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THE SIGNALLING ROLE OF SUPEROXIDE ANION IN VASCULAR SMOOTH MUSCLE CELLS

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THE SIGNALLING ROLE OF SUPEROXIDE ANION IN VASCULAR SMOOTH MUSCLE CELLS

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RÉSUMÉ

L'anion superoxyde peut agir comme une molécule de signalisation ou comme un facteur préjudiciable selon sa concentration, l'organe cible, et selon la présence ou non d'antioxydants neutralisants. Actuellement, dans les cellules musculaires lisses (CMLs) vasculaires, les effets de l'anion superoxyde sur les différentes voies de transduction du signal et sur les interactions croisées entre ces voies ne sont pas encore définies. Par conséquent, une meilleure connaissance des effets de l'anion superoxyde sur les différentes voies de signalisation pourrait fournir une meilleure compréhension des mécanismes sous-jacents aux fonctions altérées des CMLs vasculaires observées dans des conditions pathologiques. L'objectif général de cette étude était de caractériser et d'évaluer le rôle modulateur de l'anion superoxyde, produit par la réaction de l'hypoxanthine avec la xanthine oxidase, sur les activités de différentes voies de signalisation dans les CMLs vasculaires, et de déterminer si la sensibilité de différentes voies de signalisation à l'anion superoxyde était altérée dans l'hypertension artérielle. Le projet de ce programme de recherche était basé sur les principaux postulats suivants : (1) l'anion superoxyde pourrait affecter sélectivement la production d'inositol 1,4,5-triphosphates (IP_3), de GMPc, ou d'AMPc dans les CMLs vasculaires; (2) le rôle modulateur de l'anion superoxyde pourrait être dû à une altération des interactions croisées entre différentes voies de signalisation; et (3) les anomalies observées dans les CMLs vasculaires chez le rat spontanément hypertendu (SHR) pourraient être reliées à des altérations des différentes voies de signalisation induites par l'anion superoxyde.

Une production augmentée d'IP_3 induite par l'anion superoxyde dans les CMLs d'aorte de rat ou d'artère mésentérique en culture a été démontrée pour la première fois dans cette étude. L'anion superoxyde a augmenté la formation d'IP_3 d'une manière concentration-dépendante et

temps-dépendante. La superoxyde dismutase (SOD), mais non la catalase, a inhibé significativement la formation d'IP3 induite par l'anion superoxyde. L'inhibition de la phospholipase C (PLC) a aboli l'effet de l'anion superoxyde sur la formation d'IP_3. La génistéine et la tyrphostine A25, deux inhibiteurs de la tyrosine kinase, ont aussi inhibé significativement la formation d'IP_3 induite par l'anion superoxyde. L'utilisation d'anticorps anti-PLCγ a atténué significativement la formation d'IP_3 induite par l'anion superoxyde. De plus, le taux d'expression des protéines de la PLCγ a été augmenté après l'exposition des CMLs à l'anion superoxyde. Ces observations suggèrent donc que dans les CMLs vasculaires la formation d'IP_3 induite par l'anion superoxyde pourrait être en grande partie secondaire à une augmentation de l'activité de la tyrosine kinase liée aux voies de signalisation de la PLCγ.

En ce qui concerne la voie du GMPc, l'anion superoxyde a diminué significativement les niveaux de base de GMPc et supprimé aussi l'augmentation des niveaux de GMPc induite par des stimulateurs de la guanylyl cyclase, le nitroprussiate de sodium (NPS) ou la s-nitroso-n-acétylpénicillamine (SNAP). La formation d'IP_3 stimulée par l'anion superoxyde a été significativement inhibée par le NPS ou la SNAP, mais potentialisée de façon importante par un inhibiteur de la guanylyl cyclase l'ODQ ou par le KT5823 (un inhibiteur de la protéine kinase dépendant du GMPc). Cependant, l'anion superoxyde n'a pas eu d'effet sur les niveaux de base d'AMPc ou sur la production d'AMPc induite par la forskoline et de plus, l'inhibition de l'adénylyl cyclase ou de la protéine kinase dépendante de l'AMPc n'a pas affecté la formation d'IP_3 stimulée par l'anion superoxyde. Ces données, par conséquent, suggèrent que l'inhibition de la formation de GMPc par l'anion superoxyde contribue probablement à l'activation de la formation d'IP_3 induite par l'anion superoxyde en atténuant le rétrocontrôle inhibiteur du GMPc

sur les voies de signalisation liées à la PLC, tandis que la voie de signalisation de l'AMPc ne serait pas impliquée dans la formation d'IP_3 induite par l'anion superoxyde.

Dans les CMLs vasculaires de rat SHR, les effets de l'anion superoxyde ont été plus puissants que dans les CMLs de rat WKY, en ce qui concerne l'augmentation de formation d'IP_3, la diminution des taux de GMPc et la facilitation induite par l'anion superoxyde des interactions croisées entre les voies du GMPc et de l'IP_3. Dans les CMLs vasculaires des deux souches de rat, la formation d'IP_3 induite par l'anion superoxyde a été inhibée par une variété d'antioxydants, dont la N-acétylcystéine, l'acide α-lipoïque, la mélatonine et la SOD. Il apparaît donc vraisemblable que l'hypersensibilité à l'anion superoxyde des voies de l'IP_3 et du GMPc puissent contribuer à l'augmentation du tonus vasculaire et de la réactivité des CMLs dans l'hypertension artérielle.

Nous avons aussi investigué si l'effet de la mélatonine était dû à ses propriétés antioxydantes. Un effet inhibiteur plus important de la mélatonine sur la contraction aortique induite par la norépinéphrine (NE) a été observé chez les rats SHR en comparaison avec les rats Wistar-Kyoto (WKY). L'inhibition par la mélatonine de la formation d'IP induite par la NE a été aussi plus importante dans les CMLs aortiques de rat SHR que dans celles de rat WKY. Les effets plus puissants de la mélatonine chez le rat SHR, qui ont été aussi observés avec la SOD, mais non avec la catalase, ne sont pas dûs à l'activation des récepteurs à la mélatonine ou des récepteurs α-adrénergiques. Ces résultats indiquent que les effets anti-hypertenseurs de la mélatonine sont largement dûs à l'inactivation de l'anion superoxyde, et que les niveaux endogènes d'antioxydants ne parviennent pas à contrecarrer les niveaux accrus d'anion superoxyde produits chez le rat SHR.

En conclusion, cette étude révèle une variété de nouveaux mécanismes de signalisation de l'anion superoxyde. Pour la première fois, il a été démontré que l'anion superoxyde active l'hydrolyse des phosphoinositides et augmente les taux d'IP_3 dans les CMLs vasculaires, principalement par la stimulation de la tyrosine kinase liée à la voie de signalisation de la PLCγ. Il a aussi été observé que l'anion superoxyde réduit la formation de GMPc et supprime l'inhibition croisée de l'IP_3 par le GMPc, facilitant ainsi la formation d'IP_3. Les effets sélectifs de l'anion superoxyde sur les voies de l'IP_3 et du GMPc, ainsi que l'existence d'une inhibition croisée de la formation d'IP_3 par la voie du GMPc, révèlent des mécanismes nouveaux pour expliquer le rôle modulateur de l'anion superoxyde sur les voies de signalisation dans les CMLs. Par conséquent, les effets plus puissants de l'anion superoxyde sur la signalisation de la voie de l'IP_3 et de la voie du GMPc dans les CMLs vasculaires de rat SHR, effets qui ont été démontrés pour la première fois dans cette étude, pourraient être responsables des altérations des mécanismes de transduction du signal cellulaire chez le rat SHR et ainsi contribuer au développement et/ou au maintient de l'hypertension artérielle. Ces observations permettent donc d'imaginer de nouvelles orientations pour le développement de nouvelles stratégies pour la prévention ou le traitement de l'hypertension artérielle.

SUMMARY

Superoxide anion can act as a signalling molecule or a detrimental factor depending on its concentration, the targeted organ, and the presence of counteracting antioxidants. The effects of superoxide on different signal transduction pathways and on the cross-talk interactions among these pathways in vascular smooth muscle cells (SMCs) are presently still unsettled. Therefore, a better knowledge on the effects of superoxide on different signalling pathways may provide a better understanding of the mechanisms underlying the altered functions in vascular SMCs observed in pathological conditions. The general objective of this study was to characterize and evaluate the modulating role of superoxide generated by the hypoxanthine and xanthine oxidase reaction on the activities of different signalling pathways in vascular SMCs and to investigate whether the sensitivities of different signalling pathways to superoxide were altered in hypertension. The design of the present research program was based on the following major postulates. (1) superoxide might selectively affect the generation of inositol 1,4,5-triphosphates (IP_3), cGMP, or cAMP in vascular SMCs; (2) the modulating role of superoxide might be mediated by alteration in the cross-talk interactions among different signalling pathways; and (3) the abnormalities observed in vascular SMCs from spontaneously hypertensive rats (SHR) might be related to the alterations induced by superoxide on different signalling pathways.

An enhanced production of IP_3 induced by superoxide in cultured SMCs from rat aorta or mesenteric artery was demonstrated, for the first time, in this study. Superoxide increased IP_3 formation in a concentration- and time-dependent manner. Superoxide dismutase (SOD), but not catalase, significantly inhibited the superoxide-increased IP_3 formation. The inhibition of phospholipase C (PLC) abolished the effect of superoxide on IP_3 formation. Genistein and tyrphostin A25, two tyrosine kinase inhibitors, also significantly inhibited the superoxide-

induced IP_3 formation. The application of antibody against PLC_γ significantly attenuated the superoxide-induced IP_3 formation. Moreover, the expression level of PLC_γ proteins was increased after exposing SMCs to superoxide. These observations thus suggest that superoxide-induced IP_3 formation may be in a great part secondary to an increase in the activity of tyrosine kinase-link PLCγ signalling pathways in vascular SMCs.

Concerning the cGMP pathway, superoxide significantly decreased the basal levels of cGMP and also suppressed the rise in cGMP levels induced by guanylyl cyclase stimulator sodium nitroprusside (SNP) or s-nitroso-n-acetylpenicillamine (SNAP). The superoxide-induced IP_3 formation was significantly inhibited by SNP or SNAP, but markedly potentiated by a guanylyl cyclase inhibitor ODQ or KT5823 (a cGMP-dependent protein kinase inhibitor). However, superoxide had no effect on the basal levels of cAMP or the forskolin-induced cAMP production and moreover, the inhibition of adenylyl cyclase or cAMP-dependent protein kinase did not affect the superoxide-enhanced IP_3 formation. These data, therefore, suggest that the reduced cGMP formation by superoxide probably contributes to the superoxide induced activation of IP_3 formation by lifting the inhibitory feedback of cGMP on the PLC pathway(s), whereas, the cAMP pathway may not be involved in the superoxide-induced IP_3 formation.

In vascular SMCs from SHR, the effects of superoxide were more potent than in SMCs from WKY, including the increase in IP_3 formation, the decrease in cGMP levels, and the superoxide-induced facilitation of the cross-talk interaction between cGMP and IP_3 pathways. The superoxide-induced IP_3 formation was inhibited by a variety of antioxidants, including n-acetylcysteine, α-lipoic acid, melatonin and SOD, in vascular SMCs from both strains. It thus appears that the hypersensitivity of IP_3 and cGMP pathways to superoxide is likely to contribute to the increased vascular tone and reactivity of SMCs in hypertension.

Whether the effect of melatonin is due to its antioxidant properties was also explored. A greater inhibitory effect of melatonin on the norepinephrine (NE)-induced aortic contraction was observed in SHR than in Wistar-Kyoto rats (WKY). The inhibition of the NE-induced IP formation by melatonin was also greater in aortic SMCs from SHR than that from WKY. The enhanced effects of melatonin in SHR, which were found to be similarly enhanced with SOD but not with catalase, were not mediated by melatonin receptors or α-adrenoceptors. These results indicate that the anti-hypertensive effects of melatonin are largely due to the scavenging of superoxide, and that the levels of endogenous antioxidants may not counteract the levels of over-produced superoxide in SHR.

In conclusion, this study reveals a variety of novel signalling mechanisms for superoxide. For the first time, it was demonstrated that superoxide activates the hydrolysis of phosphoinositides and increases IP_3 levels in vascular SMCs mainly through the stimulation of tyrosine kinase-link PLCγ signal pathway. It was also found that superoxide reduces cGMP formation and suppresses the cross-inhibition of IP_3 by cGMP, thus facilitating IP_3 formation. The selective effects of superoxide on IP_3 and cGMP pathways as well as the existence of a cross-inhibition of IP_3 formation by cGMP pathway provide novel mechanisms for the signalling role of superoxide in vascular SMCs. Therefore, the altered signalling effects of superoxide on the IP_3 pathway and the cGMP pathway, which were demonstrated in vascular SMCs from SHR for the first time in this study, could thus be responsible for the alterations in cellular signal transduction mechanisms in SHR and might contribute to the development and/or maintenance of hypertension. These observations could provide new avenues for the development of new strategies for the prevention or treatment of hypertension.

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DEDICATION

To: My dear husband **RUI**

And lovely daughters **JENNY** and **JESSY**.

LIST OF ABBREVIATIONS

AC :	Adenylyl cyclase;
ACE:	Angiotensin converting enzyme;
AII:	Angiotensin II;
ANP:	Atrial natriuretic peptide;
ADP:	Adenosine diphosphate;
ATP:	Adenosine triphosphate;
βARK:	β-adrenergic receptor kinase;
cAMP:	Adenosine-3',5'-cyclic monophosphate;
CAT:	Catalase;
cGMP:	Guanosine 3',5'-cyclic monophosphate;
DAG:	Diacylglycerol;
DOCA:	Deoxycorticosterone acetate;
DTT:	Dithiothreitol;
EDHF:	Endothelium-derived hyperpolarising factor;
ER:	Endoplasmic reticulum;
G protein:	GTP-binding protein;
GC:	Guanylyl cyclase;
GDP:	Guanosine diphosphate;
GTP:	Guanosine triphosphate;
GSH:	Reduced glutathione;
GSH-Px:	Glutathione peroxidase;

GSSG: Oxidized glutathione;

H_2O_2: Hydrogen peroxide;

HX: Hypoxanthine;

IP_3: Inositol 1,4,5-trisphosphate;

MAP: Mitogen-activated protein;

MLC: Myosin light chain;

NAC: N-acetylcysteine;

NADP: Nicotinamide adenine dinucleotide phosphate;

NADPH: Dihydronicotinamide adenine dinucleotide phosphate;

NE: Norepinephrine;

NO: Nitric oxide;

NOS: Nitric oxide synthase;

$O_2^{\bullet-}$: Superoxide anion;

OFR: Oxygen-derived free radicals;

$OH^{\bullet}$: Hydroxyl radical;

PDE: Phosphodiesterase;

PIP_2: Phosphatidylinositol 4,5-biphosphate,

PKA: cAMP-dependent protein kinase;

PKC: Protein kinase C;

PKG: cGMP-dependent protein kinase;

PLA: Phospholipase A;

PLC: Phospholipase C;

PLD: Phospholipase D;

ROS:	Reactive oxygen species;
SHR:	Spontaneously hypertensive rats;
SMCs:	Smooth muscle cells;
SNAP:	S-nitroso-N-acetyl-D,L-penicillamine;
SNP:	Sodium nitroprusside;
SOD:	Superoxide dismutase;
SR:	Sarcoplasmic reticulum;
WKY:	Wistar-Kyoto rats;
XO:	Xanthine oxidase.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

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1. CELLULAR METABOLISM AND FUNCTIONS OF REACTIVE OXYGENE SPECIES.

