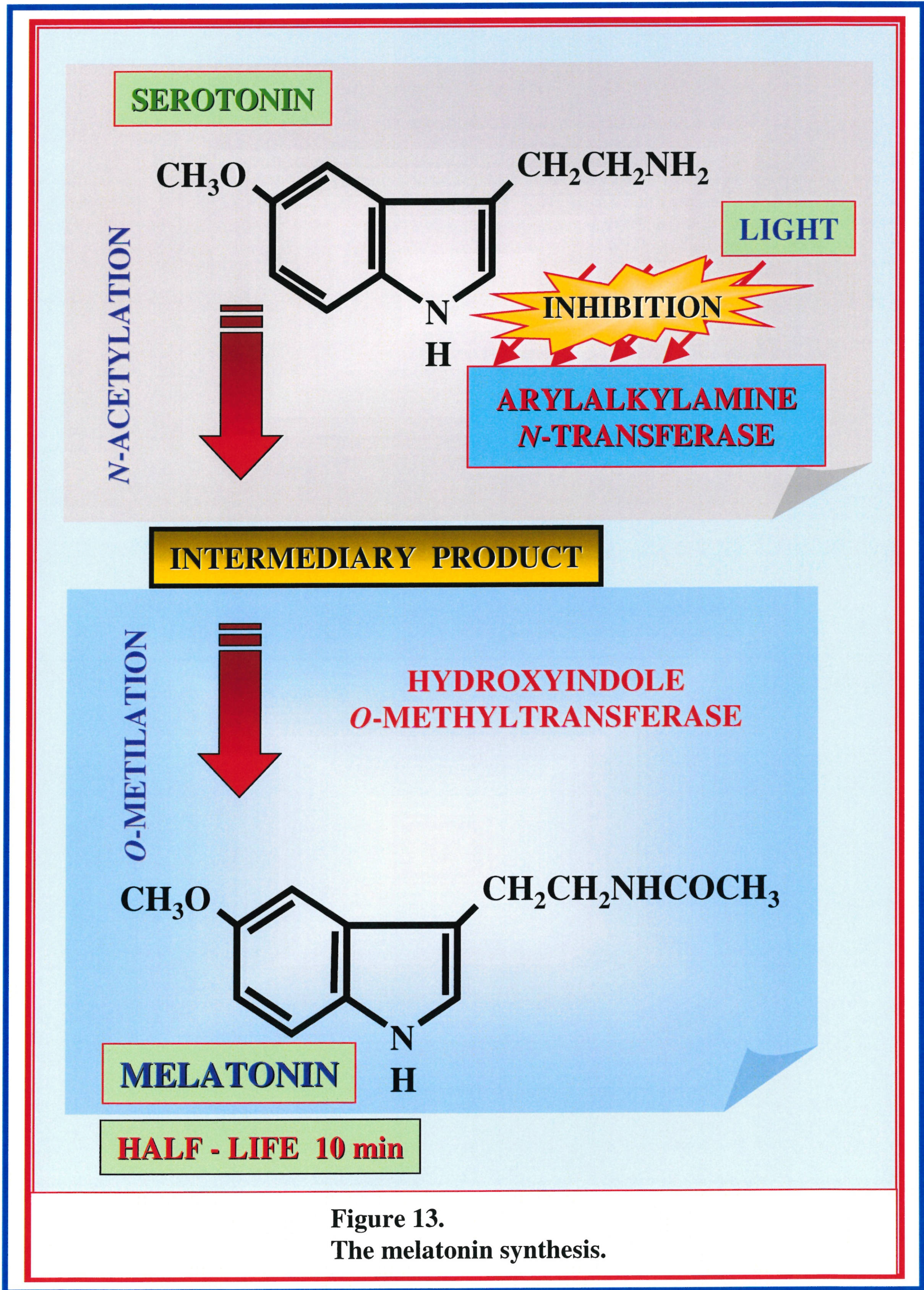


Figure 13.
The melatonin synthesis.

Melatonin binds three pharmacologically and kinetically distinct receptors, defined as MT1 (with picomolar affinity), MT2 (with nanomolar affinity) and MT3 sites [229]. The existence of MT3, a novel putative melatonin receptor, was described in nervous and peripheral tissues of Syrian hamster and recently recognized as being a hamster homologue of the human quinone reductase 2 [230]. MT1 was the first melatonin receptor found in the rabbit retina [231].

The binding of 2-[^{125}I]-iodomelatonin to the MT1 site is reversible, saturable, and show high affinity (< 300 pM), slow association and dissociation kinetics, as well as temperature dependence (affinity increases with temperature). The main signal transduction pathway of MT1 receptors in both neuronal and non-neuronal tissues is the inhibition of cAMP formation through a pertussis toxin-sensitive inhibitory G protein [232].

To date three subtypes of the MT1 receptor have been identified. $MT1_A$, expressed in the brain of mammals and birds and $MT1_C$, expressed mainly in the mammalian retina, are almost indistinguishable (Kd ≈ 20-40 pM), whereas $MT1_B$ has a wider distribution in the animal kingdom and a higher Kd (Kd ≈ 160 pM) [218].

A putative MT2 receptor has been identified so far in avian and mouse brain, kidney, and testes along with in hamster melanoma cell line. The binding of 2-[^{125}I]-iodomelatonin to MT2 site is reversible, saturable, and shows lower affinity than MT1 (Kd ≈ 0.9-10 nM), fast association and dissociation kinetics, and temperature dependence (affinity decrease at higher temperature).

The specific antagonists are luzindole for MT1, N-acetyltryptamine for both, and prazosin for MT2, but their affinity and pharmacological profile are complicated by interference with melatonin active-metabolites into a structure-activity relationship dependent of tissue

specificity. Specific antagonists are capable of antagonizing the melatonin-mediated stimulation of phosphoinositide hydrolysis. The physiological role of MT2 is still unknown [233]. The putative binding site of so-called MT3 receptor and its pharmacological profile proved to be completely different from MT1 and MT2. It can be described as sensitive to 2-iodomelatonin > 6-chloromelatonin > methyl-isobutyl-amiloride > acridine orange > 5-methylcarbonylamino-N-acetyltryptamine > prazosin > N-acetylserotonin > melatonin, and possessing very rapid kinetics of binding and ligand exchange compared with MT1 and MT2 [234].

The pattern of receptor distribution varies on a species-to-species and even a tissue-to-tissue basis but it is considered ubiquitous, with a larger distribution in the central nervous system, endocrine glands, and retina. The most well known mechanism of melatonin action is described in pituitary cells. In these cells, melatonin binds to high affinity receptors coupled with the PTX-sensitive G proteins, most likely G_i/G_o. These inhibitory effects seem to reach several downstream intracellular messengers expressed by an accumulation of cAMP and cGMP, an increase of diacylglycerol synthesis and a release of arachidonic acid.

Previous studies have underlined the existence of melatonin receptors in endothelial cells from cerebral and rat caudal arteries [235]. In the later, melatonin potentiates contractile responses to exogenous noradrenaline as well as nerve stimulation through activation of a site with the pharmacology of an MT1 receptor [235]. This effect of melatonin was blocked by luzindole (K_b = 370 nM), but not by the 5-HT_2 receptor antagonist ketanserin [236]. The pharmacological differences in the potency of agonists and the affinity of the receptor antagonist luzindole in the rabbit retina MT1 receptor suggest that its molecular structure, its coupling mechanism, or both, may differ from that of the non-neuronal melatonin receptor of amphibian melanophores and of rat caudal artery [237]. Finally, nuclear receptors RZR_β, retinoic acid and

retinoid-X receptor, which are present in virtually all cells, show melatonin binding and were recently isolated and cloned [235].

Indeed, due to its high lipophilic property, evidence suggests that melatonin can access cytosolic and nuclear sites to mediate a variety of cellular effects. One of the mechanisms of melatonin action may be through specific binding to CaM. Melatonin binds to CaM with a high affinity, and has been shown to act as a powerful, specific, and natural calmodulin antagonist [238]. Melatonin is capable of preventing cell proliferation and apoptosis by regulating the cell cycle. In relation with oxidative stress, melatonin can regulate mRNA for an enhanced synthesis of antioxidant enzymes [95].

I.7.3. Melatonin's antioxidant activity

The indole structure of melatonin can exert direct antioxidant effects by scavenging free radicals and/or inhibiting their formation. As an antioxidant, it was demonstrated to act efficiently against DNA damages and to decrease of lipid peroxidation. This effect has been demonstrated *in vitro* [239] against lipid peroxidation [240] and oxygen free radical production [241] in a wide variety of cellular preparations [242], even though the mechanism of scavenging remains obscure and controversial [240]. *In vitro* experiments, with systems for evaluating the radical scavenging capacity, have demonstrated that melatonin was 2 times more active than vitamin E in scavenging $ROO^\bullet$, a by-product of lipid peroxidation. It has been demonstrated in ECs from human umbilical arteries that melatonin significantly suppresses the H_2O_2-induced inhibitory effect on $NO^\bullet$ production through its ability to scavenge $^\bullet OH$ [93].

I.7.4. Melatonin's vascular activity

In the last decade, an important amount of evidence was accumulated supporting the implication of melatonin in the regulation of vascular smooth muscle cell tone, vascular reactivity, and arterial pressure. The hypotensive efficiency of melatonin was observed in patients with essential hypertension [96] and in genetically hypertensive rats [97]. The hypotensive action of i.v. infused melatonin appears to be associated with an inhibition of the basal sympatho-adrenal tone, mediated partly by the blockade of postsynaptic α1-adrenergic receptor-induced inositol phosphate formation [243].

However, several studies have reported contradictory results about the interaction between melatonin and vascular reactivity. In acute administration on different vascular preparations, melatonin was demonstrated to relax isolated rat and rabbit aorta [244] as well as iliac and renal arteries [245]. In rat caudal artery, melatonin potentiates contractile responses to exogenous noradrenaline as well as nerve stimulation, through a site whose pharmacology resembles that of an MT1 receptor [235]. Potentiation of contractile responses induced by electrical stimulation, KCl-induced depolarization [246], phenylephrine and/or serotonin, were observed in rat cerebral artery [98], rat caudal artery and porcine coronary artery. Both, melatonin and/or its active analogs induced these effects [247].

CHAPTER II. RATIONALES AND HYPOTHESIS

II.1. RATIONALES OF THE LITERATURE REVIEW

Our laboratory has been involved in the study of Ca^{2+} homeostasis in several cell lines, including endothelial cells. A comparison between ECs from SHR and WKY rats has demonstrated an altered Ca^{2+} signaling in ECs from hypertensive rats. These results emphasize the dimension of Ca^{2+} responses induced by different agonists [47,248]. The actual knowledge and the actual specialized literature provide only a few data regarding the oxidative stress effects on ECs Ca^{2+} homeostasis in response to Ca^{2+} mobilizing agents. There was therefore a need to investigate the status of Ca^{2+} homeostasis under the effect of oxidative stress in comparison with the Ca^{2+} homeostasis of ECs from SHR and the overall effect of antioxidant treatment on Ca^{2+} homeostasis.

Normal endothelial Ca^{2+} responses to hormones, autacoids, and agonists represent the key modulatory event of vascular reactivity. An altered Ca^{2+} responses of ECs may thus be involved in the impaired endothelium-dependent vascular modulation and consequently, in the pathogenesis of numerous cardiovascular diseases, including idiopathic hypertension. The ECs' Ca^{2+} homeostasis is altered overwhelmingly and often irreversibly by artificial and/or pathological oxidative stress. Therefore, an endogenous overproduction of free radicals by the endothelium of SHR, associated with an abnormal Ca^{2+} signaling mechanism and an impaired paracrine function, might be positively correlated with the pathogenesis of this model of hypertension. A review of the literature reveals that, despite the crucial importance of Ca^{2+} signaling in the control of endothelium-dependent vascular reactivity, the homeostasis of Ca^{2+} in primary endothelial cells from normotensive and hypertensive rats remains poorly described and understood. The complex and intricate chemistry of free radicals and their implication in

general physiology and pathology, especially in the context of the endothelium remain ambiguous and often controversial. Furthermore, the effect of oxidative stress and the manner by which the free radicals are affecting Ca^{2+} signaling was never experimented in primary culture of rat endothelial cells. A parallel between Ca^{2+} homeostasis of ECs from SD rats under oxidative stress with global Ca^{2+} homeostasis of ECs from SHR was never investigated. There is substantial evidence that oxidative stress has deleterious effects on Ca^{2+} signaling in many strains of endothelial cells. Several questions remain unanswered such as how the cascade of events responsible of Ca^{2+} signaling is affected by free radical production, which free radical is mostly implicated in the poisonous effects, and which components of the signaling cascade are most vulnerable and affected. The antioxidant treatment has revealed multi-beneficial effects in many experimental protocols, *in vitro*, in different living cell preparations, in various pathological animal models, as well as diverse human clinical trials of a range of pathological conditions related to oxidative stress. However, there are not yet presented assays of specific and non-specific antioxidant effects on Ca^{2+} signaling under oxidative stress of ECs from normotensive rats in comparison with Ca^{2+} signaling in ECs of hypertensive rats.

The magnitude and the availability of Ca^{2+} pools, the manner in which internal Ca^{2+} stores are organized along with their sensitivities and responses to $InsP_3$-generating and non generating agonists are otherwise poorly understood. Furthermore, the dynamics of intracytosolic Ca^{2+} pools and their availability in physiological conditions as compared to oxidative stress have never been studied in primary cultures of aorta ECs from normotensive and hypertensive rats.

Melatonin was successfully used in lowering blood pressure in genetically hypertensive rats. The effect of melatonin in lowering blood pressure was also signaled in human

hypertensive subjects. Beside other effects, the antioxidant property of melatonin has been recognized and its mechanism was relatively characterized even though it is still under enormous controversy, primarily because of the complexity of free radical reactions and the difficulty to provide evidence for these reactions. However, there is no description in the literature yet with reference to the hormonal effects of melatonin on endothelial cells. Whether the aorta ECs have melatonin specific receptors related to the Ca^{2+}-signaling cascade is unknown. How melatonin influences the Ca^{2+} homeostasis in ECs and what is the effect of melatonin under the oxidative stress conditions in normotensive and hypertensive rat ECs has not been yet investigated or reported.

II.2. HYPOTHESIS

These considerations led to the following hypotheses:

1. The Ca^{2+} signaling transduction process in ECs from SHR is affected by an overproduction of free radicals;
2. ECs have several types of internal Ca^{2+} pools with differential sensitivity to oxidative stress and antioxidants;
3. Melatonin affects differently the Ca^{2+} homeostasis of ECs from SHR and normotensive rats, partially through free radical scavenging properties.

II.3. EXPERIMENTAL APPROACHES

Therefore, our experimental approaches will consist of:

1. Measurement of the Ca^{2+} responses in different endothelial cell lines, with emphasis on the Ca^{2+} responses obtained from rat ECs in primary cultures after stimulation with $InsP_3$-dependent and $InsP_3$-independent agonists;

2. Evaluation of the modulatory effects of several different level of oxidative stress on the different mechanisms of Ca^{2+} release in ECs;

The treatment of ECs from normotensive rats with specific and/or non-specific antioxidants should be without effect, whereas the antioxidative treatment of normotensive ECs submitted to oxidative stress must protect the Ca^{2+} responses against the harmful effects of free radicals. In addition, an artificial oxidant stress imposed to ECs from normotensive rats should mimic the deleterious effects of free radical overproduction on Ca^{2+} homeostasis of SHR.

3. Evaluation of the effect of acute and chronic treatment with specific and non-specific antioxidant enzymes, on agonist-induced Ca^{2+} release in normotensive and hypertensive ECs with and without the effect of induced oxidative stress;

According to the first hypothesis, the Ca^{2+} signaling mechanism in SHR and not in SD rats should be sensitive to antioxidant treatment and the agonist-induced Ca^{2+} release from ECs of hypertensive rats should be improved.

4. Evaluation of the effect of acute and chronic treatment with specific and non-specific antioxidant enzymes on agonist-induced Ca^{2+} release in ECs from young spontaneously hypertensive rats;

5. Measurement of $InsP_3$ production in normotensive and hypertensive ECs, under normal, oxidative stress, and antioxidant conditions;

The oxidative stress may stimulate the activity of PLC, resulting in an overproduction of $InsP_3$ and thereby increase the activity of $InsP_3R$. Alternatively, the increase in activity of $InsP_3R$ may come about directly through the $InsP_3R$ oxidation.

6. Evaluation by Western blot of the specific antioxidant enzymes' expression;

7. Measurement of the expressed enzymes activity in both, normotensive and hypertensive ECs;

The increased generation of FRs in ECs from SHR may arise from both, a genetic modification of the specific enzymes directly implicated in the free radical scavenging and detoxification, (particularly a change in the expression of SOD and catalase) and/or from an alteration of the activity of these specific antioxidative enzymes.

8. Evaluation of the intracytosolic $InsP_3$-sensitive Ca^{2+} pools, TG-sensitive Ca^{2+} pools and ryanodine-sensitive Ca^{2+} pools in ECs, in the presence and in the absence of antioxidants;

We expect to find $InsP_3$-sensitive pools as well as Ry-sensitive pools beside the TG-sensitive pools in all ECs. We postulate that the architecture of these pharmacologically distinctive pools might form a single and unitary Ca^{2+} pool, represented by the endoplasmic reticulum.

9. Evaluation of capacitative Ca^{2+} entry obtained from normotensive and hypertensive ECs, in all experimental conditions;

The oxidative stress may alter the regulation of Ca^{2+} influx, Ca^{2+} sequestration and the availability of Ca^{2+} in Ca^{2+} reservoirs. Conversely, the antioxidant treatment should restore Ca^{2+}

influx and should protect the storage capability, the availability of Ca^{2+} as well as the integrity of the intracytosolic Ca^{2+} release/recapture mechanisms.

10. Evaluation of the melatonin effect as agonist on Ca^{2+} release from all ECs used in this study, as well as the melatonin preconditioning effect on agonist-induced Ca^{2+} mobilization through $InsP_3$-dependent and independent mechanisms;

As other hormones recognized to be actively implicated in the modulation of vascular tone, melatonin should be endowed with membrane specific receptors linked to the $InsP_3$ signaling pathway.

11. Evaluation of the melatonin effect on agonist-induced Ca^{2+} evolution under oxidative stress;

The treatment of ECs from spontaneously hypertensive rats with melatonin should improve the general Ca^{2+} homeostasis and particularly, the agonists-induced Ca^{2+} responses.

12. The effect of melatonin on capacitative Ca^{2+} entry in all ECs in this study;

13. Evaluation of the melatonin effect on agonist induced Ca^{2+} response and CCE in paired agonist response protocols in ECs from BAE and SD rats.

CHAPTER III. Article I

ABNORMAL Ca^{2+} SIGNALING IN VASCULAR ENDOTHELIAL CELLS FROM SPONTANEOUSLY HYPERTENSIVE RATS: ROLE OF FREE RADICALS

Article published in Journal of Hypertension
2001, 19:1-10

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Running title: **FREE RADICAL EFFECTS ON ENDOTHELIAL CELLS.**

Key Words: Endothelium, hypertension, SHR, Sprague-Dawley, Ca^{2+}, Fura-2

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ABSTRACT

Objective. The main object of this work was to test the hypothesis that the Ca^{2+} signal transduction process in endothelial cells from genetically hypertensive rats (SHR) is affected by an overproduction of free radicals.

Method. The Ca^{2+} response to the inositol 1,4,5-triphosphate ($InsP_3$) mobilizing agonist ATP was measured using the fluorescence probe Fura-2 in endothelial cells from Sprague Dawley (SD) rats, and in young and age-matched (SHR). The effect of free radicals and reducing agents on the intracellular Ca^{2+} release and $InsP_3$ production was determined in resting and ATP-stimulated cells. Experiments were also performed to compare the expression level and enzymatic activity of catalase and superoxide dismutase (SOD) in endothelial cells from SHR and SD rats.

Results. The exposure of aortic endothelial cells from SD rats to the free radicals generating system hypoxanthine + xanthine oxidase (HX/XO) caused a time and concentration dependent inhibition of the ATP-induced Ca^{2+} response. A similar HX/XO-dependent inhibition was also observed in SD cells stimulated with the endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, thapsigargin. The incubation with the antioxidative enzymes catalase and SOD had no effect on the ATP-induced Ca^{2+} release in SD cells, but led to a strong increase of the internal Ca^{2+} release in cells from adult (12 weeks old) or young (3 weeks old) SHR. The effect of antioxidants was not related to an enhancement of the ATP-induced $InsP_3$ production, nor to a lower expression and activity of SOD and catalase.

Conclusion. The present work provides evidence that the Ca^{2+} signaling process in SHR endothelial cells is affected by an overproduction of free radicals resulting in a depletion of releasable Ca^{2+} from $InsP_3$-sensitive and insensitive Ca^{2+} pools. These results point towards a

beneficial action of antioxidants on Ca^{2+} signaling in endothelial cells from models of hypertension.

III.1. INTRODUCTION

Endothelial cells play an important role in the regulation of vascular tone by secreting a variety of vasoactive factors in response to hormonal and/or physical stimuli. These factors include vasorelaxing agents such as the endothelium-derived hyperpolarizing factor (EDHF), prostacyclin (PGI_2) and nitric oxide ($NO^•$) [1], and vasoconstrictors like endothelin-1 (and perhaps other endothelins), endoperoxides (PGH_2), and thromboxane A_2. There is now increasing evidence for an overproduction of reactive oxygen species (ROS) in endothelial cells from animals with hypertension, whether primary or induced by a salt-rich diet (see McIntyre et al., 1999 [2]). Direct measurements with lucigenin have provided evidence for a 250% increase in superoxide anion ($^•O_2^-$) concentration in endothelial cells from stroke-prone spontaneously hypertensive rats (SHRSP) compared to Wistar-Kyoto (WKY) [3]. Because $^•O_2^-$ is known to react rapidly with $NO^•$ to form peroxynitrite, an increased scavenging of $NO^•$ by $^•O_2^-$ may result in a decreased $NO^•$ availability despite increased synthesis [4]. In support of this proposal is the observation that the endothelium of genetically hypertensive rats (SHR) releases 40% less $NO^•$ that that of normotensive rats due to a higher $^•O_2^-$ production [5,6]. Similarly, an increased $NO^•$ decay induced by the spontaneous reaction between $NO^•$ and $^•O_2^-$ was reported in mesenteric arteries of SHRSP, despite a $NO^•$ production identical to WKY [7].

In addition to an effect on $NO^•$ availability, an enhanced production of reactive oxygen species (ROS) such as $^•O_2^-$ is likely to affect other processes essential the endothelium control of vascular tone. In particular free radicals have been shown to inhibit the Ca^{2+} response of

endothelial cells to Ca^{2+} mobilizing agonists [8-10]. A large body of evidence has confirmed that Ca^{2+} signaling in vascular endothelial cells consists of initial inositol 1,4,5-triphospate ($InsP_3$) dependent release of Ca^{2+} from intracellular stores followed by a Ca^{2+} entry from the external medium. The effect of free radicals appeared time dependent and resulted in an initial inhibition of the agonist-evoked Ca^{2+} entry added to a reduction of the $InsP_3$-induced Ca^{2+} mobilization and a global cytosolic Ca^{2+} increase [11]. Because the endothelium constitutes a major source for an excess ROS production in various experimental models of hypertension [3,4,6,7], modifications of the Ca^{2+} signaling process need to be expected in these cases. For instance, the peak $[Ca^{2+}]_i$ response to acetylcholine was found to be significantly reduced in cells from aldosterone-salt hypertensive rats (AHR) compared to controls. Similarly, the bradykinin-induced release of Ca^{2+} from internal pools appeared less important in endothelial cells from SHR relative to WKY [12]. However, the relationships between Ca^{2+} signaling and ROS production in endothelial cells from models of hypertension have never been investigated. Fura-2 experiments were thus undertaken to test the hypothesis that the Ca^{2+} signaling process measured in aortic endothelial cells from the genetically hypertensive rat model SHR is affected by an overproduction of ROS. Our results show that exposure of aortic endothelial cells to antioxidative enzymes such as catalase or SOD causes an increase of the ATP-induced Ca^{2+} mobilization in SHR cells but not in cells from normotensive rats. We found in addition that the effects of antioxidants on SHR were not related to an improved $InsP_3$ production, but probably involve an enhanced Ca^{2+} recapture into internal Ca^{2+} pools.

III.2. MATERIALS AND METHODS

III.2.1. Vascular endothelial cells

Aortic endothelial cells were prepared according to the procedure described previously [12]. Briefly, aortic endothelial cells were isolated from 12 weeks old Sprague Dawley (SD) male rats and from male SHR 3 and 12 weeks of age (Charles River, St-Constant, Quebec, Canada). The 12 weeks old, male rats from both strains were at a body weight between 250-275 gr., whereas the 3 weeks old SHR rats were only at 12 gr. of body weight. The mean systolic blood pressure was 200 mm Hg and over for the 12 weeks SHR and 120 mm Hg for SD rats and 3 weeks old SHR. The isolation method was identical in all cases. After the lethal anesthesia of the rats (Phenobarbital, 50 mg/kg, IP), aortas were removed from the thoraco-abdominal cavity and cleaned of connective tissues. The vessels were cut open longitudinally in strips of 5 mm. The aortas were digested in a stepwise manner with various concentrations of dispase and collagenase in oxygenated Dulbecco's modified Eagle medium (DMEM) (Gibco BRL Products, Life technologies, Burlington, Ontario, Canada), at 37°C under mild agitation. Endothelial cells were harvested by centrifugation for 10 min at 2600 r.p.m. at room temperature. Each cell culture was prepared from a single strain of rats at once. The rat aortic endothelial cells were cultured on collagen (1%, type VII, Sigma) precoated 25 mm rounded glass cover slips in DMEM containing 10% fetal calf serum (Gibco BRL Products, Life technologies, Burlington, Ontario, Canada), 10 ng/ml endothelial cell growth factor, and 50 μg heparin/ml. The single subculture passage of each primary cell culture was realized by treatment of confluent endothelial monolayers with 0.05 % trypsin in 0.5 mM EDTA saline for 1-2 min at room temperature. The endothelial cells were identified morphologically by their "cobblestone-like" aspect when grown into a confluent monolayer and by their ultrastructure characteristics, in

particular the presence of Weibel-Palade bodies in the cytoplasm. To minimize phenotypic changes during continuous culture, only cells from the primary culture and the first passage were used in the present study. The cells were used for microspectrofluorometry after 6-8 days of culture. The exposure of endothelial cells to oxidative stress was followed of trypan blue test for cellular viability.

III.2.2. Solutions

The external bathing medium was a standard Earle's solution containing (in mM): 121 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 6 $NaHCO_3$, 1 NaH_2PO_4, 5.5 glucose and 25 HEPES buffered at pH 7.3 with 10 KOH. Calcium-free external solutions were prepared by adding 2 mM K_2 EGTA to Earle's solutions without $CaCl_2$. Acetoxymethylester Fura-2, ionomycin, hypoxanthine, SOD, deferoxamine, glucose oxidase, vitamin E, and heparin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Thapsigargin was obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada), preserved in a 1mM DMSO stock solution and used in a 1μM final solution. Endothelial growth factor, collagenase, dispase, xanthine-oxidase, catalase, and ATP were from Roche Diagnostic Canada (Laval, Quebec, Canada). Superoxide production by xanthine-oxidase was assayed by monitoring *cytochrome c* reduction at 550 nm.

III.2.3. Microspectrofluorometry

The fluorescence was recorded using an epifluorescence microscope (Nikon inverted microscope IM 35) attached to a dual-excitation spectrofluorometer (Spex Fluorolog II; Spex Industries Inc., Edison, NJ, USA). For Fura-2, the excitation wavelengths were set at 350 nm and 380 nm respectively, and a dichroic mirror (Nikon DM 400) was placed in the excitation

pathway. The emission was monitored at 505 nm with a standard bandpass filter (Andover Corporation 500FS10). We used as objective a Nikon Neofluor 40x (Nikon, Nikon Canada Instruments Inc., Montreal, Quebec, Canada).

The intracellular calcium concentration, $[Ca^{2+}]_i$ was calculated on the basis of the equation of Grynkiewicz (1985) [6]:

$$[Ca^{2+}]_i = Kd \times (R-R_{MIN})/(R_{MAX}-R) \times Sf_2/Sb_2, \qquad (1)$$

with Kd equal to 224 nM, R the ratio of the fluorescence measured at 350 nm and 380 nm, and Sf_2/Sb_2, the ratio of fluorescence at 380 nm in low and high calcium respectively. The maximal and minimal fluorescence ratios R_{MAX} and R_{MIN} were determined using 10 µM ionomicyn to permeabilize the cells first in standard Earle's conditions and second in an Earle's solution containing 10 mM EGTA at pH 8.8.

The mean increase in $[Ca^{2+}]_i$ during stimulation, $\Delta[Ca^{2+}]_i$, was estimated by integrating the function:

$$\Delta[Ca^{2+}]_i = 1/T \int_0^T ([Ca^{2+}]_i (\tau) - {}^{b}[Ca^{2+}]_i)\Delta\tau \qquad (2)$$

where $[Ca^{2+}]_{i\ basal}$ is the $[Ca^{2+}]_i$ prevailing before stimulation, $[Ca^{2+}]_i$ (τ) the time dependent variation in cytosolic Ca^{2+} concentration following cell stimulation and T, the total time of stimulation by the agonist.

III.2.4. Measurement of $InsP_3$ formation

Endothelial cells were incubated 24 hours in a serum-free and inositol free DMEM to which was added 5µ□ Ci/ml of myo-[2-^{3}H] inositol (Du Pont Canada Inc. Diagnosis and Biotechnology Systems, Ont., Canada). The culture medium containing unincorporated isotope was rinsed afterward with a warm (37° C) standard Earle's solution. The cells were further

incubated for 30 min in the same buffer containing 20 mM/l LiCl to inhibit the conversion of the inositol phosphate so that the radiolabeled inositol phosphates could accumulate within the cell. Unless stated otherwise, agonists were applied for 150 s and the reaction was terminated by adding 0.9 ml of methanol: chloroform: HCl in a 40:20:1 ratio. After the addition of 0.4 ml of chloroform plus 0.4 ml distillated water, the samples were centrifuged to separate lipid and aqueous phases. The aqueous phase was transferred to a column containing 0.8 ml of resin AG 1-X8 (200-400 mesh, formate form, Bio-Rad, Richmond, CA, USA), from which inositol phosphates were eluded sequentially with ammonium formate buffers of increasing molarity. The radioactivity was measured in a Liquid Scintillation Counter (1215 Rackbeta II, LKB Wallac, USA). The lipid phase was counted to measure the phosphatidylinositol pool (PIP). The accumulation of inositol phosphate was expressed as the ratio inositol phosphate / PIP to take into account the variations in the labeling of the lipid pool.

III.2.5. Western blot analysis

Western blots and enzymatic assays for both SOD and catalase were performed using cytosolic fractions purified from cultured cells. This purification technique was adapted from that of Güner *et al* (1996) [13] originally designed for lung tissues. Briefly, the cells cultured on 100 mm Petri dishes for 6 days were rinsed twice with cold phosphate buffered saline (PBS) and scraped using a rubber policeman. The cells were pelleted down at low speed and recovered in fresh PBS (2 ml). The tissues were homogenized using a potter (20 strokes at maximal speed) sonicated for 30 seconds and centrifuged at 100,000-x g for 60 minutes. The supernatants were collected, assayed for protein contents, and kept frozen (-20°C) until used (not more than four weeks).

Western blots were performed as described by Bissonnette *et al* (1999) [14]. For catalase, the samples were run on a 7.5% acrylamide gel and primary antibody used at a dilution of 1:2000 (# AB1212, Chemicon Intl., Temecula, CA, USA). For SOD, a 15% acrylamide gel was used and the primary antibody incubated at a dilution of 1:1000 (# AB1237, Chemicon Intl., Temecula, CA, USA). For both Western blots, the secondary antibody (anti-rabbit coupled to FITC: New England Biolabs, Mississauga, Ont., Canada) was incubated at a dilution of 1:400,000 and detected using a commercial ECL kit (Pierce, Rockford, IL, USA). Quantitation was performed by densitometry analysis of specific bands using an imaging system (Alpha Innotech Corp., San Leandro, CA, USA) along with a commercial software (Chemilmager 4000i).

III.2.6. Enzymatic assays

Activities of both catalase and SOD were performed using spectrophotometric techniques. All data are mean ± SEM of three determinations made from 5 different cultures. **Catalase**: evaluation of catalase activity was performed according to Güner *et al* (1996) [13] and data are presented in absorbance values (OD/mg protein). **SOD**: the activity of SOD is based on the competition of the SOD for superoxide generated by the catalysis of glucose by glucose oxidase (Nagi *et al*, 1995) [15]. The activity is estimated by comparing assays in absence of SOD (V) to that in presence of SOD (v). The quantitation of SOD is thus evaluated as the inhibition ratio V/v. One unit (U) of SOD represents an activity, which generates a 50% inhibition (V/v=2).

III.2.7. Protein determination

Total protein contents of supernatants were determined with a commercial BCA based technique (Pierce, Rockford, IL, USA) using bovine serum albumin fraction V as standard (Sigma, St-Louis, MO, USA).

III.2.8. Statistics

The data were expressed as means ±SEM. Statistical significance was analyzed using unpaired Student's T-test. $P < 0.01$ was considered statistically significant.

III.3. RESULTS

III.3.1. Ca^{2+} response to ATP in endothelial cells from SHR and SD rats

The Ca^{2+} response of SD and SHR aortic endothelial cells to ATP was first investigated in a series of Fura-2 experiments in which the cells were initially stimulated in external Ca^{2+}-free conditions followed by a cell superfusion with a Ca^{2+}-containing medium. This procedure allowed an estimation of the Ca^{2+} release process from internal pools while providing indications on the magnitude of the associated capacitative Ca^{2+} entry. Examples of Fura-2 signals measured on SD and SHR cells are presented in figure 1A. The resting mean $[Ca^{2+}]_i$ in primary cultured endothelial cells from normotensive SD rats and SHR was estimated at 45.0 ± 0.1 nM (n=26) and 50.2 ± 0.1 nM (n=18) respectively, indicating a small but significant difference in the basal Ca^{2+} level between the two cell populations ($P<0.01$). The average Ca^{2+} increase ($\Delta[Ca^{2+}]_i$) in SD and SHR cells for the period of ATP (10 μM) stimulation (150 s) is illustrated in figure 1B. The mean $\Delta[Ca^{2+}]_i$ for SD was estimated at 65 ± 12 nM (n=18), compared to 24 ± 12 nM (n=26) for endothelial cells coming from adult SHR. The Fura-2

recordings in figure 1 showed in addition that the readmission of Ca^{2+} into the external medium failed to initiate a large increase in $[Ca^{2+}]_i$ in both SD and SHR cells, thus providing evidence for a low agonist-induced Ca^{2+} entry in these cell types.

Experiences were next undertaken to determine the importance of $InsP_3$ production to the difference in ATP-induced Ca^{2+} mobilization presented in figure 1B. Internal Ca^{2+} release was measured in external Ca^{2+}-free conditions on SD and SHR endothelial cells following stimulation with the Ca^{2+}-pump inhibitor thapsigargin (TG, at 1 μM). This experimental procedure allowed Ca^{2+} liberation from internal pools without $InsP_3$ production. The results in figure 2 show a mean $\Delta[Ca^{2+}]_i$ of greater magnitude (45 ± 9 nM; n = 4) in SD relative to SHR (20± 6 nM; n=4) endothelial cells, suggesting that the difference in ATP-induced $\Delta[Ca^{2+}]_i$ presented in figure 1B is independent of the $InsP_3$ production cascade.

III.3.2. Effects of free radicals on the Ca^{2+} response in endothelial cells from SD rats

The possibility of a reduced Ca^{2+} response in SHR due to an overproduction of free radicals was also investigate by measuring the effects of free radicals on the Ca^{2+} signaling process in endothelial cells from normotensive SD rats, using a combination of hypoxanthine + xanthine oxidase (HX/XO) as free radical generating system. The release of Ca^{2+} from internal pools and the associated capacitative Ca^{2+} entry were measured in SD cells following pretreatment with free radicals (500 μM HX /50 mU/ml XO) for periods ranging from 30 to 120 min. The Fura-2 recordings in figure 3A show that the exposure of SD cells to HX/XO resulted in a time dependent increase of the cell basal Ca^{2+} level with an inhibition of the ATP induced Ca^{2+} mobilization process. These results are summarized in figure 3B. The mean $\Delta[Ca^{2+}]_i$ elicited by ATP after a 30 min incubation period was 28 ± 5 nM (n=3), a value corresponding to 42 % of the control response (P<0.01). The resting $[Ca^{2+}]_i$ in these cells (82 ± 1 nM; n=3) was

also statistically higher than the control value. A 90 min incubation led to a 60% increase of the cell resting Ca^{2+} level relative to control, and to a total inhibition of the $\Delta[Ca^{2+}]_i$ induced by ATP. Similar results were obtained for incubation periods up to 120 min. Figure 3C presents a dose response curve of $\Delta[Ca^{2+}]_i$ as a function of the HX concentration (with 50 mU/ml XO) for a fixed incubation time of 90 min. Half inhibition was observed at 130 μM HX with a near total inhibition at concentrations exceeding 300 μM. Finally, prolonged exposure (90 min-120 min) of SD cells to HX/XO resulted in a greater $\Delta[Ca^{2+}]_i$ increase following Ca^{2+} readmission into the external medium. Globally, these observations confirm that the main effects of free radicals on Ca^{2+} signaling in endothelial cells consist in an impairment of the internal Ca^{2+} release process with a time dependent-enhancement of the plasma membrane Ca^{2+} permeability.

The inhibitory effect of ROS on the Ca^{2+} release process triggered by ATP in SD cells was further investigated by measuring the basal and stimulated $InsP_3$ production in untreated SD cells and in cells exposed for 90 min to HX/XO (500 mM/50 mU/ml). The results presented in figure 4 show that there was 600% increase of the resting $InsP_3$ level in ROS generating system treated cells relative to control. Addition of ATP to ROS treated cells resulted in an $InsP_3$ production that was not statistically different ($p > 0.5$) from that measured following exposure to HX/XO alone. It follows from these observations that the inhibition of the ATP dependent Ca^{2+} release measured in SD cells exposed HX/XO may, in part, be related to a depletion of the $InsP_3$ Ca^{2+} pools due to a ROS related excessive stimulation of the $InsP_3$ production machinery during cell treatment.

A characterization of the effect of ROS on Ca^{2+} signaling in SD cells was also performed by measuring the release of Ca^{2+} induced by thapsigargin following exposure to HX/XO (500 mM/50 mU/ml) (figure 2). Compared to non treated SD cells, there was a drastic decrease of the Ca^{2+} response initiated by thapsigargin (1 μM) measured following a 90 min exposure to HX

/XO, with mean $\Delta[Ca^{2+}]_i$ of 45 ± 9 nM (n=4) in control conditions and 7 ± 3 nM (n=8) after ROS treatment. These results support therefore a model whereby the magnitude of the releasable Ca^{2+} in $InsP_3$-sensitive and -insensitive internal pools is decreased following ROS exposure.

III.3.3. Modulation of Ca^{2+} signaling by antioxidant enzymes in SD and SHR endothelial cells

The hypothesis of a modulatory effect of free radicals on the Ca^{2+} signaling process in SHR was next investigated in a series of experiments where the ATP-induced Ca^{2+} release was measured in cells from SD rats and SHR following treatment with antioxidants. The results presented in figure 5A confirm that the average Ca^{2+} increase triggered by ATP in SD endothelial cells was not significantly (n = 8: $P<0.01$) affected following a 48 h exposure to either catalase (50 IU/ml) or SOD (50 mU/ml). Identical findings were obtained with deferoxamine (80 μM) and the antioxidant vitamins C and E (data not shown: n=4). In contrast, SHR cells exposed to 50 IU/ml catalase for only 90 min showed a mean $\Delta[Ca^{2+}]_i$ of 57 ± 20 nM (n=18), an increase of 115% relative to controls ($P<0.01$). In SHR cells treated with SOD (50 mU/ml) for 90 min, the mean $\Delta[Ca^{2+}]_i$ was estimated at 34 ± 13 nM (n=11), a value statistically different from control ($P<0.01$) (Data not shown). The beneficial effect of antioxidants appeared time-dependent as chronic treatments of SHR cells for 48 hr with catalase and SOD led to ATP-induced mean $\Delta[Ca^{2+}]_i$ of 82 ± 22 nM (n=9) and 78 ± 37 nM (n=9) respectively. These values represent increases of more than 200 % of the mean $\Delta[Ca^{2+}]_i$ measured for untreated cells (27 ± 12 nM). Similarly, chronic incubations in deferoxamine (80 μM) and

vitamin E (800 U/ml) caused an 88% augmentation of to mean ATP-induced Ca^{2+} release relative to controls with values of 50 ± 9 and 50 ± 11 nM, respectively (n=9).

The appearance of a hypertensive state in SHR is a slow and continuous process with SHR of less than 4 weeks being normotensive. Experiments were performed to determine if the effect of antioxidative agents on SHR was age related. The results of these experiments confirmed that the cells coming from normotensive 3 weeks old SHR were already sensitive to antioxidants with mean ATP-dependent $\Delta[Ca^{2+}]_i$ increases of 150% and 50% following a 90 min treatments with catalase or SOD respectively (n=9) (Data not shown). Similar results were obtained following treatments of the cells for 48 h with either catalase or SOD (n=9). The effect of non-specific antioxidants was less important, but the observed potency profile corresponded to that measured on adult SHR (Data not shown).

A possible explanation for the observed beneficial effect of antioxidant enzymes on the Ca^{2+} response to ATP in SHR concerns a modification of the $InsP_3$ production process by antioxidants. The results presented in figure 6 show that the incubation of SHR cells with catalase (CAT) has no significant effect on the resting and ATP-induced $InsP_3$ production relative to non-incubated cells. The improved Ca^{2+} response to ATP observed under catalase conditions is not likely therefore to be consequent to an increased $InsP_3$ production.

III.3.4. Expression and activity of catalase and SOD in SD and SHR endothelial cells

Another explanation to account for the stimulatory effect of catalase and SOD on the Ca^{2+} mobilization in SHR (figure 5) involves a deficient production in endogenous antioxidant enzymes in SHR endothelial cells. Experiments were carried out in which the expression level and functional activity of catalase and SOD were measured in cultured aortic endothelial cells from adult SD and SHR rats respectively. The Western blot analysis presented in figure 7

shows that there were no significant differences in the expression level of catalase and SOD between SD and SHR cells as evidence by similar densitometry values for both enzymes in the two cell lines. Accurate determinations of enzyme activities performed by spectrophotometric assays show equivalent values for both SOD and catalase thus failing to provide evidence for a down regulation of these enzymes in SHR compared to SD (see table 1). These results strongly suggest that the results in figure 6 are not related to a lower content of antioxidant enzymes in SHR.

III.4. DISCUSSION

Our results demonstrate that a pretreatment of aortic endothelial cells with antioxidant enzymes such as catalase or SOD leads to an increased Ca^{2+} response to ATP in SHR but not in SD cells. This effect appeared not to be age-related as it was also observed on normotensive 3 weeks old SHR. We showed furthermore that the action of antioxidants on SHR cells could not be accounted for by a change in $InsP_3$ production.

The Fura-2 results presented in figure 5B confirmed that the chronic treatment of SHR cells with the antioxidant enzymes catalase or SOD leads to a significant increase of the Ca^{2+} response to ATP, an effect not observed in SD cells. These observations strongly suggest that the Ca^{2+} response in SHR endothelial cells is endogenously depressed due to an overproduction of free radicals. There is currently clear evidence supporting an enhanced production of free radicals in endothelial cells from several genetic models of hypertension [3,16,17]. For instance, direct measurements of $^{\bullet}O_2^-$ concentration by a chemiluminescence method have demonstrated the presence of 68 nM of $^{\bullet}O_2^-$ in endothelial cells from SHRSP compared to 27 nM in WKY [3]. Similarly, the basal and internal Ca^{2+}-stimulated production of $^{\bullet}O_2^-$ and H_2O_2 was reported to be higher in SHR aortas than in WKY [18]. The overproduction of $^{\bullet}O_2^-$ was

found to precede the increase in blood pressure in this genetic model of hypertension [18]. These findings would be in accordance with the data presented in this work where the beneficial effect of catalase and SOD on the ATP-induced Ca^{2+} release was equally apparent in cells from normotensive 3 weeks hold SHR. Several sources of $^{\bullet}O_2^-$ in the endothelium have been reported including cyclooxygenase and NADH oxidoreductase [19,20]. Recent evidence points however toward an increased expression of the endothelium constitutive nitric oxide synthase (cNOS) as one of the major sources for the excess O_2^- production measured in SHR and SRHSP endothelial cells [4,18]. It appears therefore that the impaired functional NO release reported in endothelial cells from several models of hypertension would be more compatible with an increased scavenging of NO by O_2^- rather than a deficient NO production [4]. Furthermore, since the production of $^{\bullet}O_2^-$ is calcium dependent like the production of NO by cNOS [18], an $^{\bullet}O_2^-$-mediated depression of the Ca^{2+} response in SHR cells constitutes a protective mechanism against an enhanced $^{\bullet}O_2^-$ production.