Reduction-oxidation (redox) reactions are important chemical processes that regulate cellular functions by generating reactive oxygen species (ROS). ROS act as signalling molecules at subtoxic levels to transmit biological signals from extracellular space to intracellular milieu or between intracellular organelles. ROS also have multiple detrimental effects. An overproduction of ROS exceeding cellular antioxidant capacity is associated with cellular injury including DNA damage (Chan, 1996), protein malfold (Götz *et al.*, 1994), lipid peroxidation (Busciglio & Yankner, 1995), and altered calcium homeostasis (Suzuki & Ford, 1992). Recent studies indicate that ROS may play an important role in the pathogenesis of many cardiovascular diseases such as ischemia-reperfusion heart damage, heart failure, and hypertension. The elucidation of the generation and function of ROS as well as the balance between oxidant and antioxidant systems under physiological and pathological conditions may help to better understand the regulation of cellular signal transduction pathways and to characterise the signalling role of ROS.

1.1. Generation of Reactive Oxygen Species.

By definition, reactive oxygen species (ROS) are highly reactive chemical species that differ from standard compounds in having unpaired electrons in their outer orbitals. Included in the category of ROS are free radicals that typically have an oxygen- or nitrogen-based unpaired electron, and other species (such as H_2O_2) that act as oxidants. When molecular oxygen accepts an electron, it becomes the superoxide anion ($O_2^{\bullet -}$). The sequential transfer of one electron to oxygen (i.e. the sequential reduction of oxygen) results in the formation of two intermediates

(less reactive $O_2^{\bullet-}$ and highly reactive hydroxyl radical $OH^{\bullet}$), a relatively stable and long-lived intermediate (hydrogen peroxide, H_2O_2), and a stable and harmless end product (water).

$$O_2 + 1e^- \longrightarrow O_2^{\bullet-} \xrightarrow{+ 1e^- + 2H^+} H_2O_2 \xrightarrow{+ 1e^-} OH^{\bullet} \xrightarrow{+ 1e^- + 2H^+} H_2O$$

The major ROS are as follows:

Superoxide anion ($O_2^{\bullet-}$): Superoxide anion can oxidize catechol compounds including catecholamines and can reduce metals, such as reducing ferric salt to the ferrous state and return to $O_2^{\bullet-}$.

Hydrogen peroxide (H_2O_2): Hydrogen peroxide is a non-radical species, but as an oxidizing agent it can give rise to other ROS.

Hydroxyl radical ($OH^{\bullet}$): Hydroxyl radical is generated in two reactions.

Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$

Haber-Weiss reaction: $H_2O_2 + O_2^{\bullet-} \xrightarrow{Fe3+/Fe2+} OH^{\bullet} + O_2 + H_2O$.

Actually, a two-step reaction is involved in Haber-Weiss reaction, in which $OH^{\bullet}$ is formed in a traditional Fenton reaction followed by reduction of the iron by superoxide anion ($Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$). In this sequence, recycling of the metal catalyst maintains a reaction chain.

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Alkoxyl radicals ($OR^{\bullet}$) and peroxyl radicals ($OOR^{\bullet}$): Lipid peroxidation generates two additional reactive and short-lived radicals, namely, alkoxyl radicals ($OR^{\bullet}$) and peroxyl radicals ($OOR^{\bullet}$). In addition, organic peroxides (lipid hydroperoxides, ROOH) are also formed as relatively more-stable intermediates or end products.

Nitric oxide ($NO^{\bullet}$): $NO^{\bullet}$ is also an ROS because it has an unpaired electron associated with the oxygen atom. An interaction of $NO^{\bullet}$ with superoxide anion produces a labile non-radical compound, the peroxynitrite anion ($O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$). When peroxynitrous acid is formed by acidification of the anion ($ONOO^- + H^+ \rightarrow ONOOH$), it will spontaneously decompose by homolytic scission to yield the highly reactive hydroxyl radical ($ONOOH \rightarrow OH^{\bullet} + ONO^{\bullet}$).

Oxidized glutathione (GSSG): GSSG, a disulfide-linked homodimer, is known for its action to oxidize protein thiols. Either an intramolecular disulfide bond between adjacent sulfhydryls in the protein or a mixed disulfide bond between the protein and glutathione will be formed as the result of the GSSH-induced oxidation. Many abnormal protein functions and structures are related to the formation of a disulfide bond. For instance, GSSG can directly interact with IP_3 receptors and result in a release of calcium from IP_3 sensitive pool in the absence of IP_3 formation (Suzuki *et al.*, 1997). GSSG can also mediate ion channel activities such as inhibiting K^+ channels or activating Na^+ channels, leading to an altered endothelial cell signalling (Elliott & Koliward, 1997). Being generated via the action of glutathione peroxidase (GSH-Px), GSSG is reduced back to glutathione (GSH) via the activity of GSH reductase (GSH-Rt), which serves to maintain GSSG levels between one-tenth and one hundredth those of total GSH. GSH, a tripeptide, is the single most abundant redox-active sulfhydryl compound within

the cytosol of mammalian cells. By providing reducing equivalents for the redox reaction catalyzed by GSH peroxidase, GSH represents the major antioxidant defence mechanism. Cells have several other mechanisms in place to handle GSSG, including conjugation reactions via glutathione-s-transferase and extrusion to extracellular space via active pump units (Frisher &Ahamd, 1977; Rushmore & Pickett, 1993). The oxidation of GSH to its dimer GSSG is coupled to the reduction of hydroxyperoxide (ROOH) to alcohol (ROH). The regeneration of GSH requires the transformation of NADPH to NADP.

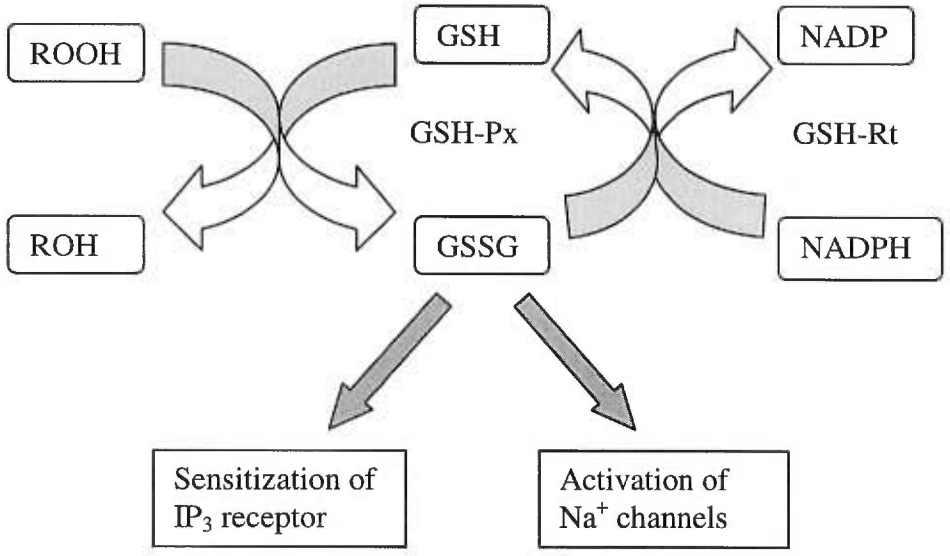

Fig. 1-1. The generation and reduction of oxidized glutathione.

1.2. Cellular Sources of Reactive Oxygen Species.

ROS can be generated by normal metabolic processes such as the reduction of oxygen to water through the mitochondrial electron transport chain. Normal cellular energy metabolism is not 100% efficient, and it is considered that between 1% to 5% of all oxygen used in energy

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metabolism escapes as ROS. Oxidative reactions are also very important in other biological reactions and many of these have the potential to generate ROS under physiological conditions (Table 1-1). For example, both cytochrome P450s and cyclooxygenase generate ROS (Katusic, 1996). Thus, the production of ROS can be a natural part of cellular functions. Two main endogenous $O_2^{\bullet-}$-generating enzyme systems are xanthine oxidase (XO) and NADH/NADPH oxidase. *In vivo*, xanthine dehydrogenase is converted to XO and which mediates the metabolism of nucleotides such as xanthine and hypoxanthine (HX) to form $O_2^{\bullet-}$, H_2O_2, and uric acid (Wession & Elliott, 1994). Inhibitors of XO, e.g. allopurinol and oxypurinol, have been used to attenuate the injury process and have permitted to identify XO as a mediator of tissue injury. NADH/NADPH oxidase is a multi-subunits enzyme, which has been claimed to be the major source of $O_2^{\bullet-}$ production in many tissues.

Table 1-1. Endogenous Generation of ROS

		ROS
Enzymatic sources	1. Cyclooxygenase	$O_2^{\bullet-}$, $OH^{\bullet}$
	2. Leakage from mitochondrial electron transport	$O_2^{\bullet-}$, H_2O_2
	3. Lipoxygenase	$O_2^{\bullet-}$, $OH^{\bullet}$
	4. Monoamine oxidase	H_2O_2, $OH^{\bullet}$
	5. NADH/NADPH oxidase	$O_2^{\bullet-}$, $OH^{\bullet}$
	6. NO synthase	$NO^{\bullet}$
	7. Xanthine oxidase	$O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$
Non-enzymatic sources	1. Auto-oxidation of catecholamines, 2. ascorbate, GSH	$O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$,
	2. Fenton-type reactions	$OH^{\bullet}$
	3. Lipid peroxidation reactions	$OR^{\bullet}$, $OOR^{\bullet}$, ROOH
	4. Redox cycling (via tissue reducing agents; e.g., ascorbate) Metals: iron, copper and Cellular toxins: 6-hydroxydopamine,	$O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$
	5. Spontaneous decomposition of peroxynitrous acid	$OH^{\bullet}$

3. MAJOR SIGNAL TRANSDUCTION PATHWAYS.

Signal transduction can be defined as the process converting extracellular stimuli to intracellular regulators, usually starting with a ligand binding to a plasma membrane receptor and ending with a physiologic event such as cell contraction. Generally speaking, a completed signal transduction system is composed of extracellular signals (being neurotransmitters or other

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substances), transducers (membrane receptors and coupling elements, such as guanosine triphosphate binding proteins - G proteins), and the second messengers such as cAMP. The best understood signal transduction mechanism is the one coupled with various G proteins.

G protein-coupled receptors, such as adrenergic receptors, AII receptors, and endothelin receptors, are all serpentine proteins that span the cell membrane seven times. Many of these receptors have been cloned and their multiple and diverse functions have been defined. In general, amino acid residues of these receptors in the third cytoplasmic loop, the one nearest to the C terminal, interact with G proteins. G proteins are heterotrimers containing α-, β-, and γ-subunits. G proteins are located at the inner leaflet of the plasma membrane, acting as a link between receptors and the enzymes that generate second messengers. Both the guanine nucleotide-binding site and the GTPase activity are associated with the α-subunit of G protein. When a ligand binds to a G protein-coupled receptor, the α-subunit releases GDP and binds GTP. The binding of GTP causes the dissociation of G protein trimer into a $G\beta\gamma$ and a $G\alpha$-GTP complex. In most cases, it is the $G\alpha$-GTP complex that brings about the biologic effects. There are a large number of different G proteins in mammalian cells. Gs and Gi ($Gi\alpha$1-3) groups couple with cAMP signal pathway. Gq and G11 groups activate phospholipase C ($PLC\beta$) - IP_3 pathway. The vasodilatory effects of atrial natriuretic peptide (ANP) and nitric oxide (NO), however, are believed to be due to the direct activation of the guanylyl cyclase-mediated cGMP signal pathway.

Various signal transduction pathways play different and specific roles in regulating the functions of the cardiovascular system, such as the regulation of cardiac output, blood vessel tone, and the proliferation of cardiac myocytes, vascular smooth muscle cells (SMCs), and endothelial cells (Griendling *et al.*, 1997; Schnabel & Böhm, 1996). Taking the blood pressure

control as an example, at least four important controlling mechanisms are involved. They are (1) the autonomic control with acetylcholine and norepinephrine (NE) as the main transmitters to modulate the vascular tone and cardiac contractility; (2) the neuro-humoral control with hormones such as angiotensin II (AII), vasopressin, kinins, ANP etc; (3) the endothelial control (endothelin versus NO); and (4) control mechanisms of the smooth muscle contractility. Each of these control mechanisms activates its corresponding signal transduction pathways and the summation of those inflences provides an integrated control of peripheral resistance and cardiac contractility.

It was postulated that alterations in signal transduction pathways, such as β-adrenoceptor desensitization, overexpression of Gi proteins and decreased cAMP formation, may be responsible for the impaired cardiovascular functions observed in hypertension (de Champlain *et al.*, 1998; Anand-Srivastava, 1996 & 1997; Böhm *et al.*, 1995; Meij, 1996).

2.1. Adenosine 3',5'-cyclic monophosphate (cAMP) pathway.

2.1.1. Signalling sequence.

The signal transduction of cAMP pathway starts when first messengers (extracellular ligands) bind to specific membrane receptors. Thereafter, the activated receptors associate with the corresponding G proteins, thus trigging their binding to GTP. Then, Gα subunit dissociates from the membrane receptor and from Gβγ subunits. This process is synchronized by the binding of Gα subunit to adenylyl cyclase (AC) that is located at the inner leaflet of the plasma membrane. The activated AC removes two phosphate groups from ATP and joins the "free end" of the remaining phosphate group to the sugar part of the ATP molecule, forming cAMP.

Downstream to cAMP formation is the activation of the cAMP-dependent protein kinase (PKA). The signal that is conveyed by this cAMP-mediated transduction pathway eventually induces cellular responses. The termination of the cAMP-mediated signal transduction is realized by the hydrolysis of GTP to GDP by Gα subunit. As such, the affinity of Gα subunit to AC is significantly reduced, and the trimeric G protein complex reforms. Finally, the extracellular ligand is dissociated from its membrane receptor to reset the signalling pathway back to the resting state.

2.1.2. G proteins and adenylyl cyclase.

It is well known that Gs protein stimulates and that Gi protein inhibits AC activity. Although these two G proteins have identical β and γ subunits, their α subunits are different. Cholera toxin induces ADP-ribosylation of Gsα, inhibiting the GTPase activity of this subunit. As a result, GTP cannot be hydrolyzed to GDP and AC is permanently activated by Gsα. Pertussis toxin, on the other hand, induces ADP-ribosylation of Giα, preventing GTP binding. Therefore, Giα cannot be activated and AC activity is not inhibited. Though acting on different G proteins, the net effects of cholera toxin and pertussis toxin result in an increased activity of AC.

Adenylyl cyclase is a membrane associated enzyme protein, containing 1100 amino acid residues. It is composed of two internal repeats of six transmembrane domains separated by an intracellular catalytic segment. Another intracellular catalytic segment is located near the C terminal (Alberts *et al.*, 1994). In mammalian tissues, at least 6 isoforms of AC have been identified. AC can be directly activated by the chemical forskolin (Hatjis, 1986). Under *in vivo*

conditions, AC activity is quickly reduced to the basal level when the association of the ligand with membrane receptor ceases.

Traditionally, the complex of βγ subunits of G protein (Gβγ) were thought to act merely as a lipid-bound docking site for the binding of Gα subunit. Since 1987, clear evidence shows that Gβγ also activates its own effectors. It has been reported that Gβγ increases the activity of PLC (Camps *et al.*, 1992), and stimulates phospholipase A_2 (Jelsema and Axelrod, 1987; Kim *et al.*, 1989). Gβγ can directly stimulate G protein-coupled receptor kinases, including the β-adrenoceptor kinase (βARK) (Clapham, 1996; Lefkowitz, 1993), a plasmalemmal calcium pump (Loteersztajn *et al.*, 1997), and phosphoinositide 3-kinase (Thomason *et al.*, 1994). Moreover, Gβγ activates the mitogen-activated protein (MAP) kinase since the reduced expression of Gβγ results in the inhibition of the Ras-dependent MAP kinase activation via a pertussis toxin-sensitive G protein (Luttrell *et al.*, 1997). It has also been reported that Gβγ subunits have regulatory effects on certain types of adenylyl cyclase since depending on the tissue type some forms of adenylyl cyclase can be inhibited, activated, or not affected, by Gβγ subunits (Tang and Gilman, 1991). Taken together, Gsα, Giα, and Gβγ subunits of G proteins are all involved in the regulation of AC.