Our results also suggest that a ROS-mediated decrease of releasable Ca^{2+} from internal Ca^{2+} stores may be responsible for the depressed Ca^{2+} response in SHR endothelial cells. The results in figure 2 where SD cells exposed HX/XO were shown to release less Ca^{2+} in response to thapsigargin stimulation than non-treated cells support an action of ROS leading to Ca^{2+} pools depletion. This mechanism would also account for the thapsigargin-induced Ca^{2+} release of smaller magnitude measured in SHR compared to SD cells (figure 2). The inhibition of SERCA pumps by thapsigargin is not however restricted to $InsP_3$-sensitive Ca^{2+} stores, but includes ryanodine-sensitive stores as well [21,22]. The thapsigargin results argue therefore for a general dysfunction of Ca^{2+} handling in SHR cells involving $InsP_3$-insensitive Ca^{2+} reservoirs as well. The observation of a reduced ATP-stimulated Ca^{2+} release despite a slight increase in ATP-dependent $InsP_3$ production in SHR (0.0028 ± 0.0006: n=10, figure 6) relative to ATP-

dependent $InsP_3$ production in SD cells (0.0021 ± 0.0004: n = 7, figure 4A) argue equally for an effect involving Ca^{2+} internal stores. A depletion of Ca^{2+} pools under oxidative stress conditions may originate from several processes including an increase in Ca^{2+} permeability of the endoplasmic reticulum (ER) membrane by opening of ion channels, a disruption of the ER membrane and/or an inhibition of the ER Ca^{2+}-ATPase. Evidence has already been provided that the oxidation of the $InsP_3$ receptor results in channel activation at lower $InsP_3$ levels [23]. Part of the action of ROS on internal Ca^{2+} signaling in SHR may thus consists in a partial depletion of $InsP_3$-sensitive pools due to an increase of the $InsP_3$ receptor activity under basal $InsP_3$ conditions. Furthermore, experiments with isolated microsomes have shown that HX/XO can empty Ca^{2+} stores by direct inhibition of the Ca^{2+}-ATPase [24,24]. The combined action of a decreased Ca^{2+} sequestration activity joined to an enhanced Ca^{2+} leak through functional $InsP_3$ receptors is likely to account for the depletion of the thapsigargin-sensitive Ca^{2+} pools measured in SHR endothelial cells, and for the impaired Ca^{2+} response to Ca^{2+} mobilizing agonists such as ATP (see figure 1).

When comparing the Ca^{2+} response of SHR and SD cells we can not however totally rule out a contribution of the of the $InsP_3$-production machinery. An effect due to the membrane receptors may in some cases explain part of the variability in the relative magnitude of the Ca^{2+} response observed between endothelial cells from normotensive and hypertensive animal models. For instance, despite of a smaller internal Ca^{2+} release by bradykinin in SHR endothelial cells compared to WKY, the Ca^{2+} response observed in these two cell preparations was not statistically different when endothelin and angiotensin II were used as agonists [12]. This observation does not rule out however the possibility that the release of Ca^{2+} by endothelin or angiotensin II may be enhanced to a greater extent in SHR compared to WKY cells following cell treatment with antioxidants. Altogether, our results strongly suggest that Ca^{2+} signaling in

SHR is endogenously depressed consequent to an overproduction of free radicals that in turn, leads to a depletion of releasable Ca^{2+} from $InsP_3$-sensitive and -insensitive Ca^{2+} pools.

III.5. CONCLUSIONS

Abnormal Ca^{2+} signaling has been reported in vascular endothelial cells from several models of hypertension. The present work provides evidence that the Ca^{2+} signaling process in SHR endothelial cells is affected by an overproduction of reactive oxygen species. These observations point therefore towards a beneficial effect of antioxidants as a mean to normalize the Ca^{2+} response of the endothelium in certain types of cardiovascular dysfunctions such as hypertension.

III.6. ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council of Canada (MT 7769) and from the Quebec Heart and Stroke Foundation. The technical assistance of Joanne Vallée is also acknowledged.

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III.8. FIGURES CHAPTER III.

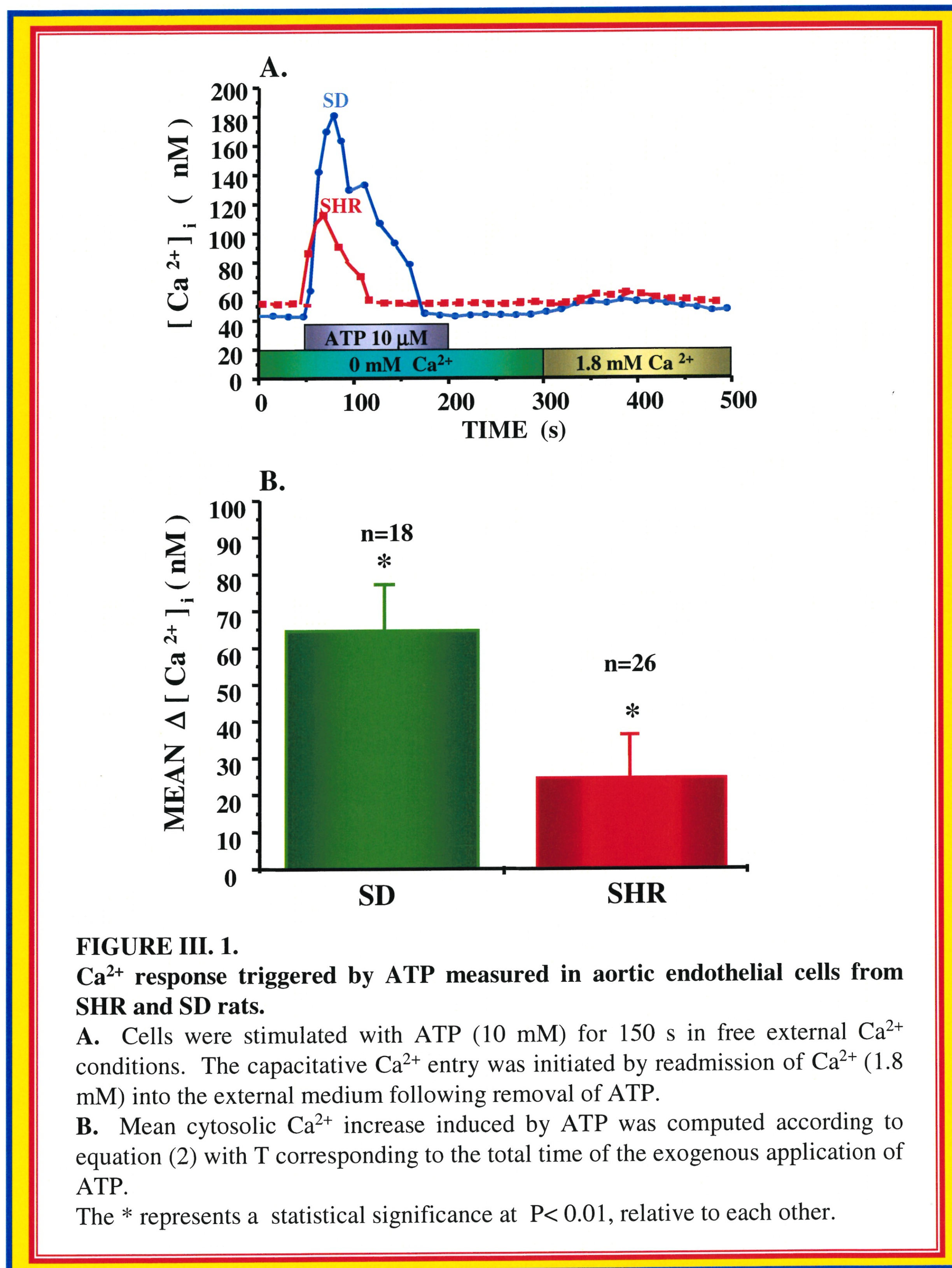

FIGURE III. 1.

Ca^{2+} response triggered by ATP measured in aortic endothelial cells from SHR and SD rats.

A. Cells were stimulated with ATP (10 mM) for 150 s in free external Ca^{2+} conditions. The capacitative Ca^{2+} entry was initiated by readmission of Ca^{2+} (1.8 mM) into the external medium following removal of ATP.

B. Mean cytosolic Ca^{2+} increase induced by ATP was computed according to equation (2) with T corresponding to the total time of the exogenous application of ATP.

The * represents a statistical significance at $P < 0.01$, relative to each other.

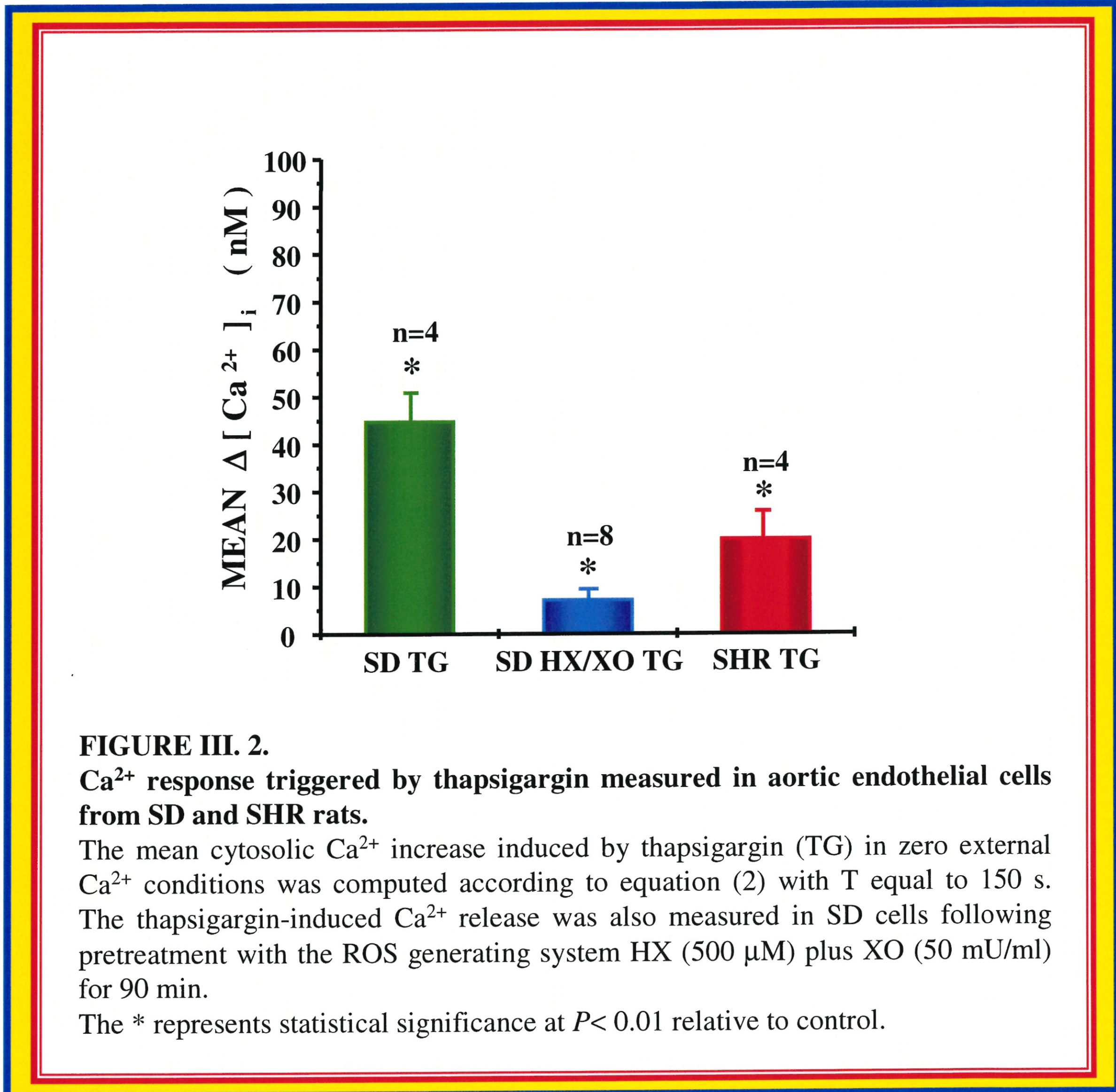

FIGURE III. 2.
Ca^{2+} response triggered by thapsigargin measured in aortic endothelial cells from SD and SHR rats.
The mean cytosolic Ca^{2+} increase induced by thapsigargin (TG) in zero external Ca^{2+} conditions was computed according to equation (2) with T equal to 150 s. The thapsigargin-induced Ca^{2+} release was also measured in SD cells following pretreatment with the ROS generating system HX (500 μM) plus XO (50 mU/ml) for 90 min.
The * represents statistical significance at $P < 0.01$ relative to control.

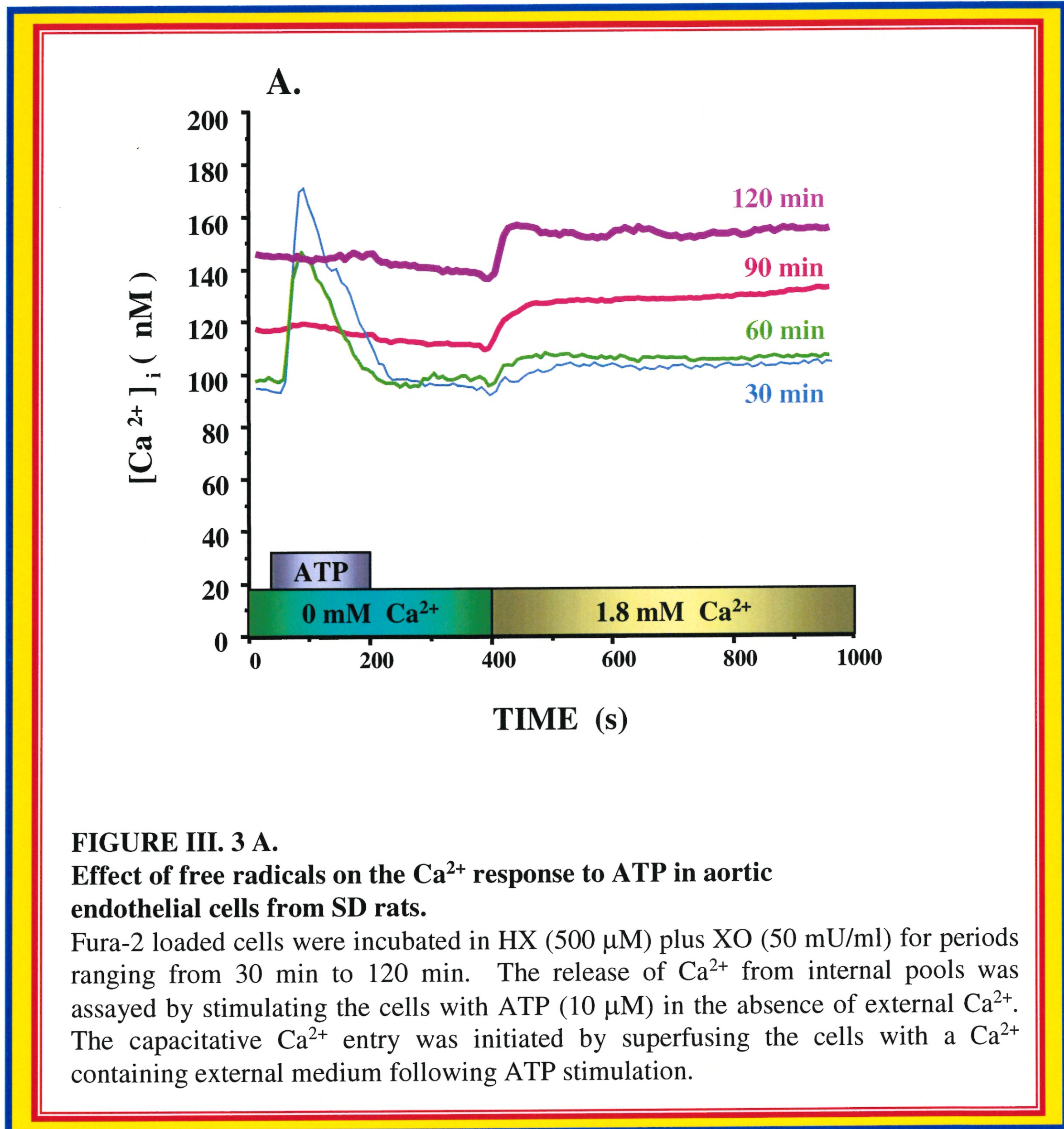

FIGURE III. 3 A.
Effect of free radicals on the Ca^{2+} response to ATP in aortic endothelial cells from SD rats.
Fura-2 loaded cells were incubated in HX (500 µM) plus XO (50 mU/ml) for periods ranging from 30 min to 120 min. The release of Ca^{2+} from internal pools was assayed by stimulating the cells with ATP (10 µM) in the absence of external Ca^{2+}. The capacitative Ca^{2+} entry was initiated by superfusing the cells with a Ca^{2+} containing external medium following ATP stimulation.

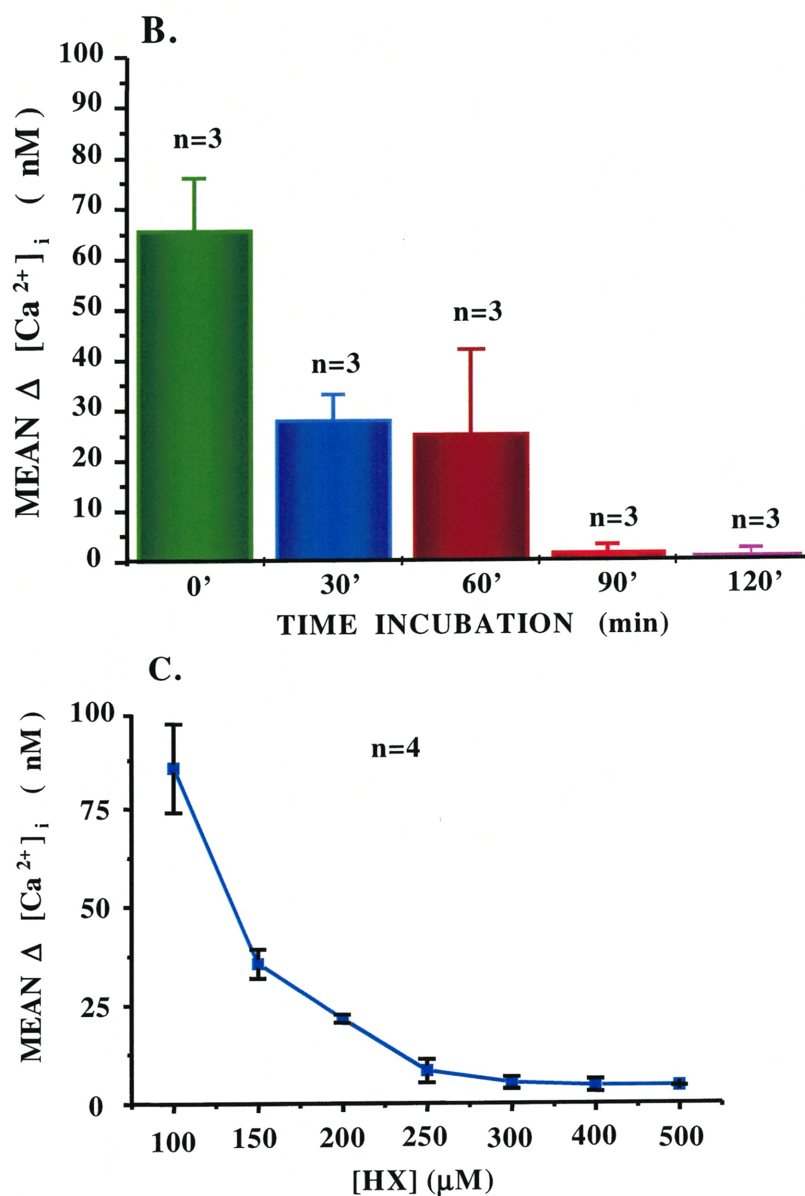

FIGURE III. 3B and 3C.
Effect of free radicals on the Ca^{2+} response to ATP in aortic endothelial cells from SD rats.

B. Mean $\Delta[Ca^{2+}]_i$ increase resulting from ATP stimulation plotted as function of the time. Aortic endothelial cells were exposed to the free radical generating system HX/XO.

C. Dose response curve of the HX/XO inhibition. The mean Ca^{2+} increase resulting from ATP stimulation after 90 min incubation with the free radical generating system HX/XO is plotted as a function of the HX concentration with XO fixed at 50 mU/ml. The mean cytosolic Ca^{2+} concentration increase was computed as described in figure 1.

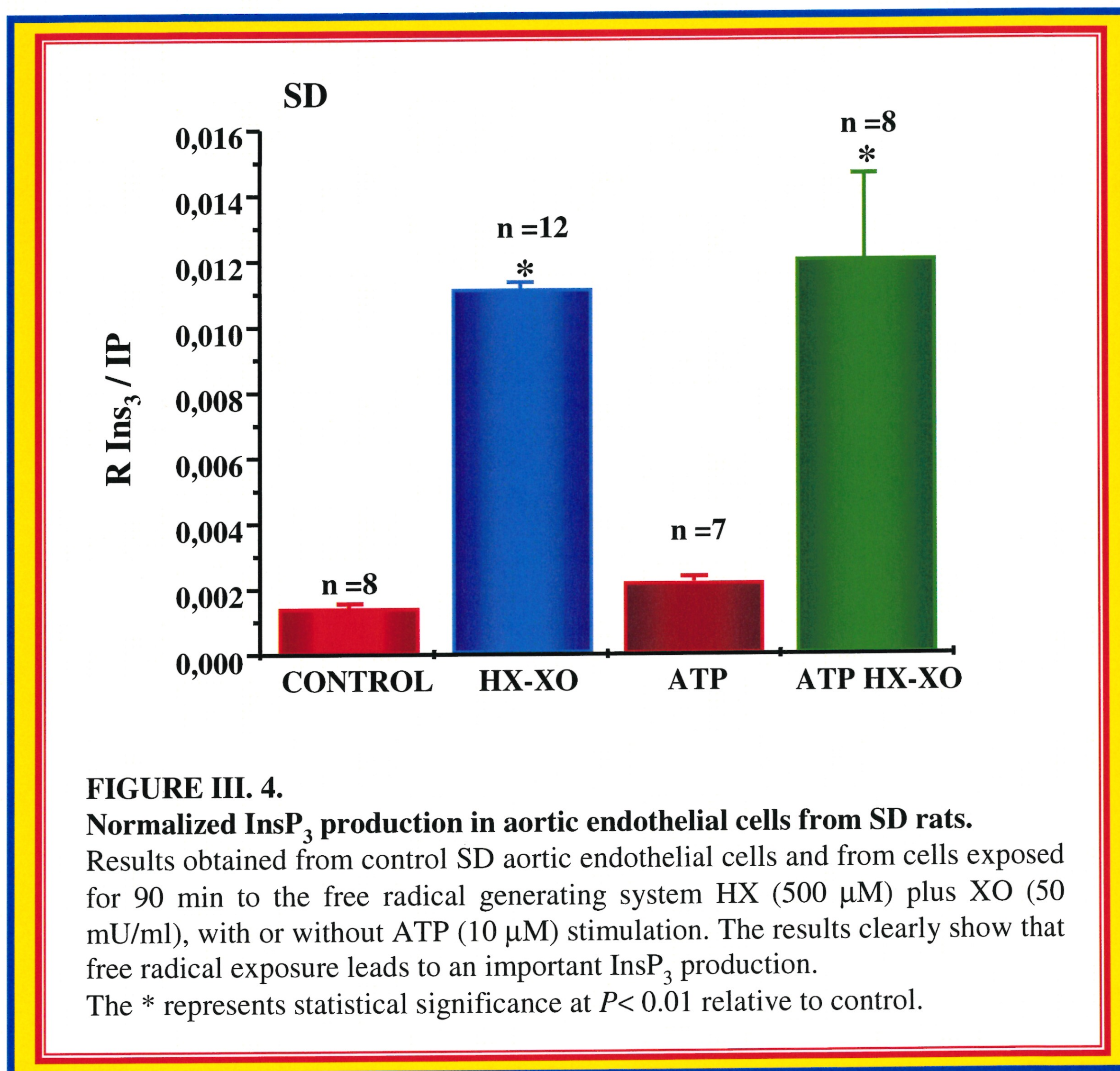

FIGURE III. 4.
Normalized $InsP_3$ production in aortic endothelial cells from SD rats.
Results obtained from control SD aortic endothelial cells and from cells exposed for 90 min to the free radical generating system HX (500 µM) plus XO (50 mU/ml), with or without ATP (10 µM) stimulation. The results clearly show that free radical exposure leads to an important $InsP_3$ production.
The * represents statistical significance at $P < 0.01$ relative to control.

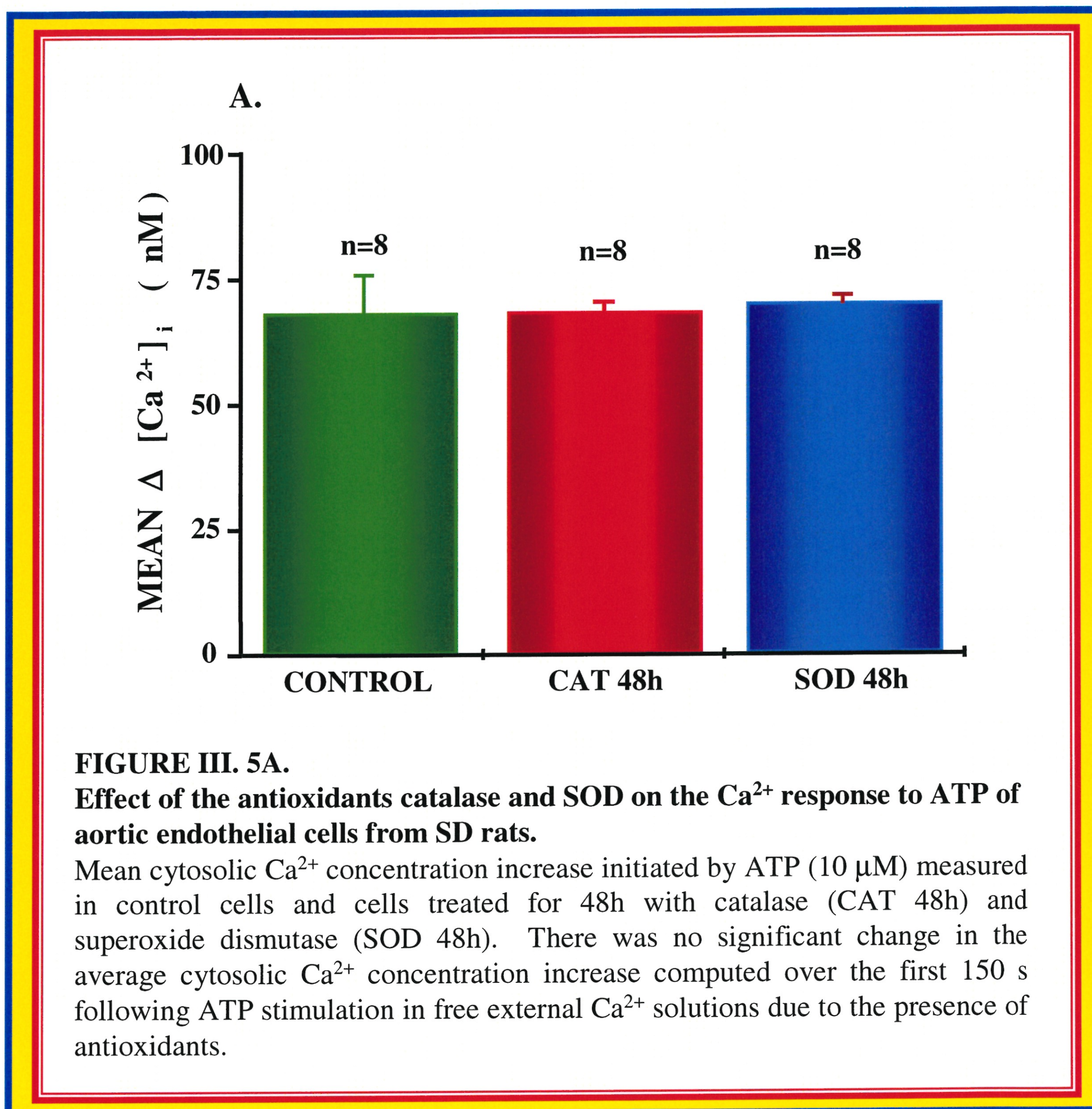

FIGURE III. 5A.
Effect of the antioxidants catalase and SOD on the Ca^{2+} response to ATP of aortic endothelial cells from SD rats.
Mean cytosolic Ca^{2+} concentration increase initiated by ATP (10 μM) measured in control cells and cells treated for 48h with catalase (CAT 48h) and superoxide dismutase (SOD 48h). There was no significant change in the average cytosolic Ca^{2+} concentration increase computed over the first 150 s following ATP stimulation in free external Ca^{2+} solutions due to the presence of antioxidants.

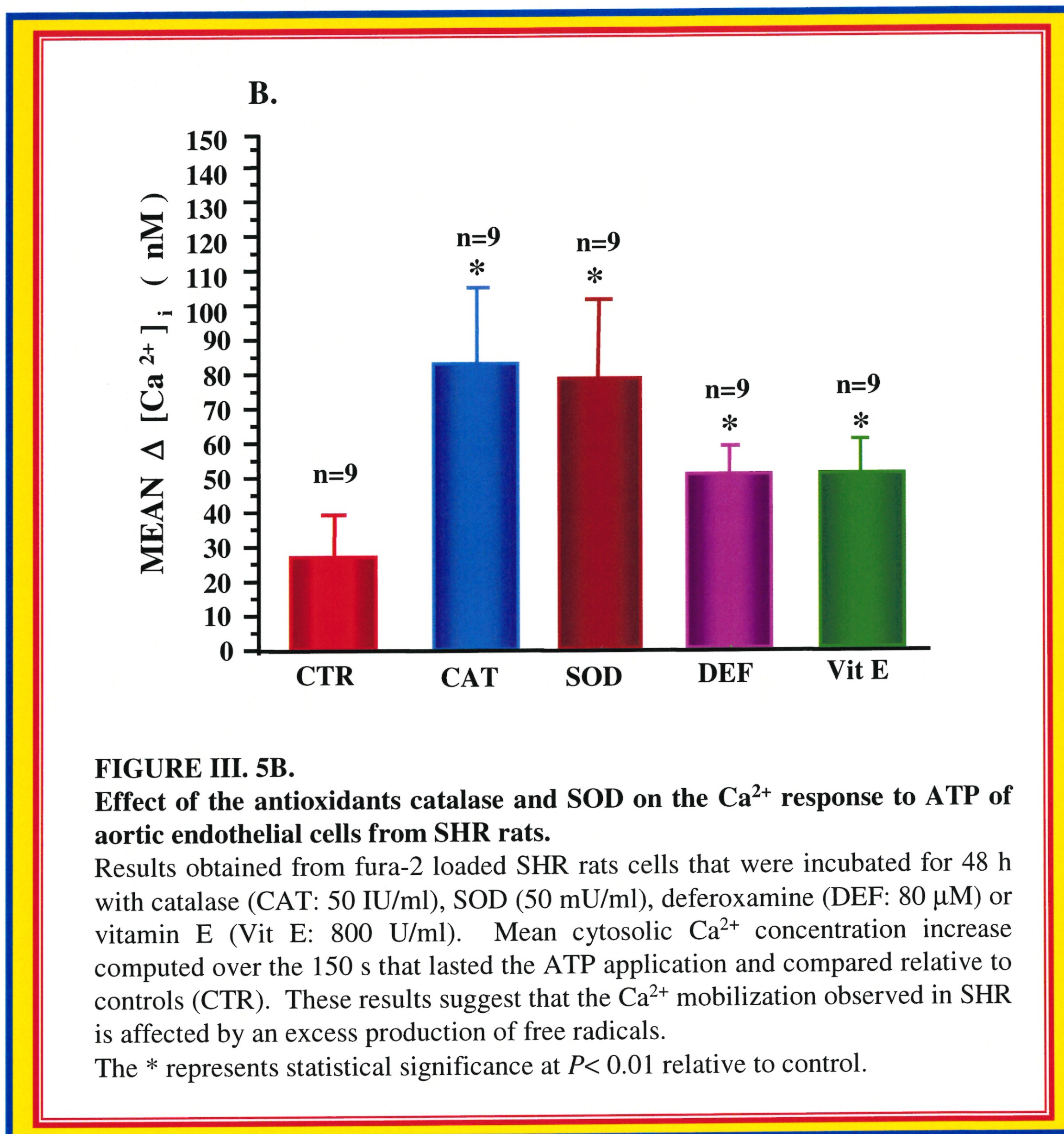

FIGURE III. 5B.
Effect of the antioxidants catalase and SOD on the Ca^{2+} response to ATP of aortic endothelial cells from SHR rats.
Results obtained from fura-2 loaded SHR rats cells that were incubated for 48 h with catalase (CAT: 50 IU/ml), SOD (50 mU/ml), deferoxamine (DEF: 80 μM) or vitamin E (Vit E: 800 U/ml). Mean cytosolic Ca^{2+} concentration increase computed over the 150 s that lasted the ATP application and compared relative to controls (CTR). These results suggest that the Ca^{2+} mobilization observed in SHR is affected by an excess production of free radicals.
The * represents statistical significance at $P < 0.01$ relative to control.

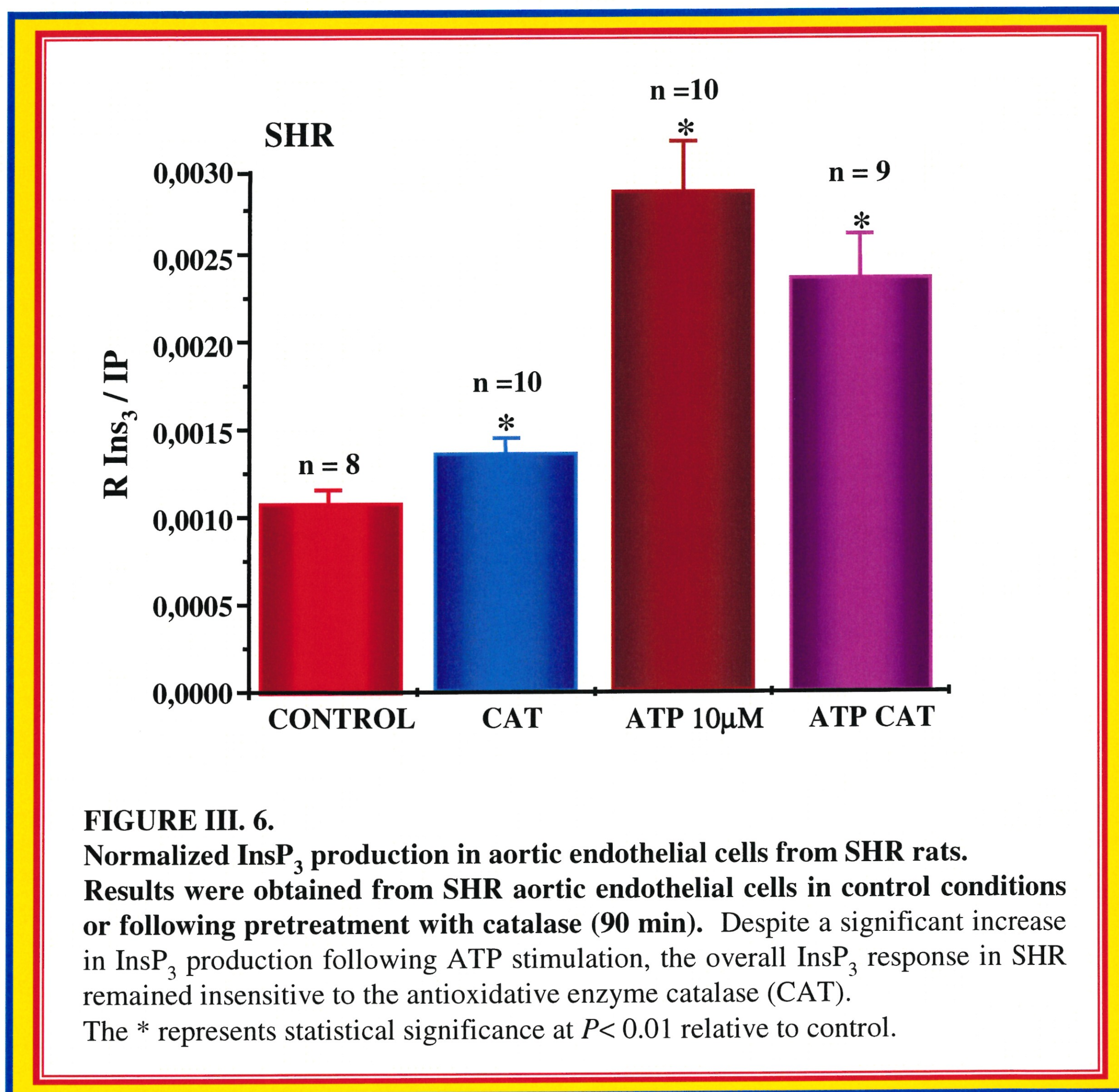

FIGURE III. 6.
Normalized $InsP_3$ production in aortic endothelial cells from SHR rats. Results were obtained from SHR aortic endothelial cells in control conditions or following pretreatment with catalase (90 min). Despite a significant increase in $InsP_3$ production following ATP stimulation, the overall $InsP_3$ response in SHR remained insensitive to the antioxidative enzyme catalase (CAT).
The * represents statistical significance at $P < 0.01$ relative to control.

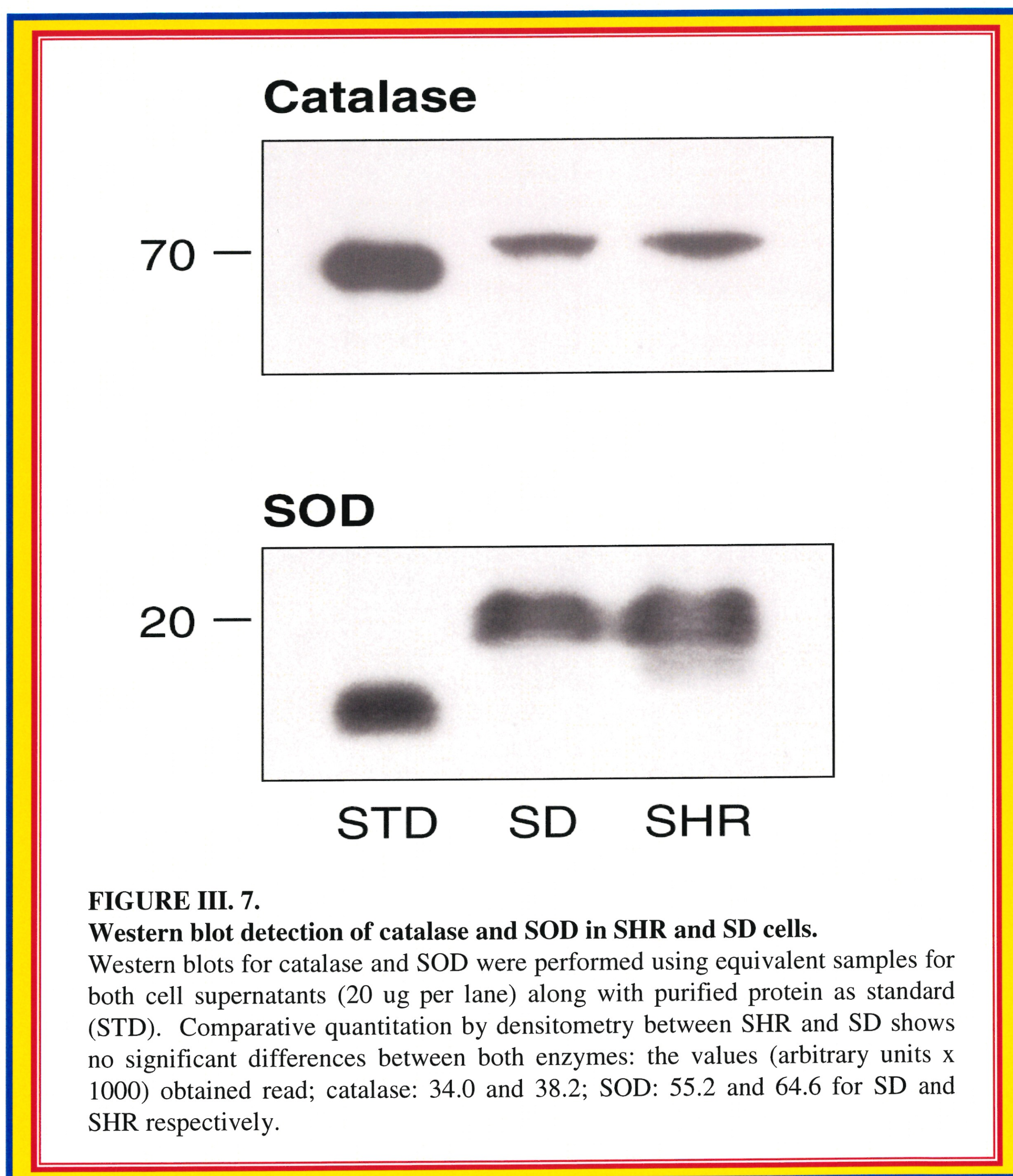

FIGURE III. 7.

Western blot detection of catalase and SOD in SHR and SD cells.

Western blots for catalase and SOD were performed using equivalent samples for both cell supernatants (20 ug per lane) along with purified protein as standard (STD). Comparative quantitation by densitometry between SHR and SD shows no significant differences between both enzymes: the values (arbitrary units x 1000) obtained read; catalase: 34.0 and 38.2; SOD: 55.2 and 64.6 for SD and SHR respectively.

	S D	**SHR**
CATALASE (OD/mg prot)	**3.4 ± 0.9**	**3.1 ± 0.8**
SOD (IU/mg prot)	**67 ± 4**	**68 ± 13**

Table III. 1.
Determination of enzymatic activities for SOD and catalase in SD and SHR cells.
The enzymatic determination for both enzymes was performed on 100,000 x g supernatants of cell homogenates as described in Methods. Experiments were performed in triplicate and the resulting values represent mean ± SEM of five cultures for both cell types.

CHAPTER IV. Article II

OXIDANT STRESS AND ANTIOXIDANT EFFECTS ON Ca^{2+} POOLS OF ENDOTHELIAL CELLS FROM NORMOTENSIVE AND HYPERTENSIVE RATS

Article in preparation for American Journal of Physiology

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Running title: OXIDANT AND ANTIOXIDANT EFFECTS ON Ca^{2+} POOLS OF ENDOTHELIAL CELLS

Key Words: Endothelium, hypertension, BAE, SHR, Sprague-Dawley, Ca^{2+}, Fura-2, thapsigargin, CCE, fluorescence,

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ABSTRACT

The evolution of intracellular calcium concentration ($[Ca^{2+}]_i$) in endothelial cells (ECs) plays a key role in endothelium production and release of vasoactive and paracrine factors. Changes in $[Ca^{2+}]_i$ depend on the releasable Ca^{2+} accumulated in intracytosolic pools and the entry of Ca^{2+} from extracellular space. The purpose of the present study is to characterize the behavior of Ca^{2+} pools in primary culture of aorta ECs from normotensive and hypertensive rats under normal, oxidative stress and antioxidative treatment conditions. The releasable Ca^{2+} was evaluated in Fura-2 loaded ECs by stimulation with $InsP_3$-generating agonist (ATP) and thapsigargin (TG). In contrast with the normotensive ECs from SD (Sprague Dawley) rats and BAE (Bovine Aorta Endothelial cells), the Ca^{2+}-pools of ECs from SHR (Spontaneously Hypertensive rats) are disproportionately smaller due to lesions inflicted by the endogenous production of free radicals. The results of this study demonstrate that despite the absence of intracytosolic caffeine-sensitive pools, there are multiple sensitivity Ca^{2+}-pools in primary cultures of rat aorta ECs. The existence of $InsP_3$-sensitive pools is complemented by TG-resistant pools in ECs from both SHR and SD rats. The results demonstrate that TG-resistant pools are insensitive to antioxidants, whereas the $InsP_3$-sensitive pools are antioxidant-sensitive and are located on ER membranes with TG-insensitive but antioxidant-sensitive SERCA isoforms. The $InsP_3$-sensitive Ca^{2+} pools in ECs from SHR are improved by antioxidative treatment in contrast with Ca^{2+} pools of ECs from normotensive rats, which remain unaffected, by antioxidant treatment. The intracellular pools of ECs from SHR are independent but not isolated because the results confirm a time dependent Ca^{2+} cross-exchange between TG- and $InsP_3$-sensitive pools. The HX/XO-induced oxidative stress generated a complete drain of Ca^{2+} from $InsP_3$- and TG-sensitive Ca^{2+}-pools in ECs from SD rats and stimulates their capacitative

Ca^{2+} entry (CCE) in a time dependent manner. The antioxidant treatment had no effect on the availability of Ca^{2+} in TG-sensitive pools, whereas the availability of $InsP_3$-sensitive pools was integrally recovered because of the TG-insensitive sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) isoform, which demonstrates to remain active under the antioxidant protection. The CCE is at the limit of detection in both strains of primary cultures of aorta ECs from rats, in comparison with the CCE of ECs from BAE. Only the double stimulation with TG and ATP induced a significant increase of CCE. Therefore, this study proves that the reduced Ca^{2+} availability of $InsP_3$-sensitive Ca^{2+} pools in SHR is the consequence of the overproduction of free radicals. It can be considered that the expression of an antioxidant-sensitive SERCA appears as an adaptation of endothelium to a potential burst of free radical overproduction.

IV.1. INTRODUCTION

The endothelial cell (EC) represents the fundamental structural unit of vascular endothelium and it is a small paracrine factory for the production and the release of a variety of autacoids and hormones [1,2]. Most of the EC's paracrine secretion is implicated in vascular modulation (permeation, vasodilatation and vasoconstriction) in the entire body, as are vascular growth factors, endothelium derived relaxing (EDRF) and/or contracting factors (EDCF).