2.1.3. Regulation of the cAMP pathway at the membrane receptor level.

Recent studies have demonstrated that a family of protein serine/threonine kinases is involved in the functional regulation of membrane receptors (Lefkowitz, 1993), namely the G protein-coupled receptor kinases (GRKs). GRKs rapidly desensitize the coupled receptors when these receptors are in an agonist-bound active conformation. Two representative prototypes of GRKs are β-adrenoceptor kinase (βARK) and rhodopsin kinase. Phosphorylation of β-

adrenoceptor by βARK promotes the binding of β-arrestin to β-adrenoceptor, preventing further activation of Gsα. The binding of β-arrestin to β-adrenoceptor also initiates the sequestration of the receptors, leading to their down-regulation (Lefkowitz, 1993). βARK, which carries no isoprenyl group, is activated by Gβγ (Inglese *et al.*, 1992). The γ-subunit is isoprenylated (or fanesylated) at its carboxyl terminal and thus might serve as an anchor for βARK (Schnabel & Böhm, 1996; Ishikawa, 1998). The discovery of βARK has thus provided a negative feedback mechanism for the G protein activation cycle. While Gsα released from G protein trimer complex by receptor activation stimulates AC, the free Gβγ dimer rapidly phosphorylates and desensitizes the receptor-G protein-effector complex.

2.1.4. Dynamic regulation of cAMP levels.

Cyclic AMP is a classical second messenger found in all prokaryotic cells. The rapid change in cAMP concentration, due either to the fast turn-on of cAMP formation or to the fast degradation of the formed cAMP, is one prerequisite for the cell that should quickly respond to extracellular signals. The half-life of the cAMP generated by AC action is short in the cytosol. It is rapidly and continuously decomposed to 5'-AMP in the presence of cAMP phophodiesterases (PDEs). Caffeine, theophylline, or isobutylmethylxanthine (IBMX) inhibits the activities of those PDEs. In addition, different PDEs have variable affinities to cGMP thus the intracellular cAMP levels could be regulated by cGMP through its effect on the activities of PDEs (Butt *et al.,* 1993; Lincoln & Cornwell TL, 1993).

2.1.5. Different combinations of ligand-receptor-G protein-AC-cAMP determine a specific cellular response.

Multiple extracellular ligands can regulate the cAMP-mediated signalling pathway in the same cell. A well-known example is the integrated effects of four hormones on cAMP pathway in adipocytes (Alberts *et al.*, 1994). The binding of adrenocorticotropic hormone (ACTH), epinephrine, glucagon, and thyroid-stimulating hormone (TSH) to their respective receptors at the surface of adipocytes activates Gs protein, increasing intracellular cAMP level. In this case, the actions of heterogeneous extracellular ligands converge to yield a homogeneous cellular response, i.e. the cAMP-mediated breakdown of triglyceride to fatty acid.

Heterogeneous ligands can also induce heterogeneous cellular responses by reciprocally regulating cAMP level in the same cell. Acetylcholine acts on muscarinic receptor (M_2) in cardiac muscle, stimulating Giα. Consequently, AC activity is reduced and intracellular cAMP level is lowered. This chain reaction underlies the cholinergic stimulation-induced bradycardia (Opie, 1998). β-adrenergic stimulation also affects intracellular cAMP levels in cardiac muscle. However, catecholamine binding to β-adrenoceptor (β_1) stimulates Gsα, activates AC, and increases cAMP levels. Consequently, the intracellular calcium levels are elevated and phospholamban is phosphorylated by cAMP, leading to increased cardiac contraction and tachycardia (Opie, 1998).

A third scenario also exists when the same ligand induces heterogeneous cellular responses in different cell types. This is largely ascribed to the presence of different membrane receptors for the same ligand in the different cells. As mentioned above, the binding of norepinephrine to β receptor in cardiac myocytes stimulates Gsα, leading to an increase in cAMP levels but the same norepinephrine decreases cAMP levels when it binds to α_2 receptor in platelet and stimulates Giα (Heufelder *et al.*, 1985). Different combinations of ligand-receptor-G protein-AC-cAMP in different cell types determine the specificity of cellular responses.

2.1.6 PKA is mainly responsible for the cAMP-mediated cellular responses.

Being water-soluble, cAMP can easily gain access to cell membrane, cytosol or the nucleus, where it can directly affect ion channel functions and protein kinase activities. PKA is the major intracellular target of cAMP (Alberts *et al.*, 1994). The inactive PKA is a tetramer, composed of two catalytic subunits and two regulatory subunits. The binding of cAMP to the regulatory subunits releases two active catalytic subunits from the tetramer. Such activated PKA phosphorylates various cytosolic and membrane proteins by transferring the terminal phosphate groups of ATP to specific serines or threonine of selected proteins. The activated PKA causes many cellular responses in various types of cells, depending on the cell type-specific distribution of different proteins. The PKA-mediated cellular responses are exemplified by cortisol secretion from adrenal cortex, glycogen breakdown in muscle and liver, triacylglycerol breakdown in adipose tissue, secretion of 17β-estradiol from ovarian follicle, bone resorption, thyroxin secretion from thyroid, and water resorption from the kidney (Alberts *et al.*, 1994).

The first line of evidence showing the presence of AC in vascular smooth muscle cells was obtained in 1962 by Klainer *et al.*. Studies in the following decades clearly demonstrated the importance of cAMP pathway in the regulation of vascular smooth muscle contractility (Bolton, 1979). The stimulation of β-adrenoceptors (β_2 sub-type) in vascular SMCs increases AC activity, elevates cAMP level, activates PKA, and decreases smooth muscle contraction force. The cellular response of SMCs to increased cAMP-PKA activity is opposite to that of cardiac myocytes since the activation of β-adrenergic (β_1 adrenoceptors) in cardiac myocytes results in a positive inotropic reaction via the activated PKA. Therefore, the same increase in cAMP levels induces opposite functional responses in cardiomyocytes and vascular SMCs

(Kawada *et al.*, 1997; Opie, 1998). The opposite cAMP effects have been ascribed to the different substrates of PKA in these different tissues. The vasodilatory effect of cAMP is mediated by the inhibition of the phosphorylation of myosin light chain kinase, which hinders the actin-myosin interaction (Bennett & Waldman, 1995). This kinase is essential for the initiation of smooth muscle contraction, but is not a prerequisite for cardiac muscle contraction, in which the actin-myosin interaction is regulated by troponin complex. In contrast, the major substrates of PKA in cardiac myocytes are calcium channels in plasma membrane and phospholamban in the sarcoplasmic reticulum (SR) membrane. The phosphorylation of the former enhances the extracellular calcium entry to produce positive inotropic effect, and the phosphorylation of the latter promotes the calcium re-uptake into SR, leading to lusitropic effect, i.e. an increase in the rate of relaxation (Opie, 1998).

In opposition to the above hypothesis, many laboratories have shown that cAMP has opposite effects on the same substrate in cardiac myocytes and vascular SMCs. For instance, it was reported that cAMP inhibits L-type calcium channel currents in vascular SMCs but stimulates them in cardiac myocytes (Wang *et al.*, 1991). In addition, a "cross-activation" of cGMP-dependent protein kinase (PKG) by cAMP was suggested. Francis *et al.* (1988), Lincoln *et al.* (1990), and Jiang *et al.* (1992) originally put this theory forward to explain the relaxation of vascular smooth muscle by cAMP. Because the affinity of PKG for cAMP is relatively high and the levels of cAMP usually exceed those of cGMP in vascular tissues, it is theoretically possible that cAMP might activate PKG. On the other hand, Cornwell *et al.* (1994) have shown that the growth inhibitory actions of NO in adult rat aortic SMCs are due at least in part to the activation of PKA by cGMP. These findings clearly indicate that each cyclic nucleotide is capable of activating both cyclic nucleotide-dependent protein kinases in the intact cell. The activation of

PKA would increase extracellular calcium entry, while the activation of PKG would decrease intracellular calcium concentration. It has been postulated that in vascular SMCs the predominant effect of cAMP is mediated by PKG (Lincoln *et al.*, 1990), whereas in cardiac myocytes the elevated cAMP levels would mainly activate PKA since PKG activity is low in that tissue (Jiang *et al.*, 1992).

The mechanisms underlying the reciprocal effects of cAMP-PKA on the contractile status of cardiac myocytes and vascular SMCs are still being debated. Fig. 1-2 summarizes the potential interactions of the cAMP- and cGMP-dependent pathways in vascular SMCs.

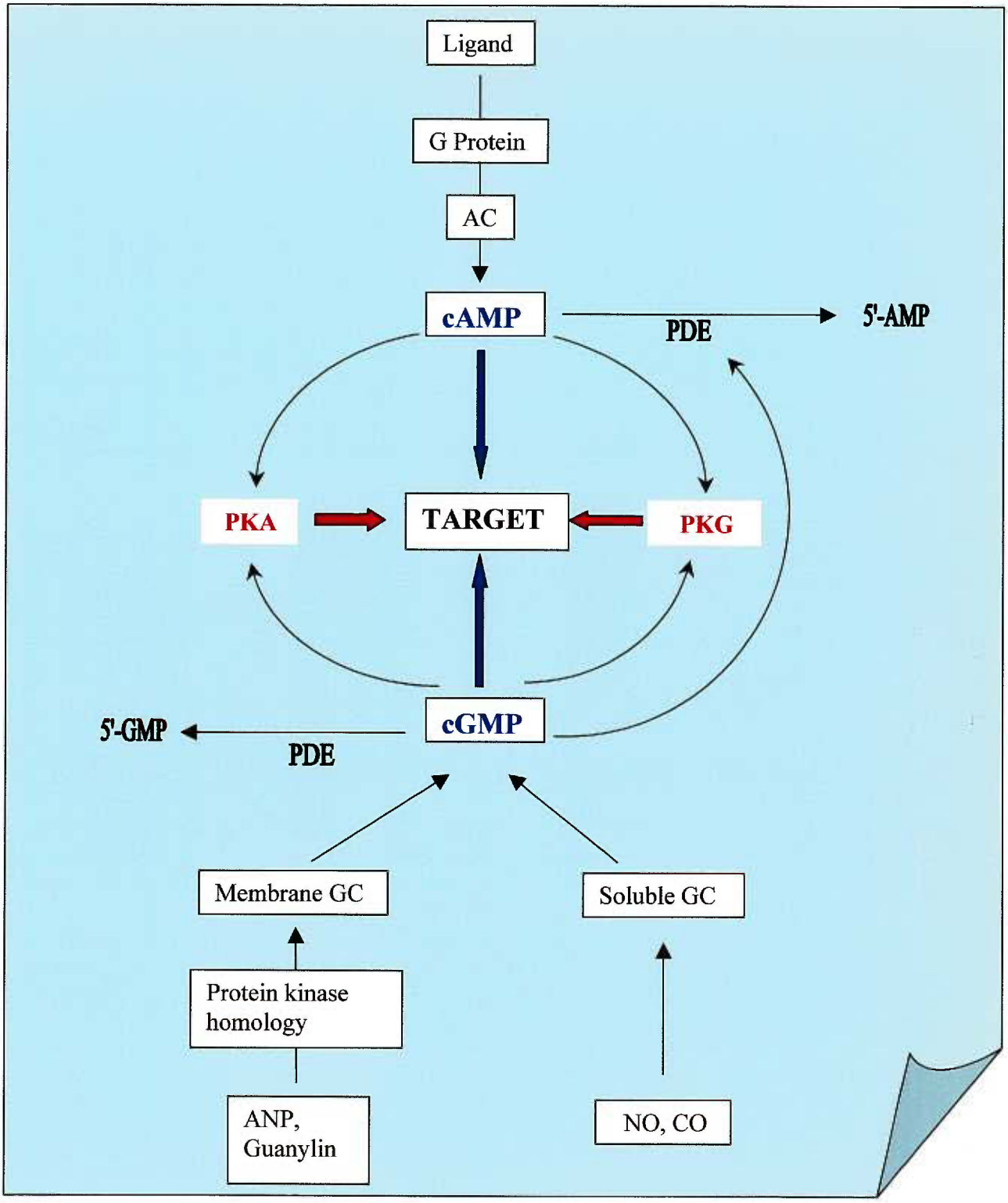

Fig. 1-2. Signal transduction in vascular SMCs mediated by the cAMP- and cGMP-dependent pathways. PDE, Phosphodiesterase.

L. Wu, Superoxide anion as a signalling molecule.

2.2. *Guanosine 3',5'-cyclic monophosphate (cGMP) pathway.*

2.2.1. Basic signalling elements.

Similar to the cAMP pathway, the ligand-receptor binding and the activation of G proteins are required for the activation of the cGMP pathway. A cholera and pertussis toxin-sensitive G protein, Gt or transducin, is involved in the activation of this pathway in vertebrate rod photoceptors (Alberts *et al.*, 1994). Other activating molecules such as NO for this pathway, however, do not involve specific membrane-bound G proteins. The synthesis of cGMP is catalyzed by guanylyl cyclase (GC) and cGMP is degraded by cGMP PDE. The biological events mediated by the cGMP signalling pathway result from the activation of many substrates of cGMP, of which the most important is cGMP-dependent protein kinase (PKG).

2.2.2. Activating molecules for cGMP synthesis.

Chronologically, the first identified modulating signal for cGMP pathway was the photon from the light. In this process, the absorption of photons by rhodopsin molecules was found to activate cGMP PDE which resulted in a fall, rather than a rise, in the cellular cGMP levels (Alberts *et al.*, 1994). As a matter of fact, NO and ANP are the best known natural stimulators for cGMP synthesis.

NO is synthesized from arginine in a reaction catalyzed by nitric oxide synthase (NOS) inside the cells. Two general classes of NOS have been identified: a constitutive (cNOS) form, which is stimulated by Ca^{2+}/calmodulin, and an inducible form (iNOS) whose expression is upregulated by cytokines and endotoxins. Three isoforms of NOS have been identified. NOS 1 is found in neurons; NOS 2 in macrophases and other immune cells; and NOS 3 in endothelial cells. NOS 3 is a Ca^{2+}-calmodulin-NADPH dependent enzyme and is activated by an increase in

intracellular Ca^{2+} concentration in response to vasodilators such as acetylcholine and bradykinin, or to shear stress acting on the cell membrane (Stoclet *et al.*, 1998). NO synthesized in the endothelium diffuses to vascular SMCs, where it activates soluble guanylyl cyclases (sGC), producing cGMP. The NO-induced vasorelaxation is mediated by cGMP through multiple mechanisms. These include the activation of myosin light chain phosphatase resulting in the desensitization of SMCs to Ca^{2+} (Lee 1997, Brophy *et al.*, 1997), the activation of K channels and membrane hyperpolarization, a decrease in extracellular Ca^{2+} entry and intracellular Ca^{2+} release, and an increase in Ca^{2+} extrusion. The vasorelaxation may also result indirectly from the inhibition of type II cyclic nucleotide phosphodiesterase (PDE-II) by cGMP, which could induced a rise in cAMP levels (Butt *et al.*, 1993; Lincoln & Cornwell, 1993).

NO is not entirely responsible for the endothelium-dependent vasorelaxation, depending on the vessel type and agonists used to activate the vasorelaxing mechanisms. In rat aorta, the endothelium-dependent vasorelaxation produced by an agonist such as acetylcholine is abolished by NOS inhibitors, indicating that it is entirely or almost entirely mediated by NO release. In contrast, NO only partially contributes to the relaxing role of endothelium in other types of blood vessels. For instance, the dilatation of human distal epicardial coronary artery in response to acetylcholine is NO-dependent, whereas the vasodilation of coronary resistive vessel is not NO-dependent (Lefroy *et al.*, 1993). Human dermal artery vasodilatation in response to acetylcholine seems to be mediated predominately by a dilator prostanoid rather than by NO generation since the NOS inhibitor, L-N(G)-monomethyl-arginine (L-NMMA), did not inhibit the vasodilation whereas the cyclooxygenase inhibitor, aspirin, did (Noon *et al.*, 1998). Moreover, a portion of the non-NO-dependent bradykinin-induced relaxation of human isolated small coronary arteries appears to be mediated by an endothelium-derived hyperpolarizing factor

(EDHF) (Kemp & Cockes, 1997). EDHF was also found to be responsible for the relaxation induced by acetylcholine and by the calcium ionophore A23187 of the guinea-pig basilar artery, since the relaxation was not completely inhibited by the NOS inhibitor, NG-nitro-L-arginine (L-NOARG) (Petersson *et al.*, 1997).

Unlike NO, ANP cannot freely diffuse across cell membrane. Its effect on cGMP formation is due to the activation of two types of ANP receptors, ANP-A and ANP-B. These receptors span the cell membrane and have cytoplasmic guanylyl cyclase domains that can directly initiate cGMP formation. ANP receptors are widely distributed in different tissues including vascular smooth muscle and glomeruli. The rise in ANP secretion is proportional to the degree of stretching the atria by an increase in central venous pressure. The activation of the third type of ANP receptors, ANP-C, does not involve cGMP formation. It was suggested that ANP-C may act via G proteins to activate PLC and inhibit AC (Anand-Srivastava, 1997). It is worth noting that the biological effects of ANP are generally opposite to those of AII, and indeed, the effects of ANP are most readily demonstrated against a background of stimulation by AII.

Guanylin and uroguanylin (Kita *et al.*, 1994; Forte *et al.,* 1993) are newly discovered endogenous stimulators for cGMP synthesis. These peptides stimulate membrane GC and regulate intestinal and renal functions via cGMP. Receptor autoradiography localized the receptors for uroguanylin and guanylin in renal proximal tubules and seminiferous tubules of testis. Synthetic guanylin and uroguanylin peptides were found to activate the receptor-GCs in opossum kidney cortex, eliciting the increase in intracellular cGMP level (Fan *et al.*, 1997).

2.2.3. Guanylyl cyclase (GC).

Two types of GCs exist: soluble GC and membrane GC (Drewett & Garbers, 1994; Garbers & Lowe, 1994). Soluble GCs are expressed in almost all cell types in the cardiovascular system, including cardiomyocytes, vascular SMCs, EC and platelets (Drewett & Garbers, 1994). Soluble GC functions as a heterodimer composed of an α and a β subunits. At least two α and β isoforms were identified by molecular cloning (Drewett & Garbers, 1994). Soluble GC contains a prosthetic heme group, which functions as a receptor for NO and carbon monoxide, the putative physiological activators (Wang, 1998). Membrane GC combines both ligand binding and catalytic activity in a single poly-peptide chain which consists of a N-terminal extracellular receptor domain, a transmembrane segment, a domain homologous to protein kinase, and a C terminal catalytic domain (Drewett & Garbers, 1994; Garbers & Lowe, 1994). Six isoforms of membrane GCs have been cloned.

2.2.4. Multiple targets of cGMP.

The regulatory function of cGMP is accomplished by its interaction with various substrate proteins. They include PKG, PKA, cGMP-dependent PDEs, and other targets such as ion channels (Vanandrager & de Jonge, 1996). The present review will focus on the cGMP–PKG signalling transduction pathway.

2.2.5. The PKG-mediated vasorelaxation.

PKG belongs to the large superfamily of protein kinases. These enzymes regulate the activity of numerous proteins by catalyzing the transfer of the γ-phosphoryl group of ATP, to the hydroxyl group of serine, threonine, or tyrosine residues of an acceptor substrate protein. Similar to PKA, PKG phosphorylates serine and threonine residues. The selective agonists of

PKG are 8-bromo-cGMP and 8-parachlorophenylthio-cGMP (8-pCPT-cGMP). PKG-specific antagonists are Rp-8-bromo-cGMPS and Rp-8-pCPT-cGMPS.

To date, only two mammalian isoforms of PKG have been identified. PKG-I, consisting of an α and a β isoform, is the most prominent PKG isoform in the cardiovascular system. PKG-II is mainly expressed in intestine, kidney and brain. High levels of PKG-I are found in vascular SMCs, EC, and platelets. In these cells, the function of PKG-I is thought to counteract the increase in contraction provoked by Ca^{2+}-mobilizing agonists, to reduce endothelial permeability, and to inhibit platelet aggregation (Butt *et al.*, 1993), respectively. Recent patch-clamp study showed that PKG-II, but not PKG-I, was able to activate CFTR (cystic fibrosis transmembrane conductance regulator)-Cl channel in excised membrane patches (French *et al.*, 1995). PKG-I and PKG-II also differ in cellular and subcellular distribution. PKG-I is predominantly a cytosolic protein, but may be targeted to specific anchor proteins of the cytoskeleton, e.g. vimentin. PKG-II is tightly bound to the plasma membrane by both hydrophobic interaction and by its association with the cytoskeleton.

The relaxation of SMCs induced by PKG bears more relevance to my current study. The cGMP-PKG -induced vasorelaxtion is largely related to the reduction of intracellular calcium concentration of vascular SMCs. The following mechanisms have been proposed to underlie the vasorelaxing effect of PKG (Butt *et al.*, 1993; Lincoln *et al.*, 1994; Lincoln & Cornwell, 1993; Hofmann *et al.*, 1992; Francis & Corbin, 1994).

a) *Inhibition of the PLC-mediated generation of IP_3 formation*: The target of PKG in this process is not clear but might be the G protein that couples the hormone receptor to PLC. Recent study has shown that PKG inhibits the thrombin-induced increase in IP_3 formation and involves in regulation of intracelluar calcium level through the phosphorylation of Giα (Pfeifer *et al.*,

1995). Thus, it suggests that in addition to the classic Giα-coupled AC-cAMP signalling pathway, Giα also involves in the PKG-mediated the modulation of IP3 signalling pathway (Also see Section 2.5 in this chapter).

b) *Activation of Ca^{2+}-ATPase in the plasma membrane as well as in ER membrane*: Phosphorylation of phospholamban might play a role in the PKG-mediated modulation of the Ca^{2+}-ATPase in the ER. Direct activation of Ca^{2+}-ATPases by PKG would increase Ca^{2+} re-uptake into ER or extrusion from the cytoplasm (Lincoln & Cornwell, 1991).

c) *Activation of Ca^{2+}-activated K channels*: PKG causes a hyperpolarization of the cell membrane by activating the Ca^{2+}-activated K channels. This hyperpolarization inhibits Ca^{2+} influx through voltage-operated Ca^{2+} channel. PKG was also suggested to activate the K channels directly by phosphorylation (Alioua *et al.*, 1995) or indirectly by activating a phosphatase, which subsequently activates the channel (White *et al.*, 1993).

d) *Decrease in $[Ca^{2+}]_i$*: Several lines of evidence have indicated that the effects of cGMP were through activation of PKG, leading to a decrease in $[Ca^{2+}]_i$ and relaxation of vascular SMCs (Cornwell & Lincoln, 1989; Lincoln & Cornwell, 1991). More specifically, it was reported that PKG inhibited the function of IP_3–sensitive calcium releasing channels.

e) *Decrease in the Ca^{2+}-sensitivity of the contractile proteins*: PKG stimulates the dephosphorylation of myosin light chain through activation of a phosphatase (Lincoln *et al.*, 1994). Using aequorin as a Ca^{2+} indicator, Defeo and Morgan (1985) demonstrated that changes in mechanical tension of vascular smooth muscles produced by vasoconstrictors were not always correlated with changes in $[Ca^{2+}]_i$. In addition, it was reported that the relaxation of vascular smooth muscles induced by nitroglycerin (Yanagisawa *et al.*, 1989), or sodium nitroprusside (SNP) (Karaki *et al.*, 1988) was not proportional to the reduction of $[Ca^{2+}]_i$. These findings

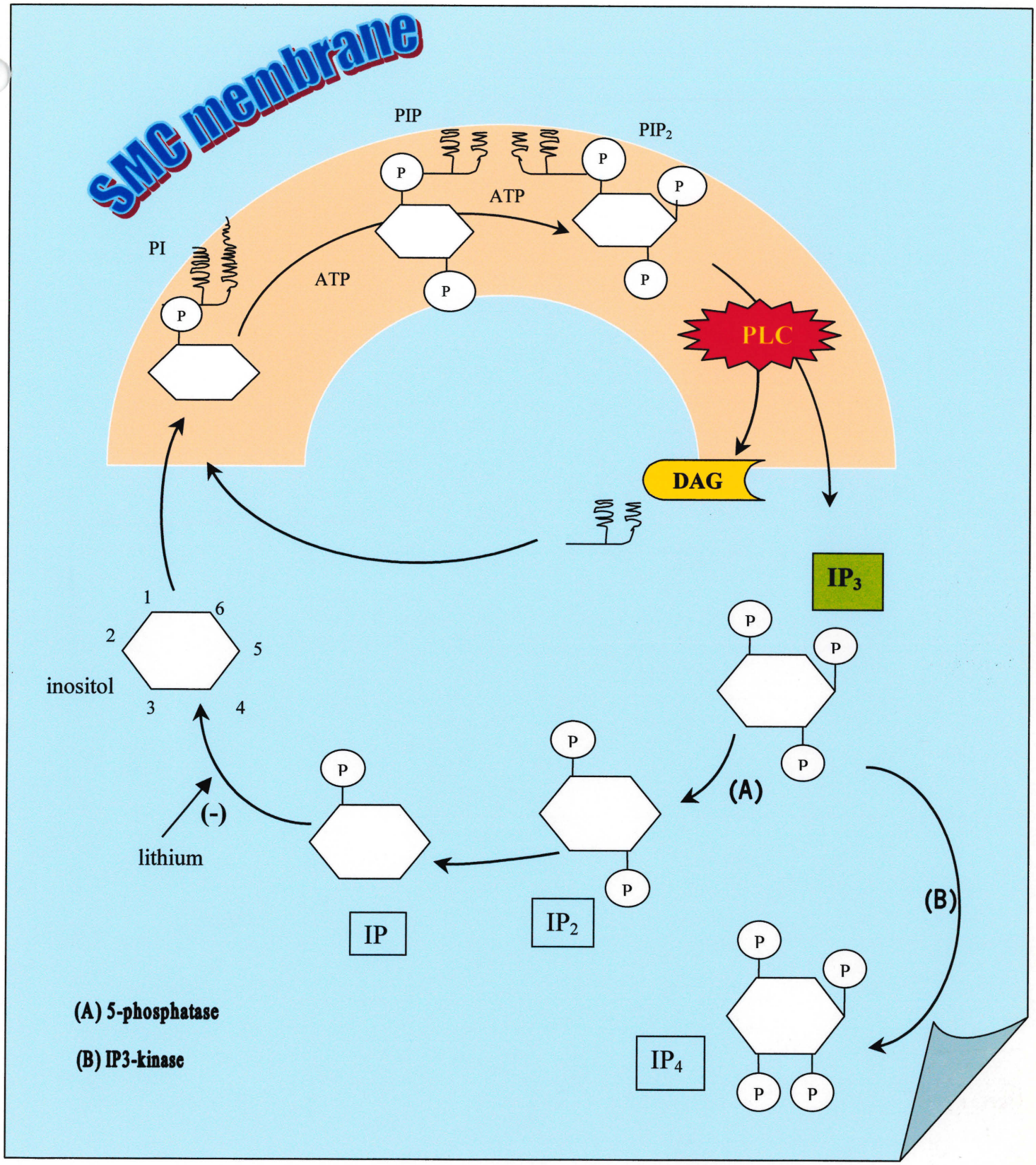

Fig. 1-3. Phosphatidylinositol cycle in vascular smooth muscle cells.

L. Wu, Superoxide anion as a signalling molecule.

Fig. 1-3. Phosphatidylinositol cycle in vascular smooth muscle cells.

2.3. Phospholipase C (PLC) pathway.

2.3.1. IP_3 is generated through the activation of G protein-coupled PLCβ and tyrosine kinase-coupled PLCγ.

The selective ligand-receptor interaction at the plasma membrane surface activates phospholipase C (PLC) that catalyzes the hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP_2) to form IP_3 and diacylglycerol (DAG) (Fig. 1-3). Three PLC isoforms (β, γ, and δ) have been identified in mammalian tissues (Rhee, 1994), PLCγ in rat vascular SMCs (Schmitz *et al.*, 1997), PLCβ and PLCγ in human aortic SMCs (Schelling *et al.*, 1997), and PLCδ in rat aorta (Kato *et al.*, 1992). PLCβ is activated by the receptor-coupled Gq, G11 (Rhee & Choi, 1992), or Go proteins (Alberts *et al.*, 1994), whereas PLCγ is activated when its tyrosine residues are phosphorylated by tyrosine kinase (Rhee & Choi, 1992). It is clear now that binding of certain ligands to receptors, such as norepinephrine (NE) to α_1-adrenoceptors and endothelin to its ET_A receptors, activates Gq, leading to activation of PLCβ. Interestingly, since angiotensin II type 1 (AT_1) receptors also contain intracellular tyrosine kinase domains, AII increases the formation of IP_3 via both the Gq-coupled PLCβ pathway and the tyrosine kinase-mediated PLCγ pathway. Whether the activation of PLC_δ elicits IP_3 production is still presently unsettled.

2.3.2. DAG and IP_3.

Two signalling molecules (DAG and IP_3) generated from the hydrolysis of PIP_2 have different intracellular targets, and induce different cellular reactions. DAG activates protein

kinase C (PKC) that increases the synthesis of *c-fos* mRNA, phosphorylates various substrate proteins, activates AC (Komalavilas & Lincoln, 1996), increases the activity of the Na^+/H^+ exchanger and activates myosin light chain kinase via the inhibition of myosin light chain phosphatase. PKC also serves as a negative feedback regulator of PLC activity, at least in part, for terminating the PLC response (Griendling *et al.*, 1997). IP_3 binds to IP_3 receptors which are located on endoplasmic reticulum (ER) membranes and function as the intracellular calcium releasing channel. This channel belongs to the superfamily of ionotropic receptors as its opening leads to the release of Ca^{2+} from ER. Increased cytosolic concentration of free Ca^{2+} activates calmodulin and subsequently increases the activity of myosin light chain kinase, resulting in vascular constriction.

2.3.3. IP_3 receptor subtypes.

The functional IP_3 receptor is homotetramerized by four identical IP_3 receptor proteins to form a Ca^{2+} conducting pore (Hille, 1992). At least three subtypes of mammalian IP_3 receptors have been cloned, to date. Different genes, designated as type 1, type 2, and type 3, encode these IP_3 receptors. Since type 1 IP_3 receptor is the best characterised and an ubiquitously expressed subtype, originally purified from cerebellum by Furuichi *et al.* (1989), the molecular structure and the functional regulation of this subtype will be reviewed here as a representative of IP_3 receptors. Type 1 IP_3 receptor is composed of 2,794 amino acids (313 kD) (Furuichi *et al.*, 1989; Mignery *et al.*, 1990). The whole molecular sequence can be divided into three segments. The NH_2-terminal segment accounts for approximately 24% of the whole sequence, which is the site for ligand binding. The COOH-terminal segment (about 60% of the whole sequence) functions as the Ca^{2+} releasing channel, which has 6 transmembrane-spanning domains. In between is the

coupling or modulatory segment (Mignery & Sudhof, 1990). The overall structural organization is similar for all 3 subtypes of IP_3 receptors. A 68% homology in amino acid composition in the ligand-binding segment, 53% in the coupling segment, and 59% in the Ca^{2+} releasing channel segment are found among these subtypes of IP_3 receptors (Blondel *et al.*, 1993). Clearly, the ligand-binding segment is the most conserved region as opposed to the least conserved coupling segment. This fact provides the molecular basis for the selective modulations of different subtypes of IP_3 receptors. The molecular complexity of IP_3 receptors is further indicated by the presence of alternative splicing variants of type 1 IP_3 receptors, but not of type 2 or type 3 receptors (Newton *et al.*, 1994). One splicing variant of type 1 IP_3 receptor is termed S1, of which 15 amino acids are altered in the NH_2-terminal ligand-binding segment. Another splicing variant (S2) has 40 altered amino acids in the coupling segment. The expression of these variants is probably cell type- and species-dependent.