The cytosolic free calcium concentration ($[Ca^{2+}]_i$) is a pivotal factor for the regulation of cellular transduction signals. In many cases, the initial response of endothelial cells to stimuli involves elevation of cytosolic Ca^{2+}. Both the release of Ca^{2+} from intracellular stores and the influx of Ca^{2+} from the extracellular space contribute to the $[Ca^{2+}]_i$ increase. There is increasing evidence that $[Ca^{2+}]_i$ and capacitative calcium entry (CCE) are involved in volume regulation, phototransduction, mitogenesis, regulation of adenylyl cyclase activity, sustained Ca^{2+} oscillations and Ca^{2+} waves in all non-excitable cells [3]. In ECs, the EDRF and EDCF release

is dependent upon the increase of $[Ca^{2+}]_i$ and the influx of extracellular Ca^{2+} [4], thus the increase of $[Ca^{2+}]_i$ is necessary and sufficient for the release of relaxing and/or contracting factors [5].

It is known that the ECs are provided with fast exchanging intracellular reservoirs of Ca^{2+}. They are able to release Ca^{2+} in response to rapidly diffusing second messengers, such as inositol 1,4,5-triphosphate ($InsP_3$) or to self-generating Ca^{2+} wave (Ca^{2+}-induced Ca^{2+} released). Therefore, a model of two intracytosolic Ca^{2+} pools has been proposed to cover the multiple Ca^{2+} manifestations of non-excitable cells [6]. ECs respond to hormones, agonists, and autacoids through a biphasic increase of $[Ca^{2+}]_i$. The most important trigger for Ca^{2+} release in ECs is $InsP_3$, which is generated by the sub-plasmalemmal enzyme phospholipase C. The first component of the biphasic mechanism of $[Ca^{2+}]_i$ increase is an $InsP_3$ mediated Ca^{2+} release from intracellular stores, directly coupled through an unknown mechanism of Ca^{2+} entry from the external medium, called CCE [7,8]. Evidence indicates that agonist-stimulated Ca^{2+} influx is dependent upon membrane potential and the degree of fullness of the endoplasmic reticulum [3,5,9]. The capacitative calcium entry might be the unique Ca^{2+} entry pathway in certain vascular endothelial cells, emphasizing the relevance of this pathway to the Ca^{2+} homeostasis [9-12]. The typical vascular endothelial cell lack voltage-gated Ca^{2+} channels and under basal conditions, a large inward rectifier K^+ current dominates the membrane potential [10,13]. Some stimuli, e.g., ATP, histamine, bradykinin, acetylcholine, are hyperpolarizing the membrane potential, thus increasing the electrochemical gradient for Ca^{2+}, which appears to be modulated by activation of Ca^{2+}-dependent K^+ and Cl^- currents [10,13-15]. Agonists and hormones in ECs activate Ca^{2+}-dependent K^+ channels ($K^+_{(Ca)}$); these are pharmacologically different from the $K^+_{(Ca)}$ channels described in other cell types [16] because the physiological role of these

channels in endothelium is to hyperpolarize the endothelial cell and to increase the driving force for Ca^{2+} entry during stimulation by agonists [15,17-19].

The free Ca^{2+} concentration in the cytosol is maintained between 0.04 and 0.1 μM [20,21]. The resting level of Ca^{2+} is balanced primarily by active Ca^{2+} extrusion systems located at the plasma membrane and by the coordinated activities of Ca^{2+} sequestering systems located in the endoplasmic reticulum (ER), mitochondria, and nuclear membranes. The sequestration of Ca^{2+} in cytoplasmic organelles forms the intracytosolic Ca^{2+} reservoirs or stores. The cytosolic stores contain three major components: 1) Ca^{2+} pumps in membranes to sequester and to extrude Ca^{2+}; 2) binding protein for an efficient and substantial sequestration (calsequestrin and calreticulin) in the matrix; and 3) specific receptors as $InsP_3R$ and ryanodine receptor (RyR) for the release of Ca^{2+} back to cytosol. Overlapping of $InsP_3$ and ryanodine-sensitive pools exist in most if not all cells at variable extent [22,23]. In ECs the major component responsible for intracytosolic Ca^{2+} release is $InsP_3R$, but reports indicate the existence of functional RyR in endothelial ER and ryanodine sensitive stores appear to overlap with $InsP_3$ sensitive stores [24,25].

The inhibition of SERCA with thapsigargin (TG), 2,5-di-t-butylhydroquinone (BHQ) or cyclopiazonic acid (CPA) induces the emptying of intracytosolic Ca^{2+}-pools through a time-dependent leak of stored Ca^{2+} from ER, and induces the signaling for the opening of CCE. For instance, there are demonstrations that not all $InsP_3$-sensitive pools are sensitive to the Ca^{2+}-ATPase's inhibitor thapsigargin. Genazzani and Galione have already demonstrated that nicotinic acid-adenine dinucleotide phosphate (NAADP) is able to mobilize Ca^{2+} from $InsP_3$- and TG-insensitive pools in sea-urchin egg [26]. Waldron et al. reported the selection of a thapsigargin-resistant strain of cells (DC-3F/TG2) in already thapsigargin-resistant pools of DC-3F cells, which is still $InsP_3$-sensitive and capable to accumulate Ca^{2+} against the presence of a

thapsigargin concentrations up to 10 μM [27-29]. The oxidant stress affects differently the intracytosolic Ca^{2+} pools of ECs. Elliott et al., report that in control calf pulmonary artery ECs (CPAECs), sequential additions of bradykinin and ionomycin produced similar increases in $[Ca^{2+}]_i$. By contrast, incubation with t-butyl hydroperoxide (t-BuOOH) progressively decreased the response of $[Ca^{2+}]_i$ to bradykinin and increased the response to ionomycin, suggesting that the total ionomycin-releasable Ca^{2+} pool remains replete during oxidant stress [30].

Our previous findings demonstrate the reduction of releasable Ca^{2+} from ECs submitted to oxidative stress. The ECs of spontaneously hypertensive rats (SHR) have a releasable Ca^{2+} sensitive to antioxidants, and antioxidant treatment improves the availability of Ca^{2+}, thus proving that oxidative stress dramatically affects the homeostasis of Ca^{2+} in ECs [21]. Because the nature of Ca^{2+} pools in ECs from SHR was never investigated and because a free radical altered Ca^{2+} homeostasis was previously demonstrated in ECs from SHR, a comparative study of intracytosolic Ca^{2+} pools of ECs from normotensive and hypertensive rats has become an imminent necessity. The aim of our study was to investigate the presence of different Ca^{2+} pools in primary cultures of ECs from normotensive and hypertensive rats and to analyze the impact of oxidative stress and antioxidant treatment in the evolution and availability of Ca^{2+} pools, corroborated with the modification of Ca^{2+} influx, under equivalent treatment conditions. Thus, a series of Fura-2 experiments have been undertaken to verify and to compare the assortment and the dimension of Ca^{2+} pools in primary cultures of ECs from SHR versus normotensive rats and ECs from BAE. The most important goal was to evaluate the effect of oxidant stress and the effect of antioxidant treatment on Ca^{2+} pools and on capacitative Ca^{2+} entry from both primary cultures of SHR and SD rats.

Our results demonstrate that both rat primary ECs cultures lack caffeine-sensitive Ca^{2+} pools. Our study demonstrates the presence of TG- and $InsP_3$-sensitive pools in both strains of

rat ECs. The dimensions of $InsP_3$- and TG-sensitive pools are disproportionately smaller in ECs from SHR, and TG-sensitive pools are relatively isolated from the $InsP_3$-sensitive pools in comparison with ECs from SD rats. While, the artificially induced oxidative stress on ECs from SD rats had a draining effect on both types of Ca^{2+} pools, the antioxidant treatment has induced a full protection merely to $InsP_3$-sensitive pools, whereas the TG-sensitive pools were insensitive to antioxidant treatment in both, artificially- and pathologically-induced oxidative stress in ECs from SD and SHR, respectively. The behavior of $InsP_3$-sensitive pools in ECs from SHR support the presence of a particular SERCA pump isoform, resistant to TG and oxidation, most likely following a genetic adaptation to the free radical overproduction. The agonist and/or TG-induced CCE in rat primary ECs cultures were at the limit of detection in comparison with the huge agonist-induced CCE in ECs from BAE. The CCE was considerably stimulated only after a powerful emptying of Ca^{2+} pools. The artificial oxidant stress induced, in a time dependent manner, a significant increase of CCE in primary cultures of rat ECs whereas the antioxidant treatment was devoid of effect vis-à-vis the CCE of both EC primary cultures.

IV.2. MATERIALS AND METHODS

IV.2.1. Vascular endothelial cells

The Bovine Aorta Endothelial cell line (BAE) is a laboratory culture maintained by successively reseeding endothelial cells originating from bovine aorta and sub-cultured according to previously described methods [31,32]. The BAE were used between passages 16-26 and verified for the presence of factor VIII to confirm the endothelial nature of cells. The culture of BAE was grown on 25 mm round coverslips coated with collagen, but without endothelial growth factor in the growing medium. Aortic endothelial cells from normotensive

and hypertensive rats were prepared according to the procedure described previously by Wang and Sauvé [33]. Briefly, aortic endothelial cells were isolated from Sprague Dawley (SD) male rats (12 weeks of age), sex, and age matched with spontaneously hypertensive rats (SHR) obtained from Charles River (St. Constant, Quebec, Canada). The 12 weeks old, male rats from both strains had a body weight between 250-275 g. The mean systolic blood pressure of SHR rats was 200 mm Hg and over, whereas the mean systolic blood pressure of SD rats was ≅ 120 mm Hg. The isolation method was identical for both rat strains. After the lethal anesthesia of rats (Phenobarbital, 50 mg/kg, IP), aortas were removed from the thoraco-abdominal cavity and cleaned of connective tissues. The vessels were cut open longitudinally in strips of 2-3 mm. The aortas were digested in a stepwise manner with various concentrations of dispase and collagenase in oxygenated Dulbecco's modified Earle's medium (Gibco BRL Products, Life technologies, Burlington, Ontario, Canada), at 37°C under mild agitation. Endothelial cells were harvested by centrifugation for 10 min at 2600 r.p.m. at room temperature. The rat aortic endothelial cells were cultured on collagen (1%, type VII, Sigma-Aldrich Canada Ltd.-Oakville, Ontario, Canada) pre-coated 25 mm round glass coverslips in Dulbecco's modified Earle's medium containing 15% fetal calf serum (Gibco BRL Products, Life technologies, Burlington, Ontario, Canada), 10 ng/ml endothelial cell growth factor and 50 μg heparin/ml. To minimize the danger of phenotypic changes during culture procedures, only ATP and BK monitored cells from the primary culture and the first passage were used. The single subculture passage of primary culture was realized by treatment of confluent endothelial monolayers with 0.05% trypsin in 0.5 mM EDTA saline for 1-2 min at room temperature. Their "cobblestone-like" aspect when grown into a confluent monolayer and the presence in their cytoplasm of the ultra-structural characteristics of Weibel-Palade bodies identified the endothelial cells

morphologically whereas the presence of factor VIII confirmed the endothelial histochemical nature of cells.

IV.2.2. Solutions

The external bathing medium was a standard Earle's solution containing (in mM): 121 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 6 $NaHCO_3$, 1 NaH_2PO_4, 5.5 glucose and 25 HEPES buffered at pH 7.3 with 10 KOH. Calcium-free external solutions were prepared by adding 2 mM K_2 EGTA to Earle's solutions without $CaCl_2$. Acetoxymethylester Fura-2, ionomicyn, hypoxanthine, SOD, deferoxamine, glucose oxidase, vitamin E, and heparin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Thapsigargin was obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada), preserved in a 1mM DMSO stock solution and used in a 1µM in final solution. Endothelial growth factor, collagenase, dispase, xanthine-oxidase, catalase, and ATP were from Roche Diagnostic Canada (Laval, Quebec, Canada). Superoxide production by xanthine-oxidase was assayed by monitoring *cytochrome c* reduction at 550 nm.

IV.2.3. Microspectrofluorometry

The fluorescence was recorded using an epifluorescent microscope (Nikon inverted microscope IM 35) attached to a dual-excitation spectrofluorometer (Spex Fluorolog II; Spex Industries Inc., Edison, New Jersey, USA). For Fura-2, the excitation wavelengths were set at 350 nm and 380 nm, respectively, and a dichroic mirror (Nikon FT 400) was placed in the excitation pathway. The emission was monitored at 505 nm with a standard band pass filter (Andover Corporation 500FS10). The objective was a Nikon Neofluor 40x (Nikon, Nikon Canada Instruments Inc., Montreal, Canada).

The intracellular calcium concentration, $[Ca^{2+}]_i$ was calculated with the Grynkiewicz (1985) equation:

$$[Ca^{2+}]_i = Kd \times (R-R_{MIN})/(R_{MAX}-R) \times Sf_2/Sb_2, \qquad (1)$$

with Kd equal to 224 nM, R the ratio of the fluorescence measured at 350 nm and 380 nm respectively, and Sf_2/Sb_2, the ratio of fluorescence at 380 nm in low and high calcium respectively. The maximal and minimal fluorescence ratios R_{MAX} and R_{MIN} were determined using 10 μM ionomycin to permeabilize the cells, first in standard Earle's solution and second in an Earle's solution containing 10 mM EGTA at pH 8.8.

The mean increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) during stimulation, was estimated by integrating the function:

$$[Ca^{2+}]_i = 1/T \int_0^T ([Ca^{2+}]_i(\tau) - {}^b[Ca^{2+}]_i)\Delta\tau \qquad (2)$$

where ${}^b[Ca^{2+}]_i$ is the $[Ca^{2+}]_i$ prevailing before stimulation, $[Ca^{2+}]_i(\tau)$ the Fura-2 derived variation in cytosolic Ca^{2+} concentration during stimulation by the agonist and T, the total time corresponding to the period of stimulation.

IV.2.4. Statistical analysis

The data were expressed as means ±SEM. Statistical significance was analyzed using unpaired Student's T-test. The P level < 0.01 was taken to reflect a significant difference. The resultant variations in calcium concentrations are presented as mean $\Delta[Ca^{2+}]_i$/s nM calculated after integration of the difference $[Ca^{2+}]_i(\tau)$ - ${}^b[Ca^{2+}]_i$ divided by the time length of each stimulation.

IV.3. RESULTS

IV.3.1. ATP AND THAPSIGARGIN INDUCED Ca^{2+} RELEASE IN AORTA ENDOTHELIAL CELLS

The Ca^{2+} responses of SD, SHR and BAE endothelial cells to 10 µM ATP and 1µM TG were investigated in a series of Fura-2 experiments in which cells were initially stimulated in external Ca^{2+}-free conditions followed by a cell perfusion with a 1.8 mM Ca^{2+} medium. This procedure allows an estimation of the amount of Ca^{2+} release from $InsP_3$-sensitive pools, whereas the readmission of Ca^{2+} in perfusion provides indications on the magnitude of the associated capacitative Ca^{2+} entry component of the whole agonist-induced Ca^{2+} response. Analysis of the results obtained from the Fura-2 experiments, were carried out in conformity with equation (1) and (2). Figure 1A shows the mean $\Delta[Ca^{2+}]_i$, induced by 10 µM ATP carried out in $[Ca^{2+}]_o$, and computed over a period of 150 s. The ATP released in ECs from SD rats had a mean $\Delta[Ca^{2+}]_i$ of 65 ± 11 nM (n=26) whereas, a mean $\Delta[Ca^{2+}]_i$ of only 24 ± 6 nM (n=18) or 170% less (P<0.01), was released in ECs from SHR. In ECs from BAE, the ATP released had a mean $\Delta[Ca^{2+}]_i$ of 152 ± 12 nM (n=10), which was 130% larger than that of ECs from SD rats and 530% higher than that of ECs from SHR (P<0.01).

ECs were stimulated with the same type of protocols, but with 1 µM of the specific SERCA inhibitor TG, a stimulation which avoids the production of $InsP_3$. As shown in Figure 1B, in primary cultures of ECs from SD rats, the TG released a mean $\Delta[Ca^{2+}]_i$ of 53 ± 6 nM (n = 6) and in ECs from SHR, the mean $\Delta[Ca^{2+}]_i$ was 23 ± 4 nM (n = 6), 112% less than in ECs from SD rats (P<0.01), but identical to the ATP-induced Ca^{2+} release from $InsP_3$-sensitive pools. In the ECs from BAE, TG has released only 98 ± 12 nM Ca^{2+} (n = 6), a significantly lower (55%

less ($P<0.01$)) amount of Ca^{2+} than that released by ATP, but statistically higher in comparison with rat ECs. It is important to note that the quantities of Ca^{2+} released from $InsP_3$- and TG-sensitive pools from primary cultured endothelial cells are much smaller than the responses of ECs from BAE. These results demonstrate also that the ECs from hypertensive rats have significantly smaller responses for both ATP and TG than ECs from SD rats. Therefore, these results confirm the presence of smaller Ca^{2+} pools and/or smaller Ca^{2+} availability in ECs from SHR, independently of $InsP_3$ production.

The behavior of TG-induced Ca^{2+} release is different if the external bathing solution contains the physiologic concentration of Ca^{2+}. Thus, if Ca^{2+} release is induced by TG in the presence of external Ca^{2+} as seen in Figure 2A, the mean $\Delta[Ca^{2+}]_i$ measured for TG will increase at 69 ± 6 nM, which is 30% higher and statistically different ($P<0.01$) from the TG-induced Ca^{2+} release in free Ca^{2+} external conditions (only 53 ± 4 nM) Figure 1B). This result indicates that in these conditions, the emptying of Ca^{2+} pools was rapidly signaled to the plasmalemma and the Ca^{2+} entry was activated and associated immediately to the entire TG-induced Ca^{2+} release.

IV.3.2. DIVERSITY OF Ca^{2+} POOLS IN AORTIC ECs FROM SHR AND SD RATS

To examine the diversity and the overlapping of the cytosolic Ca^{2+} pools underlying the ATP- and TG-dependent Ca^{2+} release in ECs from SHR and SD rats, a series of experiments was undertaken where ECs were exposed first to 1 µM TG and subsequently stimulated with 10 µM ATP in external Ca^{2+} free conditions. This experimental procedure allows first a mobilization of Ca^{2+} from internal pools avoiding the $InsP_3$ production and in the second part of the protocol, the stimulation with ATP induces a Ca^{2+} release through the $InsP_3$ formation cascade. In control experiments, TG induced a mean $\Delta[Ca^{2+}]_i$ of 43 ± 8 nM while the Ca^{2+} response to ATP after TG, corresponds to a mean $\Delta[Ca^{2+}]_i$ of 36 ± 4 nM (n=8). The results

shown in Figure 3 indicate that the mean $\Delta[Ca^{2+}]_i$ measured for ATP, following the TG stimulation, was statistically smaller in comparison with the mean $\Delta[Ca^{2+}]_i$ obtained from only ATP-stimulated cells (65 ± 11 nM) (Figure 3B). These findings indicate the presence in ECs of SD rats of at least two types of Ca^{2+} pools. The value of ATP-induced Ca^{2+} release obtained after the TG-induced Ca^{2+} release was ≈ 45% smaller than in control experiments, whereas the TG-induced Ca^{2+} release was not statistically different relative to control. This observation suggests that TG- and ATP-sensitive pools would not be completely independent, but they overlap and could exchange important quantities of Ca^{2+}.

The same types of protocols were carried out in ECs from SHR. The application of TG initially induced the release of a mean $\Delta[Ca^{2+}]_i$ of 22 ± 4 nM, whereas the consecutive ATP-stimulation caused a mean $\Delta[Ca^{2+}]_i$ of 23 ± 4 nM (n = 4). The dimension of both TG- and ATP-induced Ca^{2+} release are almost equivalent with those of control conditions (Figure 4). It is important to note that the Ca^{2+} pools in ECs from SHR returned to the basal level of $[Ca^{2+}]_i$ ($^b[Ca^{2+}]_i$) after both TG and ATP stimulation. After the preconditioning of ECs from SHR with catalase for 90 min, the same stimulation sequence induced a slight increase of TG-induced Ca^{2+} release from TG-sensitive pools (29 ± 8 nM, n=4), statistically not different in comparison with control, whereas the mean $\Delta[Ca^{2+}]_i$ released by ATP from $InsP_3$-sensitive pool increased to 63 ± 12 nM (n=4). The ATP-induced Ca^{2+} release presents a significant difference ($P<0.01$) versus catalase untreated ECs from SHR, with an increase of more than 170%. The catalase was not able to restore the recapture function of the TG-sensitive pools' SERCA pumps to the level of cells from normotensive animals but the $InsP_3$-sensitive pools' SERCA pumps were fully protected by catalase. Therefore, the $InsP_3$-sensitive pool of ECs from SHR is susceptible to the antioxidant effect of catalase and it strongly suggests that a reducing intracellular environment is

favorable to the availability of Ca^{2+} from $InsP_3$-sensitive pools due to the presence of a different SERCA isoform. These results suggest that catalase, an enzyme that catalyzes peroxides decomposition, has an important shield effect against TG inhibition of SERCA from $InsP_3$-sensitive pools. The mechanism of TG inhibition on this type of SERCAs would be realized through an organic peroxidation process, which can be prevented by the catalase activity. It is important to note that the CCE in ECs from SHR rats stimulated consecutively by TG + ATP leads to an important activation and increase of the agonist-induced Ca^{2+} influx (Figure 4A and 3A).

With ECs from SHR, additional experiments were performed to investigate the effect of TG on Ca^{2+} mobilization initiated by ATP after exposing the cells to TG preconditioning (1 μM) for a period of 1 to 6 hours before ATP (10 μM) stimulation in external free-Ca^{2+} conditions. Examples of Fura-2 recordings carried out according to this protocol are illustrated in Figure 5A and B. After 1 h of incubation in TG, the same amount of Ca^{2+} as in control conditions, a mean $\Delta[Ca^{2+}]_i$ of 22 ± 5 nM (n = 3), was released from $InsP_3$-sensitive pools. However, a prolonged TG incubation for 6 h induced a complete drain of Ca^{2+} from intracytosolic pools. The upshot of these experiments demonstrates that the mean $\Delta[Ca^{2+}]_i$ released by ATP is not statistically different in TG-treated versus control cells (from Figure 1A and 4B), strongly suggesting that the $InsP_3$-sensitive pools are relatively independent and are not overlapped with TG-sensitive pools. In contrast, the capacitative Ca^{2+} entry initiated by readmission of Ca^{2+} in the external medium appeared substantially higher in cells exposed to the combined stimulation of TG and ATP. These observations suggest that the $InsP_3$-sensitive Ca^{2+} pools are unaffected by TG, and that combined stimulation with TG and ATP is essential to generate a significant agonist-induced Ca^{2+} influx in SHR cells. However, the $InsP_3$- and TG-sensitive pools are not utterly isolated and there might be an important exchange of Ca^{2+}

between the two pools because the leak of Ca^{2+} after prolonged incubation in TG for 6 h has induced a complete emptying of $InsP_3$-sensitive pools and a consequent annihilation of ATP-induced Ca^{2+} response (Figure 5B).

The potential existence and contribution of ryanodine-sensitive Ca^{2+} pools to the cytosolic Ca^{2+} increase was investigated by measuring the sequential Ca^{2+} responses induced by caffeine and ATP in ECs from SHR and SD rats. Caffeine in doses from 10-20 mM failed to cause a detectable increase in internal Ca^{2+} under these conditions (Figure 6A). Evidence suggests that TG can unmask the caffeine-sensitive pools after depletion of intracytosolic Ca^{2+} pools [34]. Thus, ECs from SHR and SD rats that have been exposed to TG for 60 min were stimulated with 20 mM caffeine (Figure 6B). Even in these conditions, caffeine failed to induce a detectable increase in internal Ca^{2+} but, at this dose, it has substantially stimulated the Ca^{2+} mobilization triggered by ATP from $InsP_3$-sensitive pools. The mean $\Delta[Ca^{2+}]_i$ measured in this case was equal to 70 ± 10 nM (n=3) (increase of >190%)(Figure 7). This value is significantly higher ($P<0.01$) than the corresponding ATP-induced mean $\Delta[Ca^{2+}]_i$ obtained from SHR cells in control conditions (see Figure 1A and 4). These results confirmed that the $InsP_3$-sensitive stores in primary cultures of ECs from SHR and SD rats are resistant to TG while indicating an unidentified stimulatory effect of caffeine on the cytosolic Ca^{2+} release initiated by ATP.

IV.3.3. THE EFFECT OF OXIDATIVE STRESS AND ANTIOXIDANT TREATMENT ON Ca^{2+} POOLS OF ECs FROM SHR AND SD RATS

The changes in cytosolic Ca^{2+} induced by TG and ATP stimulations have been investigated under the conditions of oxidative stress, where confluent ECs from SD rats have been incubated for 90 min in the free radical generating system hypoxanthine (250 µM) and xanthine oxidase (50 mU) (HX/XO). After HX/XO-induced oxidative stress, SD cells were

consecutively stimulated by TG and ATP (Figure 8 and Figure 9A and B). Firstly, it is important to note the elevated $^{b}[Ca^{2+}]_i$ in cells exposed only to HX/XO free radical generating system (Figure 8). As it can be seen (Figure 9A) there was a slightly detectable increase in internal Ca^{2+} following the administration of 1 μM TG (the mean $\Delta[Ca^{2+}]_i$ was < 3nM). The 10 μM ATP stimulation failed to initiate a release of Ca^{2+} from $InsP_3$-sensitive pools (Figure 8 and 9B). The remarkable change to be mentioned in these conditions is the increased amplitude of the capacitative Ca^{2+} entry.

Co-incubating the cells in HX/XO plus either catalase or SOD reduced the initial $^{b}[Ca^{2+}]_i$ level but did not improve the Ca^{2+} response to TG (Figure 8 and 9A). In contrast, the results summarized in Figure 9B demonstrate that the presence of catalase and SOD has reversed the damaging effect of free radicals only for $InsP_3$-sensitive pools, with mean $\Delta[Ca^{2+}]_i$ values of 66 ± 8 nM (n=5) and 55 ± 6 nM (n=5) respectively, which are relatively equivalent with the control. This finding is very important because it corroborates positively with the possible presence of a TG-resistant SERCA isoform in ECs from SHR and demonstrates that this pump isoform is affected into a similar manner by TG treatment and artificial oxidant stress, a mechanism that actually is sensitive to antioxidant treatment.

Additional investigations about the effect of antioxidants on TG-sensitive pools were completed on ECs from SHR. The results presented in Figures 10A and B provide evidence for the existence of a TG-sensitive Ca^{2+} pool where TG, applied for 250 s, has released a mean $\Delta[Ca^{2+}]_i$ values of 27 ± 6 nM in ECs from SHR in control conditions (n=4). This TG-induced Ca^{2+} release would represent less than half of Ca^{2+} released by TG in ECs from SD rats. Experiments were performed on preconditioned ECs from SHR where ECs were incubated in the presence of catalase and SOD for 90 min. Despite a substantial recovery of the $InsP_3$-

sensitive pools in SHR cells treated with either catalase or SOD, the TG-induced Ca^{2+} release was not influenced at all by either catalase or SOD. Catalase-treated ECs have released 28 ± 3 nM (n=4) and SOD preconditioned ECs have generated only 26 ± 5 nm (n=4). Therefore, the comparison between the control and the Ca^{2+} responses generated by TG in the presence of antioxidant enzymes, demonstrates no statistical differences. This observation indicates that the beneficial effect of catalase and SOD on ATP-induced Ca^{2+} signaling measured in ECs from SHR and SD rats under artificial oxidant stress do not affect TG-sensitive pools, because the SERCA isoform of TG-sensitive pools is most likely different from that of $InsP_3$-sensitive pools.

IV.3.4. THE EFFECT OF OXIDATIVE STRESS AND ANTIOXIDANT TREATMENT ON CAPACITATIVE Ca ENTRY OF ECs FROM SHR AND SD RATS

The capacitative calcium entry (CCE) has been taken and analyzed from all experimental protocols (n > 50) of ECs from SD and SHR. The time integration of CCE curves and the statistical analysis have generated Figure 11 and 12. These data demonstrate that there is no significant difference in CCE dimension between SD and SHR cells in control conditions, showing a very weak CCE mean $\Delta[Ca^{2+}]_i$, between 2.0 and 2.5 nM (Figure 11 and 12). However, the results represented in Figure 11B, show that oxidative stress was capable of inducing a very important increase of CCE in ECs from SD. It is very difficult to prove that this increase is related only to the opening of the Ca^{2+} entry by emptying the intracellular Ca^{2+} pools. The oxidative stress and the consecutive stimulation with TG and ATP have increased the CCE by 220% versus control. But, the oxidative stress combined with catalase and SOD treatment induced a CCE equivalent with control conditions (Figure 11A) suggesting that oxidative stress-induced increase of CCE is strictly related with the deleterious effect of free radicals, which are

emptying Ca^{2+} pools and probably produce an increase of membrane permeation to cations. In SHR cells (Figure 12A), the antioxidant enzymes have an unimportant inhibitory effect over the calcium entry. The combined stimulation of TG and ATP was the only means to stimulate significantly (increased with a factor >3) the CCE in ECs from SHR, whereas the catalase was without effect on CCE in these conditions (Figure 12B).

IV.4. DISCUSSION

The results presented in this study demonstrate significant differences in the amount of releasable Ca^{2+} from $InsP_3$- and TG-sensitive Ca^{2+} reservoirs of different types of ECs. Both, the ATP- and TG-induced Ca^{2+} responses are of larger magnitude in ECs from BAE compared with the Ca^{2+} responses obtained in equivalent experimental conditions from primary cultures of rat aorta endothelial cells. The matching Ca^{2+} responses obtained from aorta ECs of hypertensive (SHR) rats are much smaller in comparison with aorta ECs from normotensive (SD) rats. The results demonstrate that ECs from SHR have 60% smaller ATP-induced Ca^{2+} responses and 55% smaller TG-induced Ca^{2+} responses than the corresponding normotensive (SD) rat strain. The smaller Ca^{2+} responses in ECs from SHR were demonstrated to be the result of a deleterious overproduction of free radicals, which dramatically affects the whole status of Ca^{2+} pools [21]. The evidence demonstrates that the behavior of Ca^{2+} homeostasis of ECs from SHR is similar with that of ECs from normotensive SD rats submitted to oxidative stress. These results provide evidence that ECs have separated intracellular Ca^{2+} pools, shown by their different sensitivity for TG, ATP, and antioxidant treatment. Our results demonstrate for the first time, the presence in endothelium from SHR rats of independent TG-resistant pools insensitive to antioxidants, and of independent $InsP_3$-sensitive pools that are sensitive to antioxidants. Our study also indicates that aortic endothelial cells from SD and SHR display a

very weak agonist-evoked Ca^{2+} influx, but the combined stimulation of TG and ATP results in a substantial increase of agonist-evoked Ca^{2+} entry.

IV.4.1. The dimension of agonist-evoked Ca^{2+} increase in endothelial cells

The huge variability of ECs agonist-induced Ca^{2+} response values reported in the literature indicates that the Ca^{2+} homeostasis of cultured endothelial cells might behave differently from ECs *in situ* [35,36]. There is accumulating evidence, including the results from Ca^{2+} measurements presented in this study, suggesting an impaired Ca^{2+} response to Ca^{2+} mobilizing agonists in endothelial cells from hypertensive animal models compared to normotensive controls [33,37,38]. For instance, a depressed Ca^{2+} response to acetylcholine was recorded in aortic endothelial cells from aldosterone-salt hypertensive SD rats compared to controls [39]. Similarly, the Ca^{2+} increase initiated by bradykinin was found to be of smaller magnitude in aortic endothelial cells from SHR compared to SD [21] and Wistar Kyoto (WKY) rats [33], although, the angiotensin-II-induced Ca^{2+} responses do not appear to be statistically different between normotensive and hypertensive rat strains [33]. Several factors may be responsible for the decreased Ca^{2+} mobilization observed in endothelial cells from a variety of hypertension models. These include a decreased expression of functional membrane receptors, depressed $InsP_3$ production machinery or a depletion of releasable Ca^{2+} from intracytosolic pools. One of the major damaging factors for the decreased agonist-induced Ca^{2+} mobilization is the production of free radicals. Several sources of $^{\bullet}O_2^-$ in the endothelium of hypertensive animal models have been reported including cyclooxygenase and NADH oxidoreductase [40,41]. Recent evidence points toward an increased expression of the endothelium constitutive nitric oxide synthase (cNOS) as one of the major sources for the excess $^{\bullet}O_2^-$ production measured in SHR and SHRSP (Stroke prone SHR) endothelial cells [42,43]. All these free

radical production sources alter the Ca^{2+} signaling mechanism in SHR and induce a depletion of releasable Ca^{2+} from intracytosolic pools [21].

IV.4.2. The nature of Ca^{2+} pools in ECs from SD and SHR

Ca^{2+} pools are most frequently characterized in terms of their dynamic properties, pharmacological characteristics, and availability of Ca^{2+} in rapidly- and slowly-exchanging Ca^{2+} pools. Nevertheless, the properties of Ca^{2+} storage compartments and their characterization are not limited only to the Ca^{2+} exchanging rates with the cytosol and/or other pools, but they pertain also to their functions. They are actively implicated in the regulation of cellular metabolic functions, cellular reactivity and most likely, through an active interaction between intracytosolic organelles, in which the inter-pool-Ca^{2+} exchange, $[Ca^{2+}]_i$ regulation, as well as the modulation of Ca^{2+} sequestration are fundamental features of Ca^{2+} pools' architecture [44,45]. Therefore, we can assume that mitochondrial Ca^{2+} is an essential dynamic regulator of metabolism; S/ER Ca^{2+} is essentially a rapidly mobilizing reservoir, whereas *trans*-Golgi network Ca^{2+} content appears to be critical for the packaging of the regulated luminal content. Unfortunately, to date, only nonspecific pharmacological tools permit a relative discrimination of the complex interrelationships between the intracytosolic Ca^{2+} pools.

As an accepted landmark, TG is a specific and irreversible inhibitor of all SERCAs from all intracytosolic pools. Through the alteration of the pump-and-leak equilibrium, it thus empties all Ca^{2+} pools. Therefore, TG became the pharmacological tool of choice to stimulate the release of Ca^{2+} from internal pools avoiding the production of $InsP_3$ [46,47]. In our experiments, carried out in free $[Ca^{2+}]_o$, TG and ATP have released an almost equivalent amount of Ca^{2+}. New evidence is demonstrating that the intracytosolic Ca^{2+} pools can be endowed with different SERCA affinities and sensitivities to TG. Waldron et al. have demonstrated that in

DC-3F/TG2 cells, which grow in 10 μM thapsigargin, a TG-resistant pool was capable of generating rapid cytosolic Ca^{2+} signals in response to a phospholipase C-coupled agonist. This resistance was attributable to the expression of a novel intracellular Ca^{2+} pump activity with 20.000-fold lower sensitivity to thapsigargin, but with a similar high affinity for Ca^{2+} (K_m 0.1 μM), detectable also in the parental thapsigargin-resistant cell line DC-3F/TG2 [47]. Nevertheless, ECs hold a plasticity of expression of various active SERCAs and $InsP_3R$ isoforms during proliferation and differentiation, and possibly different Ca^{2+} pools may play distinct roles in long-term cell physiology. This plasticity is related to the variable type of ECs [48]. Genazzani and Galione [26] demonstrate that the nicotinic acid-adenine dinucleotide phosphate (NAADP) is a novel intracellular Ca^{2+} releasing agent in sea-urchin eggs. The NAADP induces Ca^{2+} release from Ca^{2+} pools independent of those induced by $InsP_3$ or cyclic adenosine dinucleotide phosphate (cADP), which are resistant to TG and cyclopiazonic acid [26]. Corda et al. propose the existence of a distinct caffeine-sensitive pool in ECs, controlled by the status of Ca^{2+} loading of intracellular pools and expressed only in the presence of physiological $[Ca^{2+}]_o$ [34]. In human aortic ECs, 1 μM of TG induces a full emptying of Ca^{2+} pools in free $[Ca^{2+}]_o$ in less than 10 min and no further increase in $[Ca^{2+}]_i$ was elicited by ionomycin [34]. Despite this observation, our experimental data from primary cultures of ECs from SHR rats, demonstrate that no important overlap exists between the $InsP_3$- and TG-sensitive Ca^{2+} pools. An exposure of SHR cells to TG for periods up to 60 min was unable to prevent a subsequent ATP-induced Ca^{2+} release, suggesting the presence on ER of a TG-resistant SERCA, which maintains loading of Ca^{2+} in $InsP_3$-sensitive pools despite the presence of TG. However, a prolonged incubation (2-6 h) in TG has reduced in a time-dependent manner the amount of Ca^{2+} released by ATP. After 6 h of incubation in TG, the ATP was not capable to release detectable Ca^{2+}, demonstrating that Ca^{2+} pools have been completely emptied, probably

because of a communication and Ca^{2+} exchange, which exists between intracytosolic Ca^{2+} pools. Indeed, in most cells, the $InsP_3$- or TG-sensitive Ca^{2+} stores encompass only a portion of the total cellular Ca^{2+} stores, and different Ca^{2+} pools do interact actively through an important Ca^{2+} exchange, as demonstrated in these results. As it can be seen in Figure 5B, this was the condition for the stimulation of an important CCE and it was similar to the CCE measured in ECs from BAE.

The single possible experimental approach to investigate the expression of $InsP_3$- and RyR-sensitive pools through Ca^{2+} measurements is as follows: the Ca^{2+} sensitive fluorophore loaded cells are stimulated sequentially in Ca^{2+} free medium, first with caffeine and later with $InsP_3$-generating agonist. Afterwards, the order of agonist is reversed, first the $InsP_3$-generating stimulus followed by caffeine. If the two types of channels are expressed on separate pools, the rise of $[Ca^{2+}]_i$ caused by caffeine should not affect the rise of $[Ca^{2+}]_i$ induced by $InsP_3$ and vice versa [22]. Caffeine is used as a probe to investigate the presence of Ca^{2+} pools regulated by CICR mechanism. It was proposed that the action of caffeine consists essentially in increasing the Ca^{2+} affinity of the RyR channel [49]. Our laboratory has already reported the existence of caffeine sensitive pools in ECs from BAE [50]. In another study on freshly dissociated single rabbit aortic ECs, caffeine induced a transient rise of $[Ca^{2+}]_i$ in Fura-2 loaded cells in the presence and absence of extracellular Ca^{2+}. This was associated with a dose-dependent increase in unitary current activity superimposed on a large and prolonged transient outward current upon application of caffeine and was documented by whole-cell membrane currents recorded under voltage–clamp conditions [37]. Despite these observations, the present study demonstrate that the perfusion of 10-20 mM of caffeine, in Ca^{2+} free solution with the primary cultures of ECs from SHR and SD rats, has had no impact on mobilization of Ca^{2+} from internal pools. In spite of the fact that caffeine appeared ineffective in resting, TG-treated or ATP-stimulated ECs,

the absence of caffeine-induced Ca^{2+} release cannot be directly regarded as evidence for the lack of RyR or of caffeine-sensitive Ca^{2+} channels on rat ECs [51].

The pretreatment of intact cells with high concentrations of TG results in the abolition of Ca^{2+} release by both agonists coupled to $InsP_3$ generation and activators of ryanodine receptors, but in a few cell types, a residual caffeine-induced Ca^{2+} mobilization has been observed after TG treatment [52-54]. In our experiments, after prolonged TG incubation (1h), caffeine, without initiating a detectable Ca^{2+} increase, was found to potentiate the mobilization of ATP-induced Ca^{2+}-release in ECs from SHR. Caffeine has been constantly reported to behave as an inhibitor of the $InsP_3$ receptor and phosphodiesterases. Nevertheless, under these experimental conditions, the administration of caffeine in ECs from SHR is likely to promote an efficient Ca^{2+} sequestration, a protection of $InsP_3$-sensitive Ca^{2+} pools and/or a transfer of Ca^{2+} from $InsP_3$-insensitive to $InsP_3$-sensitive pools, leading to an improved Ca^{2+} mobilization by ATP. Therefore, the subsequent ATP stimulation to caffeine of ECs from SHR and SD rats induced an important increase of ATP-induced Ca^{2+} release. It was demonstrated that the histamine stimulation subsequent to caffeine administration induced a substantial increase of Ca^{2+} release in HeLa cells [51] maintained in free $[Ca^{2+}]_o$, showing that caffeine causes a refilling of the histamine-related pools. It can be argued that a more important Ca^{2+} release in response to a second application of the agonist was always measured in cells exposed to caffeine during the first application of the agonist [51].

The combined stimulation of TG with ATP led to a significant increase of the agonist-induced Ca^{2+} entry in both SD and SHR endothelial cells. Through this double stimulation, much of the Ca^{2+} pools have been emptied and the signal for the opening of the CCE for refilling the intracellular pools was generated. This indicates that only the combined stimulation

of Ca^{2+} pools was essential for the signaling process leading to the activation of an important Ca^{2+} influx in these cells.

IV.4.3. The effect of oxidative stress and antioxidant treatment on Ca^{2+} pools

Exposure of ECs from SD rats for 90 min to HX/XO-free radical generating system induced an inhibition of both, TG- and ATP-induced Ca^{2+} release from internal pools. The oxidative stress affects both $InsP_3$- and TG-sensitive pools in a similar manner, by inducing a time dependent emptying of releasable Ca^{2+}. Our data are in contradiction with the results reported by Elliott and Doan [30] who have found that t-BuOOH-induced oxidant stress in CPA cells progressively decreased the $[Ca^{2+}]_i$ induced by bradykinin and increased the response to ionomycin, suggesting that the total ionomycin-releasable Ca^{2+} pool remains replete during oxidant stress. From their proposal, the Ca^{2+} from the $InsP_3$-sensitive pool would be transferred and available to the ionomycin induced Ca^{2+} release. In our primary culture of rat ECs, Ca^{2+} has become unavailable in both $InsP_3$- and TG-sensitive pools after oxidant stress.

The antioxidant enzymes were not able to recover the function of TG-sensitive pools. This could be made possible by a large depletion of Ca^{2+} from TG-sensitive pools by deleterious effect of oxidative stress and by the presence of a different SERCA isoform on these pools. The most likely possibility is that the oxidative stress and the inhibition induced by TG affect the SERCA in a similar manner, through a chemical mechanism independent of the catalase activity. The ATP-sensitive pools seem to be well protected by the antioxidant activity of both, catalase and SOD. Even more, the ATP-induced responses are higher than in control conditions, when compared to the response that was obtained after TG-induced Ca^{2+} release in normal conditions. The ATP-induced Ca^{2+} release under the protection by catalase is statistically different from control conditions, whereas in SOD protected conditions, ATP-

induced Ca^{2+} response is higher, but not different versus control. However, the manner on which oxidant stress and antioxidant enzymes modulate the behavior of Ca^{2+} pools in ECs from SD rats support once again the notion that the altered Ca^{2+} behavior of ECs from SHR is hampered because of a sub-lethal production of free radicals. The substantially greater amount of ATP-induced Ca^{2+} release from $InsP_3$-sensitive pools can originate only from an increased availability of Ca^{2+} following the active interaction and Ca^{2+} translocation between ER's TG-sensitive and $InsP_3$-sensitive pools. Furthermore, it appears that the TG-sensitive pools were not completely depleted either by TG, or by the oxidant stress. It is clear from these results that TG has little or no effect on the Ca^{2+} mobilization process initiated by ATP from $InsP_3$-sensitive Ca^{2+} pools. In addition, it is highly probable that the inhibitory effect of oxidant stress has different mechanisms of action and/or it is induced by different free radical species for each type of Ca^{2+} pools in ECs from SD rats. The most important finding is that the $InsP_3$-sensitive pool in ECs from SHR is sensitive to the antioxidant effect of catalase whereas the TG-sensitive pool remains insensitive. This finding confirms that the sub-lethal production of free radicals in ECs from SHR diminishes the amount of available Ca^{2+} for $InsP_3$-inducing agonists.

IV.4.4. The Ca^{2+} entry pathway in rat aorta endothelial cells

Analysis of CCE in primary cultures of ECs from SHR and SD rats, following agonist-induced Ca^{2+} release, reveals an unexpected small Ca^{2+} influx (mean $\Delta[Ca^{2+}]_i \approx 2.5$ nM) but quantitatively and dynamically comparable in both EC strains. In addition, we have demonstrated in ECs from SD rats that TG-induced stimulation in physiological $[Ca^{2+}]_o$ has generated an instantaneous stimulation of CCE and an overall 30% increase of the TG-induced $[Ca^{2+}]_i$. In this study, the maximal activation of CCE was observed only under conditions where subsequent stimulation with agonist could enable further release of Ca^{2+} and/or under intense

oxidative stress. The application of oxidative injury for 90 min in ECs from SD, induced more than a 3-fold increase in CCE. As it can be seen in Figure 11, the CCE in ECs from SD increased in parallel with the increase of oxidative stress injury, culminating with a ≈ 8-fold increase after 120 min. This increase of CCE induced by oxidative stress is in contradiction with the oxidative stress induced by t-BuOOH in ECs from BAE reported by Elliott and Doan, where t-BuOOH inhibits both BK-stimulated Ca^{2+} signaling and CCE [30]. In our experiments, the oxidant stress intensity did not alter the viability of ECs (data not shown). Evidence demonstrates that the oxidative stress activates a non-selective cation channel responsible for membrane depolarization in calf vascular endothelial cells, which is responsible for a certain amount of Ca^{2+} influx but, at the same time, the activation of this channel will reduce the driving force for Ca^{2+} entry [55]. However, it is impossible to eliminate a certain participation of non-specific Ca^{2+} permeation due to the effect of free radicals, even if it was demonstrated that the plasmalemma of oxidant-treated cells did not exhibit any visible increase in permeability to cations over 2 hours of oxidative stress application [56].