2.3.4. Regulation of IP_3 receptor.

Specific gating mechanisms: Two mechanisms are responsible for the termination of the activation of IP_3 receptor. (1) IP_3 is rapidly dephosphorylated (and thereby inactivated) by specific phosphatases, and (2) Ca^{2+} that enters the cytosol is rapidly removed. Some IP_3 are phosphorylated to form inositol 1,3,4,5-tetrakisphosphate, which may mediate slower and more prolonged cellular responses or promote the refilling of the intracellular Ca^{2+} stores from the extracellular fluid, or both (Alberts *et al.*, 1994).

Modulation by cytoplasmic Ca^{2+}: Cytoplasmic Ca^{2+} at physiological levels have a biphasic effect on the IP_3-induced Ca^{2+} release, both activation and inactivation. The inhibitory effect is assumed to be due to the binding of Ca^{2+} to some associated proteins and the stimulatory effect is probably induced by the direct binding of Ca^{2+} to the IP_3 receptor protein (Callamaras & Parker, 1994). Two important issues are related to the biphasic effect of cytosolic Ca^{2+} on IP_3

receptors. First, the Ca^{2+}-dependent activation of IP_3 receptors with a cytoplasmic Ca^{2+} concentration less than 300 nM suggests that the IP_3 receptor, like the ryanodine receptor, can function as a Ca^{2+}-induced Ca^{2+} releasing channel. Second, the biphasic regulation of IP_3 receptors by cytoplasmic Ca^{2+} is an essential mechanism for the generation of Ca^{2+} waves and oscillations in many types of cells.

Modulation by ATP: The activities of the IP_3 receptors can be increased by ATP, due to the increased single channel open probability and mean open time (Yoshida & Imai, 1997).

Modulation by phosphorylation: The phosphorylation of IP_3 receptors by PKA and PKG has been demonstrated. Either an inhibition or a stimulation of the IP_3-induced Ca^{2+} release in response to the phosphorylation of IP_3 receptors by PKA and PKG can occur, depending on the tissue and preparations. In intact SMCs, PKG is mainly responsible for the phosphorylation of the IP_3 receptors in response to increased cAMP and cGMP (Komalavilas & Lincoln, 1996). The IP_3 receptor is also the substrate for PKC, calmodulin-dependent protein kinase, and tyrosine kinase as well (Yoshida & Imai, 1997).

Pharmacological tools for studying IP_3 receptor: Heparin is a potent competitive antagonist of IP_3 receptors. It inhibits IP_3 binding as well as the activation of the IP_3 receptors. However, heparin may also inhibit the formation of IP_3 and activate ryanodine receptors. Two newly discovered IP_3 receptor agonists are adenophostin A and B, which are 10 to 100-fold more potent than IP_3 in activating IP_3 receptors (Yoshida & Imai, 1997; Missiaen *et al.*, 1998).

2.4. Tyrosine kinase pathway.

The tyrosine kinase pathway may play an important role in the phosphorylation of a variety of intracellular proteins, the activation of proto-oncogenes, the regulation of gene transcription (Glenney, 1992), and the regulation of smooth muscle contractility. At the cell

membrane level, the binding of high-affinity ligands to receptor tyrosine kinases activates the tyrosine kinase located within a cytoplasmic domain of the receptor protein. Consequently, the receptor is autophosphorylated and certain intracellular target proteins are phosphorylated. Cytosolic tyrosine kinase activities have also been demonstrated in addition to membrane-associated tyrosine kinases (Alberts *et al.*, 1994).

2.4.1 Stimulatory signals for the tyrosine kinase pathway.

Many growth factors, such as platelet-derived growth factor (PDGF), EGF, transforming growth factor-β (TGF-β), and insulin, directly activate tyrosine kinase receptors (Alberts *et al.*, 1994; Opie, 1998). For instance, EGF mediates three tyrosine kinase-dependent smooth muscle response paradigms, two of which comprise a rapid increase in muscle tension and one of which is characterized by an agonist-mediated reduction in sensitivity to other agents (Hollenberg, 1994).

Some hormones, including AII, adrenaline and vasopressin, act on both tyrosine kinase-linked and G protein-linked signalling pathways (Fig. 1-4). Regarding the tyrosine kinase-linked pathway, AII has been shown to phosphorylate and activate pp60src (Linseman *et al.*, 1995; Ishida *et al.*, 1995) which inturn activates several other molecules involved in intracellular signalling, including PLC-γ, pp120, p125 ^{FAK}, paxillin, JAK2, STAT1, Gα and caveolin (Marrero *et al.*, 1995; Li *et al.*, 1996; Schieffer *et al.*, 1996). Upon phosphorylation, STATs form dimers that associate with p48 and are translocated to the nucleus where they activate gene transcription (Marrero *et al.*, 1995). It seems that AII activates multiple tyrosine kinase-linked pathways in vascular SMCs that, in analogy to other growth factors, are likely to be important in mediating the growth effects of AII.

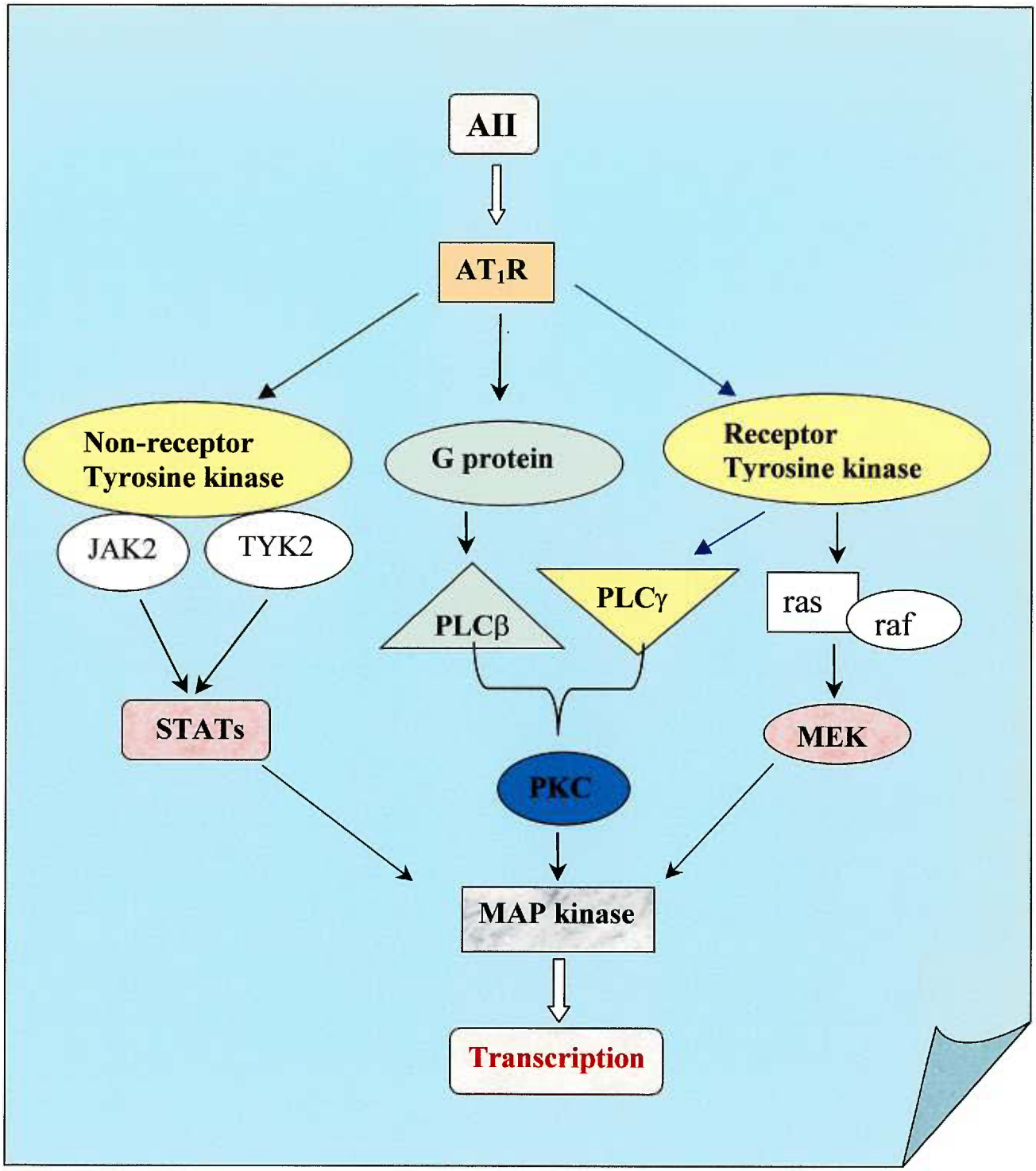

Fig. 1-4. The MAP kinase pathway activated by AII. JAK, Janus kinase; MEK, MAP kinase kinase; STAT, Signal transducer and activator of transcription.

L. Wu, Superoxide anion as a signalling molecule.

2.4.2. Subgroups of tyrosine kinases.

The first group of tyrosine kinases is constituted by membrane receptor tyrosine kinases including the insulin receptor and the receptors for EGF and PDGF. The intracellular segment of those receptor proteins contains intrinsic tyrosine kinase activity. The second group is constituted by cytosolic nuclear non-receptor protein tyrosine kinases such as the protooncogenes, Abl and Fes. These kinases do not possess intrinsic tyrosine kinase activity, but could rapidly recruit cytoplasmic tyrosine kinase (e.g. the so-called JAK-kinases) into the signal transduction pathway (Schlessinger & Ullrich, 1992; Miyajima *et al.*, 1992). The third group is constituted by membrane-associated non-receptor tyrosine kinases and is related closely to $p60^{v\text{-}src}$.

2.4.3. Intracellular effectors of tyrosine kinases.

2.4.3.1. Phospholipase Cγ (PLCγ).

PLCγ is activated when its tyrosine residues are phosphorylated by tyrosine kinases (Rhee & Choi, 1992). The activation of PLC_γ catalyzes the hydrolysis of PIP_2 to form inositol IP_3 and DAG. IP_3 serves as an intracellular signal to release calcium from IP_3-sensitive calcium pools, leading to an enhanced vascular contractility, while DAG activates PKC that involves in the modulation of different cellular activities (see 2.3.2.).

2.4.3.2. Mitogen-mediated protein kinase (MAP-kinase).

The MAP kinase cascade is the best-studied effector pathway for receptor tyrosine kinases. MAP kinases are a group of serine/threonine enzymes that function to phosphorylate serine/threonine residues of proteins in a sustained fashion. The major biological events related to this family of kinases are cell growth, differentiation, and cell transformation (Glenney, 1992;

Opie, 1998). Some MAP kinases can be activated by tyrosine kinase receptors (Hollenberg, 1994), while others can be activated by G-protein-coupled receptors (Alberts *et al.*, 1994). In addition to its role as a negative feedback regulator of PLC activity, PKC activity is also essential for MAP kinase pathway. Situated in the cytosol when inactive, PKC responds to DAG by translocation to the plasma membrane and thereby becomes active. It then initiates a signalling sequence, leading to the activation of MAP kinases. The MAP kinase pathway is thus responsible for transmitting the signal of tyrosine kinase and PLCβ coupled receptor activitions to the nucleus, influencing transcription and stimulating cell growth and proliferation.

2.4.3.3. Phosphatidylinositol-3' kinase (PI-3 kinase).

PI-3 kinase is another important effector pathway of receptor tyrosine kinases. The activation of PI-3 kinase generates phospholid products that bind to and stimulate protein kinase B (PKB) (Saltiel, 1996). PKB is related to PKA and PKC due to the partial homology in their amino acid sequences. One identified function of PKB is to inhibit cellular apoptosis, promoting cell survival (Frank *et al.*, 1997).

2.5. The cross-talk between cAMP- / cGMP-signalling pathways and IP_3 signalling pathway.

Cross-talk interactions among different signal transduction pathways have been postulated to be ubiquitous regulating mechanisms. To give a more focused and concise literature review in relation to the present studies, mainly the cross-talk between cAMP/cGMP transduction pathways and IP_3 signalling pathway will be reviewed here.

The IP_3 receptor is known to be phosphorylated by several kinases *in vitro,* including PKA (Ferris *et al.*, 1991a), PKC, Ca^{2+}-calmodulin-dependent protein kinase II (Ferris *et al.*, 1991b), and tyrosine kinases (Harnick *et al.*, 1995). Concerning the phosphorylation of the IP_3 receptor, it should be noted that few studies have demonstrated the phosphorylation of IP_3 receptor in intact cell in response to second messenger-activated pathways. Studies using a confocal laser scanning microscope to determine the cellular distribution of PKG have demonstrated that this enzyme is located in the SR (Cornwell *et al.,* 1991) where both phospholamban and the IP_3 receptor are located. The effects of cGMP and PKG on the phosphorylation of the IP_3 receptor have been examined in intact rat aorta. Aortas treated with the NO donors, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), SNP, or the selective PKG activator, 8-CPT-cGMP, exhibited increased IP_3 receptor phosphorylation *in situ.* The treatment of aortas with the adenylyl cyclase activator, forskolin was also found to increase phosphorylation of the IP_3 receptor on the PKG site, although the selective cAMP-dependent protein kinase activator, 8-CPT-cAMP, did not increase the phosphorylation of the IP_3 receptor. The PKG selective inhibitor KT 5823 was shown to inhibit both SNP- and forskolin-induced IP_3 receptor phosphorylation more potently than the selective PKA inhibitor, KT 5720. These results thus indicate that it is PKG that mediates both cAMP- and cGMP-induced increase in IP_3 receptor phosphorylation in intact aorta (Komalavilas & Lincoln, 1996). However, it is worth noting that this issue may be controversial since not all researchers agree on the phosphorylation of IP_3 receptors by PKG. In a study carried by Pfeifer *et al.* (1995), using Chinese hamster ovary cells transfected with cDNAs encoding PKG, no phosphorylation of IP_3 receptors by PKG was observed.

Few studies have also indicated that the metabolism of IP_3 may be modulated by cGMP and by PKG. Lang & Lewis (1991) have shown that the IP_3 formation can be inhibited by cGMP in cultured aortic endothelial cells of the pig since SNP and ANP as well as 8-bromo-cGMP induced a significant inhibition of the IP response following its activation by thrombin.

3. EFFECTS OF ROS ON DIFFERENT SIGNAL TRANSDUCTION PATHWAYS.

ROS actively participate in the regulation of different signal transduction mechanisms (Suzuki *et al.*, 1997; Droge *et al.*, 1994). Some types of ROS increase the cytosolic free Ca^{2+} concentration, while others either stimulate tyrosine as well as serine/threonin phosphorylation, activate protein kinases and inhibit protein phosphatases, or act on the effector molecules such as phospholipase A_2 or on oxidative stress-responsive transcription factors. Through the modulation of various signal transduction pathways, ROS function as signalling molecules to regulate transcription factor activation, apoptosis, bone resorption, cell growth, chemotaxis, and cell contraction.

For example, hydroxyl radicals which are extremely active oxidants are known to cause protein oxidation, damage DNA, and alter $[Ca^{2+}]_i$ (Opie, 1998; Suzuki *et al.*, 1997). To add to the long list of the examples of the ROS-related cellular damage it is necessary to consider the peroxidation of membrane lipids, which commonly leads to formation of hydroperoxide as the primary product. Hydrogen peroxide not only produces species that inactivate proteins but also alters membrane physical properties, due at least in part to changes in phospholipid orientational distribution and dynamics, which may significantly change the functions of membrane-spanning ion channels.

In addition, a special attention should be paid to the effects of oxidative stress on the modulation of calcium signalling. Among many theories to explain the development of ischemia-reperfusion heart damage the most plausible one involves the cytosolic calcium overload that is closely related to ROS over-production (Opie, 1998). ROS may play a major part in calcium overloading by 1) inducing lipid oxidation to alter the integrity of biological membrane; 2) damaging mitochondrial integrity to release calcium; and 3) activating calcium release channels of SR with enhanced release of calcium. ROS can also inhibit the uptake of calcium by SR, inhibit the sodium pump, stimulate the Na^+/Ca^{2+} exchanger (Reeves *et al.*, 1986), and decrease the rate of inactivation of the inward calcium current (Opie, 1991). Measurements of cytosolic calcium during early post-ischemic reperfusion have shown increased calcium levels and oscillations of calcium concentrations (Brooks *et al.*, 1995; Gao *et al.*, 1995; Meissner & Morgan, 1995). The consequence of an intracellular calcium overload is severe in that the normal physiological response of the contractile apparatus to calcium would be impaired, leading to stunned myocardium in ischemia. However, the mechanisms by which ROS regulate calcium homeostasis and the molecular nature of these ROS effects are still unclear.

3.1. The specificity of the regulatory role of ROS.

To function as "signalling molecules", ROS must have well-defined targets and specific actions. The specificity of ROS signalling is not assured through a "lock and key" recognition mechanism used for ligand binding to receptor, but through a chemical reaction system. The multiple forms of ROS (e.g., $O_2^{\bullet -}$, $HO^{\bullet}$, $NO^{\bullet}$, $ONOO^-$, NO^+) and their ability to interact with each other provide a set of redox-active molecules that can covalently modify selected targets

(such as thiols and iron). The specificity of the regulatory role of ROS is identified in different tissues at three levels (Lander, 1997), i.e. (i) generation of the specific species of ROS; (ii) target susceptibility; and (iii) scavenger activity. The endogenous scavengers of ROS are important for the regulation of the specificity.

3.2. Oxidation versus S-nitrosylation of sulfhydryl groups.

In general, SH-groups of proteins may be oxidized to disulfides, sulfenic acid, and sulfonic acid residues by ROS, including nitric oxide (NO). As is well known, NO interacts with the heme of the soluble GC, causing a conformational change and triggering cGMP synthesis. What is less known, however, is that NO also covalently modifies thiol proteins in a cGMP independent manner (Stamler *et al.*, 1992 a & b). This mechanism, termed S-nitrosylation, involves the transfer of N=O to the SH group of the protein to form a sulfur-linked nitroso compound. Protein S-nitrosylation is important under both physiological and pathophysiological conditions where large amount of NO is produced by the activation of the inducible NOS or overstimulation of the constitutive NOS (Brune *et al.*, 1994; Stamler *et al.*, 1992b).

While oxidation is relatively irreversible, S-nitrosylation occurs rapidly in a reversible manner. S-nitrosylation of proteins requires accessible reduced SH groups. In some cases, the fast S-nitrosylation creates the necessary conditions for the following more stable protein ADP-ribosylation (Brune *et al.*, 1994). At least two consequences of protein S-nitrosylation can be envisioned. (1) Protein S-nitrosylation will endow the modified protein with long-lasting NO-like effect, mediated through the release of NO from the S-nitrosylated protein to increase cellular cGMP. If a plasma protein or a membrane protein is S-nitrosylated, it may be

considered as a carrier or as an intracellular storage for NO mechanism, respectively. However, whether NO can be released and what drives this release from S-nitrosylated membrane proteins, such as the IP_3 receptors, are not clear. Whether the S-nitrosylation of IP_3 receptors is transient or can be maintained for a relatively longer time are also unknown. (2) The function of proteins after S-nitrosylation will be altered. Intracellular protein S-nitrosylation may serve as a signal transduction mechanism via covalent attachments reminiscent of the regulating process of phosphorylation (Stamler *et al.*,1992 a & b).

3.3. Effects of ROS on IP_3 pathway.

3.3.1. Effects of ROS on IP_3 generation.

The action of oxidants, or of oxidant-derived products, mimics certain agonists that result in the hydrolysis of phospholipids such as phosphatidylinositol (Wu *et al.*, 1998). Consequently, this could lead to the release of signalling molecules (arachidonic acid, diacylglycerol, and inositol phosphate), the disturbance of intracellular calcium homeostasis and abnormal activation of protein kinase C (Nishizuka, 1992). Increases in DAG, phosphatidic acid, and inositol polyphosphate levels were also observed in endothelial cells following treatment with low doses of H_2O_2 (Shasby *et al.* 1988).

3.3.1.1 Tyrosine kinase-PLCγ regulated IP_3 generation.

Since IP_3 formation can result from tyrosine kinase activation (Abe & Berk, 1998), the effects of ROS on tyrosine kinase activity were studied in several cases. Studies carried out in liver cells showed that orthovanadate-generated ROS activated the tyrosine kinase-

phosphatidyinositol kinase coupled system (Yang *et al.*, 1989; Chen & Chan, 1993). A stimulation of insulin receptor tyrosine kinase activity by hydrogen peroxide was also reported (Hayes & Lockwood, 1987; Heffetz *et al.*, 1990). In the presence of ultraviolet light, hydrogen peroxide stimulated T cell tyrosine kinase ZAP-70 (Schieven *et al.*, 1994). Hydrogen peroxide also induced tyrosine phosphorylation of the src family protein tyrosine kinase p561ck (Nakamura *et al.,* 1993), and activated p72syk tyrosine kinase (Schieven *et al.*, 1993). Lowering intracellular GSH, equivalent to increasing GSSG, induced tyrosine phosphorylation in Jurkat T cell. However, It is not yet clear whether ROS cause direct activation of tyrosine kinase or whether the increase in tyrosine phosphorylation is due to inhibition of tyrosine phosphotases by oxidants.

a) Activation of tyrosine kinases: Several tyrosine kinases in addition to c-Src may be activated by ROS. For example, hydrogen peroxide and diamide, which oxidize free sulfhydryl groups, stimulates tyrosine kinases (especially a 55-kD protein, called p56Lck) in cultured T lymphocytes (Nakamura *et al.*, 1993).

b) Inhibition of phosphotyrosine phosphatases (PTPase): All PTPases contain a redox-sensitive cysteine at their active site (Brondello *et al.*, 1995) and the oxidation of the sulfhydryl group inactivates the PTPases, thus stimulating tyrosine kinases.

Whether the activation of tyrosine kinase pathway is a common mechanism for the cellular effect of ROS is presently unsettled. At present, there is no evidence that tyrosine kinase-linked signalling pathway in vascular SMCs is modulated by superoxide anion.

3.3.1.2. G protein- PLCβ regulated IP_3 generation.

Recently, Derevianko *et al.* (1998, 1997) studied the effect of polymorphonuclear leukocyte (PMN)-derived ROS on the interleukin receptor–mediated NADPH oxidase activation. The inflammatory cytokine interleukins are the potent agonists of the PMN respiratory burst signal transduction cascade. The activation of interleukin receptor is known to activate G protein, increasing PLCβ activity. Their results showed that the expression of interleukin receptors was significantly increased in the presence of catalase and superoxide dismutase (SOD). SOD and catalase also increased PLC activity. From the stimulatory effect of antioxidants (SOD and catalase), it is postulated that superoxide anion and hydrogen peroxide significantly inhibit the G protein-PLCβ transduction pathway in human polymorphonuclear leukocytes. The direct effects of superoxide anion and hydrogen peroxide on the G protein-PLCβ pathway have not been determined, and the underlying mechanisms remain unclear.

The potential inhibitory effect of ROS on the G protein-PLCβ transduction pathway is opposite to the stimulatory effect of ROS on the tyrosine kinase-PLCγ transduction pathway. This inhibitory effect should lead to a reduction in IP_3 formation but the direct measurement of IP_3 formation following the ROS-induced inhibition of G protein-PLCβ transduction pathway has not been reported, to my knowledge.

3.3.2. Effects of ROS on IP_3 receptors.

The calcium releasing channels, including the IP_3 receptor and the ryanodine receptor, are redox responsive and can be directly affected by ROS. The oxidation of sulfhydryl groups of the IP_3 receptor leads to an altered Ca^{2+} releasing function (Wesson & Elliott, 1994). For example, oxidized glutathione (GSSG) in vascular endothelial cells oxidised protein thiol groups, changing the protein structure-function relationships (Elliott & Schilling, 1995; Koliwad *et al.*, 1996). The

oxidation of IP_3 receptors in endothelial cells by GSSG increased IP_3 receptor activity and depleted IP_3-sensitive Ca^{2+} stores, thereby attenuating the intracellular Ca^{2+} response to agonist stimulation (Elliott & Koliwad, 1997). Thrower *et al.* (1996) reported that thimerosal, a sulfhydryl-oxidising agent, enhanced the activity of type 1 IP_3 receptors by increasing their open time and conductance. Tert-butyl hydroperoxide (tBOOH) induced an increase in calcium-induced-calcium-release independent of stimulated IP_3 formation in hepatocytes (Elliott & Koliwad, 1997). This could be explained by the formation of mixed disulfides with GSSG or other oxidized thiols that interact with the IP_3 receptor/channel. Volk *et al.* (1997) recently showed that NO donors induced a transient oscillatory intracellular Ca^{2+} change in endothelial cells and that this effect was inhibited by both thapsigargin and cyclopiazonic acid, but not by ryanodine. Since this NO effect was not mediated by cGMP, a direct interaction between NO and IP_3 receptor was postulated. Either the oxidation or the S-nitrosylation of IP_3 receptors by NO could be responsible for this effect. The redox sensitive sulfhydryl groups of IP_3 receptors are probably located on two cysteines at positions 2610 and 2613 near the COOH-terminal segment of the IP_3 receptor (Kaplin *et al.*, 1994). Different subtypes of IP_3 receptors may have different redox responses since it was shown that the type 3 IP_3 receptor is not sensitive to sulfhydryl oxidation (Missiaen *et al.*, 1998). Moreover, direct evidence for the S-nitrosylation of IP_3 receptor is still missing.

It has been reported that superoxide anion generated by the hypoxanthine-xanthine oxidase reaction enhanced IP_3-induced Ca^{2+} release from the fractionated ER in vascular SMCs, but hydrogen peroxide failed to do so (Suzuki *et al*, 1997). In endothelial cells, both superoxide anion and hydrogen peroxide increased Ca^{2+} release from the IP_3-sensitive pool (Dreher *et al.*, 1995; Doan *et al.*, 1994). Whether these effects are due to an increase in IP_3 formation or to an

increase in IP_3 receptor sensitivity or both was still not clear before the undertaking of the present studies.

3.4. Effects of ROS on PKC activities.

Thiols in the regulatory domain of PKC are particularly responsive to the modulation by oxidants since treatments with low concentrations of peroxide produce transient activation of PKC, while higher concentration cause inactivation. These effects of hydrogen peroxide on PKC are inhibited by reductants such as dithiothreitol (Gopalakrishna & Anderson, 1991). The zinc-thiolate structures in the regulatory domain of PKC are also highly susceptible to regulation by oxidants and the selective modification of some cysteine residues in those structures leads to enzyme activation (Suzuki *et al*, 1997). These oxidative reactions not only influence the binding of effectors but also facilitate Ca^{2+}- and phospholipid-independent catalytic activity. For instance, redox cycling quinones such as menadione, duroquinone, and 2-3-dimethoxy-1,4-naphoquinone activated PKC in rat hepatocytes. GSSG, in the presence of GSH, caused PKC stimulation. Moreover, hydrogen peroxide has been demonstrated to activate PLA2 (Whorton *et al.*, 1985), PLC (Shasby *et al.*, 1988), and PLD (Nataranjan *et al.*, 1993). The resulting increase in DAG should activate PKC and the subsequent PKC-catalyzed reactions. The direct oxidation of the regulatory domain of PKC by ROS has also been suggested (Gopalakrishna & Anderson, 1989). Larsson and Cetutti (1989) reported in mouse epidermal cells that the phorbol ester binding to PKC was increased and the calcium and phospholipid dependence of PKC were decreased in the presence of hydrogen peroxide, but not in the presence of superoxide anion. Therefore, at least in this cell type, PKC activity was not modulated by superoxide anion.

3.5. Effects of ROS on cAMP– PKA pathway.

A mutual interaction between ROS and cAMP has been described. The generation of ROS can be inhibited by cAMP. For instance, an elevation of intracellular cAMP levels suppressed superoxide anion generation, although the exact mechanism was not clarified (Takei *et al.*, 1998). On the other hand, ROS was found to affect the activity of PKA. Dimon-Gadal *et al.* (1998) studied the action *in vitro* of hydroxyl radicals and superoxide anion on PKA-I and PKA-II, respectively. PKA-I was more sensitive than PKA-II to hydroxyl and superoxide radicals. Hydroxyl radicals decreased the kinase phosphotransferase activities stimulated either by cAMP or its site-specific analogs for both PKA-I and PKA-II, but PKA-I was more affected. The binding of cAMP to the regulatory subunits of PKA-I was decreased by superoxide anion. It was found that ROS modified both cAMP-binding sites A and B of the regulatory subunit but had a smaller effect on the catalytic subunit. These results suggested that ROS altered the structure of PKA and changed its enzymatic activities.

The cellular cAMP content was modulated by ROS in a tissue-specific manner. Basci *et al.* (1987) reported that in the presence of ROS, the cAMP content of the isolated rat glomeruli was significantly increased, but the cAMP content of the tubules and the cGMP content of the glomeruli or tubules were not altered. The cAMP response of the glomeruli to ROS was abolished in the presence of SOD (a scavenger of superoxide anion), but not in the presence of catalase (a scavenger of hydrogen peroxide) or methionine or taurine (scavengers of hypochlorous acid). These results indicated that superoxide anion, but not hydrogen peroxide, stimulated cAMP formation in glomeruli but not in tubules. Further studies by this group showed that superoxide anion increased the cAMP content of rat glomeruli by stimulating phospholipase activity, thus enhancing prostaglandin synthesis.

3.6. Effects of ROS on cGMP – PKG pathway.

The modulation of cGMP-PKG pathway by ROS is a good example of selectivity and specificity of the effects of ROS. Depending on the tissue type and on the concentration / species of ROS, the effects of ROS on cGMP metabolism differ.

When human platelets were exposed to xanthine-xanthine oxidase (X-XO), a system that generates both superoxide anion and hydrogen peroxide, the platelet aggregation in response to various stimuli was impaired (Ambrosio *et al.*, 1994). The formation of hydrogen peroxide was mainly responsible for the impairment of aggregation, since this impairment was prevented by catalase but not by SOD, and since the pure hydrogen peroxide generator glucose-glucose oxidase also markedly inhibited platelet aggregation in a dose-dependent fashion. Hydrogen peroxide also induced a greater than 10-fold increase in concentrations of platelet cGMP levels, whereas cAMP levels remained unchanged. The hydrogen peroxide -induced impairment of platelet aggregation was largely abolished when GC activation was prevented by incubating platelets with the GC inhibitor, LY-83583. This study demonstrated that high (but not toxic) concentrations of exogenous hydrogen peroxide inhibited platelet aggregation through the stimulation of GC and increased cGMP formation, while superoxide anion had no effect on cGMP formation.

In contrast, an inhibitory effect of superoxide anion on cGMP metabolism was observed in cultured rabbit pulmonary arterial SMCs (Marczin *et al.*, 1992) in which superoxide anion generated by xanthine and xanthine oxidase, like methylene blue (MB), inhibited the nitrovasodilator-induced cGMP accumulation. Furthermore, MB induced a dose- and time-dependent generation of superoxide anion from rabbit pulmonary artery SMCs, as evidenced

from the spectrophotometric determination of cytochrome c reduction. In addition, the MB-induced inhibition of cGMP accumulation was completely prevented by SOD, but not by catalase. These results are in line with the early observation of Ullrich *et al.* (1989) that superoxide anion inhibited soluble GC and decreased cGMP level, thus inducing platelet aggregation and blood vessel constriction.

Taken together, previous studies provide evidence that hydrogen peroxide either stimulated or had no effect on the soluble GC, whereas superoxide anion either inhibited or had no effect on the soluble GC in different cell preparations (Wolin *et al.*, 1998; Cherry *et al.*, 1990).