The SD cells under synchronized oxidant stress and antioxidant enzymatic treatment show a CCE similar to that in control conditions. This fact demonstrates that the oxidant stress alters the structures of Ca^{2+} entry pathways that cannot take place in the presence of specific antioxidant enzymes, supporting a direct implication of $^{\bullet}O_2^-$ and H_2O_2. Despite this evidence, the application of antioxidant enzymes on ECs from SHR did not have any effect on CCE. It is possible that in this case, the oxidative injury is not inflicted by oxygen-derived free radicals but by organic free radicals not affected by the specific antioxidant enzymes. Furthermore, in both ECs strains, the associated stimulation of TG with ATP and antioxidant enzymes did not have any effect on CCE.

In conclusion, the Ca^{2+} pools and the availability of Ca^{2+} in ECs from SHR are much smaller in comparison with ECs from SD rats and with BAECs. The primary ECs cultures lack intracytosolic caffeine-sensitive Ca^{2+} pools but, in exchange, they are provided with unoverlaped IP_3- and TG-sensitive pools. Both types of pools are susceptible to oxidative stress. The TG-sensitive pools are insensitive to antioxidant treatment, whereas the IP_3-sensitive pools are sensitive to antioxidant treatment due, most likely, to the presence of a different SERCA pump that is protected against oxidative stress and TG effect. The IP_3- and TG-sensitive Ca^{2+} pools in rat primary ECs are not completely independen and they are exchanging important amounts of Ca^{2+} into a time dependent manner.

IV.5. ACKNOWLEDGMENTS

This work was supported by a grant from Medical Research of Canada (MT 7769) and from Quebec Heart and Stroke Foundation. The technical assistance of Mrs. Joanne Vallée and Mrs. Julie Verner is also acknowledged.

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IV.7. FIGURES CHAPTER IV.

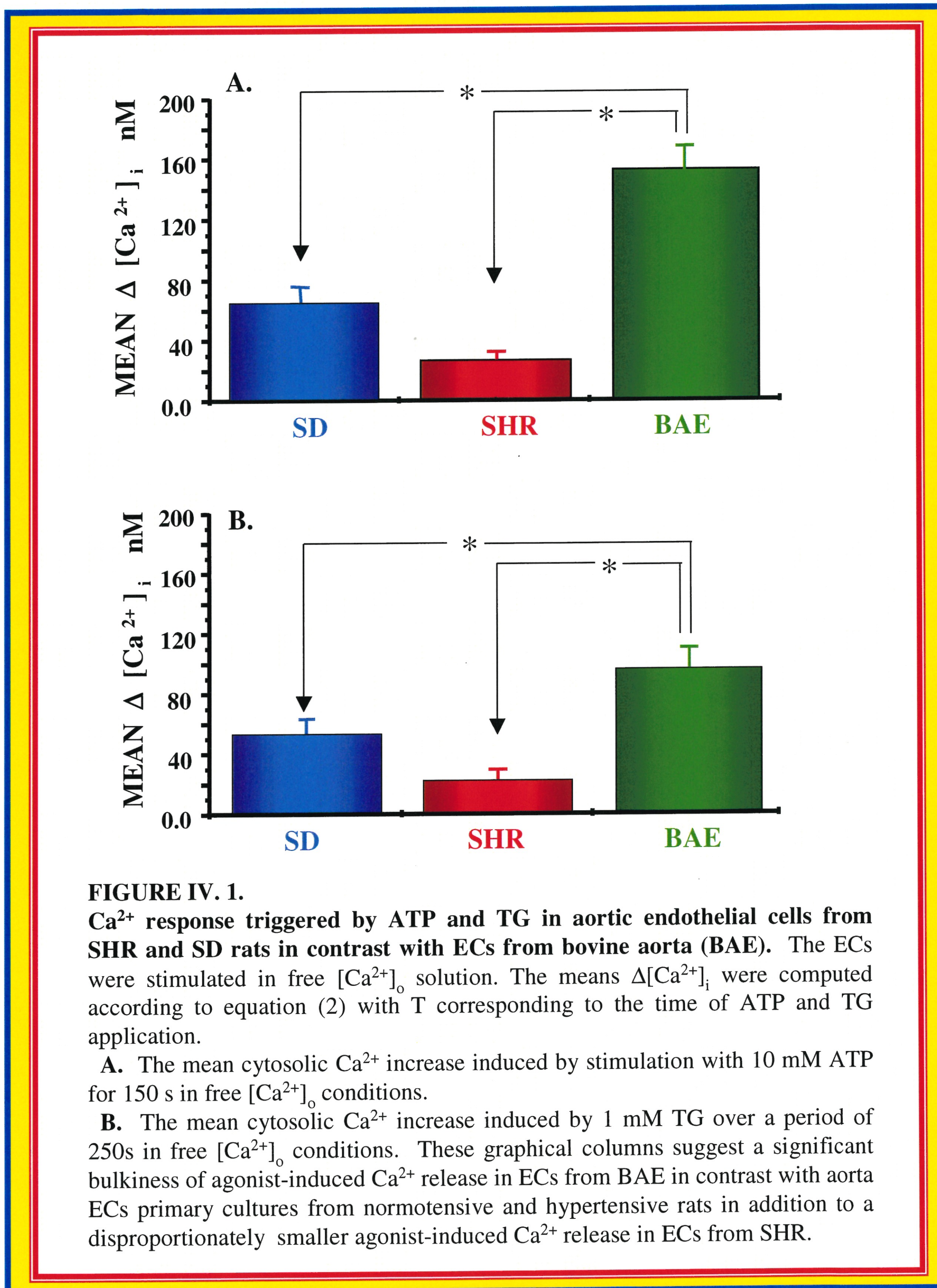

FIGURE IV. 1.
Ca^{2+} response triggered by ATP and TG in aortic endothelial cells from SHR and SD rats in contrast with ECs from bovine aorta (BAE). The ECs were stimulated in free $[Ca^{2+}]_o$ solution. The means $\Delta[Ca^{2+}]_i$ were computed according to equation (2) with T corresponding to the time of ATP and TG application.

A. The mean cytosolic Ca^{2+} increase induced by stimulation with 10 mM ATP for 150 s in free $[Ca^{2+}]_o$ conditions.

B. The mean cytosolic Ca^{2+} increase induced by 1 mM TG over a period of 250s in free $[Ca^{2+}]_o$ conditions. These graphical columns suggest a significant bulkiness of agonist-induced Ca^{2+} release in ECs from BAE in contrast with aorta ECs primary cultures from normotensive and hypertensive rats in addition to a disproportionately smaller agonist-induced Ca^{2+} release in ECs from SHR.

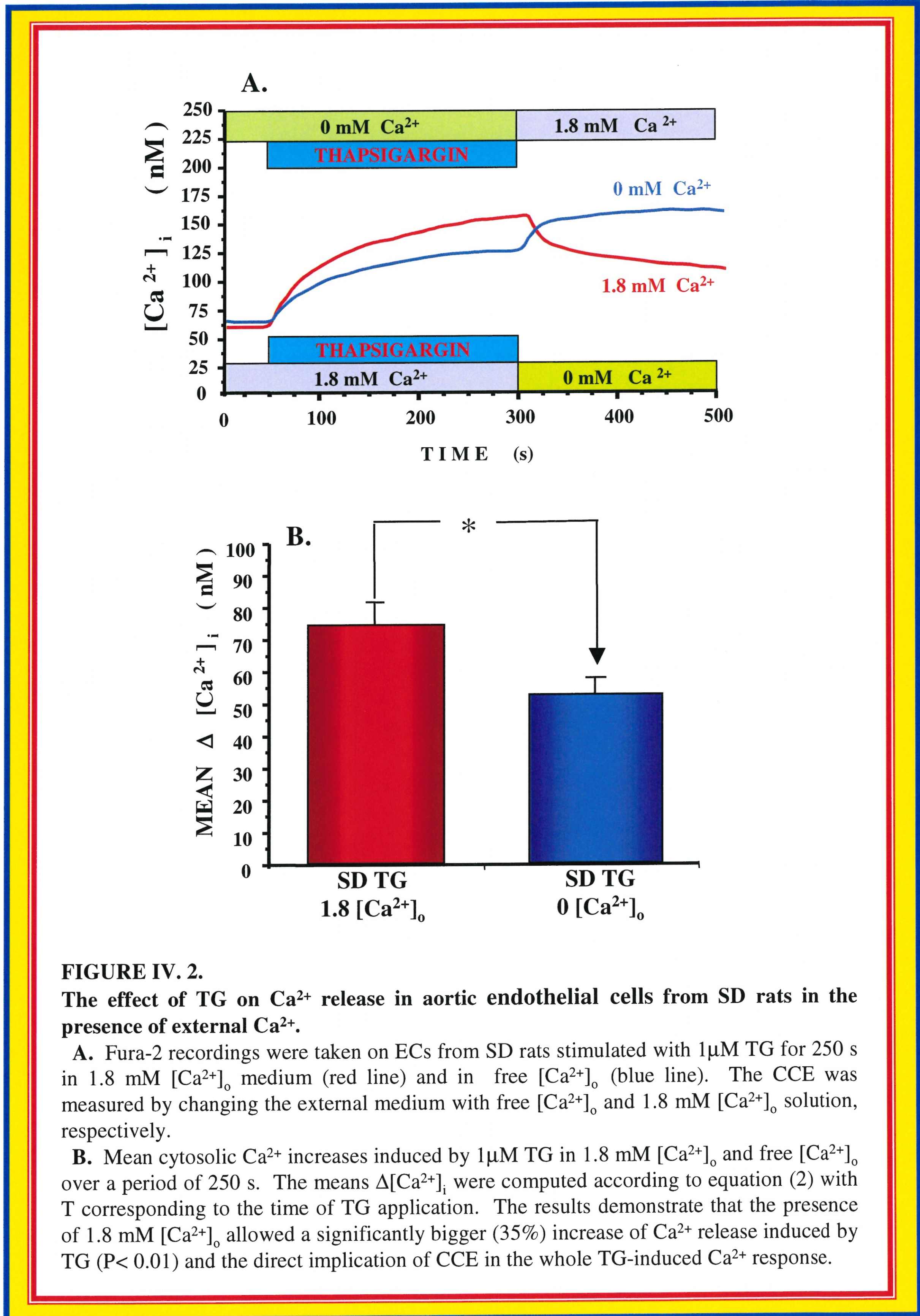

FIGURE IV. 2.

The effect of TG on Ca^{2+} release in aortic endothelial cells from SD rats in the presence of external Ca^{2+}.

A. Fura-2 recordings were taken on ECs from SD rats stimulated with 1µM TG for 250 s in 1.8 mM $[Ca^{2+}]_o$ medium (red line) and in free $[Ca^{2+}]_o$ (blue line). The CCE was measured by changing the external medium with free $[Ca^{2+}]_o$ and 1.8 mM $[Ca^{2+}]_o$ solution, respectively.

B. Mean cytosolic Ca^{2+} increases induced by 1µM TG in 1.8 mM $[Ca^{2+}]_o$ and free $[Ca^{2+}]_o$ over a period of 250 s. The means $\Delta[Ca^{2+}]_i$ were computed according to equation (2) with T corresponding to the time of TG application. The results demonstrate that the presence of 1.8 mM $[Ca^{2+}]_o$ allowed a significantly bigger (35%) increase of Ca^{2+} release induced by TG ($P < 0.01$) and the direct implication of CCE in the whole TG-induced Ca^{2+} response.

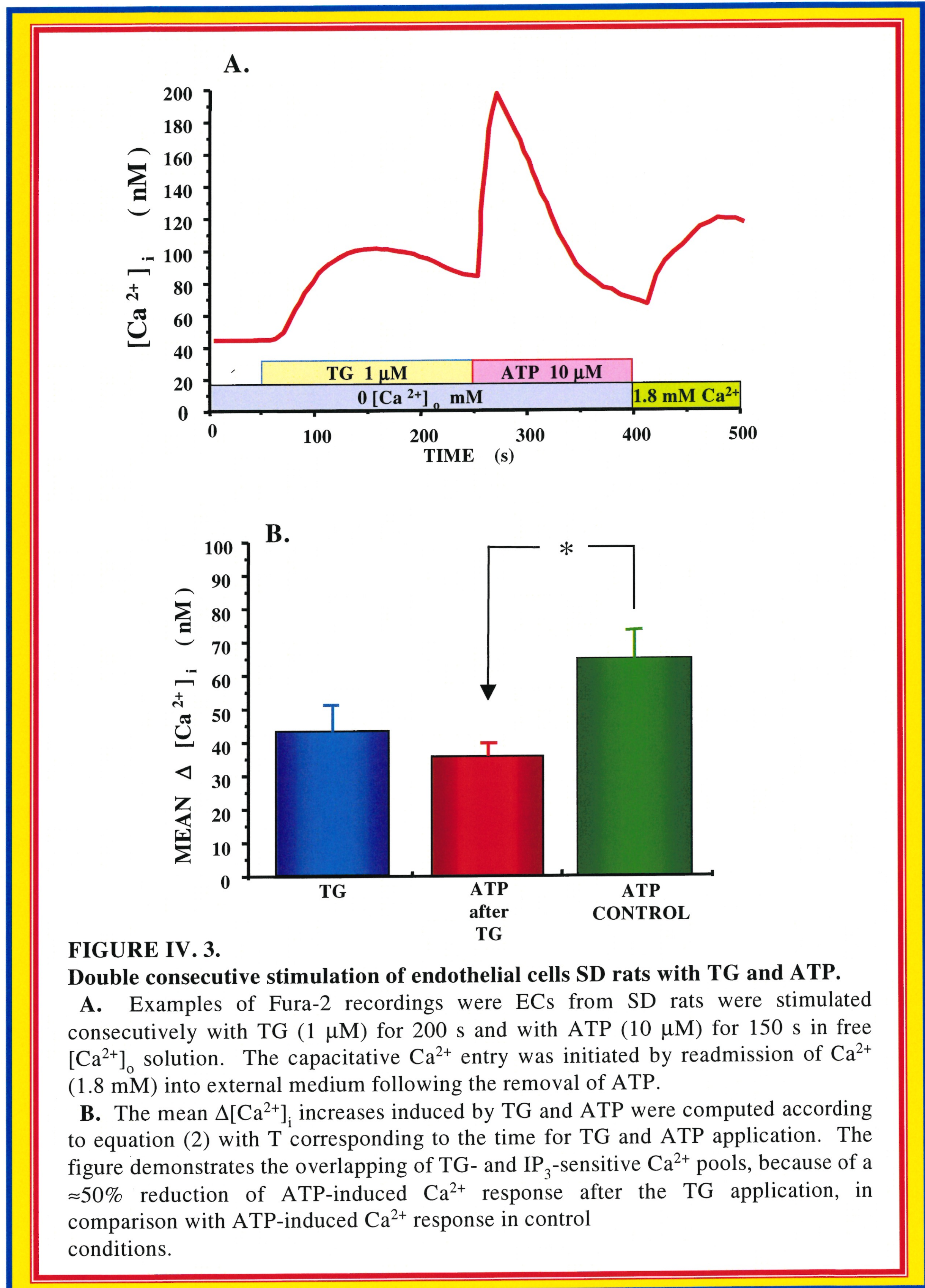

FIGURE IV. 3.
Double consecutive stimulation of endothelial cells SD rats with TG and ATP.

A. Examples of Fura-2 recordings were ECs from SD rats were stimulated consecutively with TG (1 µM) for 200 s and with ATP (10 µM) for 150 s in free $[Ca^{2+}]_o$ solution. The capacitative Ca^{2+} entry was initiated by readmission of Ca^{2+} (1.8 mM) into external medium following the removal of ATP.

B. The mean $\Delta[Ca^{2+}]_i$ increases induced by TG and ATP were computed according to equation (2) with T corresponding to the time for TG and ATP application. The figure demonstrates the overlapping of TG- and IP_3-sensitive Ca^{2+} pools, because of a ≈50% reduction of ATP-induced Ca^{2+} response after the TG application, in comparison with ATP-induced Ca^{2+} response in control conditions.

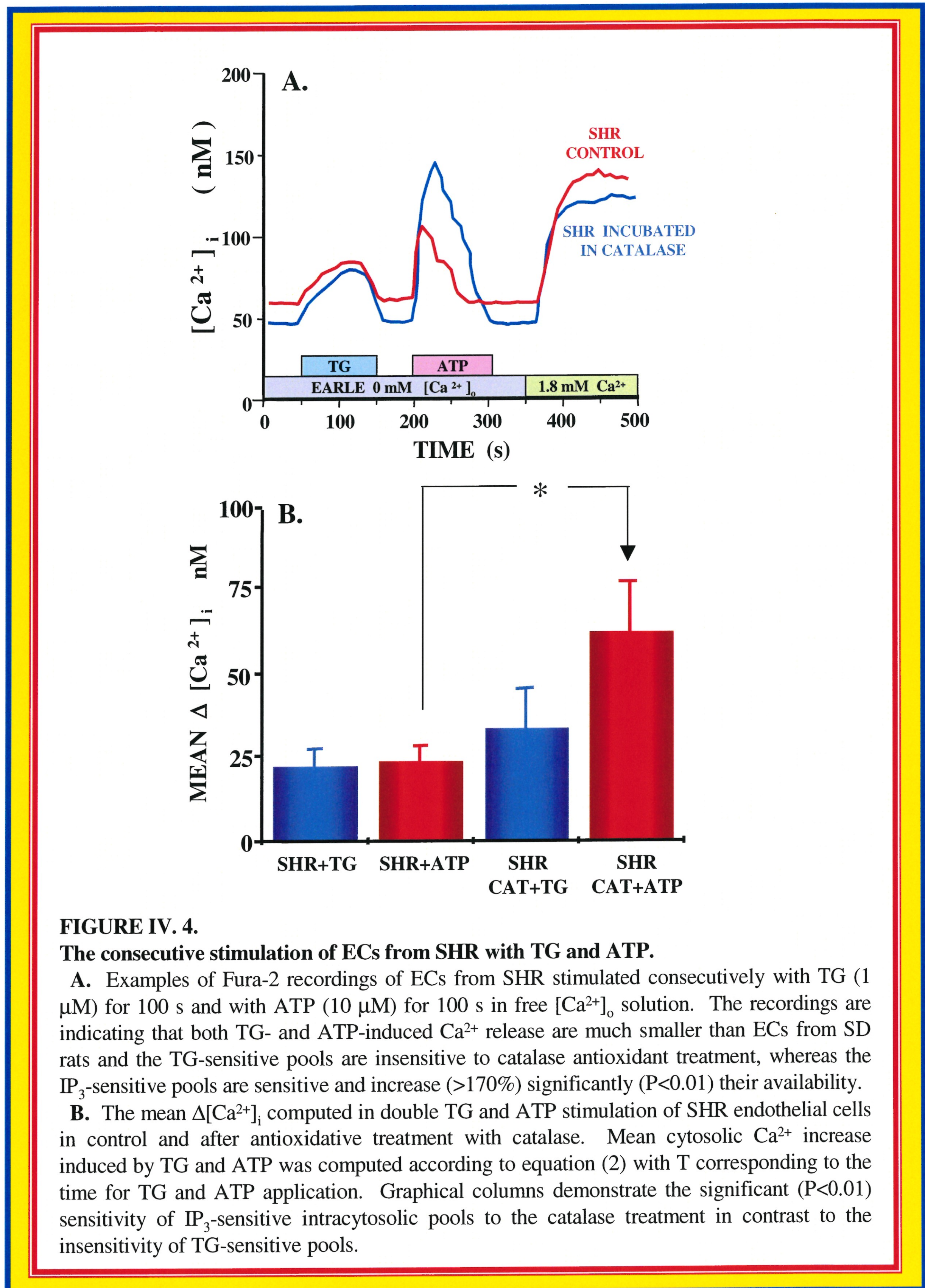

FIGURE IV. 4.

The consecutive stimulation of ECs from SHR with TG and ATP.

A. Examples of Fura-2 recordings of ECs from SHR stimulated consecutively with TG (1 μM) for 100 s and with ATP (10 μM) for 100 s in free $[Ca^{2+}]_o$ solution. The recordings are indicating that both TG- and ATP-induced Ca^{2+} release are much smaller than ECs from SD rats and the TG-sensitive pools are insensitive to catalase antioxidant treatment, whereas the IP_3-sensitive pools are sensitive and increase (>170%) significantly ($P<0.01$) their availability.

B. The mean $\Delta[Ca^{2+}]_i$ computed in double TG and ATP stimulation of SHR endothelial cells in control and after antioxidative treatment with catalase. Mean cytosolic Ca^{2+} increase induced by TG and ATP was computed according to equation (2) with T corresponding to the time for TG and ATP application. Graphical columns demonstrate the significant ($P<0.01$) sensitivity of IP_3-sensitive intracytosolic pools to the catalase treatment in contrast to the insensitivity of TG-sensitive pools.

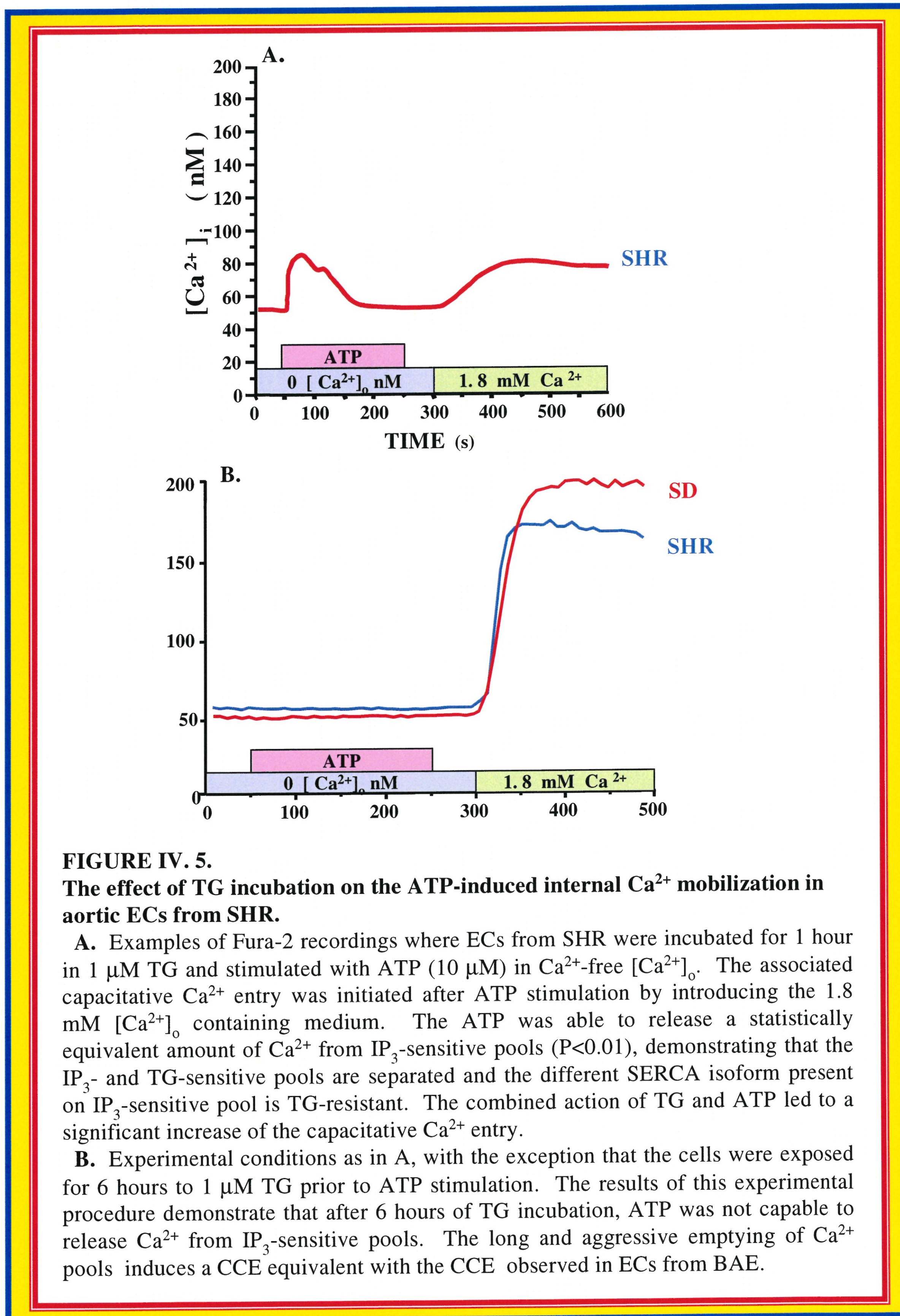

FIGURE IV. 5.
The effect of TG incubation on the ATP-induced internal Ca^{2+} mobilization in aortic ECs from SHR.

A. Examples of Fura-2 recordings where ECs from SHR were incubated for 1 hour in 1 μM TG and stimulated with ATP (10 μM) in Ca^{2+}-free $[Ca^{2+}]_o$. The associated capacitative Ca^{2+} entry was initiated after ATP stimulation by introducing the 1.8 mM $[Ca^{2+}]_o$ containing medium. The ATP was able to release a statistically equivalent amount of Ca^{2+} from IP_3-sensitive pools ($P<0.01$), demonstrating that the IP_3- and TG-sensitive pools are separated and the different SERCA isoform present on IP_3-sensitive pool is TG-resistant. The combined action of TG and ATP led to a significant increase of the capacitative Ca^{2+} entry.

B. Experimental conditions as in A, with the exception that the cells were exposed for 6 hours to 1 μM TG prior to ATP stimulation. The results of this experimental procedure demonstrate that after 6 hours of TG incubation, ATP was not capable to release Ca^{2+} from IP_3-sensitive pools. The long and aggressive emptying of Ca^{2+} pools induces a CCE equivalent with the CCE observed in ECs from BAE.

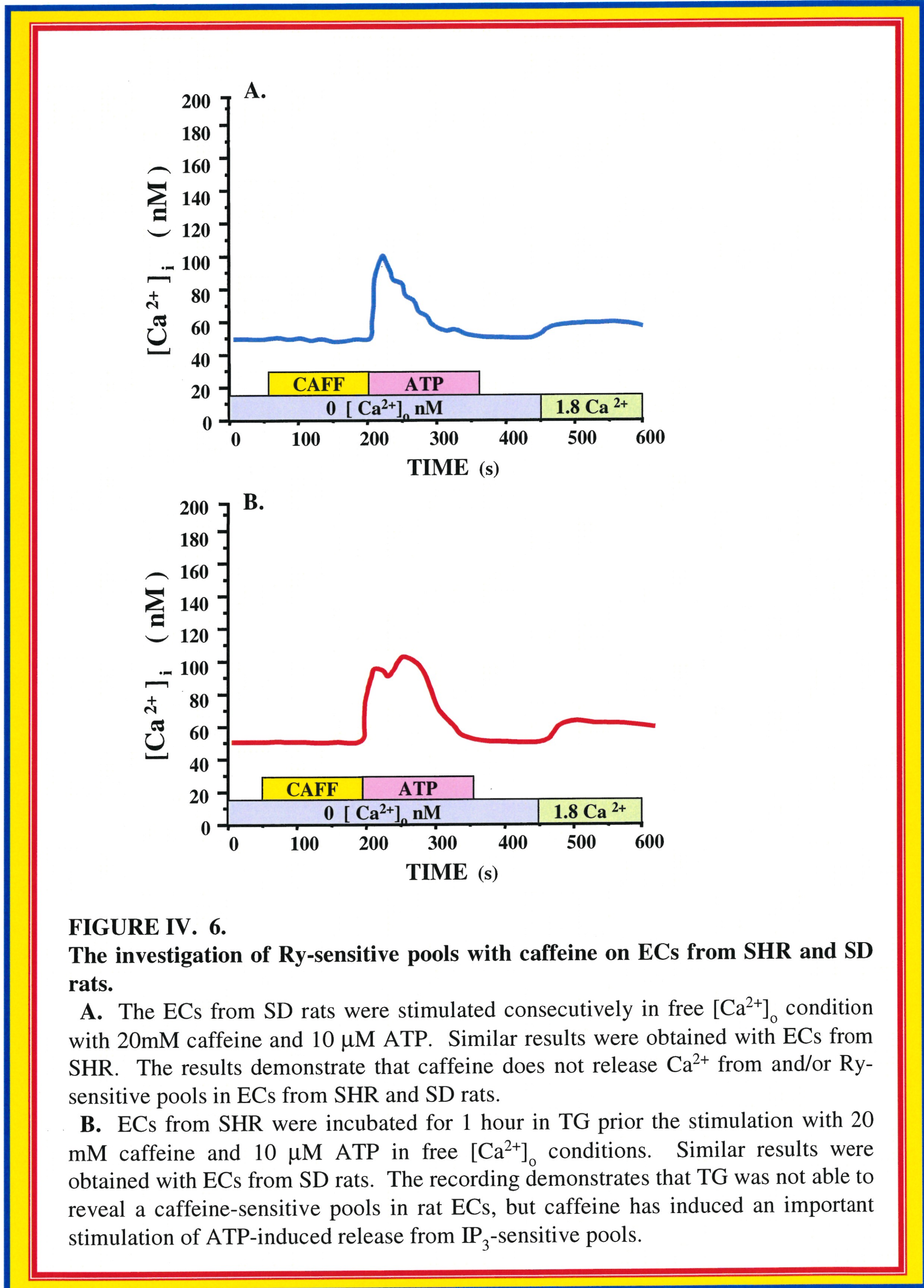

FIGURE IV. 6.
The investigation of Ry-sensitive pools with caffeine on ECs from SHR and SD rats.

A. The ECs from SD rats were stimulated consecutively in free $[Ca^{2+}]_o$ condition with 20mM caffeine and 10 μM ATP. Similar results were obtained with ECs from SHR. The results demonstrate that caffeine does not release Ca^{2+} from and/or Ry-sensitive pools in ECs from SHR and SD rats.

B. ECs from SHR were incubated for 1 hour in TG prior the stimulation with 20 mM caffeine and 10 μM ATP in free $[Ca^{2+}]_o$ conditions. Similar results were obtained with ECs from SD rats. The recording demonstrates that TG was not able to reveal a caffeine-sensitive pools in rat ECs, but caffeine has induced an important stimulation of ATP-induced release from IP_3-sensitive pools.

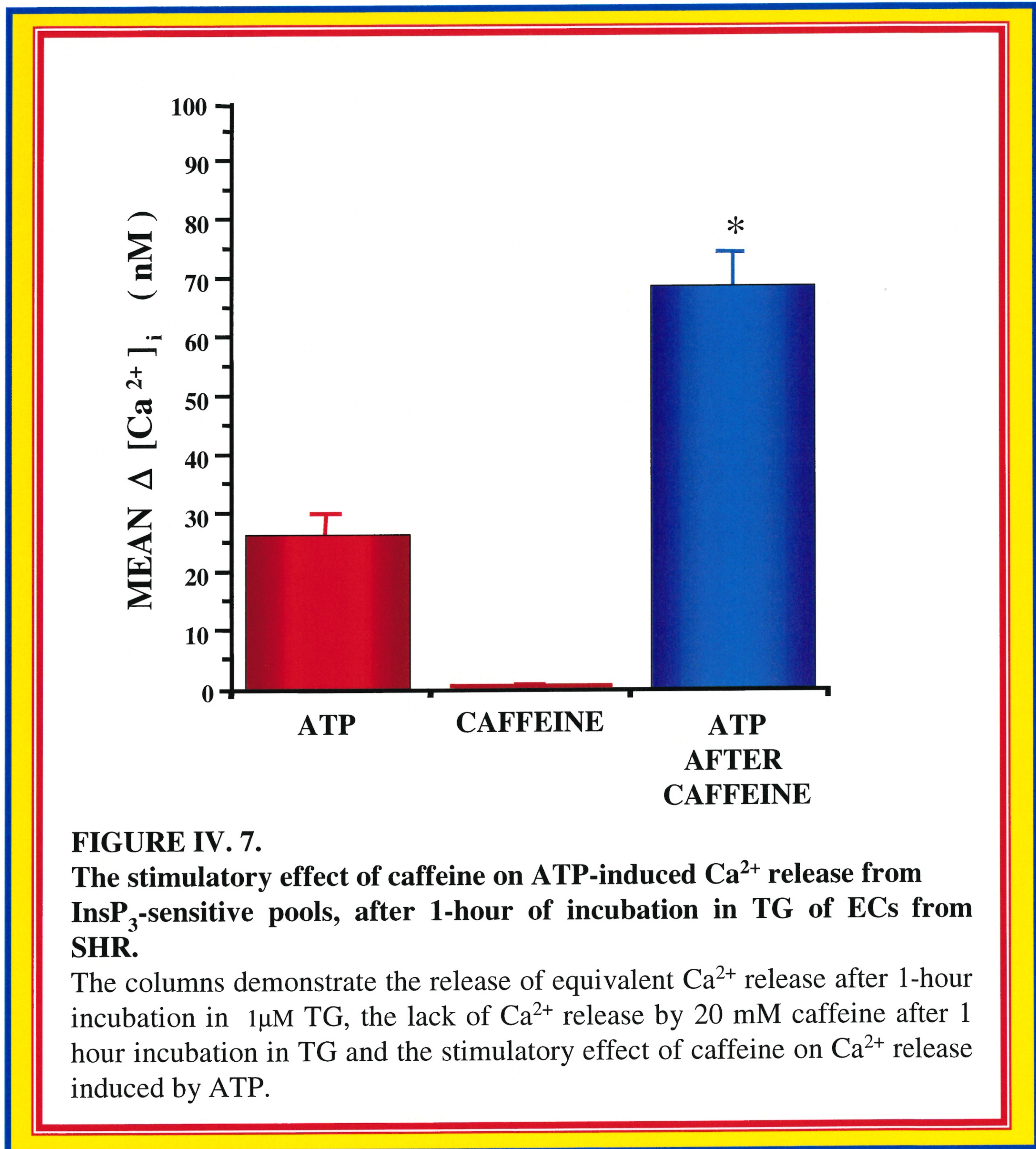

FIGURE IV. 7.
The stimulatory effect of caffeine on ATP-induced Ca^{2+} release from $InsP_3$-sensitive pools, after 1-hour of incubation in TG of ECs from SHR.
The columns demonstrate the release of equivalent Ca^{2+} release after 1-hour incubation in 1μM TG, the lack of Ca^{2+} release by 20 mM caffeine after 1 hour incubation in TG and the stimulatory effect of caffeine on Ca^{2+} release induced by ATP.

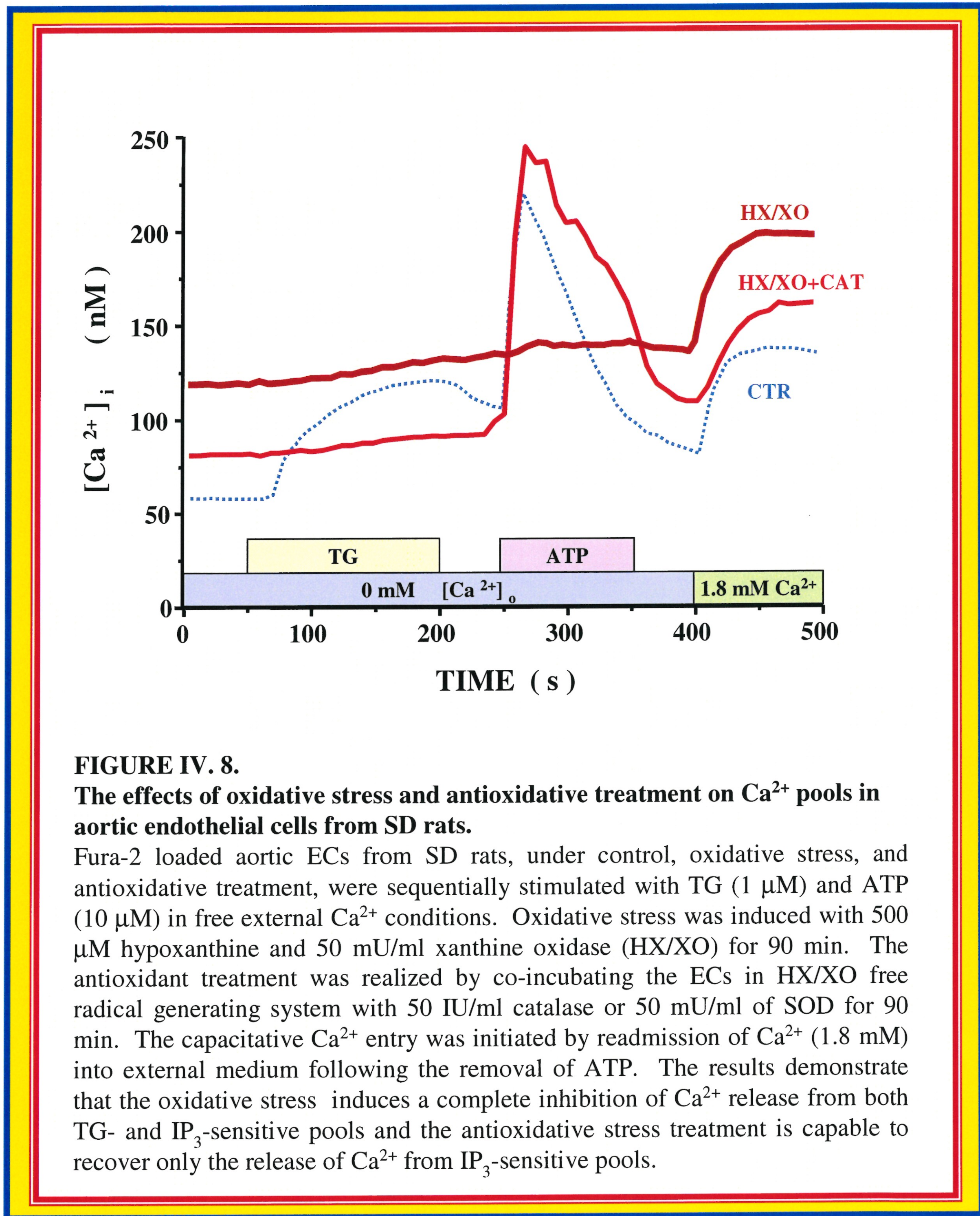

FIGURE IV. 8.
The effects of oxidative stress and antioxidative treatment on Ca^{2+} pools in aortic endothelial cells from SD rats.
Fura-2 loaded aortic ECs from SD rats, under control, oxidative stress, and antioxidative treatment, were sequentially stimulated with TG (1 μM) and ATP (10 μM) in free external Ca^{2+} conditions. Oxidative stress was induced with 500 μM hypoxanthine and 50 mU/ml xanthine oxidase (HX/XO) for 90 min. The antioxidant treatment was realized by co-incubating the ECs in HX/XO free radical generating system with 50 IU/ml catalase or 50 mU/ml of SOD for 90 min. The capacitative Ca^{2+} entry was initiated by readmission of Ca^{2+} (1.8 mM) into external medium following the removal of ATP. The results demonstrate that the oxidative stress induces a complete inhibition of Ca^{2+} release from both TG- and IP_3-sensitive pools and the antioxidative stress treatment is capable to recover only the release of Ca^{2+} from IP_3-sensitive pools.

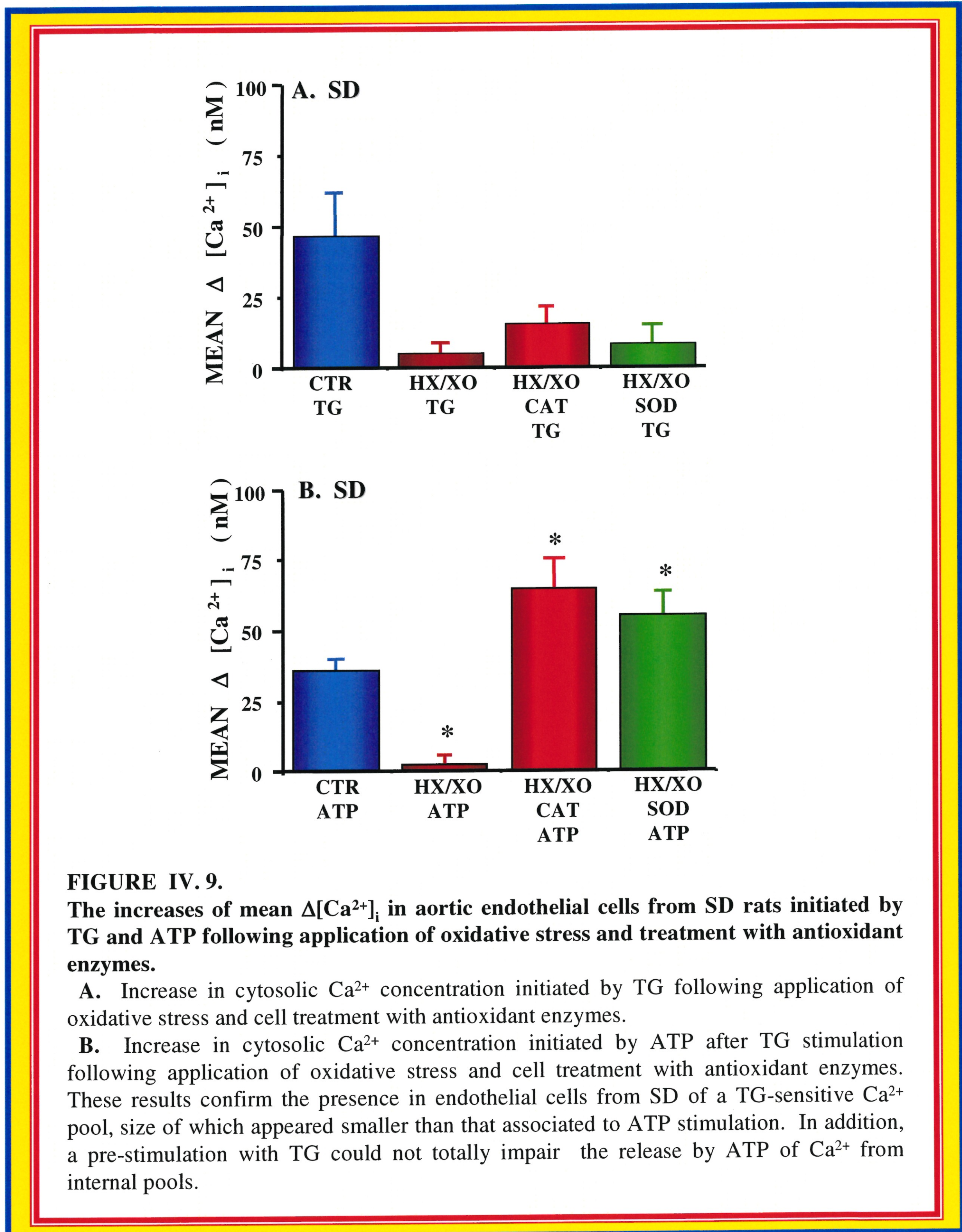

FIGURE IV. 9.

The increases of mean $\Delta[Ca^{2+}]_i$ in aortic endothelial cells from SD rats initiated by TG and ATP following application of oxidative stress and treatment with antioxidant enzymes.

A. Increase in cytosolic Ca^{2+} concentration initiated by TG following application of oxidative stress and cell treatment with antioxidant enzymes.

B. Increase in cytosolic Ca^{2+} concentration initiated by ATP after TG stimulation following application of oxidative stress and cell treatment with antioxidant enzymes. These results confirm the presence in endothelial cells from SD of a TG-sensitive Ca^{2+} pool, size of which appeared smaller than that associated to ATP stimulation. In addition, a pre-stimulation with TG could not totally impair the release by ATP of Ca^{2+} from internal pools.

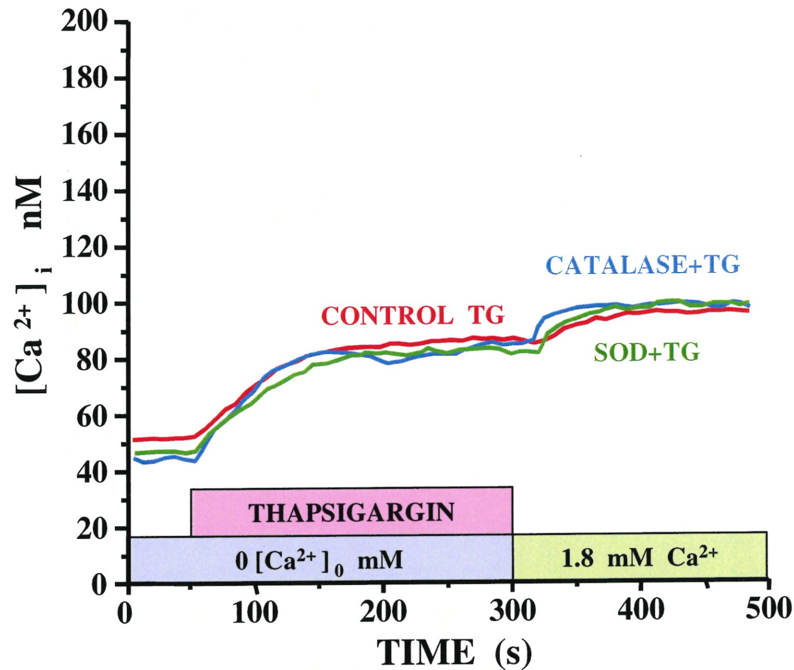

FIGURE IV. 10 A.
Effects of antioxidants on the Ca^{2+} release initiated by TG in aortic endothelial cells from SHR.
Fura-2 recordings where the release of Ca^{2+} from internal pools of ECs from SHR was assayed by stimulation with 1 μM TG for 250 s in free $[Ca^{2+}]_o$ solution. The associated capacitative Ca^{2+} entry was initiated by perfusing with 1.8 mM $[Ca^{2+}]_o$ solution, following TG stimulation. The ECs were exposed to catalase (CAT: 50 IU/ml) and SOD (50 mU/ml) for 90 min prior to TG stimulation.

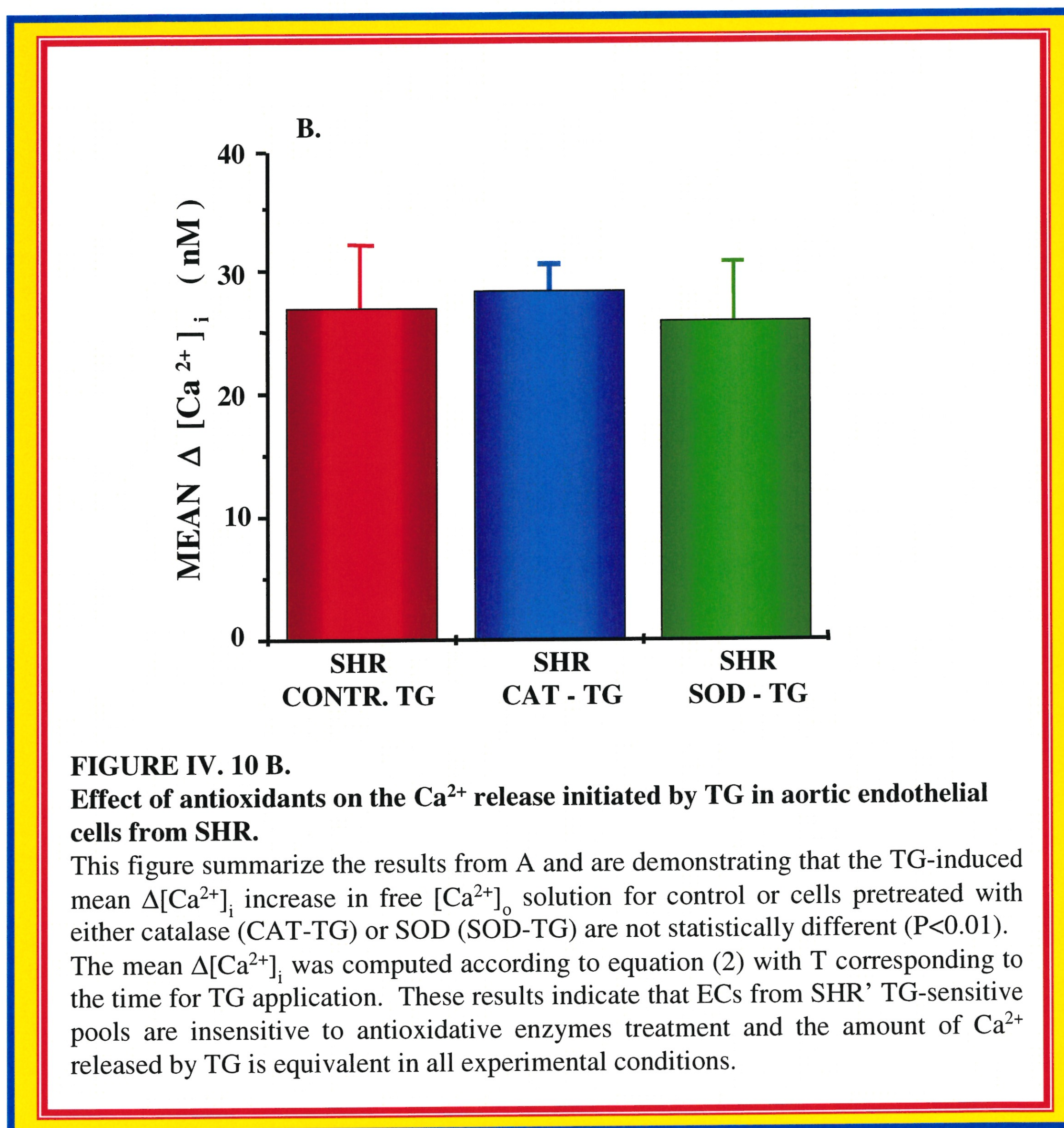

FIGURE IV. 10 B.
Effect of antioxidants on the Ca^{2+} release initiated by TG in aortic endothelial cells from SHR.
This figure summarize the results from A and are demonstrating that the TG-induced mean $\Delta[Ca^{2+}]_i$ increase in free $[Ca^{2+}]_o$ solution for control or cells pretreated with either catalase (CAT-TG) or SOD (SOD-TG) are not statistically different ($P<0.01$). The mean $\Delta[Ca^{2+}]_i$ was computed according to equation (2) with T corresponding to the time for TG application. These results indicate that ECs from SHR' TG-sensitive pools are insensitive to antioxidative enzymes treatment and the amount of Ca^{2+} released by TG is equivalent in all experimental conditions.

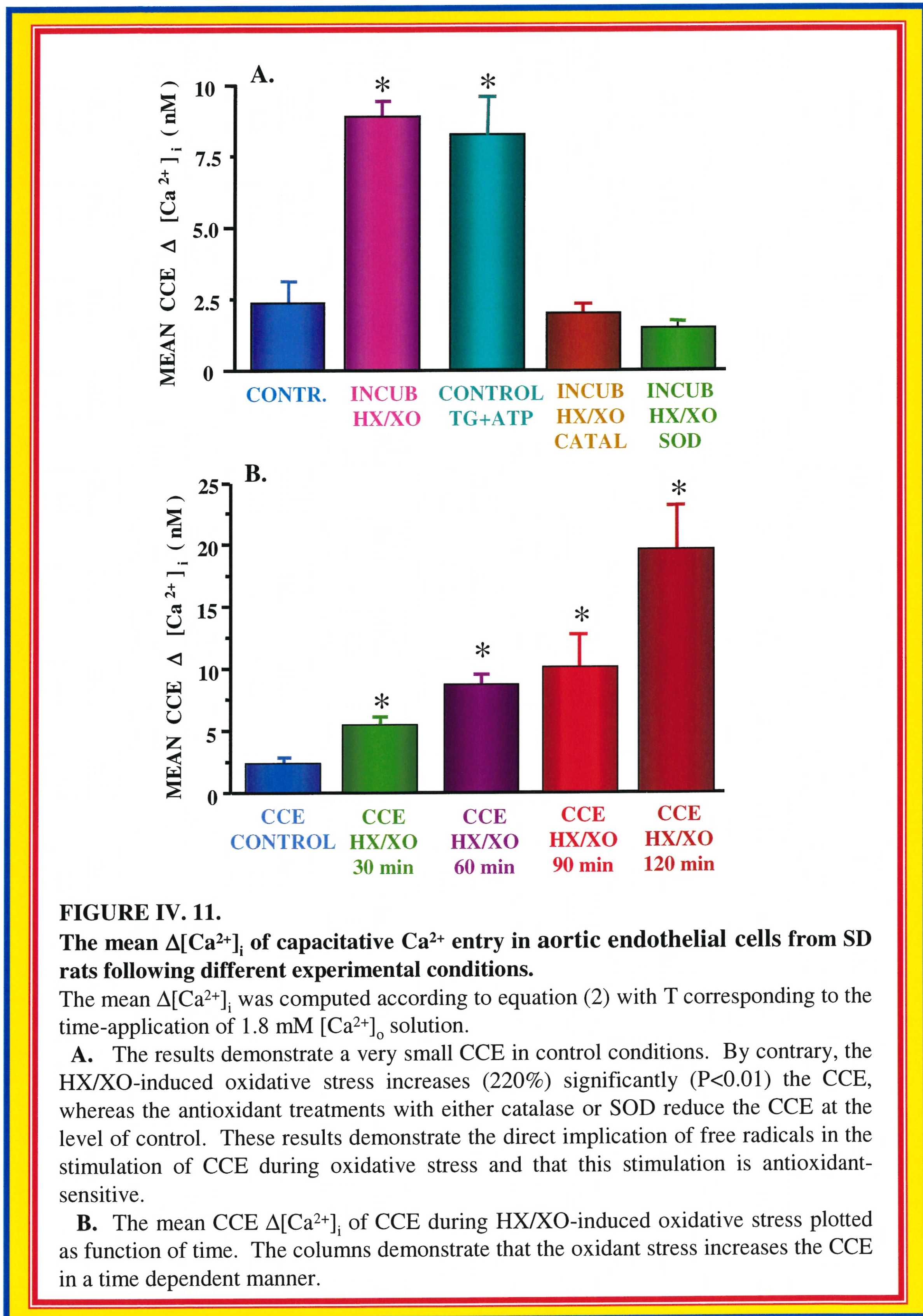

FIGURE IV. 11.

The mean $\Delta[Ca^{2+}]_i$ of capacitative Ca^{2+} entry in aortic endothelial cells from SD rats following different experimental conditions.

The mean $\Delta[Ca^{2+}]_i$ was computed according to equation (2) with T corresponding to the time-application of 1.8 mM $[Ca^{2+}]_o$ solution.

A. The results demonstrate a very small CCE in control conditions. By contrary, the HX/XO-induced oxidative stress increases (220%) significantly ($P<0.01$) the CCE, whereas the antioxidant treatments with either catalase or SOD reduce the CCE at the level of control. These results demonstrate the direct implication of free radicals in the stimulation of CCE during oxidative stress and that this stimulation is antioxidant-sensitive.

B. The mean CCE $\Delta[Ca^{2+}]_i$ of CCE during HX/XO-induced oxidative stress plotted as function of time. The columns demonstrate that the oxidant stress increases the CCE in a time dependent manner.

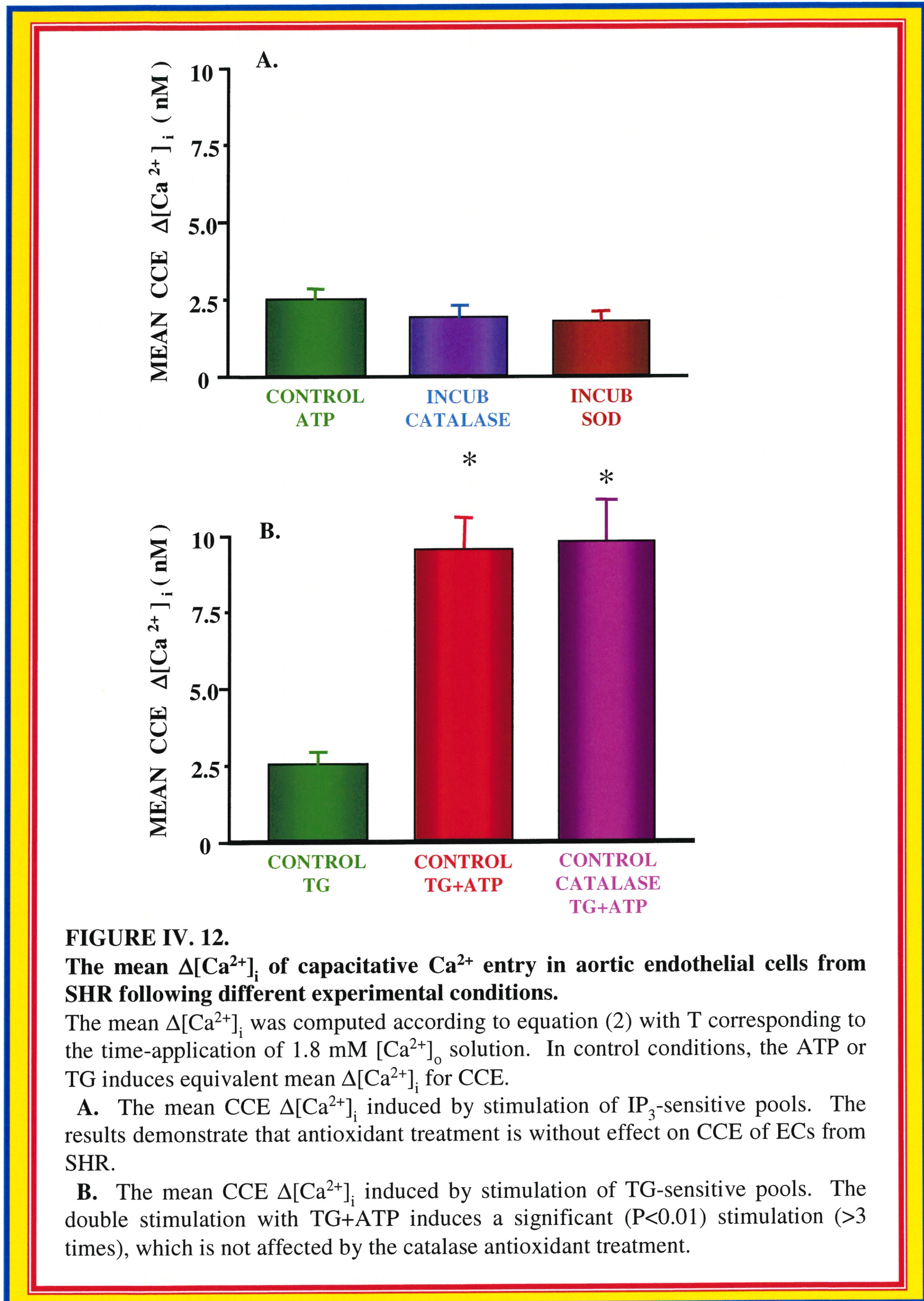

FIGURE IV. 12.

The mean $\Delta[Ca^{2+}]_i$ of capacitative Ca^{2+} entry in aortic endothelial cells from SHR following different experimental conditions.

The mean $\Delta[Ca^{2+}]_i$ was computed according to equation (2) with T corresponding to the time-application of 1.8 mM $[Ca^{2+}]_o$ solution. In control conditions, the ATP or TG induces equivalent mean $\Delta[Ca^{2+}]_i$ for CCE.

A. The mean CCE $\Delta[Ca^{2+}]_i$ induced by stimulation of IP_3-sensitive pools. The results demonstrate that antioxidant treatment is without effect on CCE of ECs from SHR.

B. The mean CCE $\Delta[Ca^{2+}]_i$ induced by stimulation of TG-sensitive pools. The double stimulation with TG+ATP induces a significant ($P<0.01$) stimulation (>3 times), which is not affected by the catalase antioxidant treatment.

CHAPTER V. Article III

THE EFFECTS OF MELATONIN ON Ca^{2+} HOMEOSTASIS OF ENDOTHELIAL CELLS

Article presented for publication in Journal of Hypertension

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Running title: **Effects of melatonin on endothelial cells**

Key words: endothelium, $InsP_3$, hypertension, free radicals, capacitative Ca^{2+} entry, Fura-2

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ABSTRACT

Objective. Melatonin is recognized for its hypotensive effect in several models of hypertension. A study was thus undertaken to test the effects of melatonin on Ca^{2+} signaling in vascular endothelial cells from genetically hypertensive rats (SHR) as compared with cells from normotensive Sprague Dawley rats (SDR) or cells from bovine aortic endothelial (BAE) cultures.

Methods. Internal Ca^{2+} variations were measured using the fluorescence Ca^{2+} indicator Fura-2 in BAE and in primary cultured endothelial cells from SHR and SDR either in response to acute stimulation by melatonin or else resulting from ATP and/or bradykinin stimulation following melatonin incubation. The presence of melatonin specific membrane receptors was assayed through binding experiments with ^{3}[H]O-methyl melatonin. Finally, the antioxidative properties of melatonin were investigated by measuring the Ca^{2+} response to ATP in BAE and SDR cells following exposure to the hypoxanthine/xanthine oxidase free radical generating system in the presence or in the absence of melatonin.

Results. The presence of melatonin specific binding sites could be confirmed in cells from SHR and SDR, but acute exposure to melatonin (0.1 to 50 μM) failed to initiate a release of Ca^{2+} from internal stores in the three cell types considered. Cell incubation with melatonin prior to receptor stimulation caused an increase in the magnitude of the internal Ca^{2+} release triggered by bradykinin and/or ATP in SHR, but not in SDR or BAE endothelial cells. Melatonin was found furthermore to reverse the inhibitory effect of free radicals on the release of Ca^{2+} from internal stores in SDR and BAE cells submitted to an oxidative stress, an indication that part of the specific action of melatonin on SHR may be related to its antioxidative properties. Finally, our results show that melatonin enhanced in a time dependent manner the capacitative Ca^{2+} entry in BAE, SHR and SDR cells following $InsP_3$-linked receptor stimulation.

Conclusion. On the basis of these observations, we concluded that melatonin potentiates the capacitative Ca^{2+} entry in endothelial cells from normotensive and hypertensive rats while enhancing the internal Ca^{2+} release in vascular endothelial cells from genetically hypertensive rats through a mechanism most likely to involve the scavenging of free radicals.

V.1. INTRODUCTION

Melatonin is known for its essential role in photic regulation, adaptation to circadian and seasonal light photoperiod, seasonal cycles of reproduction, thermoregulation, and hibernation. Most of these physiological processes have been demonstrated to involve an interaction of melatonin with specific membrane receptors coupled to G-proteins (MT_1, MT_2, and MT_3) [1]. Several studies have also provided evidence for a role of melatonin in cardiovascular regulation with melatonin causing vasorelaxation in some arteries [2,3], while potentiating the contractile responses or directly initiating contraction in others [4-6]. Acute administration of melatonin was demonstrated for instance to relax KCl precontracted vascular smooth muscles from rat aorta and to impair the contractile response initiated by α_1 and α_2 adrenergic receptor stimulation [2]. Similarly, melatonin inhibited the response to 5-HT and the contraction induced by high potassium in rabbit isolated aorta and in iliac and renal arteries [3]. In contrast, melatonin potentiated the contraction induced by exogenous noradrenaline and nerve stimulation through a luzindole-sensitive melatonin receptor in rat caudal artery [7]. A constrictive effect of melatonin was also reported in the rat middle cerebral artery pressurized *in vitro* [4]. It was proposed in this case that melatonin exerted its contractile effect through a G protein-mediated inhibition of a calcium dependent potassium channel of large conductance [4].

Melatonin is also recognized for its hypotensive effect in the genetically hypertensive rats SHR [9] and in patients with essential hypertension [8]. Of particular interest is the

observation that lack of melatonin induced a hypertensive state in pinealectomized rats [10-12], with the injection of melatonin restoring normal blood pressure [12]. It is likely that the hypotensive effects of melatonin involve a variety of regulatory systems. In SHR for instance, part of the hypotensive action of melatonin appeared to be associated with an inhibition of the basal sympatho-adrenal tone and could be mediated by the blockade of $InsP_3$ formation in response to postsynaptic α1-adrenergic stimulation [13]. Melatonin has also been described as a potent free radical scavenger [14]. The scavenging properties of melatonin have been demonstrated *in vitro* [15] against lipid peroxidation [16], and against oxygen free radical production [17-20] in different tissue preparations [21-25]. There is now increasing evidence for an overproduction of $^{\bullet}O_2^-$ in endothelial cells from animals with hypertension, whether primary or induced by a salt-rich diet [26-28]. Because $^{\bullet}O_2^-$ is known to react rapidly with $NO^{\bullet}$ to form peroxynitrite, an increased scavenging of $NO^{\bullet}$ by $^{\bullet}O_2^-$ may result in a decreased $NO^{\bullet}$ availability even under conditions that could enhance $NO^{\bullet}$ synthesis [29], thus leading to impaired endothelium-dependent vasorelaxation. The hypotensive effect of melatonin observed in several models of hypertension may therefore be consequent in part to its scavenging properties resulting in a decreased $^{\bullet}O_2^-$ production. In support of this proposal is the observation that melatonin significantly suppresses the H_2O_2-induced inhibition of $NO^{\bullet}$ production in endothelial cells from human umbilical arteries [20]. Similarly, melatonin was shown to attenuate the effect of peroxynitrite, a strong oxidant that was reported to impair Ca^{2+} signaling in endothelial cells [30]. Finally, the inhibition by melatonin of the α-adrenergic activation of the phosphoinositide-dependent signaling pathway in smooth muscle cells of SHR was found to be related to its antioxidant properties [31].

The scavenging of reactive oxygen species (ROS) by melatonin may not only insure a greater •NO availability in several models of hypertension, but is also likely to affect the intracellular Ca^{2+} signaling process essential to the endothelium control of vascular tone. Recent findings from our laboratory have indeed indicated that pretreating aortic endothelial cells with antioxidant enzymes such as catalase or SOD increases the Ca^{2+} response to ATP in cells from SHR but not in cells from Sprague Dawley rats (SDR) [32]. This observation was regarded as evidence for an altered Ca^{2+} signaling in endothelial cells from SHR consequent to an endogenous overproduction of free radicals. There is also the possibility that melatonin could affect Ca^{2+} signaling in endothelial cells through the stimulation of membrane receptors leading to the production of second messengers such as cAMP and cGMP. An activation of the agonist-induced Ca^{2+} influx was observed for instance in several cell types in response to an increase in cGMP production [2,33,34]. Furthermore, the capacitative Ca^{2+} entry in nonexcitable cells is know to be modulated by several factors, including the membrane potential, and melatonin has been reported to affect K^+ selective channels in various cellular preparations [35-37].

Despite clear evidence for a ROS-related alteration of Ca^{2+} signaling process in the endothelium of several models of hypertension and the beneficial effect of melatonin in the regulation of blood pressure, the action of melatonin on the Ca^{2+} response of vascular endothelial cells to $InsP_3$ -mobilizing agonists has never been investigated. A series of Fura-2 experiments was thus undertaken to investigate the effects of melatonin on the release of Ca^{2+} from internal pools and on the associated capacitative Ca^{2+} influx in aortic endothelial cells isolated from normotensive or hypertensive blood vessels. Our results indicate that melatonin does not behave as a Ca^{2+} mobilizing agonist, but significantly enhances the agonist induced Ca^{2+} mobilization in endothelial cells from hypertensive rats or from normotensive rats

submitted to an oxidative stress. In addition, melatonin appeared to stimulate the capacitative Ca^{2+} entry in endothelial cells from SHR and normotensive rats, through a mechanism not related to its antioxidative properties.

V.2. MATERIALS AND METHODS

V.2.1. Vascular endothelial cells

Three different endothelial cell preparations were used in this study namely: Bovine Aortic Endothelial (BAE) cells from passage 14 to 24, as well as aortic endothelial cells from normotensive Sprague Dawley rats (SDR) and genetically hypertensive rats (SHR) in primary cultures. BAE cells were cultured in a normal Dulbecco's modified Eagle's medium (DMEM: GIBCO BRL Products, Life technologies, Burlington, Ontario, Canada) supplemented with 10% newborn calf serum (Gibco BRL Products), 100 μg/ml streptomycin and 100 U/ml penicillin.

Rat aortic endothelial cells were isolated from male SDR (12 weeks of age) and sex and age-matched SHR (Charles River, St-Constant, Quebec, Canada). The isolation method was identical for both rat strains. The mean systolic blood pressure of SHR rats was 200 mm Hg and over, and the mean systolic blood pressure of SDR was ≅120 mm Hg. After lethal anesthesia with Phenobarbital (50 mg/kg, I.P.), aortas were removed from the thoraco-abdominal cavity and cleaned of connective tissue. The vessels were cut open longitudinally in strips of 2-3 mm. The aortas were digested in stepwise manner with various concentrations of collagenase and dispase in oxygenated Dulbecco's modified Earle's medium (Gibco BRL Products), at 37°C under mild agitation. Endothelial cells were harvested by centrifugation for 10 min at 2600 r.p.m., at room temperature. Each cell culture was prepared only from a single strain of rats at once. To prevent possible phenotypic changes, cells in primary cultures were never used beyond their first passage. Cells were cultured on 25 mm rounded glass coverslips precoated

with 1% collagen in a Dulbecco's modified Earle's medium containing 15% fetal calf serum (Gibco BRL Products), 10 μg/ml endothelial cell growth factor, and 50 μg/ml heparin. The endothelial cells were used at confluence and identified morphologically by their "cobblestone-like" aspect, and by the presence of Weibel-Palade bodies in the cytoplasm. Subcultures were obtained by treating confluent endothelial cells with 0,05% trypsin in 0,02% EDTA saline for 1-2 min at room temperature. Pure endothelial cells were obtained by gradient centrifugation with 50% isoosmotic Percoll solution (Pharmacia Inc., Pleasant Hill, CA, USA) in a physiological buffer and 50% DMEM. Cells which were subcultured were tested for their Ca^{2+} response to ATP (10 μM) and bradykinin BK (10 nM) and only those which yielded a response comparable to that measured on cells in primary cultures were kept for future studies.

V.2.2. Chemicals

N-[2-hydroxyethyl]piperazine-N'-[ethanesulfonicacid] (HEPES), ethylene glycol bis (β-amino-ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), acetoxymetylester Fura-2 (Fura-2 AM), ionomycin, hypoxanthine, heparin and melatonin were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). Endothelial growth factor, collagenase, dispase, xanthine oxidase, bradykinin, and ATP were obtained from Roche Diagnostics (Laval, Quebec, Canada). Thapsigargin was obtained from Calbiochem-Novabiochem Corporation (San Diego, CA, USA).

V.2.3. Solutions

The normal external solution was a Earle's medium containing (in mM): 121 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 6 $NaHCO_3$, 1 NaH_2PO_4, 5.5 glucose and 25 HEPES buffered at pH 7.35 with ≈10 ml NaOH at 1M. Ca^{2+}-free Earle's solutions were prepared from a normal

Earle's medium by replacing 1.8 mM $CaCl_2$ by 2 mM K_2EGTA. Superoxide anion production induced by the xanthine / xanthine oxidase free radical generating system was assayed by monitoring *cytochrome c* reduction at 550 nm. Melatonin was dissolved in 100% alcohol (v/v) and diluted to a stock solution of 5 mM concentration with 66% water.

V.2.4. Microspectrofluorometry

Ca^{2+} measurements were performed with Fura-2, using a standard incubation protocol as described before [38]. The fluorescence was recorded using an epifluorescent microscope (Nikon inverted microscope IM 35, Nikon Canada Instruments Inc., Montreal, Quebec, Canada) attached to a dual-excitation spectrofluorometer (Spex Fluorolog II; Spex Industries Inc., Edison, NJ, USA). For Fura-2, the excitation wavelengths were set at 350 nm and 380 nm respectively, and a dichroic mirror (Zeiss FT 400, Zeiss, North York, Ontario, Canada) was placed in the excitation pathway. Loaded cells were monitored with a 40x Nikon Neofluor objective, and the emission was measured at 505 nm with a standard band pass filter (500FS10; Andover Corporation, Salem, New Hampshire, USA).

The intracellular calcium concentration, $[Ca^{2+}]_i$ was calculated on the basis of the equation of Grynkiewicz (1985) [39]:

$$[Ca^{2+}]_i = Kd \times (R-R_{MIN})/(R_{MAX}-R) \times Sf_2/Sb_2, \qquad (1)$$

with K_d equal to 224 nM, R the ratio of the fluorescence measured at 350 nm and 380 nm, and Sf_2/Sb_2, the ratio of fluorescence at 380 nm in low and high calcium respectively. The maximal and minimal fluorescence ratios R_{MAX} and R_{MIN} were determined using 10 μM ionomycin to permeabilize the cells first in standard Earle's solution followed by an Earle's solution containing 10 mM EGTA at pH 8.8.

The mean increase in $[Ca^{2+}]_i$ during stimulation, $\Delta[Ca^{2+}]_i$, was estimated by integrating the function:

$$\Delta[Ca^{2+}]_i = 1/T \int_0^T ([Ca^{2+}]_i(\tau) - {}^b[Ca^{2+}]_i)d\tau \qquad (2)$$

where ${}^b[Ca^{2+}]_i$ is the $[Ca^{2+}]_i$ prevailing before stimulation, $[Ca^{2+}]_i$ (τ) the time dependent variation in cytosolic Ca^{2+} concentration following cell stimulation and T, the total time of stimulation by the agonist. In experiments where internal Ca^{2+} changes were related to the activation of a capacitative Ca^{2+} entry, T values ranged from 100 s to 300 s, depending on the extent of the perfusion period with a Ca^{2+} containing solution.

V.2.5. O-methyl melatonin binding studies

Binding of O-methyl melatonin was performed on confluent BAE cells and primary endothelial cells from SHR and SDR that matched the experimental conditions used for Fura-2 measurements. The Petri dishes were first rinsed three times, at room temperature, with phosphate buffered saline (PBS), to remove all traces of culture media. Binding assay was performed at 37°C for 15 minutes: the endothelial cells were incubated with 1 ml PBS containing 1 μCi of ^{3}[H]O-methyl melatonin (specific activity 83 Ci/nmol, Amersham, Baie D'Urfée, Quebec, Canada) with or without excess cold substrate (100 μM melatonin). The incubation was stopped by removing media and rinsing further four times with 2 ml of ice-cold PBS. Cell monolayers were digested with 0.5 ml NaOH (1N) for 1 hour at 60°C. Aliquots were taken for the determination of radioactivity content (0.4 ml in 5 ml scintillation cocktail (β blend, ICN, Costa Mesa, CA, USA)). Radioactivity was counted using a Beckman LS 6000SC counter. Total protein contents of supernatants were determined with a commercial BCA kit (Pierce, Rockford, Illinois, USA) using bovine serum albumin fraction V as standard. Values

were computed as fmol (10^{-15}) melatonin binding sites per mg protein and are mean ± SEM of four determinations within a same culture. Specific binding was determined by taking the difference between the bindings measured in the absence of cold melatonin minus the binding obtained in presence of cold substrate. Experiments were performed three times on different cultures.

V.2.6. Statistical analysis

The data were expressed as mean ± SEM. Unless specified otherwise, statistical significance was analyzed using unpaired Student's T-test with $P<0.01$ being considered statistically significant.

V.3. RESULTS

V.3.1. O-methyl MELATONIN BINDING STUDIES

Previous studies have underlined the existence of melatonin receptors in endothelial cells from cerebral and caudal rat arteries [40-44]. Because of a possible regulation by melatonin of the Ca^{2+} response in endothelial cells through the activation of membrane receptors, the binding properties of melatonin to specific binding sites were investigated in BAE cells and in primary cultures of aortic endothelial cells from SHR and SDR. Specific melatonin binding corresponding to 11 ± 2 (n=4) and 19 ± 2 (n=4) fmol/mg protein was respectively detected in cells from SHR and SDR, but no specific melatonin binding could be reported in BAE cells (Figure 1). These values represent approximately 32% and 39% of the total melatonin binding, the remaining binding being accounted for by non-specific interactions. These data indicate that melatonin receptors might be found in endothelial cells from SHR and SDR, but not in BAE

cells. Melatonin could hence affect Ca^{2+} signaling through a receptor-based mechanism in endothelial cells from SHR and SDR.

V.3.2. EFFECT OF MELATONIN ON INTRACELLULAR Ca^{2+} MOBILIZATION IN ENDOTHELIAL CELLS

The Ca^{2+} response of BAE cells and endothelial cells coming from SHR and SDR aortas was characterized following an acute stimulation by either melatonin or ATP. ATP was used as control agonist since its Ca^{2+} response through P2Y receptor stimulation has already been extensively studied in these cells [32]. In these experiments, cells were initially stimulated in external Ca^{2+}-free conditions followed by a cell superfusion with a Ca^{2+}-containing medium, a procedure that is typically used to estimate the $InsP_3$-dependent Ca^{2+} release from internal pools, while providing indications on the magnitude of the associated capacitative Ca^{2+} entry. Examples of Fura-2 signals measured on BAE, SDR and SHR cells are presented in Figure 2A. As seen, melatonin (0.1 to 50 μM) systematically failed to evoke a release of Ca^{2+} from internal Ca^{2+} stores in the three-cell type considered. In contrast, ATP caused under the same experimental conditions a mobilization of Ca^{2+} from internal pools and a capacitative Ca^{2+} entry. The average internal Ca^{2+} release ($\Delta[Ca^{2+}]_i$) induced by 10 μM ATP over a 100 s period ranged from 152 ± 12 nM (n=12) in BAE cells and 55 ± 9 nM (n=18) in SDR cells, to 25 ± 6 nM (n=26) in adult SHR endothelial cells (Figure 2B). Furthermore, the Fura-2 recordings in Figure 2A show that bath perfusion with a Ca^{2+} containing solution at this point failed to initiate a large $[Ca^{2+}]_i$ increase in both SDR and SHR cells, thus demonstrating that the agonist-induced capacitative Ca^{2+} entry is small in these cells. While confirming the binding results presented in figure 1 for BAE cells, these observations do not support the presence of melatonin receptors

linked to an $InsP_3$-dependent release of internal Ca^{2+} in aortic endothelial cells from SHR and SDR.

V.3.3. EFFECT OF MELATONIN INCUBATION ON INSP$_3$-INDUCED Ca^{2+} RELEASE

The modulatory effect of melatonin on the Ca^{2+} response induced by $InsP_3$-dependent Ca^{2+} mobilizing agents was next evaluated in experiments where the ATP-mediated Ca^{2+} release from internal stores was quantified in control conditions (ATP only) and following a 20 min incubation in 250 μM melatonin. Reports have already indicated that melatonin at this concentration inhibits the formation of $InsP_3$ triggered by norepinephrine in smooth muscle cells from SHR and Wistar-Kyoto rats [31]. Examples of Fura-2 recordings obtained from SHR endothelial cells stimulated with 10 μM ATP are presented in figure 3A. Melatonin incubation significantly altered the time-course and amplitude of the internal Ca^{2+} release process as indicated by the increase in $\Delta[Ca^{2+}]_i$ values from 25 ± 6 nM (n=20) in the absence of melatonin, to 42 ± 7 nM (n=20) after a 20 min melatonin pretreatment ($P<0.01$) (figure 3B). Similar results were obtained for the bradykinin (BK) mediated Ca^{2+} release with an average $\Delta[Ca^{2+}]_i$ value of 34 ±6 nM (n=20) in the absence of melatonin pretreatment and 47 ± 5 nM (n=20) ($P<0.01$) after a 20 min melatonin exposure (figure 3B). Incubating SHR cells with melatonin was also found to modify the basal internal Ca^{2+} level, $[Ca^{2+}]_i$, with a lower $[Ca^{2+}]_i$ value in cells exposed to melatonin relative to non exposed cells. In contrast, the average internal Ca^{2+} release ($\Delta[Ca^{2+}]_i$) evoked by 10 nM BK in BAE and SDR endothelial cells was unaffected by a pretreatment with melatonin (figure 3B) with average $\Delta[Ca^{2+}]_i$ values of 143 ± 7 nM (n=14) and 55 ± 6 nM (n=20) respectively after melatonin exposure as compared with 152 ± 12 nM (n=12) and 65 ± 9 nM (n=18) for non pretreated cells. These results confirm previous findings from our laboratory, which demonstrated that the agonist-induced Ca^{2+} response increased in endothelial cells from

SHR and not SDR [32] following incubation with antioxidant enzymes. Altogether, our data suggest that the action of melatonin on SHR may be related to its antioxidant properties.

The effect of incubation with melatonin on the Ca^{2+} release from internal pools in the three endothelial cell-strains was last evaluated using the SERCA inhibitor thapsigargin (TG). This procedure is classically used to mobilize Ca^{2+} from internal pools while bypassing $InsP_3$ production. As shown in Figure 4, the TG-induced release of Ca^{2+} was not significantly affected by melatonin incubation suggesting that a melatonin based modulation of Ca^{2+} release in SHR requires to some extent a production of $InsP_3$.

V.3.4. EFFECT OF MELATONIN ON OXIDATIVE STRESS IN ENDOTHELIAL CELLS

To evaluate the antioxidative properties of melatonin, experiments were performed in which superoxide anions ($^-O_2^\bullet$) were generated using the hypoxanthine/xanthine oxidase (HX/XO) system. The Ca^{2+} response was first characterized in the absence of melatonin in BAE and SDR endothelial cells exposed to 250 μM HX + 50 mU XO for 90 min and stimulated by ATP in Ca^{2+}-free conditions. Capacitative Ca^{2+} influx was then evaluated by replacing the bathing medium with a normal Ca^{2+} Earle's solution. The Fura-2 recordings in figure 5A and 5B illustrate the typical changes observed in the Ca^{2+} response of endothelial cells following an oxidative stress. As seen, a 90 min exposure to free radicals resulted in a global increase of the cell basal Ca^{2+} level, $[Ca^{2+}]_i$, a depression of the agonist-induced Ca^{2+} mobilization from internal stores and an enhancement of the capacitative Ca^{2+} influx (dash line). The basal Ca^{2+} concentration in BAE and SDR cells increased from 68 ± 2 nM and 55 ± 3 nM respectively to values of 112 ± 5 nM and 124 ± 5 nM, following pretreatment with HX/XO. Figure 5C summarizes the $\Delta[Ca^{2+}]_i$ values obtained in BAE and SDR cells in responses to a 10 μM ATP

stimulation after preincubation with HX/XO. In BAE cells exposed to HX/XO, ATP stimulation led to a $\Delta[Ca^{2+}]_i$ of 9 ± 5 nM (n=8), which amounts to less than 15% the control $\Delta[Ca^{2+}]_i$ value. Similarly, exposure of SDECs to identical oxidative stress conditions almost completely inhibited the ATP induced Ca^{2+} release. Our results in figure 5 thus support a model whereby free radicals somehow inhibit the agonist-induced Ca^{2+} mobilization process and contribute to enhance the plasma membrane permeability to Ca^{2+}.

The protective action of melatonin against the deleterious effects of free radicals on the Ca^{2+} response in BAE and SDR cells is illustrated in figure 5A and B, superimposed to the data previously discussed. As seen, co-incubation with melatonin + HX/XO successfully countered the effect of oxidative stress on the ATP-induced Ca^{2+} release in BAE cells with a mean $\Delta[Ca^{2+}]_i$ equal to 92 ± 15 nM (n=8), a value nearly identical to that measured on cells not submitted to an oxidative stress. Similar results were obtained in SDR cells with a mean $\Delta[Ca^{2+}]_i$ value of 53 ± 5 nM (n=12) after a 90 min exposure to HX/XO + melatonin, as compared with 63 ± 6 nM (n=8) in cells never exposed to the HX/XO free radical generating system (figure 5C). In addition, these data confirmed that melatonin incubation lowers the cell basal Ca^{2+} level, $[Ca^{2+}]_i$, with values of 74 ± 3 nM in BAE and 52 ± 2 nM for SDR cells as compared with 112 ± 5 nM and 124 ± 5 nM under HX/XO conditions, statistically different ($P<0.01$).

V.3.5. EFFECT OF MELATONIN INCUBATION ON CAPACITATIVE Ca^{2+} ENTRY IN ENDOTHELIAL CELLS

As discussed in figure 2, the typical Ca^{2+} response of aortic endothelial cells to Ca^{2+}-mobilizing agonists consists of an initial release of Ca^{2+} from internal stores, followed by a capacitative Ca^{2+} influx. Experiments were thus undertaken to examine the effects of melatonin

on the capacitative Ca^{2+} entry in BAE cells and in primary cultured endothelial cells from SDR and SHR. The results obtained for BAE and SDR cells are summarized in figure 6A and B respectively. In the absence of melatonin, the capacitative Ca^{2+} entry measured in BAE cells after a single BK stimulation averaged 78 ± 10 nM (n=14). Incubating BAE cells for 20 min with melatonin, resulted in a small (15%) increase of the capacitative Ca^{2+} influx with a $\Delta[Ca^{2+}]_i$ value of 92 ± 10 nM (n=14). To overcome the known desensitization of BK receptors [45] an additional series of experiments was undertaken in which BAE cells were stimulated in nominally free external Ca^{2+} conditions with two sequential BK stimulation periods applied 2 minutes apart. The capacitative Ca^{2+} entry resulting from the readmission of Ca^{2+} into the external medium amounted to 123 ± 8 nM (n=14) in cells exposed for 20 min to melatonin prior to the double BK stimulation, as compared with 75 ± 3 nM (n=14) for non-exposed cells ($P<0.01$) (figure 6A).

The mean capacitative $\Delta[Ca^{2+}]_i$ value of SDR endothelial cells without melatonin pretreatment was estimated to 8 ± 3 nM (n=20) and 14 ± 3 nM (n=20) when using the single or double BK stimulation protocol respectively (figure 6B). Incubation with melatonin caused an enhancement of the capacitative Ca^{2+} entry leading to mean capacitative $\Delta[Ca^{2+}]_i$ values of 10 ± 1 nM (n=20) for the single and 21 ± 1 nM (n=20) ($P<0.01$) for the double BK stimulation protocol.

Figure 7 summarizes the effects of melatonin on the capacitative Ca^{2+} influx in SHR endothelial cells. As we have previously reported [32], SHR cells are characterized by a small capacitative Ca^{2+} entry under control conditions. As seen, BK stimulation yielded a capacitative $\Delta[Ca^{2+}]_i$ of 5 ± 3 nM (n=8) in the absence of melatonin. ATP stimulation in turn generated a barely detectable capacitative $\Delta[Ca^{2+}]_i$ of 2.4 ± 0.4 nM (n=8). Incubating SHR cells with

melatonin caused a 430% increase of the BK-generated capacitative $\Delta[Ca^{2+}]_i$ to a value of 28 ± 4 nM (n=20). The capacitative $\Delta[Ca^{2+}]_i$ in response to ATP amounted to 10 ± 2 nM (n=8) under the same melatonin incubation conditions, an increase of more than 330% relative to control. A prolonged melatonin incubation (48 hr) of SHR cells did not significantly improve the Ca^{2+} response to ATP, with a mean capacitative $\Delta[Ca^{2+}]_i$ equal to 8 ± 2 nM (n=8), as compared with 10 ± 2 nM after a 20 min incubation.

V.3.6. MELATONIN-DEPENDENT MODULATION OF THE CAPACITATIVE Ca^{2+} ENTRY IN ENDOTHELIAL CELLS

To gain more information on the effect of melatonin on the capacitative Ca^{2+} entry in endothelial cells, a series of experiments was undertaken in which the BK-induced Ca^{2+} response (BK2) was measured on cells that were stimulated once with BK (BK1) before the incubation period of 20 or 40 min with 50 µM melatonin (see figure 8A). This procedure insures that the capacitative Ca^{2+} influx machinery is activated prior to melatonin exposure, while allowing comparing the Ca^{2+} response coming from exactly the same cell population. Melatonin was used at a concentration of 50 µM to avoid effects related to its antioxidative properties [31]. Control experiments were performed using an identical perfusion protocol in the absence of melatonin. Typical recordings obtained from SDR cells are presented in figure 8A. In the absence of melatonin (control), the magnitude of the capacitative Ca^{2+} entry remained constant for BK stimulations delivered 20 min or 40 min apart (figure 8B). Exposure to melatonin between the first (BK1) and the second BK (BK2) stimulation however caused a marked increase in the BK2-induced capacitative $\Delta[Ca^{2+}]_i$ / BK1- induced capacitative $\Delta[Ca^{2+}]_i$ ratios (CCE2 / CCE1) with values of 4 ± 1 (n=4) and 8 ± 2 (n=4) when measured after a 20 min and 40 min perfusion with melatonin respectively. The release of internal Ca^{2+} by BK in SDR

cells was equally affected after exposure to melatonin. Under control conditions, the ratio BK2-induced Ca^{2+} release / BK1-induced Ca^{2+} release (BK2 / BK1) was estimated at 0.5 ± 0.2 (n=4) and 0.4 ± 0.2 (n=4) for the 20 min and 40 min protocols respectively, indicating a time dependent decrease in the Ca^{2+} mobilization process. A 20 min exposure to melatonin increased the BK2-induced Ca^{2+} release by a factor of 1.6 leading to a BK2 / BK1 ratio of 0.8 ± 0.1 (n=4). A 40 min exposure to melatonin led to an even more substantial improvement with a BK2 / BK1 ratio of 0.9 ± 0.1 (n=4), a 100% increase relative to control (figure 8B).

Similar experiments were performed using BAE cells (Table 1). In this particular case, the BK2-induced Ca^{2+} release / BK1-induced Ca^{2+} release ratio measured in response to BK stimulation was not significantly affected with values of 0.5 ± 0.1 (n=4) following a 20 min exposure to melatonin as compared with 0.4 ± 0.2 (n=4) for non-exposed cells. In contrast the CCE2 / CCE1 ratio increased from 1.0 ± 0.2 (n=4) without melatonin exposure to 1.4 ± 0.3 (n=4) after a 20 min melatonin treatment. These results indicate an important stimulation of the capacitative Ca^{2+} entry as a result of melatonin exposure.

V.4. DISCUSSION

This study documents the effect of melatonin on the Ca^{2+} response of vascular endothelial cells to Ca^{2+}-mobilizing agonists. Our results indicate that acute exposure of vascular endothelial cells to melatonin does not lead to a release of Ca^{2+} from internal pools. However, melatonin incubation was found to reverse the inhibitory effect of free radicals on the release of Ca^{2+} from internal stores, and to improve the mobilization of internal Ca^{2+} in aortic endothelial cells from SHR. Melatonin also appeared capable of stimulating in a time dependent manner the capacitative Ca^{2+} entry in BAE cells and in primary cultured endothelial cells from SHR and SDR.