3.7. Effects of ROS on ion channels and transporters.

The electrophysiological effects of ROS are multi-faceted, affecting many different ion channels and transporters. This complexity can be exemplified by the effect of hydroxyl radicals. In isolated guinea pig ventricular myocytes, Jabr and Cole (1993) showed that the resting inward rectifying K channels and Na^{+}/Ca^{++} exchanger were inhibited, while a nonselective cation current and ATP-sensitive K channels were activated, by hydroxyl radicals. ROS generated by the HX-XO reaction inhibited inward rectifier K channel currents and the L-type voltage-dependent calcium channel currents in guinea pig myocytes (Coetzee & Opie, 1992). In rat ventricular myocytes, hydrogen peroxide increased plasma membrane Ca^{2+} leak channel activity which may contribute to the increased $[Ca^{2+}]_i$.

As mentioned before, GSSG is endogenously formed within cells. The bioactivity of GSSG results in the oxidation of protein thiol groups, leading to changes in protein structure-function relationships. When ion channel protein thiols are the target of oxidation by GSSG,

important changes in channel conductance, activity, and gating occur. In the whole-cell patch-clamp studies, the introduction of GSSG to the cytosol of endothelial cells by a micro-pipette resulted in a progressive shift in the resting membrane potential toward a depolarizing direction, which could lead to the inhibition of capacitative Ca^{2+} entry (Elliott & Koliwad, 1997). Moreover, GSSG was shown to deplete IP_3-sensitive Ca^{2+} stores, thereby attenuating the intracellular Ca^{2+} response to agonist stimulation. When GSSG accumulated within the cell, the IP_3 receptor became sensitized to IP_3, such that the release of stored Ca^{2+} was accentuated even by the basal level of IP_3 (Missiaen *et al.*, 1991).

3.8. Effects of ROS on SMC contraction and proliferation.

ROS generated in the vascular wall may act directly on SMCs, or affect the production and/or biological activity of endogenous vasoactive mediators formed in endothelial cells. It has become increasingly clear that both endothelial and vascular smooth muscle cells have the potential to produce superoxide anion and other metabolites of superoxide anion (Pagano *et al.*, 1995; Mohazzab & Wolin, 1994; Katusic & Vanhoutte, 1989). One major source of superoxide anion in vascular tissues is a membrane-bound oxidase that uses NADH predominantly as a substrate for electron donation to molecular oxygen (Mohazzab *et al.*, 1994; Katusic & Vanhoutte, 1989). The generation of superoxide anion occurs (Rosen & Freeman, 1984) both under basal condition (Matsubara & Ziff, 1986a) and during stimulation such as reperfusion (Schinetti *et al.*, 1989; Zweier *et al.*, 1994), or treatment with bradykinin (BK), calcium ionophore A23187, interferon-γ and interleukin-1 (Matsubara & Ziff, 1986b). Cyclooxygenase, XO, and NADH oxidoreductase have also been identified as sources of superoxide anion in the endothelium (Kontos, 1985; Mohazzab *et al.*, 1994; Cosentino *et al.*, 1994). Prolonged

incubation of the cells with AII stimulated superoxide anion formation as detected by monitoring chemiluminescence of lucigenin (Griendling *et al.*, 1994). PDGF (platelet-derived growth factor) stimulated intracellular hydrogen peroxide formation as monitored by detecting the oxidized 2',7'-dichlorofluoresin (Sundaresan *et al.*, 1995).

3.8.1. ROS effects on the contractility of vascular SMCs.

As shown in Fig. 1-5, multiple signal transduction pathways regulate the contractility of vascular SMCs. ROS regulate the contractility of vascular smooth muscle through mechanisms including the enhancement of intracellular calcium level, interaction with NO, stimulation of the production of vasoactive prostaglandins, inhibition of sGC, activation of PKC, and modulation of the release of vasoactive factors from the endothelium.

(i) The contractility of vascular SMCs is regulated by intracellular free calcium concentration, which is mainly controlled by the interaction of various ion channels, transporters and exchangers. Calcium can enter the cell through the voltage-dependent or through the receptor-operated channels. Calcium can also be released from intracellular calcium pools through the IP_3-sensitive or ryanodine-sensitive calcium releasing channels. The removal of calcium from the cytosolic space is accomplished by the reuptake into ER and mitochondria and by the transfer to the extracellular space. ROS act on multiple targets on the calcium homeostasis mechanisms to increase intracellular calcium concentration. The functional impairment of calcium homeostasis under the ROS influence includes the decrease in calcium uptake into ER (Rowe *et al.*, 1983), the attenuation of the Na^+/Ca^{2+} exchanger activity (Tani & Neely, 1989), the reduction in Ca^{2+} pump activity (Kaneko *et al.*, 1989), and increase in

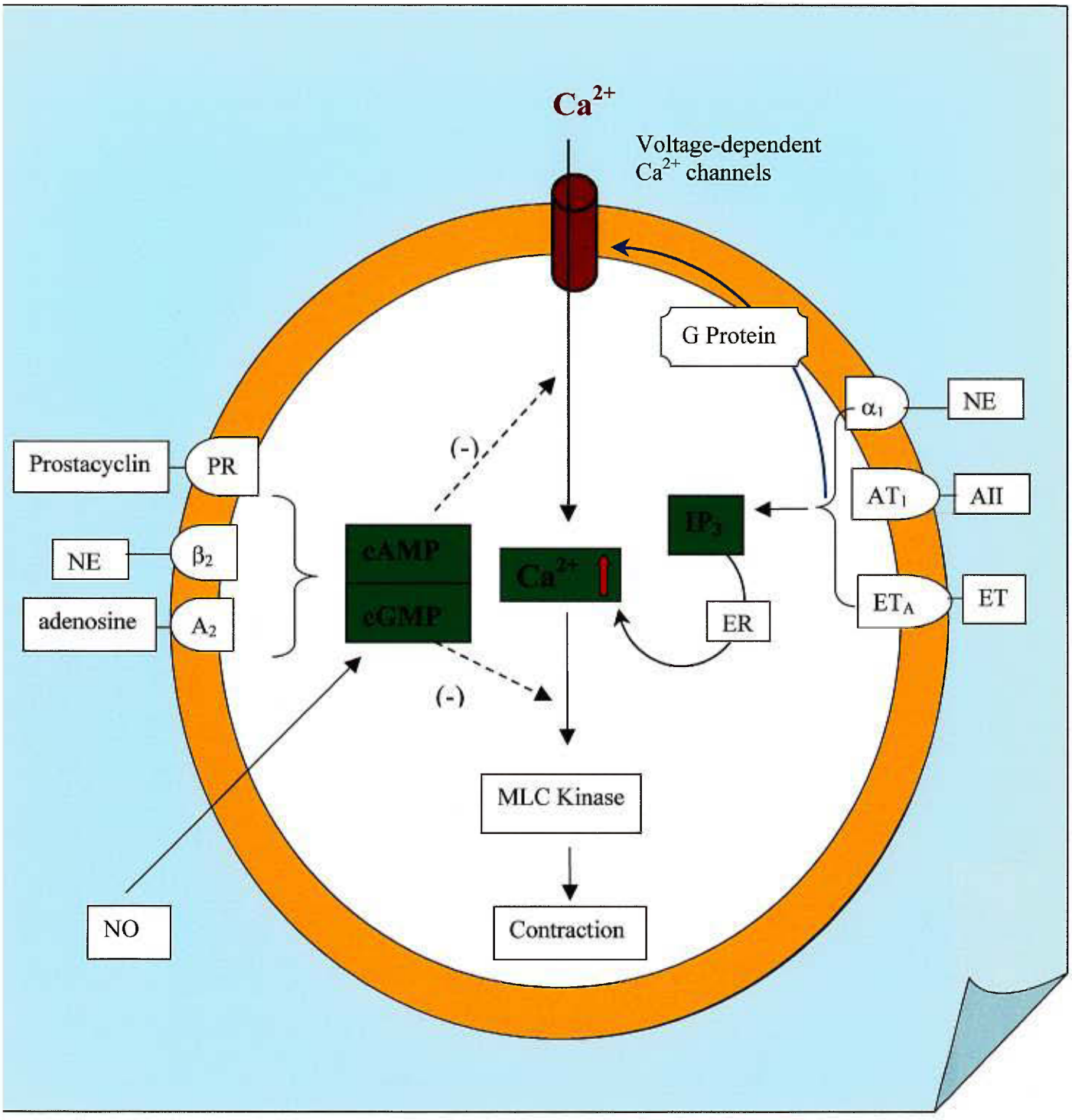

Fig. 1-5. Regulation of vascular smooth muscle contraction by different signal transduction pathways. MLC, myosin light chain; PR, prostacyclin receptor; ET, endothelin.

Fig. 1-5. Regulation of vascular smooth muscle contraction by different signal transduction pathways. MLC, myosin light chain; PR, prostacyclin receptor; ET, endothelin.

for these ROS effects. Protein oxidation, such as the oxidation of sulfhydryl groups of the proteins, is another putative mechanism (Coetzee *et al.*, 1994). Whatever the mechanisms might be the increase in intracellular calcium concentration in vascular SMCs leads to the enhanced contractility of these cells. Although this effect of ROS seems to be universal, exceptions do exist, however, since a decrease in L-type calcium channel currents by hydrogen peroxide has been reported (Coetzee & Opie, 1992) and since superoxide anion inhibits calcium current in rabbit atrioventricular nodal cells (Martynyuk *et al.*, 1997). Therefore, when evaluating the effect of ROS on intracellular calcium concentration, caution should be exerted regarding the types of ROS and the types of tissues on which those effects were studied.

(ii) The interaction of superoxide anion with NO results in the shortening of the half-life of NO and in the production of a strong oxidant, peroxynitrite ($ONOO^-$) (Beckman *et al.*, 1990). In oxygenated media, the half-life of NO is about 6 seconds due to the presence of superoxide anion (Butler *et al.*, 1995) and the addition of SOD to the media prolonged the half life of NO (Diederich *et al.*, 1994). In addition, peroxynitrite further metabolizes to hydroxyl radical and nitrogen dioxide, leading to lipid peroxidation and cytoxicity (Radi *et al.*, 1991). Thus, superoxide anion may decrease the concentration and efficacy of NO, favoring an increase in arterial tone and the formation of potentially toxic free radical that may cause tissue injury. Decreased concentration of NO may also favor the Haber-Weiss and Fenton reaction.

(iii) The increased production of superoxide anion in the blood vessel wall inhibits the synthesis of prostacyclin, but not that of thromboxane A2 (Katusic & Vanhoutte, 1989; Moncada

et al., 1976). This effect, together with the chemical inactivation of NO, may contribute to the impairment of the endothelium-dependent relaxation, resulting in vasoconstriction.

3.8.2. ROS effects on cell proliferation.

Hydrogen peroxide and xanthine/xanthine oxidase were shown to stimulate the proliferation of cultured vascular SMCs (Rao & Berk, 1992). ROS were also found to stimulate the expression of the vascular cell adhesion molecule-1 (VCAM-1) on vascular endothelial cells, which permits monocyte binding, and the expression of monocyte chemotactic peptide-1 (MCP-1), thus allowing macrophages to migrate within the vessel wall (Marui *et al.*, 1993). These effects of ROS were reversed by the antioxidants pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC). ROS caused oxidative modification of LDL, promoting its uptake by the scavenger receptor and favoring foam cell formation (Berliner & Heinecke 1996). In addition, mild oxidative stress has been reported to stimulate cell proliferation (Nose *et al.*, 1991) and MAP kinase has been demonstrated to be activated by hydrogen peroxide (Stevenson *et al.*, 1994; Fialkow *et al.*, 1994). It is thus plausible that under an enhanced oxidative stress, vascular SMCs or cardiac myocytes will proliferate, leading to remodeling and the development of cardiac and vascular wall hypertrophy.

4. ALTERED SIGNAL TRANSDUCTION PATHWAYS IN HYPERTENSION.

Extensive experimental and clinical evidences have demonstrated the increased activities of the sympathetic and renin-angiotensin systems and the altered signal transduction pathways in hypertension (de Champlain *et al.*, 1998; Schnabel & Böhm, 1996).

L. Wu, Superoxide anion as a signalling molecule.

4.1. Decreased activity of cAMP pathway.

An attenuation and blunting of β-adrenoceptor functions has been observed in animal hypertension and in human hypertension (Feldman, 1987). Several hypertension models in rats have been used for those studies, including constriction of one renal artery and removal of the other kidney (1-clip-1-kidney; 1C-1K) (Gende *et al.*, 1985), deoxycorticosterone acetate (DOCA) salt hypertensive rats (Eid & de Champlain, 1988), the L-NAME induced hypertension (K-Laflamme *et al.*, 1998), and the spontaneously hypertensive rat (SHR) (Kurtz *et al.*, 1989). Interestingly, most of those models exhibited a desensitization of the AC pathway. For instance, the isoproterenol-stimulated AC activity was depressed in aortic SMCs from SHR (Wu & de Champlain, 1996), and in cardiovascular tissues from both 1C-1K and DOCA-salt hypertensive rats (Böhm *et al.*, 1992). In 12-week-old SHR, a reduced β-adrenoceptor-mediated vasodilation in isolated mesenteric arterial beds and a reduced binding affinity to β-adrenoceptors in heart membranes were reported (K-Laflamme *et al.*, 1997). Moreover, the treatment of SHR with angiotensin converting enzyme (ACE) inhibitors resulted in a decrease of blood pressure, a restoration of AC activity (Pandey & Anand-Srivastava, 1993), and the normalization of the binding affinity to β-adrenoceptors (K-Laflamme *et al.*, 1997).

An enhanced sympathetic activity as reflected by increased circulating catecholamine levels, NE spillover rate, and increased nerve activity has been demonstrated in experimental and human hypertension (de Champlain *et al.*, 1998). Chronic exposure to the high level of catecholamines could lead to a marked decrease in myocardial responsiveness through the process of β-adrenoceptor desensitization. This type of desensitization or the uncoupling of the β-adrenoceptor from G proteins is due to the activation of β-adrenoceptor kinase (βARK), a G protein-coupled receptor kinase. The phosphorylation by βARK promotes the interaction of the

receptor with β-arrestin, which binds to the phosphorylated receptor to prevent further activation of Gsα. The phosphorylation of β-adrenoceptors followed by the binding of β-arrestin also initiates the sequestration of the receptor, leading to the down-regulation of the receptors (Lefkowitz, 1993). An increase in βARK activity linked to an impaired G-protein coupling and associated with a decrease in AC activation has been demonstrated *in vitro* and *in vivo* (Koch *et al.*, 1995; Pippig *et al.*, 1993) without any changes in the expression of Gsα and the catalytic subunits of AC. However, an increase in the expression of Giα was observed in heart and aorta from the genetic and other experimental hypertensive models (Marcil *et al.*, 1998; Anand-Srivastava, 1996; Schnabel & Böhm, 1996). This raises the possibility that an increase in βARK activity may down-regulate β-adrenoceptors or uncouple these receptors from Gsα. The AC desensitization-related decrease in intracellular cAMP levels, consequently, may contribute to the impaired cardiac contractility and increased vascular tone in hypertension.

4.2. Enhanced activity of IP_3 pathway.

It appears that the IP_3-dependent vasoconstriction becomes dominant in hypertension (Wu & de Champlain, 1996). An increased phosphoinositide metabolism during the activation of α_1-adrenoceptors in vascular tissues has also been reported in DOCA salt hypertensive rats (Eid & de Champlain, 1988) and in SHR (Turla & Webb, 1990). The enhanced IP_3 pathway in hypertension could be explained by several mechanisms. 1) The release of vascular constricting factors may be increased. For instance, an increase in NE release from the nerve endings could over-stimulate α_1-adrenoceptors. Moreover, an enhanced AII formation and endothelin release could also stimulate the corresponding receptors, activate Gq, leading to the overproduction of IP_3. 2) The density of α_1-adrenoceptors may be increased. However, at least in heart, it has been

demonstrated that the number of α_1-adrenoceptors was normal even though the activation of α_1-adrenoceptors increased IP_3 formation in hypertension (Eid & de Champlain, 1988). 3) Since a cross-inhibition of IP_3 formation by cAMP has been observed in SMCs of SHR (Wu & de Champlain, 1996), a decrease in cAMP by either an attenuation and blunting of β-adrenoceptor functions or reduced expression of Giα proteins might contribute to the activation of IP_3 formation in hypertension. In addition, It has also been reported that stimulated IP_3 response can be inhibited by cGMP or by the guanylyl cyclase stimulator (Lang & Lewis, 1991). Therefore, a change of cGMP activity such as a decrease in the intracellular cGMP levels could facilitate the IP_3 formation by lifting the negative feedback exerted by cGMP on the PLC pathway (s). This suggests that additional mechanisms beyond the receptor site may be involved in the modulation of IP_3 pathway.