V.4.1. Effects of melatonin on internal Ca^{2+} release in vascular endothelial cells

There is currently no data on the modulatory effect of melatonin on Ca^{2+} signaling in vascular endothelial cells. The results presented in figure 2 indicate that melatonin does not behave as a Ca^{2+} mobilizing agonist in BAE cells and primary cultured endothelial cells from SHR and SDR, ruling out a contribution of either MT1, MT2 or MT3 receptors to a phosphatidylinositide hydrolysis process in these cells [46,47]. Our assays were performed under conditions chosen to reveal most binding sites since a very high concentration of cold melatonin (100 μM) was used to displace the labeled substrate (12 nM). Despite these experimental conditions, our results could not confirm the presence of melatonin binding sites in BAE cells, indicating that the observed effect of melatonin on the Ca^{2+} response in this case cannot be consequent to the activation of specific melatonin receptors. There is however, a possibility of a receptor mediated effect of melatonin on Ca^{2+} homeostasis in SHR and SDR cells. Nonetheless, the observation that both SDR and BAE cells are similarly affected by melatonin does not support an important contribution of a receptor-based mechanism to the action of melatonin in SDR despite the presence of specific binding sites in this case.

Melatonin incubation on the other hand clearly increased Ca^{2+} release induced by BK and ATP in endothelial cells from SHR. This effect appeared to be SHR specific, since the incubation with melatonin failed to modify the internal Ca^{2+} mobilization process in BAE cells and in primary cultures of SDR endothelial cells. Such a response could be explained by the antioxidant properties of melatonin since we previously reported that pre-treating endothelial cells with antioxidant enzymes caused a significant increase of the Ca^{2+} mobilization triggered by ATP in SHR but not in SDR cells [32]. It was argued that the sensitivity of the Ca^{2+} response to antioxidant enzymes in SHR endothelial cells was attributable to an overproduction

of ROS which in turn led to a depletion of releasable Ca^{2+} from $InsP_3$-sensitive and -insensitive Ca^{2+} pools [32]. There are numerous reports confirming that melatonin is a highly efficient scavenger of the hydroxyl ($OH^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) [16,17,48,49]. Melatonin was found to significantly suppress the H_2O_2-induced inhibition of $NO^{\bullet}$ production in endothelial cells from human umbilical arteries [20] and to attenuate the effect of peroxynitrite in vascular endothelial cells [30]. Evidence was also provided that melatonin can stimulate the activity of the glutathione peroxidase (GSH-Px), thus enhancing its antioxidative property [50]. Similarly, a protective effect of melatonin was documented against ischemia reperfusion injury [51] and age-related diseases, two physiological states involving an increased production of free radicals [52]. Altogether, these observations strongly suggest that the melatonin-induced increase in Ca^{2+} release from internal pools in SHR endothelial cells could be closely related to its antioxidant properties.

The hypothesis of melatonin acting on SHR endothelial cells via an antioxidant effect is further supported by the fact that melatonin could prevent the deleterious effects of the HX/XO ROS generating system on internal Ca^{2+} mobilization in BAE and SDR endothelial cells. ROS are known to affect a variety of regulatory mechanisms responsible for Ca^{2+} signaling in several cell types, including endothelial cells. In the present case, the HX/XO free radical generating system annihilated the ATP induced Ca^{2+} release from both BAE and SDR endothelial cells. As discussed in an earlier work, ROS are most likely to deplete the cell $InsP_3$-sensitive Ca^{2+} reservoirs through a complex mechanism involving an effect on the production of $InsP_3$, a sensitization of the $InsP_3$ receptor itself and/or an inhibition of the endoplasmic reticulum Ca^{2+}-ATPase [32]. A co-incubation with melatonin plus HX/XO resulted in a partial recovery of the Ca^{2+} response, a clear indication that melatonin can restore normal Ca^{2+} signaling through an antioxidant effect. Melatonin remained however less potent than other antioxidants such as

catalase, SOD, and desferioxamine in preventing a dysfunctional $InsP_3$-induced Ca^{2+} release in endothelial cells under oxidative stress conditions (data not shown). This observation could suggest that hydrogen peroxide and superoxide anion and not the hydroxyl radical are the main free radicals involved in the deleterious effects of ROS on the Ca^{2+} response in endothelial cells. However, Zang at al. (1998), have recently provided evidence for melatonin not interfering with $OH^{\bullet}$ but acting as a scavenger of H_2O_2 in a dose-dependent manner [19]. These results would be in agreement with our previous observations confirming the key role of H_2O_2 in the generation of a dysfunctional Ca^{2+} homeostasis in vascular endothelial cells [32].

V.4.2. Effect of melatonin on the capacitative Ca^{2+} entry in endothelial cells

In addition to restoring the Ca^{2+} mobilization process in endothelial cells submitted to an oxidative stress, melatonin was also found to stimulate the capacitative Ca^{2+} entry in BAE and SDR endothelial cells. For instance, an 8-fold increase of the capacitative Ca^{2+} entry ratio was observed in SDR endothelial cells exposed for 40 min to a melatonin containing solution. The melatonin-induced enhancement in Ca^{2+} influx is also likely to be responsible for the 2-fold increase in the BK ratio value (BK2-Ca^{2+} release / BK1-Ca^{2+} release) observed for the 40 min melatonin perfusion protocol. However, the exact nature of the mechanism underlying the stimulating effect of melatonin on the agonist-induced Ca^{2+} entry in endothelial cells remains to be established. Nonetheless, there are clear indications that the action of melatonin on the capacitative Ca^{2+} influx in BAE and SDR endothelial cells is more important in cells that were first stimulated with the agonist prior to melatonin exposure, than in cells treated with melatonin without previous agonist stimulation. For instance, in experiments where SDR cells were not

stimulated with BK before melatonin treatment, the capacitative $\Delta[Ca^{2+}]_i$ increased 1.3 fold as a result of a 20 min melatonin exposure. In contrast, a mean capacitative Ca^{2+} entry ratio of 4 ± 1 was measured in experiments where cells were stimulated with BK at the beginning of the perfusion protocol. These observations point towards a mechanism whereby melatonin is acting through a time dependent process on capacitative Ca^{2+} influx machinery that is operational, rather than facilitating the onset of a capacitative Ca^{2+} influx pathway. This could also explain the more important capacitative $\Delta[Ca^{2+}]_i$ values obtained in SDR and BAE cells when a double stimulation protocol was used following a 20 min melatonin incubation period. Because the binding study presented in herein failed to provide evidence for specific melatonin binding sites in BAE cells, the melatonin-dependent increase in capacitative Ca^{2+} influx measured in this case cannot readily be accounted for by an interaction of melatonin with membrane receptors. This conclusion is likely to remain valid for the melatonin-dependent increase in BK-evoked Ca^{2+} influx measured in endothelial cells from SDR. Interestingly, incubating endothelial cells from SHR with melatonin without prior agonist stimulation, caused a significant increase of the capacitative Ca^{2+} entry leading to mean capacitative $\Delta[Ca^{2+}]_i$ values of 28 ± 4 nM and 10 ± 2 nM for melatonin preconditioned cells stimulated with BK and ATP respectively, compared with 5 ± 3 nM and 2.4 ± 0.4 nM for non treated cells. These results differ from those presented in figure 6 for BAE and SDR cells and point toward a specific action of melatonin on the capacitative entry of Ca^{2+} in SHR cells.

Several factors have been reported to modulate the capacitative Ca^{2+} entry in endothelial cells. There is for instance a large body of evidence indicating that the agonist-evoked Ca^{2+} influx in vascular endothelial cells is increased with a hyperpolarization of the cell potential [34,53]. Melatonin is already known to modulate the Ca^{2+} influx in gonadotroph cells [54] through an increase of the cell membrane potential. Similarly, the results obtained in rat ileal

smooth muscle cells suggest that melatonin activates an apamin sensitive $K_{(Ca)}$ channel causing an inhibition of muscle contraction via a hyperpolarization of the cell membrane [36]. The melatonin-dependent relaxation of rat gastric fundus smooth muscle cells was also reported to be apamin sensitive, an indication that $K_{(Ca)}$ channels of small conductance may be activated following melatonin exposure [55]. An action of melatonin on Ca^{2+} influx through the activation of K^+ selective channels could constitute therefore an important mechanism susceptible to account for the results presented in present study. In support of this proposal is the observation that small conductance $K_{(Ca)}$ channels are present in endothelial cells from rabbit, pig and rat aortas [56-58] and in primary cultures of SDR (Sauvé et al., unpublished data). Apamin-insensitive intermediate and large conductance $K^+_{(Ca)}$ channels have also been characterized in several vascular endothelial cell preparations including BAE cells [59] and cells in primary cultures from SHR and SDR (Sauvé et al., unpublished data). A direct stimulatory effect of melatonin on these $K_{(Ca)}$ channels remains however to be established, and melatonin may not lead to the activation of K^+ channels in all cases. For instance, it was proposed recently that the endothelium-dependent potentialization by melatonin of the contractile response to phenylephrine in rat-tail arteries was partly the result of an inhibition of endothelial K^+ channels [35]. Melatonin was also reported to block Kv 1.3 channels at high concentrations (20 mM) [37]. The action of melatonin on K^+ selective channels may differ therefore depending on the type of vessels considered.

Alternatively, melatonin could act on the structures responsible for the capacitative Ca^{2+} influx. Whole cell recordings have revealed that the store-operated Ca^{2+} influx in BAE cells triggered by thapsigargin is dose dependently inhibited by calmodulin [60,61] to which melatonin could bind with a high affinity [62]. Thus, it is most likely that melatonin may potentiate the BK-induced Ca^{2+} influx in endothelial cells by inhibiting the action of calmodulin

on the capacitative Ca^{2+} entry. Finally, theg results of our binding studies do not entirely rule out a contribution of second messengers in SDR and SHR cells. An increase in cGMP has been associated to the activation process of the capacitative Ca^{2+} entry in several cell types including pancreatic acinar cells [63], colonic epithelial cells [33] and pituitary GH3 cells [64]. Some reports indicate however, that melatonin could exert an inhibitory effect on cGMP production thus ruling out this mechanism as a potential candidate to explain an observations [65].

Altogether, our results suggest that melatonin can restore Ca^{2+} signaling in vascular endothelial cells from SHR by overcoming the deleterious effects of an overproduction of free radicals. Such a mechanism may contribute to the known hypotensive effect of melatonin observed in SHR, and points towards a beneficial contribution of melatonin in the control of blood pressure in animal models and in human hypertension.

V.5. ACKNOWLEDGMENTS

We are grateful to L. Garneau for critical reading of the manuscript. We thank Joanne Vallée for technical assistance in cell culture. This work was supported by grants from the Canadian Institutes of Health Research, MT 7769, and from the Canadian Heart & Stroke Foundation to RS.

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V.7. FIGURES CHAPTER V.

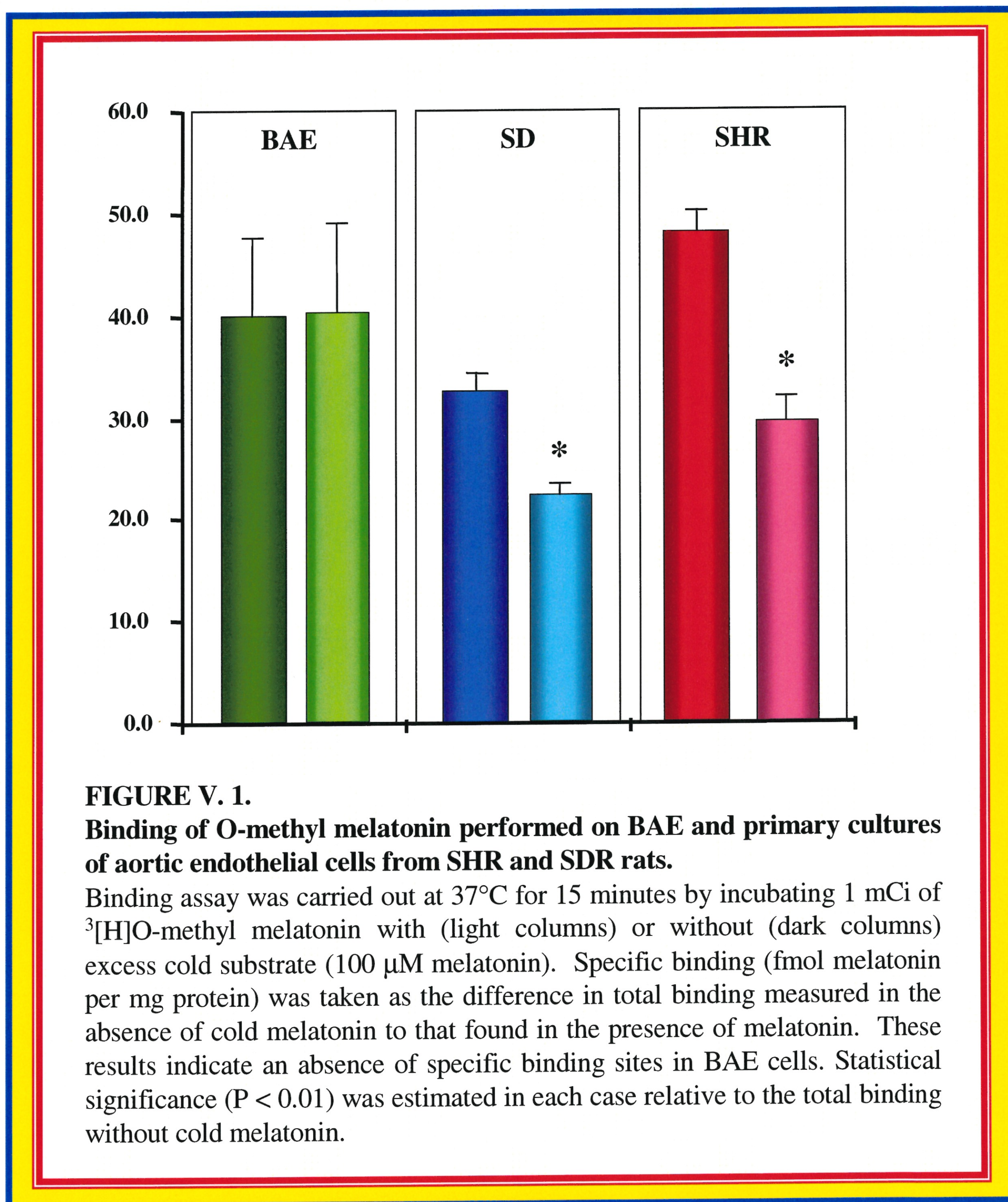

FIGURE V. 1.
Binding of O-methyl melatonin performed on BAE and primary cultures of aortic endothelial cells from SHR and SDR rats.
Binding assay was carried out at 37°C for 15 minutes by incubating 1 mCi of ^{3}[H]O-methyl melatonin with (light columns) or without (dark columns) excess cold substrate (100 µM melatonin). Specific binding (fmol melatonin per mg protein) was taken as the difference in total binding measured in the absence of cold melatonin to that found in the presence of melatonin. These results indicate an absence of specific binding sites in BAE cells. Statistical significance ($P < 0.01$) was estimated in each case relative to the total binding without cold melatonin.

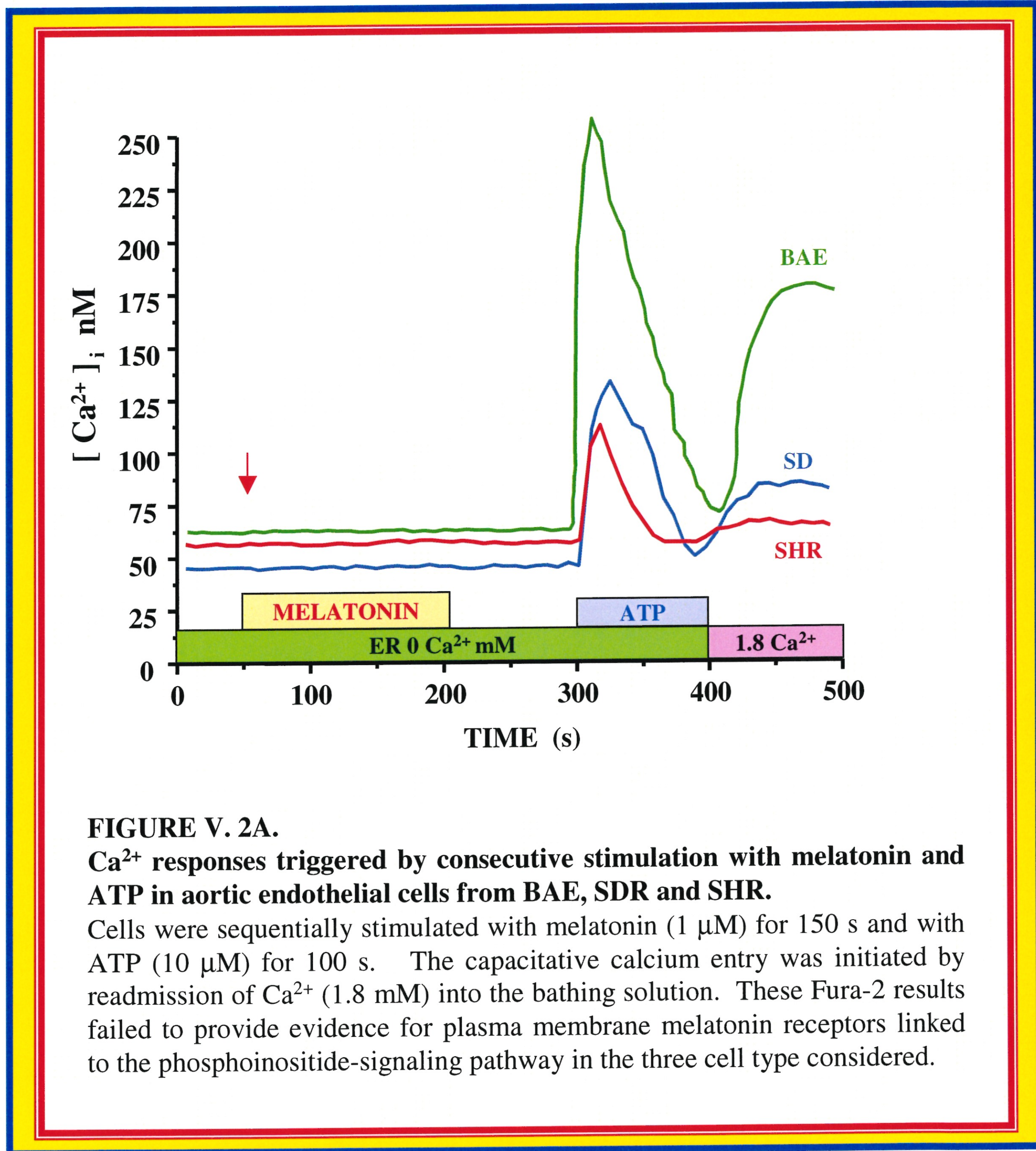

FIGURE V. 2A.

Ca^{2+} responses triggered by consecutive stimulation with melatonin and ATP in aortic endothelial cells from BAE, SDR and SHR.

Cells were sequentially stimulated with melatonin (1 μM) for 150 s and with ATP (10 μM) for 100 s. The capacitative calcium entry was initiated by readmission of Ca^{2+} (1.8 mM) into the bathing solution. These Fura-2 results failed to provide evidence for plasma membrane melatonin receptors linked to the phosphoinositide-signaling pathway in the three cell type considered.

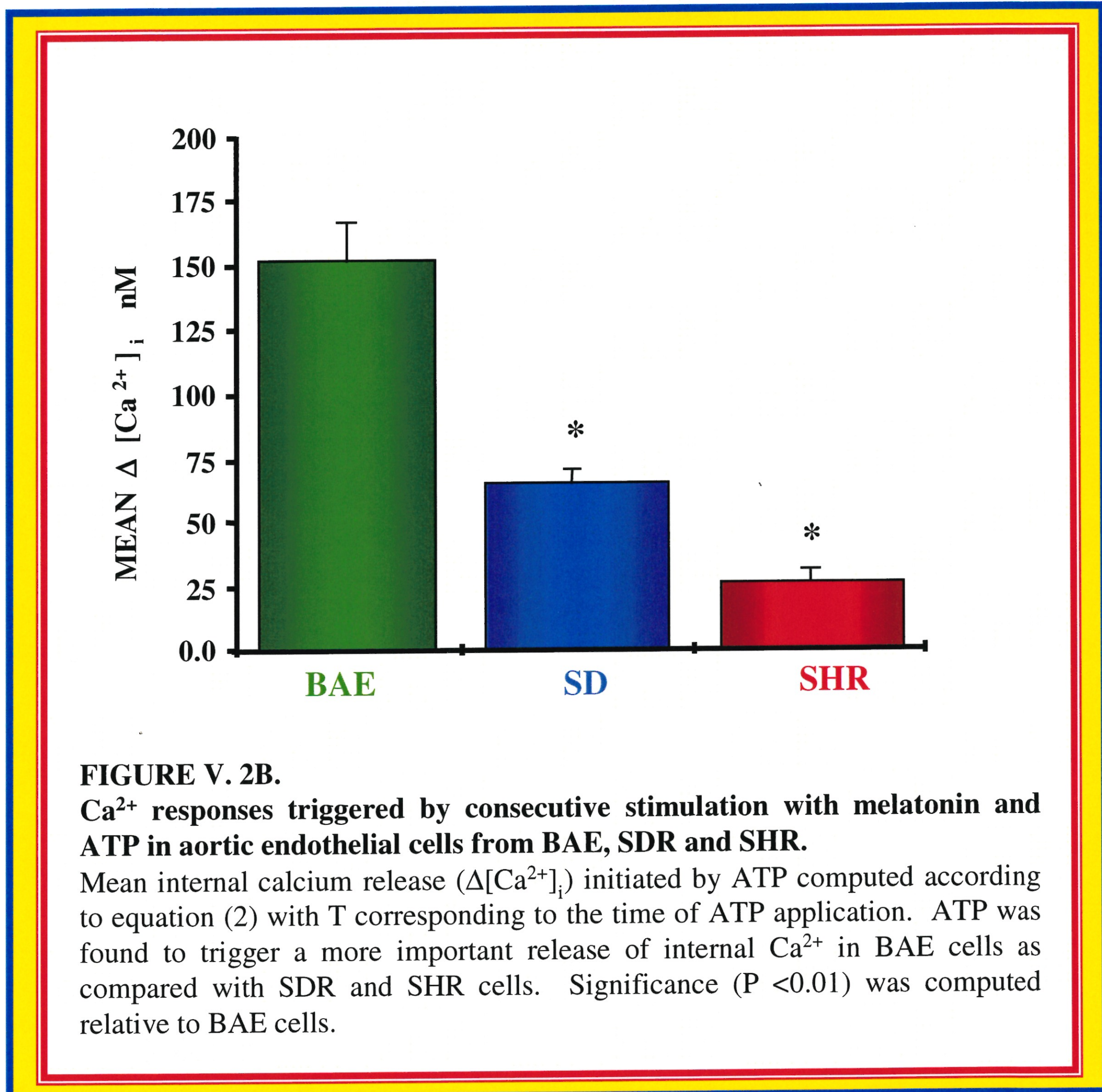

FIGURE V. 2B.
Ca^{2+} responses triggered by consecutive stimulation with melatonin and ATP in aortic endothelial cells from BAE, SDR and SHR.
Mean internal calcium release ($\Delta[Ca^{2+}]_i$) initiated by ATP computed according to equation (2) with T corresponding to the time of ATP application. ATP was found to trigger a more important release of internal Ca^{2+} in BAE cells as compared with SDR and SHR cells. Significance ($P < 0.01$) was computed relative to BAE cells.

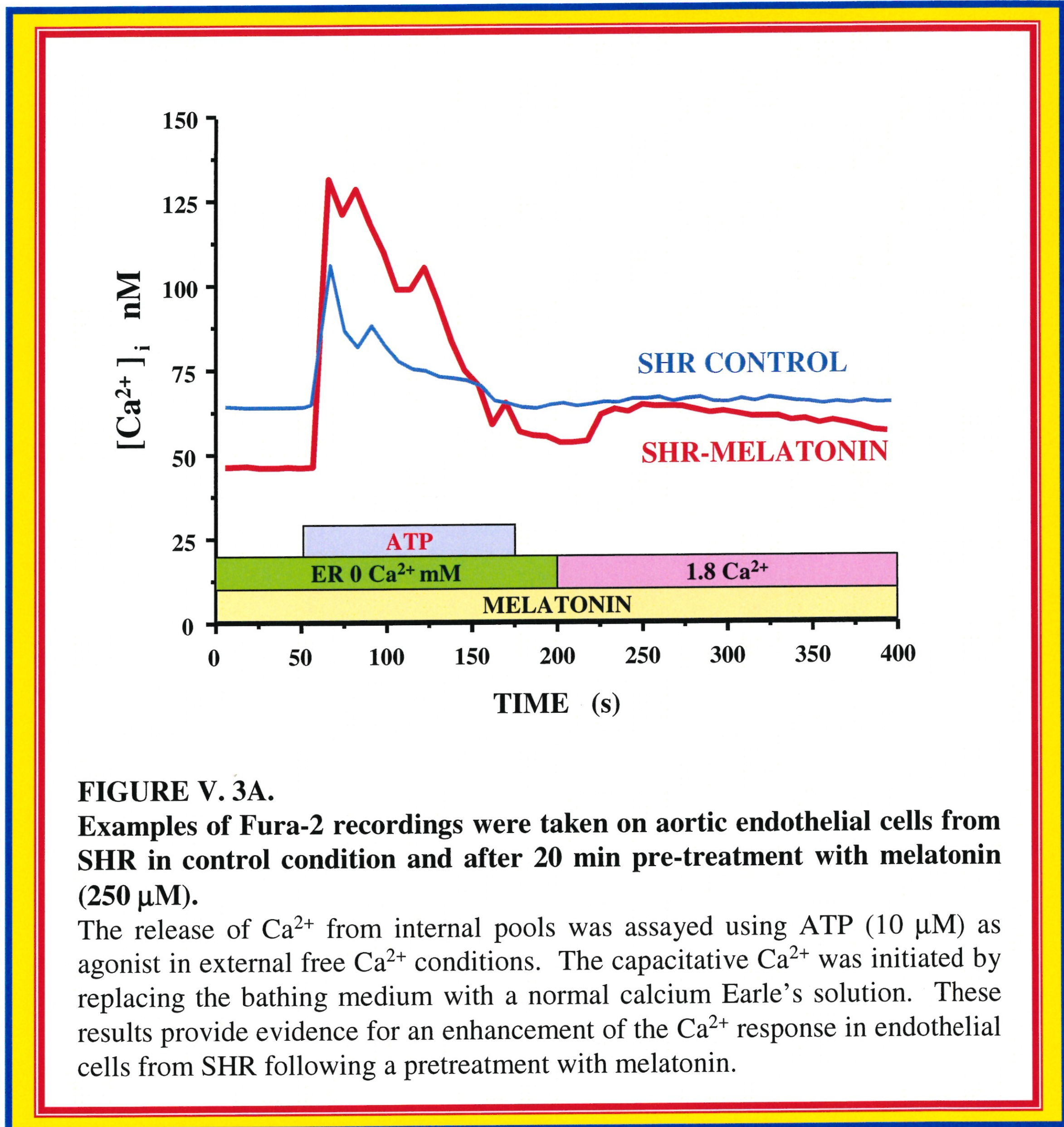

FIGURE V. 3A.
Examples of Fura-2 recordings were taken on aortic endothelial cells from SHR in control condition and after 20 min pre-treatment with melatonin (250 µM).
The release of Ca^{2+} from internal pools was assayed using ATP (10 µM) as agonist in external free Ca^{2+} conditions. The capacitative Ca^{2+} was initiated by replacing the bathing medium with a normal calcium Earle's solution. These results provide evidence for an enhancement of the Ca^{2+} response in endothelial cells from SHR following a pretreatment with melatonin.

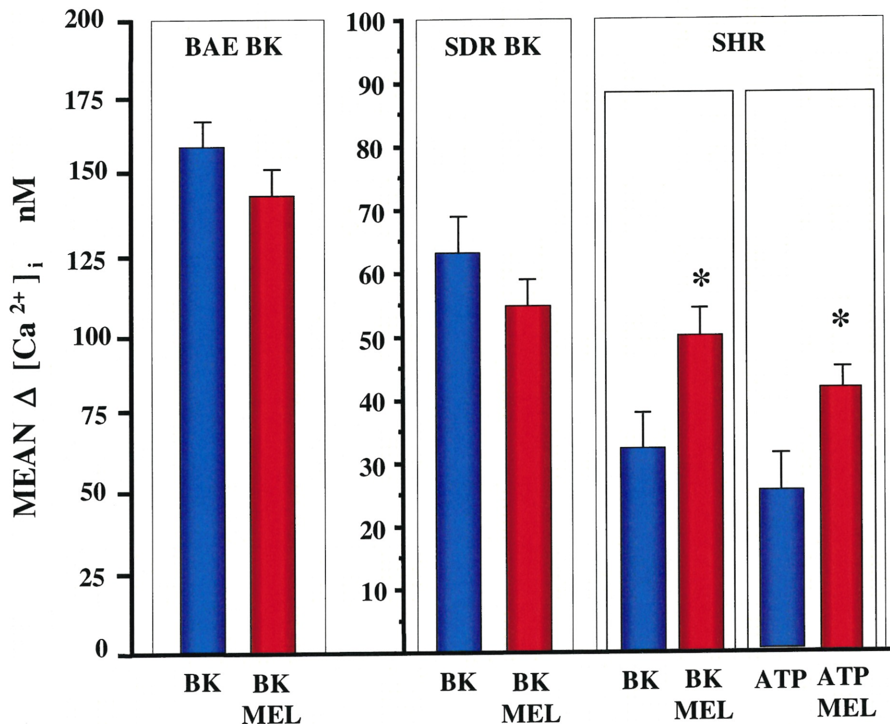

FIGURE V. 3B.
A graphical comparison of melatonin effects on mean $\Delta[Ca^{2+}]_i$ responses induced by BK in aortic endothelial cells from BAE, SDR and SHR versus the control conditions.
Effect of a 20 min pretreatment with melatonin on the magnitude of the Ca^{2+} release initiated either by BK (10 nM) or by ATP (10 μM). $\Delta[Ca^{2+}]_i$ values were computed according to equation 2 with T equal to 150 s. In contrast to the results obtained from SHR endothelial cells, there was no significant effect of melatonin incubation on the BK-evoked Ca^{2+} mobilization process in BAE and SDR endothelial cells. Significance ($P < 0.01$) was estimated in each cases relative to the mean $\Delta[Ca^{2+}]_i$ value obtained in the absence of melatonin (MEL).

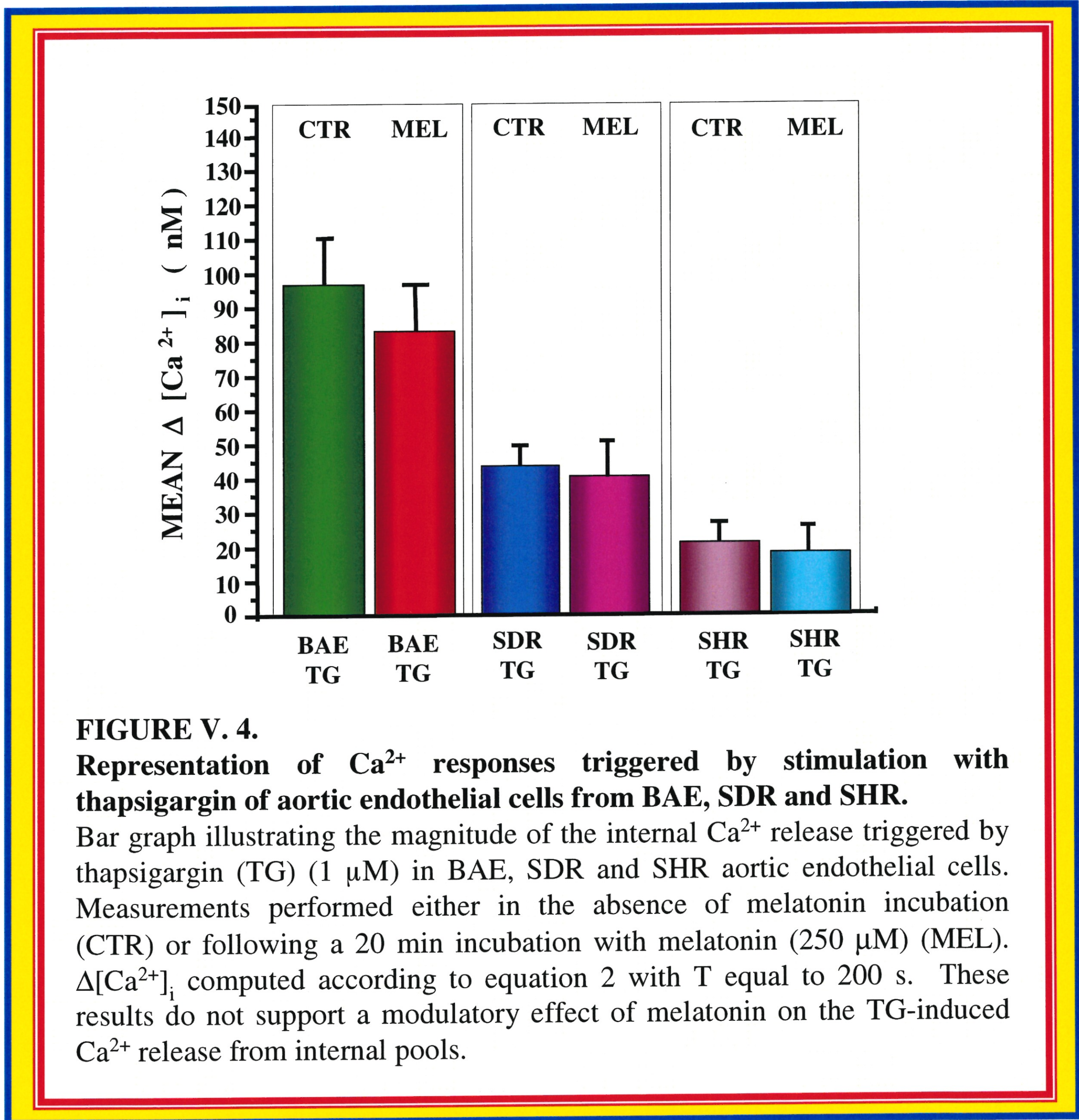

FIGURE V. 4.

Representation of Ca^{2+} responses triggered by stimulation with thapsigargin of aortic endothelial cells from BAE, SDR and SHR.

Bar graph illustrating the magnitude of the internal Ca^{2+} release triggered by thapsigargin (TG) (1 μM) in BAE, SDR and SHR aortic endothelial cells. Measurements performed either in the absence of melatonin incubation (CTR) or following a 20 min incubation with melatonin (250 μM) (MEL). $\Delta[Ca^{2+}]_i$ computed according to equation 2 with T equal to 200 s. These results do not support a modulatory effect of melatonin on the TG-induced Ca^{2+} release from internal pools.

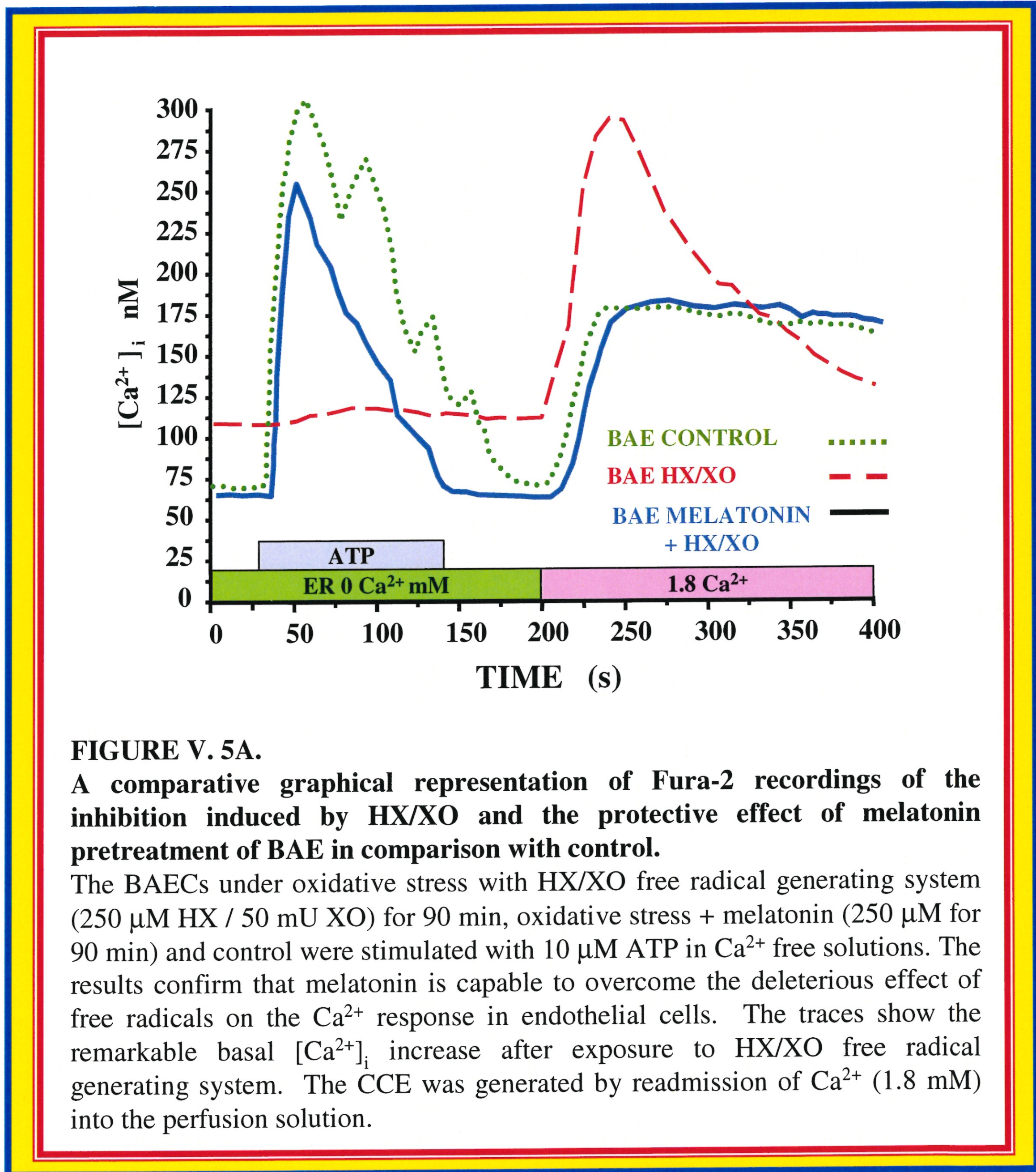

FIGURE V. 5A.

A comparative graphical representation of Fura-2 recordings of the inhibition induced by HX/XO and the protective effect of melatonin pretreatment of BAE in comparison with control.

The BAECs under oxidative stress with HX/XO free radical generating system (250 μM HX / 50 mU XO) for 90 min, oxidative stress + melatonin (250 μM for 90 min) and control were stimulated with 10 μM ATP in Ca^{2+} free solutions. The results confirm that melatonin is capable to overcome the deleterious effect of free radicals on the Ca^{2+} response in endothelial cells. The traces show the remarkable basal $[Ca^{2+}]_i$ increase after exposure to HX/XO free radical generating system. The CCE was generated by readmission of Ca^{2+} (1.8 mM) into the perfusion solution.

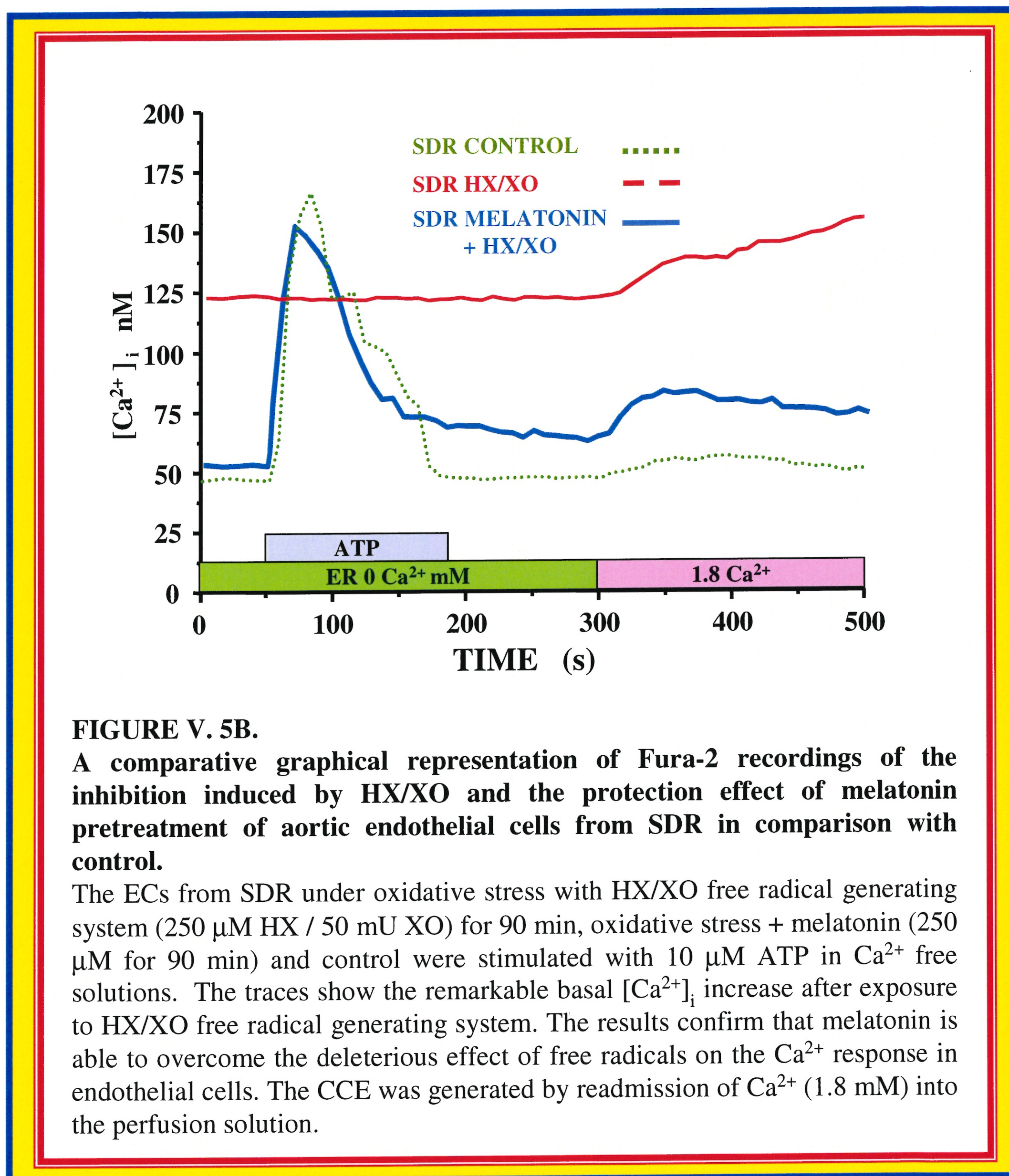

FIGURE V. 5B.

A comparative graphical representation of Fura-2 recordings of the inhibition induced by HX/XO and the protection effect of melatonin pretreatment of aortic endothelial cells from SDR in comparison with control.

The ECs from SDR under oxidative stress with HX/XO free radical generating system (250 μM HX / 50 mU XO) for 90 min, oxidative stress + melatonin (250 μM for 90 min) and control were stimulated with 10 μM ATP in Ca^{2+} free solutions. The traces show the remarkable basal $[Ca^{2+}]_i$ increase after exposure to HX/XO free radical generating system. The results confirm that melatonin is able to overcome the deleterious effect of free radicals on the Ca^{2+} response in endothelial cells. The CCE was generated by readmission of Ca^{2+} (1.8 mM) into the perfusion solution.

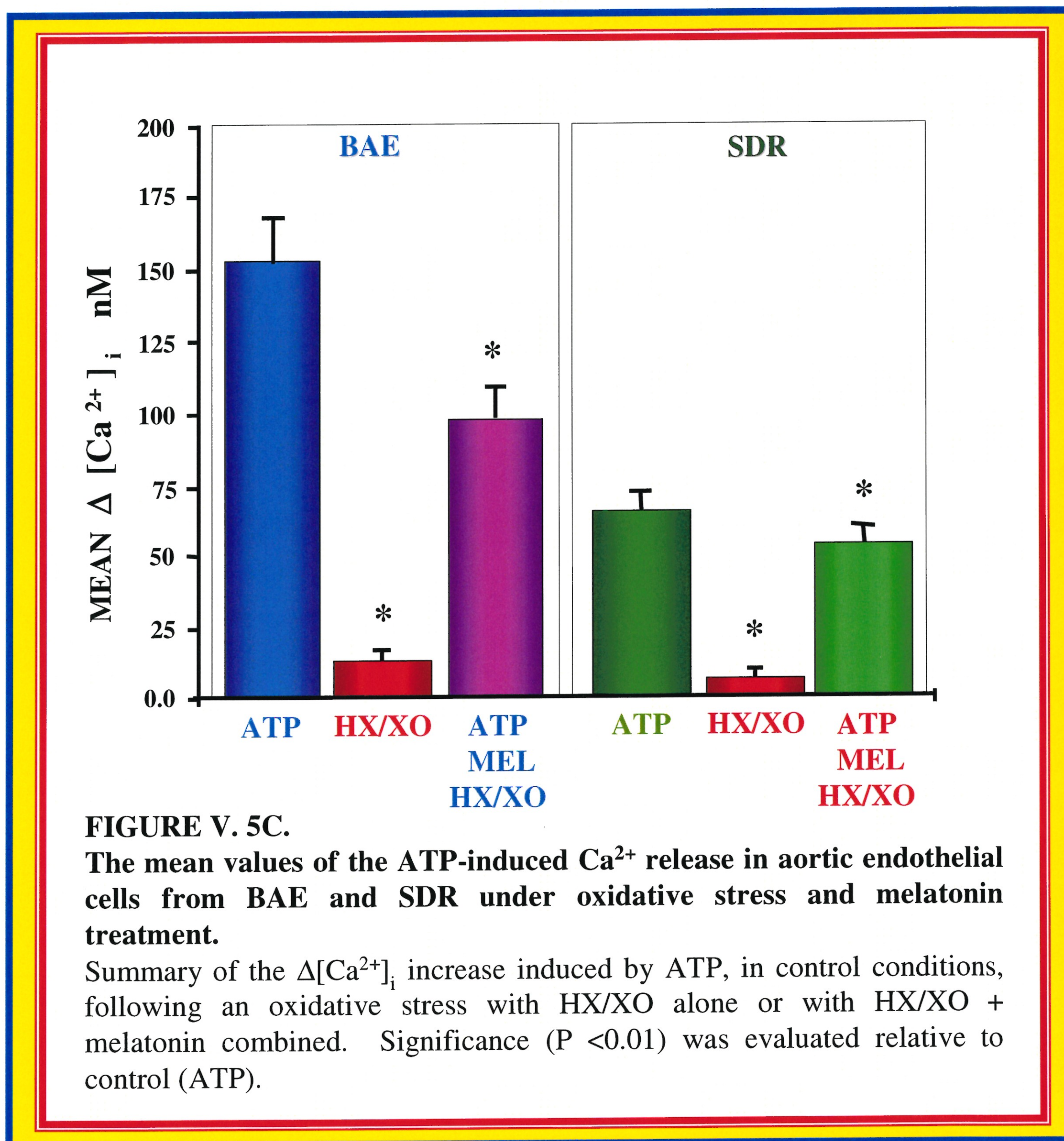

FIGURE V. 5C.
The mean values of the ATP-induced Ca^{2+} release in aortic endothelial cells from BAE and SDR under oxidative stress and melatonin treatment.
Summary of the $\Delta[Ca^{2+}]_i$ increase induced by ATP, in control conditions, following an oxidative stress with HX/XO alone or with HX/XO + melatonin combined. Significance ($P < 0.01$) was evaluated relative to control (ATP).

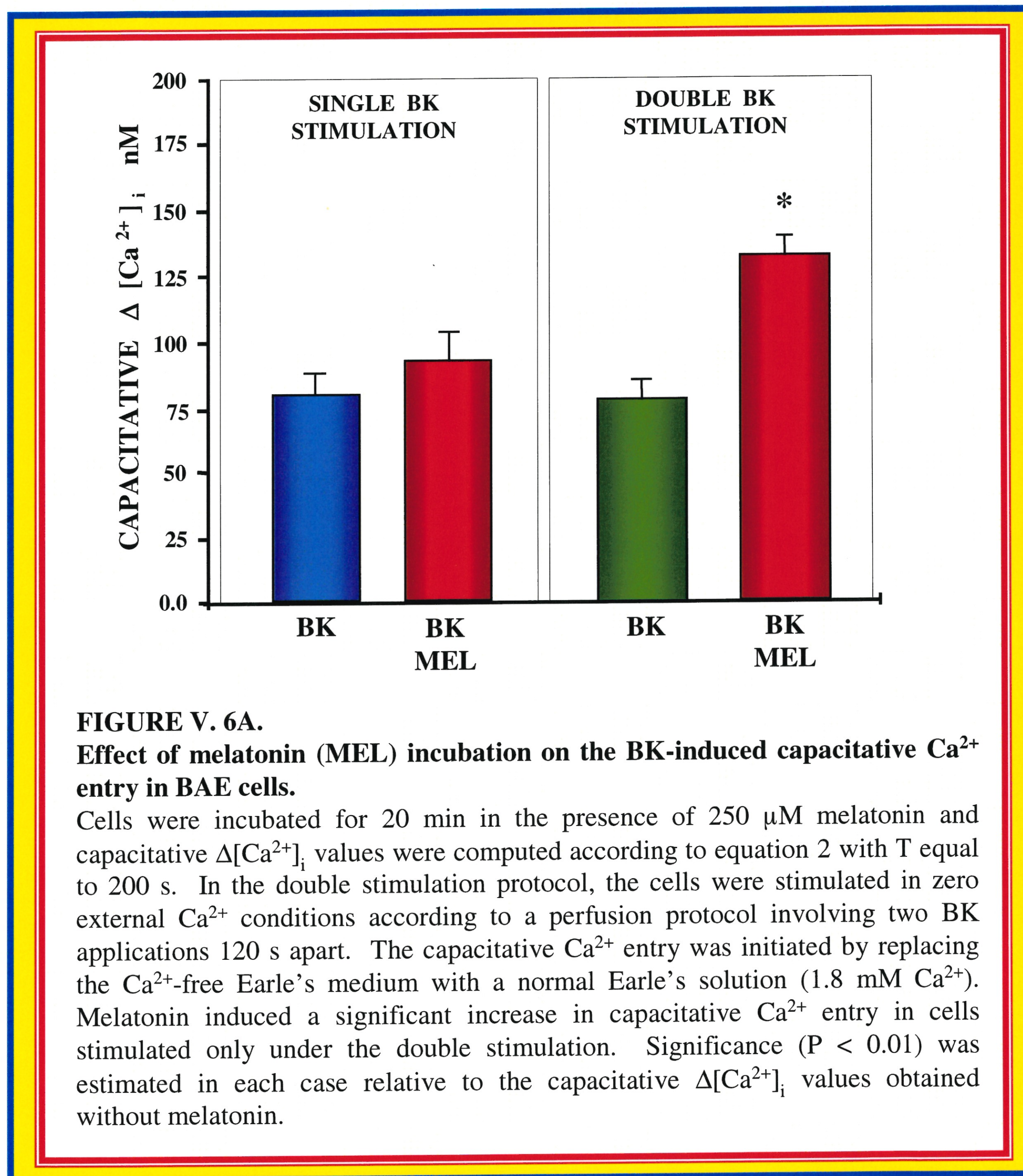

FIGURE V. 6A.
Effect of melatonin (MEL) incubation on the BK-induced capacitative Ca^{2+} entry in BAE cells.
Cells were incubated for 20 min in the presence of 250 μM melatonin and capacitative $\Delta[Ca^{2+}]_i$ values were computed according to equation 2 with T equal to 200 s. In the double stimulation protocol, the cells were stimulated in zero external Ca^{2+} conditions according to a perfusion protocol involving two BK applications 120 s apart. The capacitative Ca^{2+} entry was initiated by replacing the Ca^{2+}-free Earle's medium with a normal Earle's solution (1.8 mM Ca^{2+}). Melatonin induced a significant increase in capacitative Ca^{2+} entry in cells stimulated only under the double stimulation. Significance ($P < 0.01$) was estimated in each case relative to the capacitative $\Delta[Ca^{2+}]_i$ values obtained without melatonin.

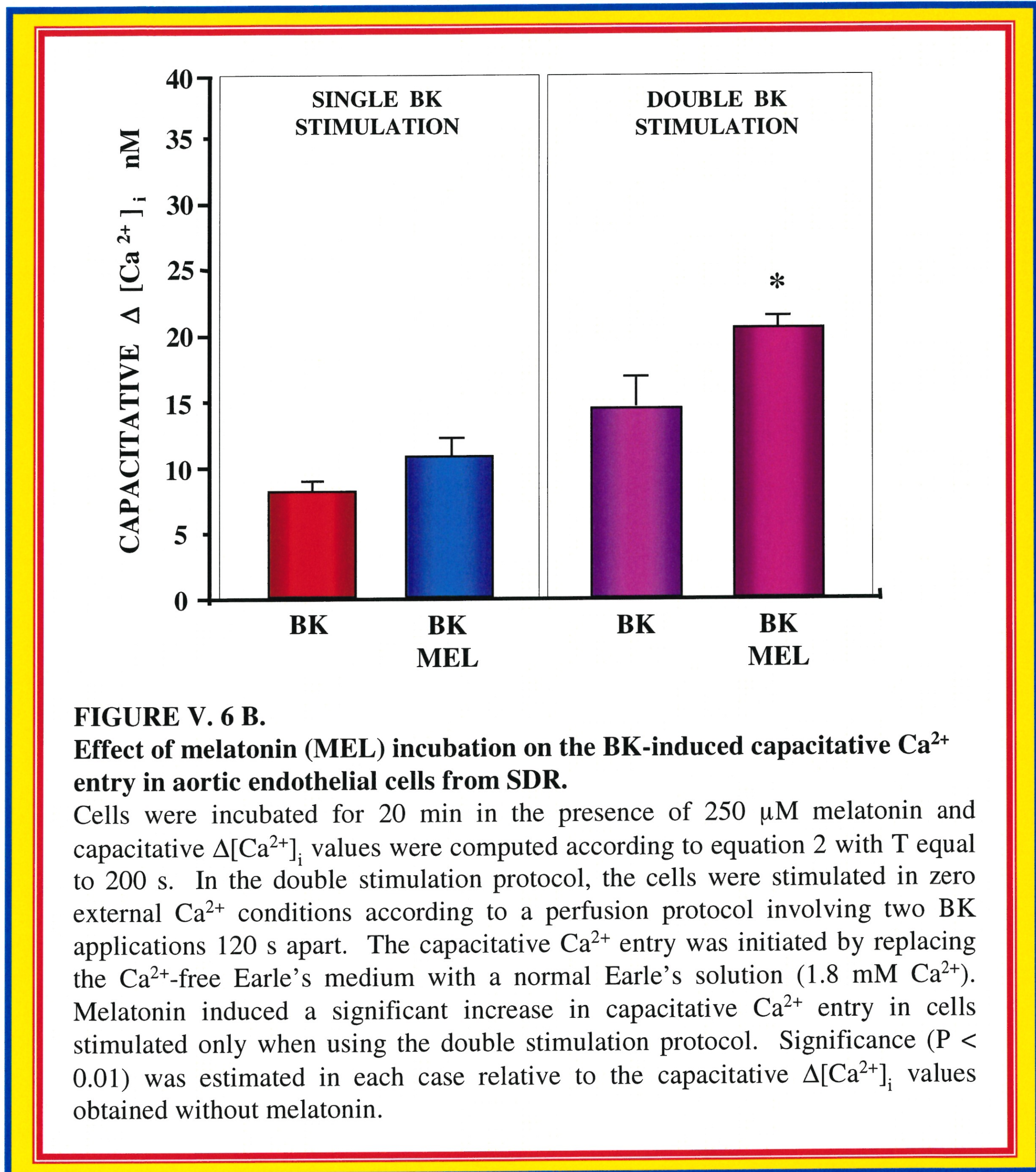

FIGURE V. 6 B.
Effect of melatonin (MEL) incubation on the BK-induced capacitative Ca^{2+} entry in aortic endothelial cells from SDR.
Cells were incubated for 20 min in the presence of 250 μM melatonin and capacitative $\Delta[Ca^{2+}]_i$ values were computed according to equation 2 with T equal to 200 s. In the double stimulation protocol, the cells were stimulated in zero external Ca^{2+} conditions according to a perfusion protocol involving two BK applications 120 s apart. The capacitative Ca^{2+} entry was initiated by replacing the Ca^{2+}-free Earle's medium with a normal Earle's solution (1.8 mM Ca^{2+}). Melatonin induced a significant increase in capacitative Ca^{2+} entry in cells stimulated only when using the double stimulation protocol. Significance ($P < 0.01$) was estimated in each case relative to the capacitative $\Delta[Ca^{2+}]_i$ values obtained without melatonin.

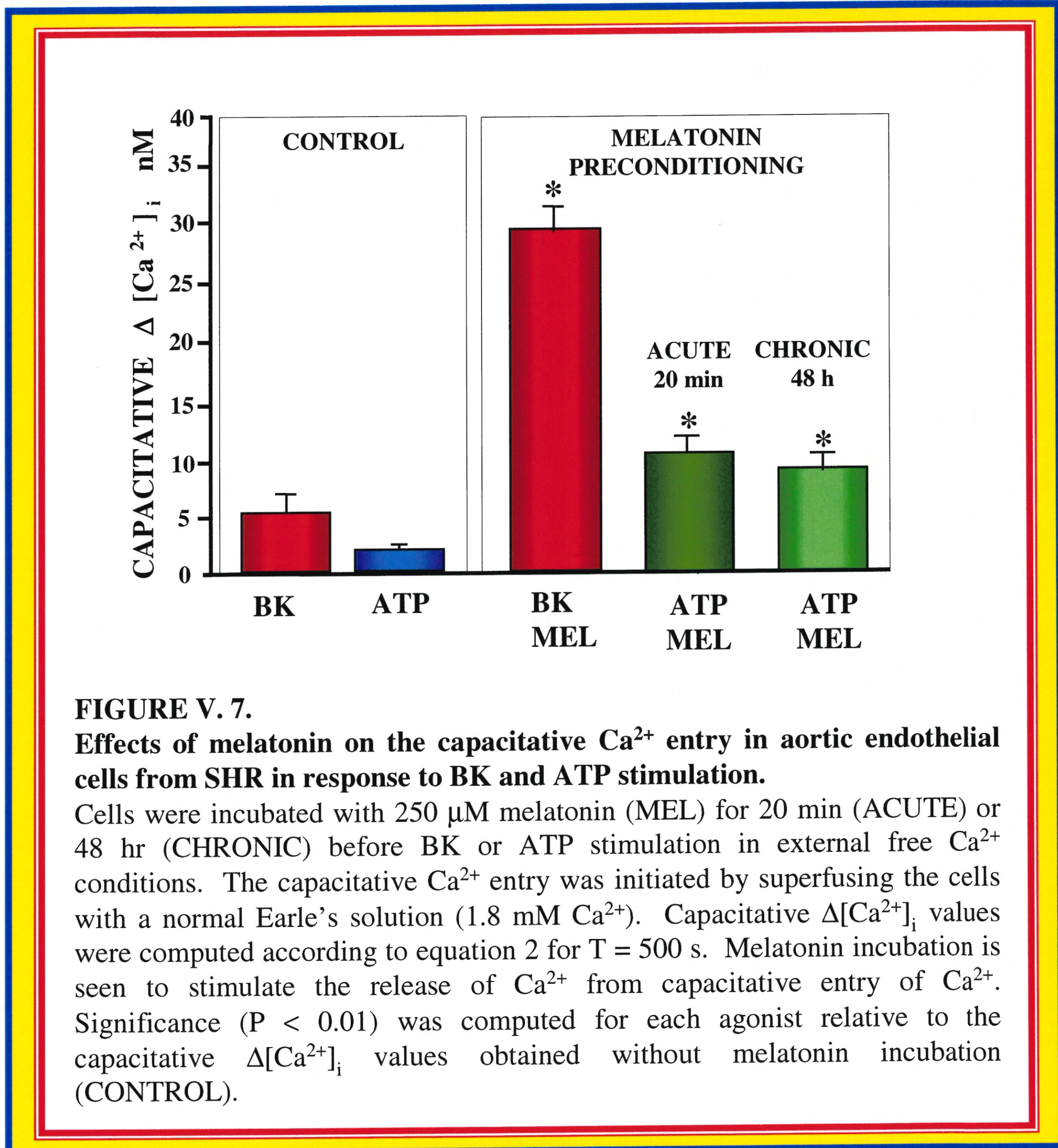

FIGURE V. 7.

Effects of melatonin on the capacitative Ca^{2+} entry in aortic endothelial cells from SHR in response to BK and ATP stimulation.

Cells were incubated with 250 μM melatonin (MEL) for 20 min (ACUTE) or 48 hr (CHRONIC) before BK or ATP stimulation in external free Ca^{2+} conditions. The capacitative Ca^{2+} entry was initiated by superfusing the cells with a normal Earle's solution (1.8 mM Ca^{2+}). Capacitative $\Delta[Ca^{2+}]_i$ values were computed according to equation 2 for T = 500 s. Melatonin incubation is seen to stimulate the release of Ca^{2+} from capacitative entry of Ca^{2+}. Significance ($P < 0.01$) was computed for each agonist relative to the capacitative $\Delta[Ca^{2+}]_i$ values obtained without melatonin incubation (CONTROL).

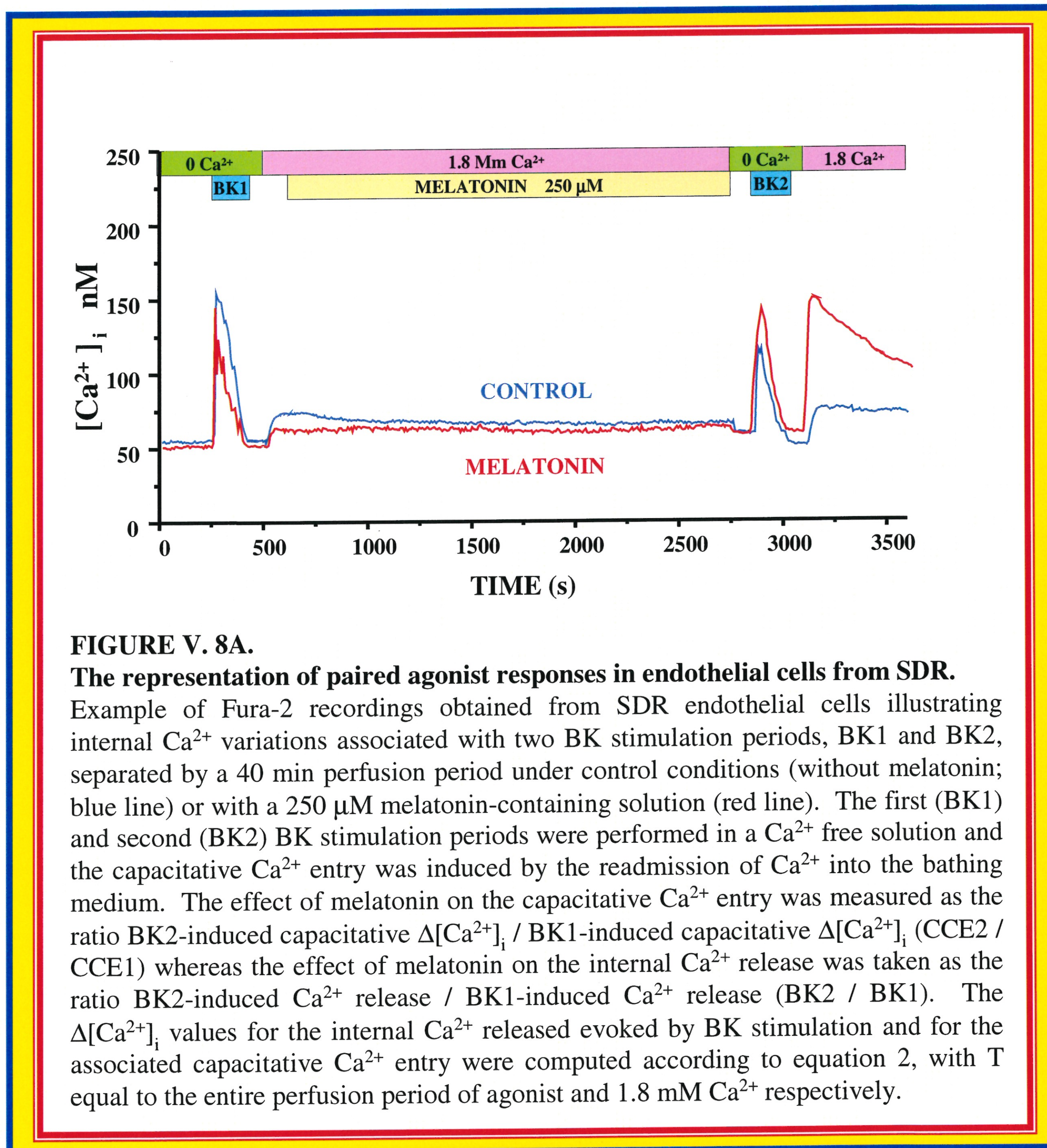

FIGURE V. 8A.

The representation of paired agonist responses in endothelial cells from SDR.

Example of Fura-2 recordings obtained from SDR endothelial cells illustrating internal Ca^{2+} variations associated with two BK stimulation periods, BK1 and BK2, separated by a 40 min perfusion period under control conditions (without melatonin; blue line) or with a 250 µM melatonin-containing solution (red line). The first (BK1) and second (BK2) BK stimulation periods were performed in a Ca^{2+} free solution and the capacitative Ca^{2+} entry was induced by the readmission of Ca^{2+} into the bathing medium. The effect of melatonin on the capacitative Ca^{2+} entry was measured as the ratio BK2-induced capacitative $\Delta[Ca^{2+}]_i$ / BK1-induced capacitative $\Delta[Ca^{2+}]_i$ (CCE2 / CCE1) whereas the effect of melatonin on the internal Ca^{2+} release was taken as the ratio BK2-induced Ca^{2+} release / BK1-induced Ca^{2+} release (BK2 / BK1). The $\Delta[Ca^{2+}]_i$ values for the internal Ca^{2+} released evoked by BK stimulation and for the associated capacitative Ca^{2+} entry were computed according to equation 2, with T equal to the entire perfusion period of agonist and 1.8 mM Ca^{2+} respectively.

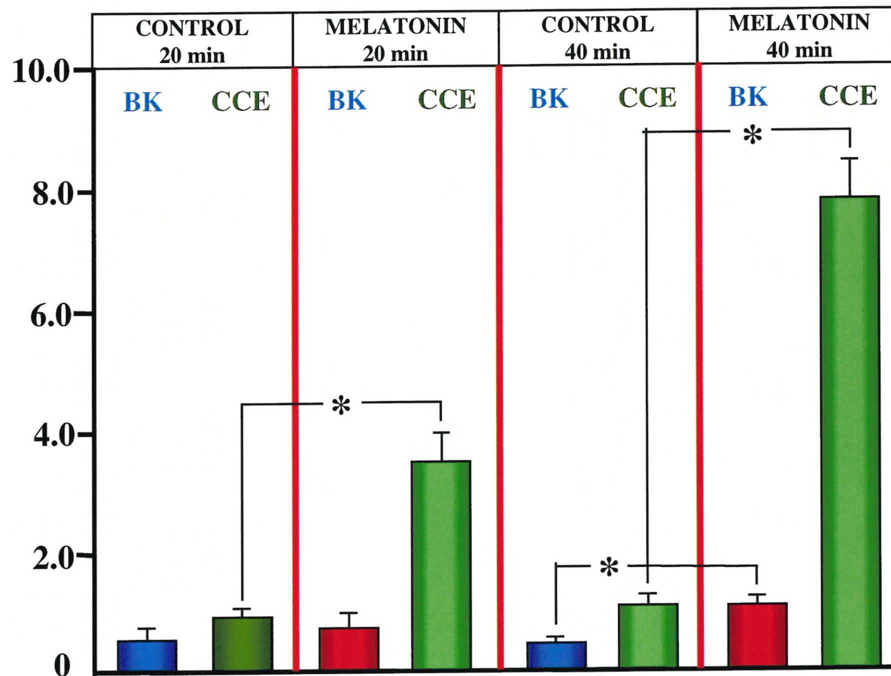

FIGURE V. 8B.

The representation of paired agonist responses in aortic endothelial cells from SDR.

Bar graphs representing the effect of melatonin measured according to the perfusion protocol illustrated in **A**. BK2 / BK1 and CCE2 / CCE1 refer respectively to the ratio of the BK2-induced Ca^{2+} release / BK1-induced Ca^{2+} release and to the ratio BK2-induced capacitative $\Delta[Ca^{2+}]_i$ / BK1-induced capacitative $\Delta[Ca^{2+}]_i$. For control experiments, the same perfusion protocol was used without melatonin.

Significance ($P < 0.01$) was established relative to the BK2 / BK1 and CCE2 / CCE1 of homologue control protocols for the 20 min or 40 min perfusion period respectively.

		20 minutes		40 minutes	
		BK	CCE	BK	CCE
SDR	CTR	0.5 ± 0.2	1.0 ± 0.2	0.4 ± 0.2	1.0 ± 0.2
	MEL	0.8 ± 0.1*	4 ± 1*	0.9 ± 0.1*	8.0 ± 2*
BAE	CTR	0.4 ± 0.2	1.0 ± 0.2	ND	ND
	MEL	0.5 ± 0.1*	1.4 ± 0.3*	ND	ND

Table V. 1.
Table representation of the modulatory effect of melatonin on the Ca^{2+} release and the capacitative Ca^{2+} entry measured in aortic endothelial cells from BAE and SDR cells.
Experimental conditions as described in figure 8. BK2 / BK1 and CCE2 / CCE1 refer respectively to the ratio of the BK2-induced Ca^{2+} release / BK1-induced Ca^{2+} release and to the ratio BK2-induced capacitative $\Delta[Ca^{2+}]_i$ / BK1-induced capacitative $\Delta[Ca^{2+}]_i$. For control experiments (CTR), the same perfusion protocol was used without melatonin. MEL refers to experiments where melatonin was applied (50 μM) either for 20 min or 40 min between the BK1 and BK2 stimulation periods. Significance ($P < 0.01$) was established relative to the BK2 / BK1 and CCE2 / CCE1 control values for the 20 min or 40 min perfusion protocol respectively. ND = not determined.

CHAPTER VI. DISCUSSION

In this study have been analyzed comparatively the relationships between Ca^{2+} homeostasis in ECs from BAE, normotensive and hypertensive rats in association with oxidative stress and antioxidant treatment. The results demonstrate that the artificially induced oxidative stress inflicts fundamental changes in Ca^{2+} homeostasis of ECs from normotensive rats and these changes mirror and parallel in part the pattern of Ca^{2+} homeostasis of ECs from SHR. The ECs from SHR possesse an abnormal Ca^{2+} signaling mechanism due to an overproduction of free radicals, which is sensitive to and restored by antioxidant treatment, although the expression and/or the activity of the fundamental antioxidant enzymes (SOD and catalase) in SHR are identical with the enzymatic expression and/or activity of normotensive rats. In the same time, the amount of $InsP_3$ production is identical under similar circumstances in both ECs lines and the oxidative stress substantially stimulates $InsP_3$ production. From the spectrum of the possible oxygen derived free radicals the most deleterious effect is induced by the accumulation H_2O_2 and, most likely by the organic peroxides.

The ECs in primary cultures express beside the $InsP_3$-sensitive pools, two other types of Ca^{2+} pools, TG-sensitive and TG-insensitive pools which are provided with a special TG-resistant SERCA isoform, but they do not express Ry-sensitive pools. In ECs are present antioxidant-sensitive and insensitive Ca^{2+} pools, which most likely occur as an adaptation to oxidative stress factors.

BAE and rat aorta ECs do not possess membrane specific receptors for melatonin coupled to the cascade of $InsP_3$ production and Ca^{2+} signaling. Nevertheless, melatonin, through its antioxidant properties, inhibits the deleterious effect of artificially induced oxidative stress in ECs from SD rats. The melatonin treatment of ECs from genetically hypertensive rats

improves their Ca^{2+} homeostasis to a level comparable to the Ca^{2+} homeostasis of normotensive rats. Moreover, the antioxidant properties of melatonin insure a protection only for the $InsP_3$-sensitive pools, but not for TG-sensitive pools and stimulate the CCE in primary rat ECs cultures while increasing the availability of Ca^{2+} in intracytosolic pools.

Some of the most important characteristics of ECs, as the Ca^{2+} homeostasis and the pharmacological design of Ca^{2+} pools to the most significant effects of oxidative stress, antioxidant, and drug treatment, demonstrated by the results of this study, are represented in Figure 14.

VI.1. THE CHARACTERISTICS OF Ca^{2+} HOMEOSTASIS IN ENDOTHELIAL CELLS

Existing knowledge about $[Ca^{2+}]_i$ and signaling has been obtained from studies using ECs cultured *in vitro*, for variable number of passages. The culture conditions for cloned endothelial cells not only alter some of their native cellular characteristics but also selectively maintain the fastest growing clone from a cell population, hiding the heterogeneity of Ca^{2+} behavior. It represents a shortcoming that strongly hampers our understanding and evaluation of endothelial Ca^{2+} behavior and homeostasis. Only in recent years, primary cultures and freshly dissociated ECs from different sources have been used. The special features and specific properties of ECs determine the functional heterogeneity in the same type of vessels in different organs. Therefore, vascular heterogeneity associated with specific Ca^{2+} responses, induced by special features and properties among the same type of vessels in different organs, have been recognized and scrutinized [249].

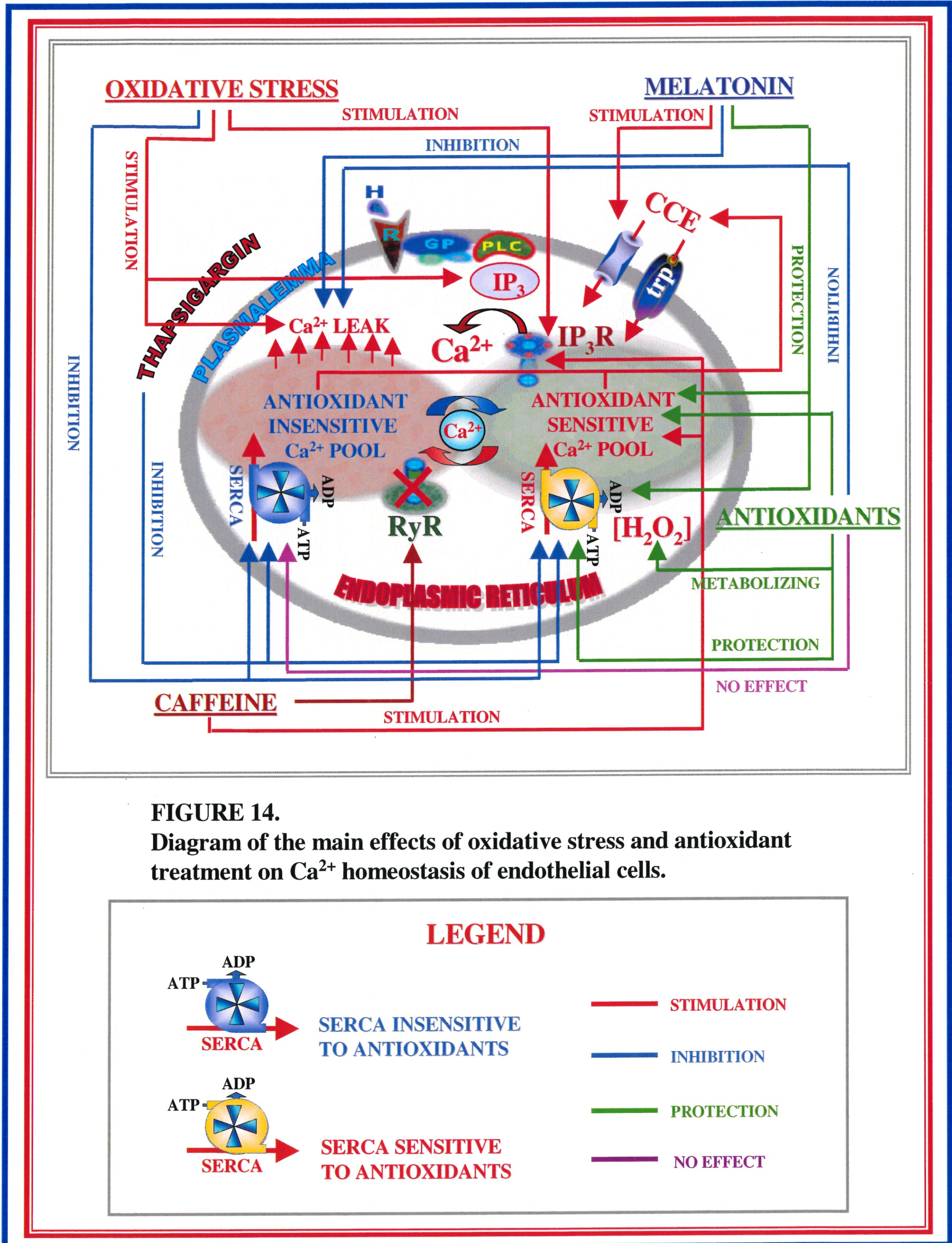

FIGURE 14.
Diagram of the main effects of oxidative stress and antioxidant treatment on Ca^{2+} homeostasis of endothelial cells.

Furthermore, significant variations may be generated by the set of technical, optical, and chemical (solutions, calibration technique) features of instruments and substances employed.

The ECs used in these study show a low level of $^b[Ca^{2+}]_i$, between 45 and 65 nM of Ca^{2+}. Similar values have been reported in other studies [122,250,251] from the same BAE or calf pulmonary artery cloned EC culture. Actually, the low $^b[Ca^{2+}]_i$ is a valuable indicator for a good culture condition during the growing period. The $^b[Ca^{2+}]_i$, in ECs from SHR was significantly higher than in ECs from normotensive SD rats and $^b[Ca^{2+}]_i$ is higher in ECs from adult SHR than in young SHR. Since the ECs from young SHR have the same $^b[Ca^{2+}]_i$ as ECs from SD rats, the elevated mean $^b[Ca^{2+}]_i$ in adult SHR indicates a time dependent alteration of Ca^{2+} leak from intracytosolic pools along with the Ca^{2+} homeostasis due to the effect of FR overproduction.

VI.1.1. Differential Ca^{2+} responses between ECs from SHR and normotensive rats

There is accumulating evidence, including the Fura-2 Ca^{2+} measurements illustrated in this study, that suggest an impaired Ca^{2+} response to Ca^{2+} mobilizing agonists in endothelial cells from hypertensive animal models compared with normotensive controls [47,248]. From the comparative evaluation of ATP and BK induced Ca^{2+} responses between ECs from BAE, and ECs from primary cultures, it is very evident that the Ca^{2+} responses of ECs from primary cultures are much smaller than ECs from BAE. The magnitudes of Ca^{2+} responses of ECs from BAE are within the limits reported in the literature, with Ca^{2+} peaks between 200-300 nM. The amplitude of Ca^{2+} responses in EC primary cultures from normotensive and hypertensive rats has been remarkably stable over the experimentation period and confirms precedent values reported by our laboratory in SHR and WKY rats [47,248].

For instance, a depressed Ca^{2+} response to acetylcholine was recorded in aortic endothelial cells from aldosterone-salt hypertensive SD rats compared to controls [48]. Similarly, the Ca^{2+} increase initiated by bradykinin was found to be of smaller magnitude in aortic ECs from SHR compared to WKY rats [47].

Several factors may be responsible for the decreased Ca^{2+} mobilization observed in endothelial cells from various models of hypertension. These include a decreased expression of functional membrane receptors, depressed $InsP_3$ production machinery or a depletion of releasable Ca^{2+} from $InsP_3$-sensitive and insensitive Ca^{2+} pools. Comparing the Ca^{2+} response of ECs from SHR and SD rats, we cannot totally rule out a contribution of the $InsP_3$-production cascade. The evaluation of ATP-induced $InsP_3$-production demonstrates that there is no significant difference between the amounts of $InsP_3$ produced following ATP stimulation in ECs from normotensive SD rats compared with SHR. An effect due to the membrane receptor expression may explain in some cases part of the variability in the relative magnitude of the Ca^{2+} response observed between endothelial cells from normotensive and hypertensive animal models. The fact that in control-conditions, the BK-induced Ca^{2+}-release in ECs from SHR is greater than ATP-induced Ca^{2+}-release was clearly demonstrated. Higher BK-induced Ca^{2+} responses in comparison with ATP-induced Ca^{2+} responses in ECs from SHR can be explained by a more representative expression of BK receptors in ECs from hypertensive rats. For instance, despite of a smaller internal Ca^{2+} release by bradykinin and ATP in SHR endothelial cells compared to WKY, the Ca^{2+} responses observed in these two cell preparations were not statistically different when endothelin and angiotensin-II were used as agonists [47]. This observation does not rule out however the possibility that the release of Ca^{2+} by endothelin or angiotensin-II may be enhanced in SHR compared to WKY cells following cell treatment with antioxidants.

Discussion

VI.1.2. Nature of Ca^{2+} pools in endothelial cells

Ca^{2+} pools are characterized by their different dynamic properties and availability of Ca^{2+} in rapidly and slowly exchanging pools. Differences among the various Ca^{2+} storage compartments are not limited to rates of exchange with other pools, but pertain also to their functions. Therefore, we can say that mitochondrial Ca^{2+} is a dynamic essential regulator of metabolism and S/ER Ca^{2+} is essentially a rapidly mobilizable reservoir. The *trans*-Golgi network Ca^{2+} content appears to be critical for the packaging of the regulated luminal content, a process leading to the formation of secretory granules and the $[Ca^{2+}]_i$-dependent assembling and disassembling of cytosolic organelle sub-domains [145].

One of our principal objectives was the investigation of Ca^{2+} pools of ECs in primary culture from normotensive and hypertensive rats. Several agonist-induced Ca^{2+} influx pathways have already been described in endothelial cells [252]. In non-excitable cells and in ECs, the initial response to the stimulation induced by $InsP_3$-generating agonists is a rapid increase in Ca^{2+} efflux from non-mitochondrial intracellular stores. This initial response is followed by more complex Ca^{2+} signals, like regenerative Ca^{2+} waves, Ca^{2+} spikes, and Ca^{2+} entry across the plasma membrane [161]. The mechanism underling these complex Ca^{2+} signals remains obscure and unknown, although $InsP_3$ is certainly implicated.

Overlapping of $InsP_3$ and ryanodine-sensitive pools exist in most if not all cells to a variable extent. This situation could be due not only to a partial co-localization of the $InsP_3$ and ryanodine receptor in individual area of ER, but also to the proximity or the distance of $InsP_3$ and ryanodine receptor on specialized membrane areas [253,254].

The most common experimental approach used to investigate the pool interaction in cells suspected to express both $InsP_3$ and RyR is as follows: the Ca^{2+} sensitive fluorophore loaded cells are stimulated sequentially in Ca^{2+} free medium, first with caffeine and then with an $InsP_3$-generating agonist. Afterwards, the order of agonist is reversed. If the two types of channels are expressed on separate pools, the rise in $[Ca^{2+}]_i$ caused by caffeine should not affect the rise of $[Ca^{2+}]_i$ induced by $InsP_3$ and vice versa [253].

Although the effects of caffeine on $[Ca^{2+}]_i$ in excitable cells are well established, the role of caffeine in Ca^{2+} regulation in EC has only recently been examined. In the present study, a perfusion with a solution having 10-20 mM caffeine, without Ca^{2+}, over primary cultures of ECs from SHR and SD rats, had no impact on Ca^{2+} mobilization from internal pools. The subsequent stimulation of ECs from SD rats with ATP induced an important Ca^{2+} release. Our laboratory has already reported the existence of caffeine sensitive pools ECs from BAE. Thuringer and Sauvé have demonstrated a caffeine-induced Ca^{2+} release from an $InsP_3$-insensitive pool in BAE cells. Application of caffeine opened Ca^{2+}-activated K^+ channels in cell-attached patch-clamp experiments in both Ca^{2+}-free and Ca^{2+}-containing external solutions [255]. Corda et al. describe the existence of caffeine sensitive pools in human aortic ECs but the amount of Ca^{2+} mobilized by caffeine in these experiments was dependent on external Ca^{2+} and on the depletion of intracytosolic Ca^{2+} pools by TG [256]. Despite these observations, in our experiments, even after 60 min incubation in TG, caffeine failed to release Ca^{2+} from intracellular pools. In another study of freshly dissociated single rabbit aortic ECs, caffeine induced a transient rise in $[Ca^{2+}]_i$ in Fura-2 loaded cells in the presence and absence of extracellular Ca^{2+}. This was associated with a dose-dependent increase in unitary current activity superimposed over a large and prolonged transient outward current upon application of caffeine [257]. However, Schilling and Elliott have found that that neither ryanodine nor

caffeine affects $^{b}[Ca^{2+}]_i$ in ECs from BAE. Besides, membrane preparations isolated from cultured ECs do not exhibit specific high- or low-affinity $[^3H]$-ryanodine binding [122]. In addition, in our experiments, after prolonged TG incubation (1h), caffeine was found to potentate the mobilization of Ca^{2+} by ATP in ECs from SHR, without initiating a detectable Ca^{2+} increase. Caffeine has been reported to behave as an inhibitor of the $InsP_3$ receptor and of phosphodiesterases. It is recognized that either the $InsP_3$-sensitive Ca^{2+} stores can respond to a rise in the level of $InsP_3$ or they can release their Ca^{2+} in the presence of a constant level of $InsP_3$ through CICR [149]. However, an interesting result using caffeine has been reported in ECs from human umbilical vein. The administration of caffeine on quiescent HUVEC-confluent monolayers pre-stimulated with histamine, generates spontaneous $[Ca^{2+}]_i$ oscillations [258].

To verify the presence of overlapping Ca^{2+} pools and to induce a CCE on ECs in our study, the first stimulation was induced with TG (to avoid the production of $InsP_3$) and the subsequent stimulation with ATP (to stimulates the production of $InsP_3$). TG blocks the SERCAs of all intracytosolic pools, thus emptying all Ca^{2+} pools. If the Ca^{2+} pool is unique, after TG treatment, all the amount of Ca^{2+} would leak out from intracytosolic pool and the subsequent $InsP_3$-generating stimulation would be without effect. In fact, in our protocols, each agonist has released a specific amount of Ca^{2+}, with ATP stimulation being statistically smaller in comparison with the single stimulation performed with ATP, but proving the existence of two separate Ca^{2+} pools. Furthermore, the combined stimulation of TG + ATP led to a significant increase of the agonist-induced Ca^{2+} entry in both SD and SHR endothelial cells. Through this double stimulation, much of the Ca^{2+} stores were emptied and the signal for opening the CCE and refilling the intracellular pools was generated. A combined stimulation of Ca^{2+} pools is essential for the signaling process leading to the activation of the Ca^{2+} influx in these cells. Our experimental data from primary cultures of ECs in both SHR and SD rats demonstrate that no

important overlap can be found between the ATP- and TG- sensitive Ca^{2+} pools. An exposure of SD and SHR cells to TG for periods up to 60 min was unable to prevent a subsequent ATP-induced Ca^{2+} release, suggesting the presence on ER of a TG-resistant SERCA isoform. However, the same experimental data confirm a time dependent communication and exchange of Ca^{2+} between the two pools.

Waldron et al. in a selected line of Chinese hamster lung fibroblasts, describe a TG-resistant fibroblasts cell line (DC-3F/TG2) capable of growing in 10 μM TG, due to a highly TG-resistant intracellular Ca^{2+} pumping activity and capable of accumulating Ca^{2+} within an $InsP_3$-releasable Ca^{2+} pool. The resistance is attributed to the expression of a novel intracellular Ca^{2+} pump activity with a significant lower sensitivity to TG. In spite of this difference in TG-sensitivity, the resistant pump has the same high affinity for Ca^{2+} (K_m 100 nM), ATP dependence, and sensitivity to vanadate as a normal SERCA pump [139].

However, a prolonged incubation in TG reduced the amount of Ca^{2+} released by ATP in a time dependent manner. After 6 h of incubation in TG, ATP was not capable of releasing detectable Ca^{2+}, demonstrating that Ca^{2+} pools had been completely emptied. This was the condition found to induce the stimulation of a huge CCE, similar to the one seen in ECs from BAE. In most cells, $InsP_3$- or TG-sensitive Ca^{2+} stores represent only a fraction of the total cellular Ca^{2+} stores. Different Ca^{2+} pools interact actively through an important Ca^{2+} exchange, as demonstrated in these results. Although the underlying mechanism of Ca^{2+} exchange between pools is highly speculative because of the limited evidences, it seems that when Ca^{2+} is released from stores, the re-uptake starts instantly. Even than TG is used as a Ca^{2+}-releasing agent, the existence of a TG-resistant SERCA will favour an important amount of Ca^{2+} re-uptake. In addition, Ca^{2+} may pass from one store to another without traversing the cytosol.

Substantial evidences suggest that the communication and Ca^{2+} exchange between discrete Ca^{2+} pools is induced and regulated by a small GTP-binding protein [259,260].

Therefore, it appears that Fura-2 detectable Ca^{2+} signaling in ECs from SHR and SD rats, even if they are provided with both $InsP_3R$ and RyR on their ER membranes, involves only $InsP_3$-sensitive intracytosolic Ca^{2+} reservoirs. However, from the point of view of a Ca^{2+} sequestration mechanism in ER, our results demonstrate the presence of an important pool containing Ca^{2+} releasable only by TG, which is most likely likewise $InsP_3$-insensitive.

An interesting observation from our Fura-2 Ca^{2+} measurements is the almost complete absence of a capacitative Ca^{2+} increase following the readmission of Ca^{2+} into the external medium. These results were confirmed in experiments in which Ca^{2+} influx was measured using the Mn^{2+}-quenching method and by performing an $InsP_3$-independent induced Ca^{2+} release with the SERCA pump inhibitor TG. Such Ca^{2+} measurements do not support an important Ca^{2+} entry in response to ATP or BK stimulation [47] in SD, WKY, and SHR endothelial cells. Otherwise, TG was not able to release a higher amount of Ca^{2+} from internal pools and experimental recordings demonstrate a serious resistance to TG-induced Ca^{2+} release in ECs from SHR and SD rats. However, when Ca^{2+} was present in the external bathing solution, TG released 40% more Ca^{2+} in comparison with free-Ca^{2+} solution protocol, demonstrating that the emptying of pools is rapidly signaled to the plasmalemma to open the CCE. The 40% of more Ca^{2+} released can be attributed only to the Ca^{2+} entry from extracellular space. In both permeabilized and intact cells, $InsP_3$ or receptors stimulating the formation of $InsP_3$, usually mobilize only a fraction of the intracellular Ca^{2+} stores [261]. The low capacitative Ca^{2+} entry may be explained by a small Ca^{2+} pool in these ECs or by a direct association of $InsP_3$-sensitive stores with the plasma membrane. Evidence are suggesting that $InsP_3$-sensitive Ca^{2+} stores can

refill with Ca^{2+} directly from the extracellular space with no apparent accompanying changes in cytosolic free Ca^{2+} [165,262], possibly through a direct association of $InsP_3R$ with the cytoskeleton [263]. Perhaps, the most likely conclusion would be that the major $InsP_3$-sensitive Ca^{2+} stores in ECs are likely to be a specialized component of ER. Due to enormous diversity of cellular responses controlled by Ca^{2+}, it should perhaps come as no surprise if the specialization of ECs demonstrates different properties. It has been observed that the same agonist or different agonists evoke heterogeneous receptor-mediated $[Ca^{2+}]_i$ responses and that ECs *in situ* are intrinsically different in terms of their responses toward agonists [249].

Whatever the association between $InsP_3$ sensitive Ca^{2+} stores and the plasma membrane, it is very likely that the stores lying closest to the plasma membrane are the ones most likely to respond to $InsP_3$. $InsP_3$ is synthesized and, to a considerable extent, degraded at the plasma membrane level [264]. Pool repletion appears in this case to occur directly at the membrane interface, without a global increase in intracellular Ca^{2+}. A similar mechanism has been reported in several cell preparations including the human fibroblasts HSWP cells [265]. It becomes also increasingly apparent that Ca^{2+} pool depletion does not lead *per se* to a store operated Ca^{2+} influx. For instance, depletion of an acetylcholine-sensitive Ca^{2+} pool could be achieved in isolated rabbit aortic endothelial cells, using either the Ca^{2+} pump inhibitor cyclopiazonic acid (CPA), caffeine or ryanodine, but CPA only succeeded in initiating a Ca^{2+} influx [266].

VI.1.3. The effect of melatonin on Ca^{2+} homeostasis of endothelial cells

The effects of melatonin have been investigated on neuronal cells from suprachiasmatic nucleus, retina, gonads, pituitary cells, amphibian melanophores, caudal artery, anterior cerebral artery and transfected cells [218]. The picture of melatonin effects on $[Ca^{2+}]_i$ evolution of

different cells is complex and variable from cell to cell type. In pinealocytes, the production and the release of melatonin are dependent on $[Ca^{2+}]_i$ increase in all studied species. The $[Ca^{2+}]_i$ increase is managed by $InsP_3$-sensitive internal stores and by voltage-dependent L-type Ca^{2+} channels. Melatonin has an inhibitory effect to the mobilization of Ca^{2+} from intracellular stores as well as the influx through the voltage-sensitive Ca^{2+} channels mediated by a membrane hyperpolarization in gonadotroph cells [267]. In caudal artery and in anterior cerebral artery, melatonin produces vasoconstriction through a potentiation of contractile responses to adrenergic nerve stimulation [98] and through modulation of K^+-channels [236], respectively.

The plasmalemmal receptors mediate the effects of melatonin by coupling to pertussis toxin-sensitive G-proteins. However, evidence suggests that melatonin can access cytosolic and nuclear sites, to mediate a variety of cellular effects, in tissues devoid of membrane-bound receptors. Melatonin, through binding to calmodulin, may directly affect Ca^{2+} signaling by interaction with essential target enzymes, structural proteins and indirectly, via specific protein kinases [268,269].

Our Fura-2 experiments demonstrate that the stimulation of ECs with melatonin, superfused in external free-Ca^{2+} solutions or in physiological $[Ca^{2+}]_o$ did not have any impact on the release of Ca^{2+} from internal pools. This is a clear evidence that aorta endothelial cells do not have plasmalemmal receptors related with the cascade of $InsP_3$ production and an increase of $[Ca^{2+}]_i$. Despite the fact that melatonin does not induce a Ca^{2+} release from internal pools, the binding of melatonin was evident in both ECs from SHR and SD rats but not in BAE. There is a single report about an increase of phosphoinositide breakdown and accumulation of $InsP_3$ following the administration of melatonin in the presence of lithium, in chick brain slices [233]. This phenomenon happens in the presence of Ca^{2+} in external bathing solutions and at melatonin

doses of 10^{-5} M. In our experiments, we have administered melatonin in the presence of $[Ca^{2+}]_o$ at 1.8 mM and at doses of melatonin up to 250 μM, but we did not observed any modification in $[Ca^{2+}]_i$. As it can be seen, in the short as well as in the paired agonist responses, subsequent stimulation with other agonists (BK and ATP) activating the $InsP_3$ cascade production, we have observed significant cellular $[Ca^{2+}]_i$ increases in the same cells where melatonin was superfused. Therefore, the binding assay we performed is believed to account for all of them, since a very high concentration of cold melatonin (100 μM) was used for displacing the labeled substrate (12 nM). The possibility that MT2 receptors are present cannot be entirely ruled out in ECs from SHR and SD rats, since binding of melatonin to specific sites was confirmed in these cells; but the presence of binding sites on BAE cells could not be demonstrate by these experimental conditions. We did not try to determine in these experiments the total number of binding sites for each cell line since the evaluation of B_{max} increases in difficulty due to the presence of multiple binding sites. To the best of our knowledge, this is the first report about the effect of melatonin on Ca^{2+} mobilization as compared to melatonin binding studies in cloned and primary cultures of endothelial cells.

The modulation of Ca^{2+} release is dependent upon several effector systems such as adenylyl cyclase, phospholipase C, K^+ channels, Ca^{2+} channels, etc. In pituitary cells, melatonin affects several intracellular messengers through specific receptors by inhibiting the accumulation of cAMP and cGMP, inhibiting the increase of intracellular calcium, the synthesis of diacylglycerol and the release of arachidonic acid. In pituitary gland, the hypothalamic releasing hormones are activating pinealocytes' endocrine functions by an increase in $[Ca^{2+}]_i$. However, this process is substantially inhibited by melatonin, the pineal gland creating an important physiological negative feedback loop [267,270]. The short protocols in our results

suggest that melatonin did not interfere with the agonist-induced increase in $[Ca^{2+}]_i$ in BAE and SD cells. But in endothelial cells from genetically hypertensive rats (SHR), melatonin has an important effect on the increase of $[Ca^{2+}]_i$. This effect is related almost certainly with a genetically established oxidative stress in ECs from SHR and with the free radical scavenger potential of melatonin.

Surprisingly, the results of paired agonist responses seem to be in contradiction with the previous statement. If ECs from BAE are pre-stimulated with BK and treated with melatonin for 20 to 40 min, the second BK stimulations have a significant but limited increase (≈30%) of the second BK induced Ca^{2+} release. By contrary, the results obtained from ECs from SD rats demonstrate a second BK induced Ca^{2+} release increase of >160% after 20 min and more than 200% after 40 min melatonin treatment. Therefore, an agonist pre-stimulation would be favorable to the melatonin potentiating effect for the subsequent agonist stimulations. These paired agonist responses might be very similar with the physiological condition of an organism, where the release of melatonin and the omnipresence of hormones and autacoids are simultaneously affecting endothelial cells. The reason for which this second BK induced Ca^{2+} release increases in primary ECs from SD and not in ECs from BAE, is very difficult to explain but, most likely, it is related with the origin of ECs (clone versus primary culture). Moreover, the results of Zemkova and Vanecek demonstrate that melatonin can have inhibitory effects on GnRH-induced mobilization of Ca^{2+} from intracellular stores as well as on Ca^{2+} influx only in neonatal but not in adult rat gonadotrophs [270,271].

VI.2. THE EFFECTS OF OXIDATIVE STRESS ON Ca^{2+} HOMEOSTASIS OF ENDOTHELIAL CELLS

In this study, we have analyzed the effects of oxidative stress induced with the HX/XO free radical generating system, using ECs from normotensive SD rats and from the BAE clone. The results from ECs of SD rats demonstrate clearly the time and dose dependent inhibition of $InsP_3$-dependent Ca^{2+} release from intracytosolic Ca^{2+} pools following the artificially induced oxidative stress with HX/XO. In both ECs lines, the applications of HX/XO-induced oxidative stress completely annihilated the ATP or the BK-induced Ca^{2+} release from $InsP_3$-sensitive pools. Calcium is required for numerous physiological functions, including control of metabolic processes, cell differentiation, proliferation, and paracrine function. Hormones and growth factors tightly control these Ca^{2+}-dependent processes. Loss of the ability to respond to such hormones and growth factors will not only deprive the cell of trophic stimulus but may also result in the activation of suicide process characteristic of apoptotic cell death [272-274].

VI.2.1. The effect of oxidative stress on Ca^{2+} signaling pathways of endothelial cells

The entire pathway of Ca^{2+} signaling is exposed to oxidant injuries induced by free radicals. G-protein, the transducer for $InsP_3$-dependent agonist effect, has been reported to be susceptible to inactivation by oxidants. Lethal doses of free radicals and a prolonged increase in $[Ca^{2+}]_i$ may obliterate the Ca^{2+} transients normally evoked by physiological agonists, thereby resulting in impairment of cell signaling [211]. On the other hand, it was demonstrated that low doses of oxidants could stimulate cell proliferation [275]. Modulation of kinase activity, involved in signal transduction and cellular metabolism, could constitute a mechanism by which a nontoxic oxidative stress can have implications on cell division and differentiation. For

instance, the exposure of rat hepatocytes to low level of oxidants resulted in a rapid 2- to 3-fold increase in the specific activity of protein kinase C [133].

Current data suggest that FRs can affect a large variety of regulatory mechanisms responsible for Ca^{2+} signaling in several cell types, including endothelial cells [23,250,276]. Wesson and Elliott [251] have shown for instance that treatment of calf pulmonary endothelial cells with HX/XO (30 min to 180 min) causes initially an inhibition of the agonist-stimulated Ca^{2+} influx, with a gradual decrease of Ca^{2+} release from internal stores coupled to a global increase of the basal intracellular Ca^{2+} level. This latter effect was attributed to a dysfunction of the plasma membrane Ca^{2+} pump in the presence of FRs. These results are in agreement with the observations reported herein on the effect of free radicals on the ATP-induced Ca^{2+} release in primary cultures of aortic ECs from BAE and SD rats. An inhibition of the bradykinin-stimulated Ca^{2+} signaling was also observed in vascular ECs treated with *t*-butyl hydroperoxide (*t*-BuOOH) [250]. However, because *t*-BuOOH failed to affect the release of Ca^{2+} triggered by BHQ, an inhibitor of microsomal Ca^{2+}-ATPase pumps, it was suggested that the action of FRs in that case consisted in the inhibition of one or more of the steps responsible for activation of Ca^{2+} release (e.g. $InsP_3$ production), rather than a depletion of Ca^{2+} from the intracytosolic stores. These observations differ from the results of our experiments and from the Ca^{2+} measurements performed on CPEC [277] where the release of Ca^{2+} from either TG- or BHQ-sensitive Ca^{2+} pools were found to be attenuated following cell exposure to HX/XO. However, the incubation time for oxidative stress-induced inhibition in CPEC was more prolonged when BHQ was used as a Ca^{2+} mobilization agent compared to $InsP_3$-induced agonist mobilization.

Evidence has already been provided indicating that the oxidation of the $InsP_3$ receptor modifies the sensitivity of the receptor to $InsP_3$, resulting in Ca^{2+} release at lower $InsP_3$ levels [210,278,279].

In addition, the $InsP_3$ measurements carried out in our studies clearly indicate that the exposure of ECs from SD to HX/XO leads to a 15-fold increase of $InsP_3$ production in resting cells and in cells stimulated with HX/XO + ATP. The combination of ATP and HX/XO induced oxidative stress was not able to induce a higher $InsP_3$ production than the HX/XO-induced oxidative stress alone. This suggests that in the first 90 min of oxidative stress, almost all the inositidae pool has been converted to the IP cascade byproducts, probably due to an uncontrolled activation of PLC. These observations are in agreement with the report that ROS (hydrogen peroxide, 0.1 mmol/l) cause a significant rise in inositol phosphate and diacylglycerol levels in endothelial cells from human umbilical vein, while inhibiting the Ca^{2+} release from internal stores [277].

Other findings suggest that high oxidative stress alters the endothelial cell morphology through alterations on the cytoskeleton, loss of plasma membrane integrity, and permeability, as well as the onset of lethal cell injury. In our experimental procedure, based on the HX/XO free radical generating system, it was found that the trypan blue exclusion test was negative at 99% for the first 3 hours of incubation, excluding alterations of the plasma membrane permeability and cell viability. Oxygen derived free radicals are present during the early phase of injury and little or no $^{\bullet}O_2^-$ production occurs later when $[Ca^{2+}]_i$ begins to increase, suggesting that the ROS are produced only at the beginning of the oxidative treatment. The HX, as substrate for XO is integrally consumed in the first 10-15 min of the HX/XO reaction. After that, the organic FR formation and lipid peroxidation are initiated and they are responsible for the destruction of

plasma membrane integrity and permeability, as corroborated by the observed increase of external Ca^{2+} influx and/or leak from intracytosolic pools [280]. Actually, the amount of substrate given to XO in our experiments might be consumed in less than 15 min, whereas the complete inhibition of Ca^{2+} signaling is induced after more than 60 min of incubation in HX/XO FR-generating system, letting us to suspect that the FR injury is realized practically by organic FRs.

In the early phase (30-60 min) of oxidative stress, a decrease of the Ca^{2+} response to ATP is very evident alongside with a more rapid return of $[Ca^{2+}]_i$ to basal levels following stimulation. This process can be explained in our experimental conditions by a reduction of Ca^{2+}-release from internal stores. The oxidative injury implies direct modifications of channel proteins, alteration of the coupling mechanism between intracytosolic Ca^{2+} stores and Ca^{2+}-influx, and an inhibition of the Ca^{2+} influx pathway due to membrane depolarization. In support of this statement, it was demonstrated that during the first 60 min of incubation with H_2O_2, the resting membrane potential of calf vascular ECs, changes from -60.7 mV to -27.6 mV [217].

After 90 min incubation with HX/XO the oxidant stress completely inhibited the agonist-activated release of Ca^{2+} from internal stores, either by inhibition of the signaling mechanism responsible for Ca^{2+} release or by a complete depletion of internal stores. In addition, an increase of $^{b}[Ca^{2+}]_i$ of more than 100% is observed, suggesting that the mechanism responsible for the removal of Ca^{2+} from the cytosol is at least altered but most likely inhibited. In CPA cells, Schilling et al. have already demonstrated that agonists induced a 12-fold increase in $^{45}Ca^{2+}$ unidirectional efflux in normal conditions, and this efflux was reduced by 50% during the intermediate phase of oxidative stress. The inhibition of agonist-stimulated $^{45}Ca^{2+}$ efflux may reflect an inhibition of membrane Ca^{2+}-ATPase and/or the endoplasmic reticulum SERCA

[155]. Inhibition of SERCA pumps will lead to depletion of internal stores, a mechanism that may be implicated in HX/XO-induced inhibition of receptor-activated Ca^{2+} release. The mechanism by which oxidative stress inhibits the Ca^{2+}-ATPases appears to be via oxidation of critical sulfhydryl groups [213,214]. However, the production of $InsP_3$ has increased 15-fold in these conditions, demonstrating indirectly that the PLC activity is not inhibited. After 2 hours of oxidative stress, $^b[Ca^{2+}]_i$ is increased almost 3-fold and ATP failed to induce an additional increase in $[Ca^{2+}]_i$. It is important to note that during these experiment, EC membranes resist to a large inward Ca^{2+} gradient and express an important but controlled CCE increase that can be stimulated by oxidative stress and/or by the oxidative stress-induced emptying of intracytosolic Ca^{2+} pools.

Thus, our results support a cellular model for the oxidant stress-induced alteration of Ca^{2+} homeostasis in ECs, whereby free radicals can deplete $InsP_3$-sensitive Ca^{2+} pools, by both sensitizing the $InsP_3$ receptor to substantially increased availability of $InsP_3$, while PLC activation increased the production of intracellular $InsP_3$ level. Moreover, these effects are coupled to a potential SERCA and PMCA-ATPase pump inhibition by OFR. Because catalase provided a near complete protection to the HX/XO-induced oxidative stress injury, it is suggested additionally that H_2O_2 is the main byproduct of the O_2 reduction cycle involved in this case. Our findings are corroborated by those of Wesson et al. [277] and suggest that the inhibition of signal transduction represents an early and critical mechanism of ECs dysfunction, an event responsible for many pathophysiological conditions of cardiovascular disease, in which H_2O_2 is the principal and most powerful aggressor.

However, in the wide variety of free radical reactions, some contradictory effects are reported. For example, it has been observed that the chronic addition of H_2O_2 to isolated

perfused vessel rings causes endothelium-dependent and -independent vascular relaxation [281]. These data are suggesting a non-specific vasodilator effect of H_2O_2, but most likely, it might be correlated to an increase of $NO^{\bullet}$ synthesis, as described by Zembowicz et al. [282] and/or to the release of vasodilator prostacyclin PGI_2, as demonstrated by Ager et al. in porcine aortic ECs [283]. All these contradictory effects might be related to the activation of a receptor operated Ca^{2+} influx pathway, described in canine systemic venous endothelial cells [284]. Therefore, the H_2O_2 produced by HX/XO-induced oxidative stress in this case does not act alone; it is associated with important deleterious effects induced by organic free radicals, mostly organic peroxides.

VI.2.2. The effect of endogenous free radical production on Ca^{2+} signaling pathways of endothelial cells from SHR

Overproduction of free radicals in ECs from SHR has already been reported in several studies [285-287], supporting the concept of a greater effect of antioxidants on Ca^{2+} signaling in SHR compared to WKY endothelial cells. In our experiments, the ECs from both, young and adult SHR were sensitive to the antioxidant treatment, demonstrating that Ca^{2+} signaling is altered due to the production of FRs. Our results demonstrate a 60% smaller agonist–induced Ca^{2+} release in ECs from SHR in comparison with ECs from SD rats, whereas the $^{b}[Ca^{2+}]_i$ is significantly higher in SHR. For instance, direct measurements of $^{\bullet}O_2^-$ concentration by a chemiluminescence method have demonstrated the presence of 68 nM of $^{\bullet}O_2^-$ in endothelial cells from SHR-stroke prone compared to 27 nM in WKY [286]. Similarly, the basal and internal Ca^{2+}-stimulated production of $^{\bullet}O_2^-$ and H_2O_2 was reported to be higher in SHR aortas than in WKY [288]. The overproduction of $^{\bullet}O_2^-$ was found to precede the increase in blood pressure in this genetic model of hypertension [288]. Several sources of $^{\bullet}O_2^-$ in the endothelium

have been reported, including cyclooxygenase and NADH oxidoreductase but the precise source of FRs in different pathophysiological circumstances is still a subject to debate [289]. However, recent evidence include new potential sources of FRs and points toward an increased expression of the endothelium constitutive nitric oxide synthase (cNOS) as one of the major sources for the excess $^{\bullet}O_2^-$ production measured in SHR and SHR-stroke prone endothelial cells [39,288]. It appears therefore that the impaired functional $NO^{\bullet}$ release reported in endothelial cells from several models of hypertension would be more compatible with an increased annihilation of $NO^{\bullet}$ by $^{\bullet}O_2^-$ rather than a deficient $NO^{\bullet}$ production [39]. Furthermore, since the production of $^{\bullet}O_2^-$ is Ca^{2+}-dependent like the production of $NO^{\bullet}$ by cNOS [288], an $^{\bullet}O_2^-$-mediated depression of the Ca^{2+} response in SHR cells should constitute a protective mechanism against an enhanced $^{\bullet}O_2^-$ production. However, the $InsP_3$ measurements indicate that despite an important FR overproduction, the $InsP_3$ production is not statistically different in comparison with either the control or the ATP-stimulated ECs from SD rats and the antioxidant treatment does not interfere with the $InsP_3$ production in SHR.

VI.2.3. The effect of oxidative stress on Ca^{2+} pools of endothelial cells

Exposure of ECs from SD rats for 90 min to the HX/XO-free radical generating system inhibited both TG- and ATP-induced Ca^{2+} release from internal pools. These observations suggest an effect of free radicals on the sequestration of Ca^{2+} in both $InsP_3$- and TG-sensitive Ca^{2+} pools in endothelial cells from normotensive rats. The inhibition was associated with a slight increase of the TG plus ATP initiated Ca^{2+} entry. The stimulation of ECs from SD with ATP alone, following HX/XO induced oxidant stress, showed a greater mean $\Delta[Ca^{2+}]_i$ for CCE. Because SD cells showed a reduced agonist-evoked Ca^{2+} entry, the main effect of FRs in this case would be to initiate a Ca^{2+} leak, as demonstrated by FR effects on calf pulmonary

endothelial cells [210]. However, such an increased Ca^{2+} leak does not seem to prevail in SHR cells, despite an overproduction of free radicals. The application of antioxidant enzymes to ECs from SD rats exposed to a sequential stimulation with TG and ATP has a spectacular effect in recovering the response to ATP stimulation, although the TG-sensitive pools remain insensitive to antioxidant treatment. The amount of Ca^{2+} released by ATP after a treatment with catalase or SOD is roughly double that of the control, probably because TG was not able to release much Ca^{2+} and more Ca^{2+} was available for the stimulation induced by ATP. This brings a supplementary argument that Ca^{2+} pools are not isolated and Ca^{2+} can be transferred from one pool to another. On other hand, it is also conceivable that the SERCA pump present on $InsP_3$-sensitive pools is always sensitive to antioxidant enzymes and capable to recapture instantaneously an important amount of Ca^{2+} released from the TG-sensitive pool to increase substantially the available Ca^{2+} in the $InsP_3$-sensitive pools. On the other hand, the oxidative stress may substantially stimulate the $InsP_3R$ activity, reflected into an increase of Ca^{2+} response to ATP and not to TG. Generally, these results support a model in which the action of FRs on Ca^{2+} signaling in ECs involves the exhaustion of internal Ca^{2+} stores.

VI.2.4. The effect of oxidative stress on capacitative Ca^{2+} entry of endothelial cells

In parallel to the hormonal stimulation, Ca^{2+} pool depletion is possible following a treatment with SERCA inhibitors or Ca^{2+} ionophores, which leads to activation of a Ca^{2+} entry. It appears that the loss of Ca^{2+} from internal stores is the fundamental cause of the original signal for the opening of CCE. Analysis of CCE in primary cultures of ECs from SHR and SD rats, following agonist-induced Ca^{2+} release, revealed an unexpected small Ca^{2+} influx (mean $\Delta[Ca^{2+}]_i \approx 2.5$ nM), which was equivalent in both cellular strains.

The application of oxidant stress injury with HX/XO for different time periods induced an increase of $^{b}[Ca^{2+}]_i$ and a Ca^{2+} influx under basal (unstimulated) conditions, suggesting that the oxidative stress injury induced an increase of membrane permeability and an inhibition of Ca^{2+}-ATPase pumps. The application of oxidant injury for 90 min in ECs from SD rats induced a >3-fold increase in CCE. The CCE increased in parallel with the application period of the oxidative stress injury, culminating with an ≈ 8-fold increase after 120 min. This increase of CCE induced by oxidative stress is in contradiction with the oxidative stress induced by t-BuOOH in ECs from BAE reported by Elliott and Doan, where t-BuOOH inhibits both the BK-stimulated Ca^{2+} signaling and the CCE [250]. Our experiments with HX/XO-induced oxidant stress did not alter the viability of ECs, as confirmed by the trypan blue exclusion test. Moreover, measurements were carried out only on Fura-2 loaded ECs able to maintain a concentration gradient for both Fura-2 and Ca^{2+} across the cell membrane. However, it is impossible to eliminate completely some Ca^{2+} entry due to the effect of free radicals. It cannot be ruled out that this might be part of the mechanism responsible for oxidant injury in ECs. In ECs from SHR, a TG-stimulation was able to induce a CCE twice that of ATP stimulation. The CCE has been increased 3-fold following a sequential stimulation with TG and ATP. Therefore, an increased and sustained CCE can be induced only after a powerful emptying of intracytosolic Ca^{2+} pools in both EC strains from SHR and SD rats.

VI.3. THE EFFECTS OF ANTIOXIDATIVE TREATMENT ON Ca^{2+} HOMEOSTASIS OF ENDOTHELIAL CELLS

To provide maximum protection, cells contain a variety of substances capable of scavenging many different types of free radicals, including lipid peroxides and carbon-centered free radicals. However, endothelial cells contain few antioxidant enzymes and the production of

large quantities of ROS may cause a rapid depletion of their antioxidant buffer capacity. The specific antioxidants or the antioxidant enzymes are strategically compartmentalized in sub cellular organelles within the cell to provide maximum protection. For instance, SOD, catalase, and GSH-Px are not only distributed in the cytosol but are also localized in mitochondria, where most of the intracellular free radicals are produced. All the antioxidants (specific and non-specific) are localized also in the extracellular space, where they confer protection against exogenously produced free radicals and those diffusing across membranes from the extracellular space.

VI.3.1. The effect of antioxidant treatment on Ca^{2+} signaling of ECs

Our results show that in ECs from normotensive rats, specific and non-specific antioxidant pre-treatments do not have any effect on agonist-induced Ca^{2+}-release from intracytosolic Ca^{2+} pools. Catalase together with GSH-Px catalyses the decomposition of H_2O_2 into water and O_2, but both can also metabolize organic peroxides. In the presence of low H_2O_2 levels, organic peroxide detoxification is preferentially catalyzed by GSH-Px, whereas at high H_2O_2 the organic peroxide detoxification is catalyzed by catalase [73]. The positive effect of catalase has demonstrated the potential to increase Ca^{2+} release from internal stores and the induction of an enhanced capacitative Ca^{2+} influx in canine venous endothelial cells following acute exposure to H_2O_2 [284]. Superoxide dismutase is probably the most specific enzyme of the primary antioxidant defense system that catalyzes the transformation of $^{\bullet}O_2^-$ into H_2O_2, thus representing also one of the most important sources of free radicals. The $^{\bullet}O_2^-$ dismutation into H_2O_2 occurs spontaneously but under the effect of SOD, the dismutation rate is $\approx 10^4$ times higher. The protection against an oxidant stress induced by $^{\bullet}O_2^-$ is much smaller than in the case of catalase. However, in contrast to the data obtained herein, the co-incubation of calf pulmonary

endothelial cells (CPEC) with HX/XO + SOD had a potentiating effect on oxidative injury rather than recovering the injury of Ca^{2+} homeostasis induced by HX/XO [251].

In addition, the results of the present study confirm that the acute and chronic treatment of SHR cells with catalase and SOD led to a significant increase of the Ca^{2+} response to ATP, in contrast with a lack of effect in ECs from normotensive rats. Exposure of ECs from SHR to antioxidant enzymes reveals an impressive recovery of agonist-induced Ca^{2+} responses reaching values similar to those of normotensive rats. Surprisingly, when ECs from SHR are chronically treated with antioxidants, the effect of SOD is equivalent with that of catalase. Our results strongly suggest that in ECs from SHR, the deleterious effect of free radicals is merely initiated by $^{\bullet}O_2^-$. If SOD is present in the system for a long-time, all the $^{\bullet}O_2^-$ overproduction is transformed instantly in H_2O_2, whereas the constant production of H_2O_2 is rapidly buffered by the cumulative action of constitutive catalase and GSH-Px. In this manner, ECs from SHR will overcome the deleterious effect of organic peroxides and will repair the membrane's peroxidized lipids and lipoproteins present in cells, reestablishing the full physiological parameters of membrane transport, and Ca^{2+} homeostasis. It is also important to note the positive effect of chronic incubation with vitamin E and the free metal chelator deferoxamine, which favor a full recovery of the agonist-induced Ca^{2+} release from internal pools of SHR. The protective effect of non-enzymatic antioxidants has not been demonstrated to be very powerful in acute treatment of induced oxidant stress injury to ECs, either from SD rats or from SHR. In addition, the observation that both SOD and catalase succeeded in increasing the Ca^{2+} response of SHR cells to ATP, suggests a prominent collateral contribution of $OH^{\bullet}$ in impairing Ca^{2+} release from internal pools in SHR cells. The fact that chronic incubation with vitamin E and the free metal chelator deferoxamine produce full recovery of the agonist-induced Ca^{2+} release

from internal pools of SHR, place both substances as very effective antioxidant treatment against free radical damages.

The development of a hypertensive state in SHR is a time dependent process and its exact causes and pathophysiological pathway remain unknown and highly speculative. The clinical and macroscopic events, correlated with a time-dependent increase in the resistance of peripheral vessels of the genetic hypertension rat, are very similar to those of human essential hypertension. The blood pressure of 3-week-old SHR is similar to their age-matched SD and WKY [290] but it is significantly higher for rats of 4 weeks and older. The exposure of ECs from young SHR to acute and chronic antioxidant treatment demonstrates antioxidant sensitivity and an improvement of agonist-induced Ca^{2+} response, with an identical pattern as in adults SHR (data not shown). Again, the most active enzyme in the acute antioxidant treatment is shown to be catalase, with a recovery effect two times higher in comparison with the effect of SOD. The chronic treatment has given the same pattern of agonists-induced Ca^{2+} responses, with catalase and SOD having an antioxidant potential similar to that observed in adult SHR. Our findings are positively corroborated by the results of Marcil et al., who have demonstrated significantly superior expression levels of $PG_{i\alpha}$-2 and $PG_{i\alpha}$-3 in hearts and aorta of 2 weeks old SHR, in comparison with normotensive WKY rats [290].

VI.3.2. The effects of antioxidant treatment on Ca^{2+} pools of endothelial cells

The fact that catalase failed to improve the release of Ca^{2+} from TG-sensitive Ca^{2+} pools in SHR would suggest that the beneficial effect of catalase is linked in this case to the protection of a SERCA isoform, the $InsP_3R$ itself and/or the production of $InsP_3$. The results of the present study clearly demonstrate that $InsP_3$ production is not involved in the beneficial effect of antioxidant enzymes in recovering the agonist-induced Ca^{2+} release in ECs from SHR.

Evidence has been provided already that the oxidation of the $InsP_3R$ results in $InsP_3R$ channel activation at lower $InsP_3$ levels [279]. The $InsP_3R$ activation has a multi-factorial dependence and is very complex phenomenon. It was already demonstrated that a lower $[Ca^{2+}]_i$, less than 300 nM, enhanced the $InsP_3R$ sensitivity to $InsP_3$ whereas, a higher concentration, over >300 nM, has an inhibitory effect. Even the redox state and the nature of molecules can affect the sensitivity of $InsP_3R$ to $InsP_3$. The $InsP_3$ binding and $InsP_3$-Ca^{2+} mobilization is generally inhibited by agents that cause oxidation of sulfhydryl groups [291]. Yet under appropriate conditions, oxidized glutathione and/or thimerosal increase the sensitivity of $InsP_3R$ to Ca^{2+} mobilizing agents [292], while other sulfhydryl reagents (e.g. N-ethylamaleimide) inhibit $InsP_3R$ sensitivity.

Experiments revealing the expression level and functional activity of catalase and SOD, measured in cultured aortic endothelial cells from adult SD and SHR rats respectively, demonstrated an equivalent enzymes expression. Moreover, accurate determinations of enzyme activities performed by spectrophotometric assays show statistically equivalent activity values for both SOD and catalase. Thus, our results failed to provide evidence for an up or down regulation of the expression and/or activity of catalase and SOD in SHR compared to SD.

The $InsP_3$ measurements do not support an important change in ATP-induced $InsP_3$ production between control SHR cells and cells chronically treated with catalase. The observation of a reduced ATP-induced Ca^{2+} release, despite a slight increase in ATP-dependent $InsP_3$ production in SHR relative to ATP-dependent $InsP_3$ production in SD cells argue equally for an effect involving Ca^{2+} internal stores. This will be materialized into a low-level content of Ca^{2+} availability in internal pools. Part of the action of ROS on internal Ca^{2+} pools in SHR may consist in a partial depletion of $InsP_3$-sensitive pools due to an increase of the $InsP_3$ receptor activity under an elevated basal $InsP_3$ level and a slight but continuous loss of Ca^{2+} from internal

pools. A depletion of Ca^{2+} pools under oxidative stress conditions may originate from several processes, including an increase in Ca^{2+} permeability of the endoplasmic reticulum (ER) membrane, by opening of ion channels, by a disruption of the ER membrane or an inhibition of the SERCA/PMCA Ca^{2+}-ATPase.

The insensitive nature of TG-sensitive pools to antioxidants is further demonstrated in ECs from SHR, where the treatment of ECs with SOD and catalase had no impact on TG-induced Ca^{2+} released from TG-sensitive pools. The consecutive stimulation of ECs from SHR with TG and ATP follows the same pattern observed in ECs from SD rats. The pretreatment of ECs from SHR with catalase induced an important recovery of $InsP_3$-sensitive Ca^{2+} pools, which increases from simple to double, but the catalase had no effect on TG-sensitive pools. The mechanism by which TG induced the specific inhibition of SERCA remains unknown. It is suggested that TG binds stoichiometrically with high affinity and high specificity to all SERCAs and causes an essentially irreversible inhibition of their activity by blocking the enzyme in a Ca^{2+}-free conformation [293]. The irreversible binding would suggest the implication of a covalent bond, which would have a powerful oxidant characteristic. Because catalase and SOD are not able to overcome this oxidative reaction on SERCA, this suggests that the oxidant process does not involve oxygen based oxidation or peroxidation reactions. The type of oxidant reaction involved in this process is more likely an organic oxidant that is out of the range of catalase and SOD enzymatic capabilities. Thus, the antioxidant treatment is not capable of restoring the responsiveness of TG-sensitive pools. However, the TG-blocking effect of SERCAs on $InsP_3$-sensitive Ca^{2+}-pools has a different chemical pattern, since the antioxidant enzymes overcome the blocking effect. Thus, this interaction would represent direct evidence that in the case of these particular SERCAs, the binding of TG to the pumps is a chemical

reaction, which implies most likely an oxygen-based FR and/or a peroxidation process, which always enter in the spectrum of activity of catalase.

VI.3.3. The antioxidant effect of melatonin

Our results demonstrate and plead furthermore for a non-specific activity of melatonin in ECs, because its antioxidant capacity can influence the behavior of Ca^{2+} in endothelial cells. The antioxidant capacity of melatonin is highly expressed on all endothelial cells, but principally in cells from spontaneously hypertensive rats.

VI.3.3.1. The antioxidant effect of melatonin on endothelial cells from SD rats

The application of melatonin treatment concomitantly with the induction of oxidative stress by HX/XO produces a complete protection of agonist induced Ca^{2+} responses and melatonin overcomes the inflicting effect of ROS. These results corroborate those obtained by many laboratories having considered antioxidative activity of melatonin [242,294-299]. Despite antioxidant efficiency of melatonin in our study, the comparison between melatonin and other antioxidant enzymes (catalase, SOD, deferoxamine) in HX/XO-induced oxidative stress, demonstrates that melatonin has the weakest antioxidant activity in ECs. This observation gives a much more deleterious importance to H_2O_2 and $^{\bullet}O_2^-$ than to $OH^{\bullet}$ during the induced oxidative stress in endothelial cells. However, in 1998, Zang et al. have reported that melatonin does not interfere with $OH^{\bullet}$ to form intermediary and final metabolites but does interfere with H_2O_2 and $^{\bullet}O_2^-$ production [299]. For instance, it was demonstrated that melatonin was less effective than vitamin E in inhibiting *in vitro* the LDL oxidation and the cytotoxicity of oxidized LDL in cultured ECs [300]. Moreover, melatonin did not react at significant rates with $^{\bullet}O_2^-$ but it was an excellent peroxyl radical scavenger despite the fact that melatonin was much less effective in

the inhibition of lipid peroxidation [239]. Some antioxidants (e.g. vitamin C) can exert pro-oxidant activity in *vitro* and/or in *vivo*. Melatonin is a compound apparently devoid of pro-oxidant effects but capable to stabilize membranes against FR damages. In contrast, its broad spectrum of antioxidative efficiency is suggesting that melatonin antioxidative capability is realized, more likely, through both a direct effect on up-regulation of intrinsic cellular antioxidative capability and through a direct free radical-melatonin reaction [239].

VI.3.3.2. The antioxidant effect of melatonin on endothelial cells from SHR

Extensive works have demonstrated an overproduction of free radicals [286,301] and our observations indicate an over-accumulation of H_2O_2 in endothelial cells from SHR [302]. The improved agonist-induced Ca^{2+} mobilization in ECs from SHR is related with melatonin antioxidant effect. In control-conditions, the BK-induced and the ATP-induced Ca^{2+}-release in ECs from SHR are smaller than in SD. Nevertheless, the melatonin treatment produced a significant increase in Ca^{2+} release from internal stores for both, BK, and ATP stimulations. This would be in favor of a contribution of $OH^{\bullet}$ in impairing Ca^{2+} release from internal pools. Melatonin was shown to be a highly efficient scavenger of both the hydroxyl radical ($OH^{\bullet}$) and peroxyl radical ($ROO^{\bullet}$). The antioxidative properties of melatonin are enhanced by its effects on stimulation of glutathione peroxidase (GSH-Px) activity and the inhibition of nitric oxide synthase (cNOS). Considering its multiple actions, melatonin is certainly one of the most versatile natural antioxidants thus far discovered. This attribute is confirmed by the results of this study, where the effect of melatonin on reducing the $^{b}[Ca^{2+}]_i$ in melatonin-treated ECs from SHR is very visible in comparison with that of other antioxidants in control. The melatonin treatment is affecting the activity of plasmalemmal and endoplasmic reticulum Ca^{2+}-ATPases, improving the activity and the efficiency of pumps for the recapture and the extrusion of

improving the activity and the efficiency of pumps for the recapture and the extrusion of cytosolic free Ca^{2+}. Our results are supported by the report of Chen et al. who has demonstrated that alloxan induced an inhibition of Ca^{2+}/Mg^{2+}-ATPase on cardiac sarcolemma, which was reversed by a melatonin treatment [303]. It was also demonstrated that the treatment of SHR with melatonin significantly reduced the blood pressure [97]. Therefore, the lowering of blood pressure in SHR by melatonin can be correlated with the beneficial effect of melatonin in EC Ca^{2+} homeostasis.

VI.3.3.3 The antioxidant effect of melatonin on CCE of endothelial cells

In this study, the CCE in primary cultures of endothelial cells was very weak, even at the limit of detection in comparison with the CCE of ECs from BAE that is greatly larger. The treatment of ECs from BAE, SD and SHR rats with melatonin, after simple or double agonist stimulation, substantially stimulated the CCE. The mechanism by which the CCE is modulated remains an open question but it is clear that melatonin interferes and stimulates the CCE. This phenomenon is important in primary endothelial cells, where the stimulatory effects of melatonin are time dependent and increase CCE by 600% after 40 min of treatment.

This stimulation cannot be related with the antioxidative property of melatonin because it appears in ECs from BAE and SD rats, which were not under oxidative stress and were not characterized by free radical overproduction. When ECs from SD rats were "pre-incubated" in melatonin for 20 min, the CCE generated was substantially smaller than in the same cells "superfused" for 20 min with melatonin. At least we can speculate that the presence of melatonin induced a process that is time dependent, because the 40 min superfusion has doubled the CCE in the same protocol of stimulation. This particular Ca^{2+} manifestation in primary cultures of rat endothelial cells, in comparison with the cloned (BAE) cells, might have

important physiological repercussions in a normal or pathological state, keeping in mind that Ca^{2+} entry is critical for the development and physiology of virtually all cells.

In conclusion, the important perturbations of the agonist-induced Ca^{2+} responses of ECs from SHR is due to a continuous low-level of oxidant stress which causes a significant depletion of the Ca^{2+} availability in intracytosolic pools. The reduced Ca^{2+} availability is demonstrated through the size of agonist-induced Ca^{2+} responses as well as through the capacity of Ca^{2+} pools. The low-level free radical production is clearly demonstrated by the insensitivity of ECs from SD rats to antioxidant treatment whereas the agonist induced Ca^{2+} responses of ECs from SHR are antioxidant-sensitive. The results from artificially induced and genetically established oxidative stress demonstrate clearly that the most aggressive oxygen derived free radical is the H_2O_2 and potentially organic peroxides. For the first time is evidenced the presence in ECs of a TG-insensitive Ca^{2+} pool which shows a sensitivity to oxidative stress but an insensitivity to antioxidant treatment. However, the presence in these cells of antioxidant-sensitive pools, which are mostly represented by the $InsP_3$-sensitive pools, demonstrates a genetic adaptation to the incessantly occurring oxidative stress to which endothelial cells are exposed. Even tough the presence of membrane melatonin receptors cannot be ruled out entirely, it is clear that these receptors are not related to the $InsP_3$ production and the Ca^{2+} signaling in aorta ECs. The stimulatory effect of melatonin on CCE occurs most likely through a stimulation of $IK_{(Ca)}$ channels and/or through antagonizing specifically the calmodulin. This effect has a direct positive consequence on the availability of Ca^{2+} in the intracytosolic pools and indirectly, on the dimension of agonist induced Ca^{2+} responses. Therefore, the complete recovery of Ca^{2+} homeostasis induced by treatment with specific antioxidative treatment and/or melatonin opens an important curative strategy for pathologies in which the oxidative stress participates as a fundamental pathogenic factor.

Discussion

CHAPTER VII.

VII.1. SIGNIFICANCE OF THE PRESENT STUDY

Despite the fact that the endogenous overproduction of free radicals by the hypertensive endothelium has been postulated and demonstrated to be associated with an abnormal Ca^{2+} signaling mechanism and positively correlated with a general impaired vasodilatation, the homeostasis of Ca^{2+} in normotensive endothelium and hypertensive models is still poorly described and understood. Moreover, the manner in which the oxidative stress and the antioxidant treatment affect the Ca^{2+} homeostasis in primary endothelial cells from normotensive rats has never been described. A parallel between the Ca^{2+} homeostasis of normotensive rats and the effect of the free radical overproduction in ECs from genetically hypertensive rats was never investigated. Therefore, the aim of this study was to bring a parallel between the artificially induced oxidative stress and the oxidative stress generated by the free radical overproduction in the SHR endothelium and to evaluate the impact of several families of antioxidants, such as specific antioxidant enzymes, non-specific antioxidants and melatonin on Ca^{2+} homeostasis of ECs. This is the first study that brings a parallel between the alterations of Ca^{2+} signaling induced by artificial oxidative stress and the homeostasis of Ca^{2+} in ECs from hypertensive animal model. Furthermore, melatonin was successfully used in lowering the blood pressure in genetically hypertensive rats and in human hypertensive subjects. The antioxidant mechanism of melatonin has been recognized and described. Nonetheless, there is no report yet in the literature about the hormonal and antioxidant effect of melatonin on endothelial cells. The presence in aortae ECs of melatonin specific receptors associated with Ca^{2+}-signaling cascade is unknown. Similarly, how melatonin influences Ca^{2+} homeostasis in

Reference List

ECs and the effect of melatonin on the oxidative stress in normotensive and hypertensive ECs has never been investigated.

The results of the present study provides new data which will help to understand the physiology and physiopathology of endothelial cells while confirming the important impact of oxidative stress on the regulation of Ca^{2+} homeostasis, the paracrine and vasoactive function of the endothelium. This novel data brings a definitive answer to the question of dysfunctional agonist-induced Ca^{2+} responses of ECs from hypertensive rats. In addition, the present study underlines the enormous importance of antioxidant-based treatments in all cardiovascular pathologies related to oxidative stress and opens new possibilities and avenues for therapeutic and curative usage of specific antioxidant enzymes and melatonin in the treatment of human idiopathic hypertension.

VII.2. DEVELOPMENTAL PERSPECTIVES

The data reported in this study confer an important opening potential for development.

As long as the evolution of the metabolic oxido-reductive state remains a fundamental characteristic of the dynamic steady state of the living cells, the production of free radicals will constitute an obvious and usual occurrence. Therefore, the evaluation of the Ca^{2+} homeostasis in all types of endothelial cells in the entire pathophysiological spectrum of cardiovascular diseases would be a scientific obligation, which would provide further fundamental understandings for cardiovascular physiology and pathophysiology.

In the perspective of the new definition for apoptosis, the intimate molecular and biological implication of apoptosis and to analyze in minute details the apoptotic signals and the signaling pathways at structural, molecular and biochemical levels in which, the free radicals are

always fundamentally implicated (most likely as regulators, signaling and/or restrictive molecules), will constitute an important field of research for the years to come in the realm of pathophysiology.

The experimental procedures described in this study must be extended to the evaluation of the Ca^{2+} homeostasis in vascular smooth muscle and cardiac cells coming from different pathological states in which the oxidative stress and the generation of free radicals occur.

The identification of the free radical sources in endothelial cells from SHR is "a must" for a definitive comprehension of the fundamental pathophysiological mechanism of genetic and idiopathic hypertension.

The effect of oxidative stress and the effect of antioxidant treatment as well as the effect of melatonin should be evaluated in molecular biology and electrophysiology, approaches that would provide evidences and advance to the intimate structures affected by oxidative stress as well as for the structures able to recover under antioxidant treatment.

The appraisal of melatonin effects must be conducted into a biological and molecular basis approach to elucidate the structures implicated in the cell regulatory mechanisms of this conspicuous and prominent hormone for the physiology and biology of every cell.

To analyze in minute details the effect of oxidative stress on CCE are considered necessary more experimental models, at cellular, molecular, and electrophysiological level and the identification of the molecular structures implicated and their behaviour under oxidative stress. Therefore, endothelial cells transiently and/or permanently transfected with the constituents of CCE (for instance the *trp* and *trp*L channels) should be evaluated under the

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effect of oxidative stress and antioxidant treatment. The effect of melatonin on CCE should be investigated at electrophysiological and molecular level.

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