4.3. Decreased activity of cGMP pathway.

Dysfunction of the endothelium dilatory mechanisms has been implicated in the pathogenesis of hypertension. A decrease in NO synthesis, or an increase in NO inactivation by superoxide anion, may partially account for the increase in peripheral vascular tone associated with hypertension. Indeed, long term administration of an inhibitor of NOS, such as N^G-nitro-L-arginine methyl ester (L-NAME), produces a hypertensive response both in normotensive animals (Rees *et al.*, 1989) and in humans (Haynes *et al.*, 1993). Several *in vitro* studies using large conduit and resistance arteries taken from different animal models of hypertension have demonstrated an impaired endothelium-dependent dilatory response to acetylcholine, but not to an endothelium-independent response of NO donors such as SNP (Wu *et al.*, 1996). This suggests a decreased NO production by endothelial cells rather than a decreased smooth muscle responsiveness to NO. In addition, an overproduction of superoxide anion has been reported in

human hypertension (Kumar & Das, 1993) and in SHR (Ito *et al.*, 1995). A recent study has also shown that AII is a powerful oxidative agent that could induce the formation of superoxide anion (Griendling *et al.*, 1994). The decreased NO level either by impaired synthesis or enhanced NO inactivation leads to a decreased formation of cGMP. Consequently, the vasodilatory mechanisms are impaired and peripheral vascular resistance is increased. However, whether endothelial dysfunction is a primary or secondary feature of hypertension is still unsettled.

As mentioned above, AII, ANP and endothelin (ET) could play an important role in regulating the blood pressure level. Even though increased plasma levels of ANP have been observed in various hypertensive models, the vasodilatory effects of ANP are, however, counteracted by the enhanced vasoconstriction induced by NE and AII. In addition, the increased ANP levels may down-regulate ANP receptors in hypertension. This may explain the fact that ANP receptor density and related activity of guanylyl cyclase were decreased in most organs from SHR (Anand-Srivastava, 1997; Garcia *et al.*, 1989). Therefore, the decreased anti-hypertensive effect of ANP could be due to the down-regulation of its receptors and the attenuated cGMP formation.

Changes in cAMP and cGMP levels in hypertension are tissue dependent. This is exemplified in the transgenic TGR(mREN2)27 rat strain. This rat strain was developed as a genetic hypertension model, carrying an additional mouse salivary REN-2 renin gene in its genome. However, as compared with the normotensive Sprague-Dawley strain, this transgenic model has an inverse circadian blood pressure profile and an unchanged rhythmic pattern of heart rate (Pons *et al.*, 1996). Witte *et al.* (1998) showed that the basal and stimulated adenylyl cyclase activity in the heart was significantly lower in transgenic hypertensive TGR(mREN2)27 rats than in normotensive Sprague-Dawley rats. In contrast, aortic cAMP formation did not differ between the two strains. Vascular cGMP formation was significantly reduced in TGR(mREN2)27 rat aortae under basal conditions and after stimulation with SNP. It was,

therefore, speculated that vasodilating second messenger pathways in this transgenic hypertensive strain were selectively desensitized.

4.4. Enhanced activity of MAP kinase pathway.

Enhanced activity of the G protein-coupled PKC pathway has been observed in hypertension. Increasing the activity of α_1-adrenoceptors as well as ET_A-coupled PLCβ, or AT_1-coupled PLCγ-mediated pathways, could enhance the hydrolysis of PIP_2 and increase the formation of DAG. The resultant activated PKC can stimulate proto-oncogenes and promote cell proliferation (Opie, 1998). AII induced a greater activity of extracellular signal-regulated kinases (ERK) in SMCs from SHR as compared to SMCs from WKY (Touyz *et al.*, 1999). The AII-induced hypertrophy of cardiac myocytes and vascular SMCs has also been demonstrated. This hypertrophy may result from the AII effect on MAP kinase pathway, as well as the AII-induced activation of Janus Kinase/signal transducers, activators of transcription (JAK/STAT) pathway, and NADPH/NADH oxidase pathway (Griendling *et al.*, 1997). It has been observed that AII stimulation induces proto-oncogenes within 15 minutes both in cardiac myocytes and nonmyocytes (Kent & McDermott, 1996; Sadoshima *et al.*, 1995). After treatment with ACE inhibitor, the left ventricular hypertrophy in SHR was markedly reduced, and myocardial fibrosis also regressed (Nagano *et al.*, 1991; Pahor *et al.*, 1991). These changes did not correlate with plasma AII levels, but correlated well with a fall in local cardiac AII concentration (Nagano *et al.*, 1991). Although these studies are far from being complete, the substantial involvement of AII effect on cell remodeling in the pathogenesis of hypertension is strongly suggested.

Many abnormalities of signal transduction pathways are encountered in hypertension. Those abnormalities are present at all three major signalling levels. The loss of balance, in terms of synthesis and release, between vasoconstrictors (NE, AII, endothelin, etc.) and vasodilators

(ANP, NO, etc.) alters the equilibrium among first messengers. A dominant α-adrenoceptor activity over β-adrenoceptor, associated to the altered G protein expression and function, may represent a major abnormality in hypertension at the level of coupling element. At the second messenger levels, impaired cAMP and cGMP signal pathways, a hypersensitivity of the PLC pathway and an overactive tyrosine kinase-MAP kinase pathway were observed in hypertension (Fig. 1-6).

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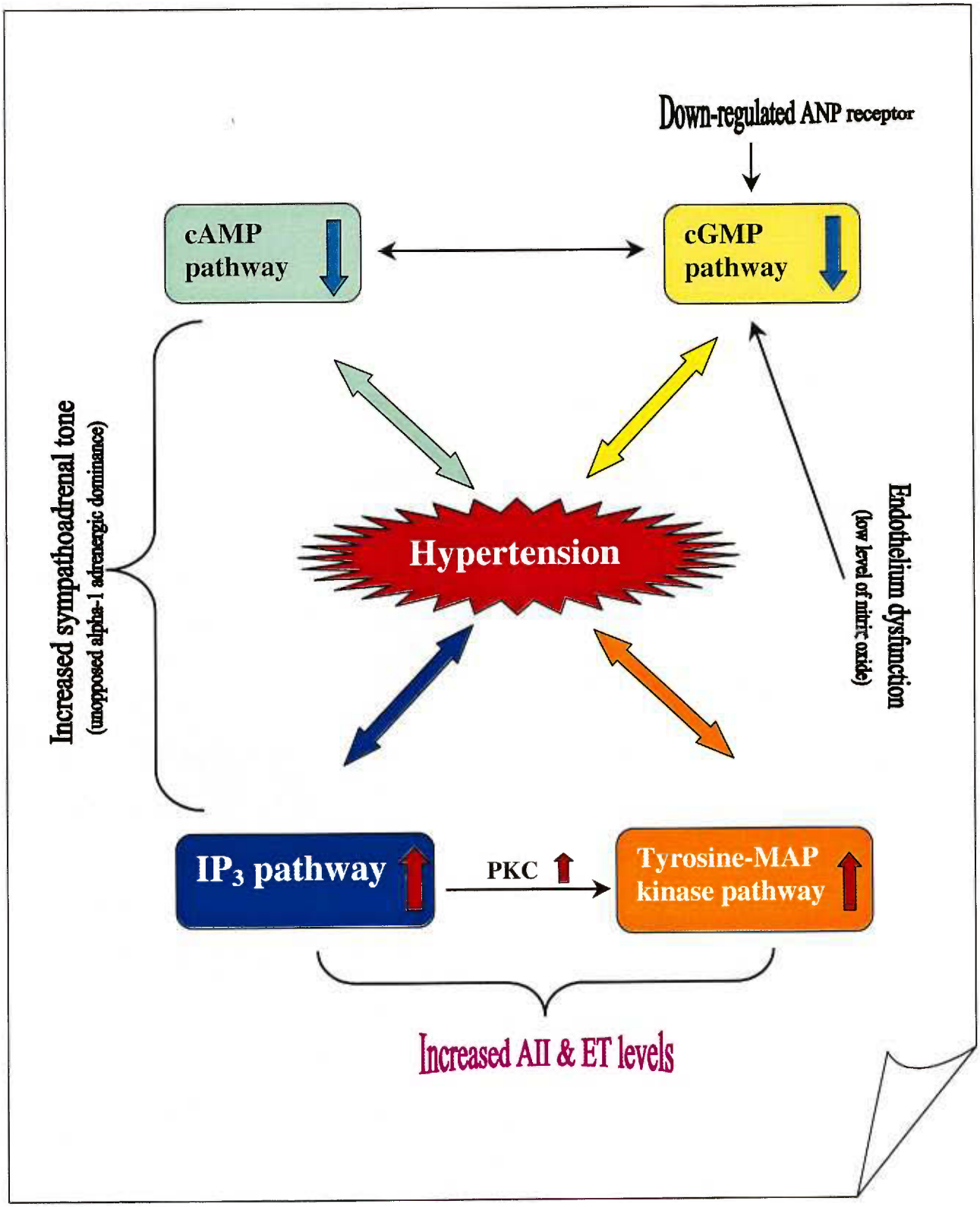

Fig. 1-6. Altered signal transduction pathways in vascular SMCs in hypertension.

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5. ALTERED CELLULAR METABOLISM AND ACTIONS OF REACTIVE OXYGEN SPECIES IN HYPERTENSION.

The relationship between ROS and the pathogenesis of hypertension has been a topic of debate for a long time. It is still not clear whether the unbalanced overproduction of ROS is the cause of hypertension or a secondary consequence of hypertension. However, the importance of an altered ROS metabolism and actions in the maintenance, if not in the etiology, of hypertension is well perceived (Alexander, 1995).

5.1. Overproduction of ROS in Hypertension.

An overproduction of ROS has been reported in cardiovascular diseases such as ischemia-reperfusion heart damage, heart failure, and hypertension (Opie, 1998; Chahine *et al.*, 1997; Oskarsson & Heistad, 1997; Richard *et al.*, 1996; Singh *et al.*, 1995; Kumar & Das, 1993). The first evidence that superoxide anion may play an important role in hypertension was provided by experiments in cerebral arterioles, in which an acute increase in arterial blood pressure caused excessive activation of arachidonic acids via the cyclooxygenase pathway with subsequent increased production of superoxide anion (Kontos, 1985). Superoxide anion may undergo dismutation to form hydrogen peroxide and then to form hydroxyl radical. The formation of hydroxyl radical was demonstrated to cause prolonged vasodilatation and oxidative vascular injury (Marshall & Kontos, 1990). In addition, hydroxyl radical-induced injury of endothelial cells was shown to inhibit both the production and release of NO. Thus, in small cerebral arteries, the impairment of endothelial function due to excessive production of ROS

during acute hypertension sets the stage for the increased reactivity to vasoconstrictor stimuli. Whether a similar scenario could contribute to the increased vascular resistance in chronic hypertension is still under investigation. The production of superoxide anion by SHR aortae was reported to be significantly increased as compared to WKY aortae (Mesaros *et al.*, 1995). In addition, superoxide anion markedly constricted the isolated endothelium-denuded SHR aortic tissues (Auch-Schwelk & Katus, 1989). These findings suggest a close relationship between oxidative and hypertension which may be characterize by an overproduction of superoxide anion as well as by a hypersensitivity of vascular SMC to superoxide anion.

Recent studies also showed that AII-induced hypertension was associated with increased vascular superoxide anion production (Griendling *et al.*, 1994); and that treatment with liposome-encapsulated SOD reduced blood pressure by 50 mm Hg in hypertensive rats receiving a 5-day infusion of AII (Laursen *et al.* 1997). Rajagopalan *et al.* (1996) reported that infusion of AII (0.7 mg/kg per day) increased systolic blood pressure and doubled the vascular superoxide anion production. The predominant source of superoxide anion activated by AII infusion was an NADH/NADPH-dependent, membrane-bound oxidase. SOD partially reversed the impaired vasorelaxing response, and administration of AII receptor (AT_1) inhibitor Losartan normalized the vascular superoxide anion production and the vasorelaxant responses, indicating that angiotensin type-1 receptor was invovled.

In aortic rings from SHR or renal and deoxycorticosterone salt hypertensive rats, oxygen consumption was significantly increased (Seidel & Strong, 1986). Xanthine oxidoreductase activity was shown to increase with age in SHR heart, but the activity of that enzyme remained constant in age-matched WKY rats (Janseen *et al.*, 1993), indicating a greater susceptibility to generate superoxide anion in SHR. An enhanced production of superoxide anion was also

postulated to play a major role in the pathogenesis of hypertensive cerebral vascular injury in acute experimental hypertension in cats (Kontos, 1985).

Abnormalities in ROS metabolism were observed in human hypertension. In uncontrolled human hypertension the production of superoxide anion and hydrogen peroxide by polymorphonuclear leukocytes as well as the plasma levels of lipid peroxide were enhanced, while the formation of NO was low (Kumar & Das, 1993; Prabha *et al.*, 1990). In another study, the NOS activity was found to be decreased and superoxide production increased in neutrophils of essential hypertensive patients (Mehta *et al.*, 1994).

In short, previous studies on experimental animals or human patients all demonstrated the increased ROS production in hypertension. Anti-hypertensive treatments in many cases partially restored blood pressure level, in parallel, increased NO levels, and decreased ROS levels (Opie, 1998).

5.2. Lack of compensation of the overproduced ROS by endogenous antioxidants.

Antioxidant defense mechanisms occur naturally and protect cells against the damage of ROS (Table 1-2). The integrated effect of endogenous antioxidants, such as vitamin C (a cytosolic antioxidant), vitamin E (a membrane antioxidant), and glutathione (a cytosolic and membrane antioxidant), protects both cytosol and membranes against ROS attack. Also present are the glutathione-dependent enzymes, glutathione peroxidase (GSH-Px), glutathione reductase, and glutathione transferase, catalase (breaking down hydrogen peroxide to oxygen and water), and the enzyme SOD (converting $O_2^{\bullet-}$ into H_2O_2). Some of these antioxidants exist in several forms. Membrane, cytosolic and plasma isoforms of GSH-Px have been reported. Similarly,

Table 1-2. Sources of Antioxidants

Enzymes	Cu/Zn-SOD (cytosol) Mn-SOD (mitochondria) SOD (extracellular) GSH peroxidase (GSH-Px) GSH-transferase GSSG reductase Serum amine oxidase Catalase (peroxisome)
Endogenous Substances	Ascorbate GSH Urate Catecholoamines Thioctic acid Vitamin E Ceruloplasmin Beta-carotene Melatonin
Exogenous Agents	Ethanol Mannitol Dimethylsulfoxide Dimethylurea Dimethylurea

Note:

* SOD catalyzes the reaction: $2\ O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$
* Catalase catalyzes the reaction: $2H_2O_2 \rightarrow O_2 + 2H_2O$
* GSH-Px catalyzes the reaction: $GSH + H_2O_2 \rightarrow 2H_2O + GSSG$
* No enzymes are known for scavenging hydroxyl radicals.
* DMSO and ethanol can scavenge hydroxyl radicals.

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there are mitochondrial, cytosolic, and extracellular isoforms of SOD. The vast network of intracellular and extracellular antioxidants strongly suggests that the levels of endogenous antioxidants must be tightly regulated for cell survival (Floyd, 1993).

The delicate balance between ROS and endogenous antioxidants is disturted under some pathophysiological conditions either by the overproduction of ROS or by the reduced production of antioxidants. Significant changes in the production or the function of endogenous antioxidants have been observed in acute and chronic myocardial dysfunction as well as in heart failure. For instance, a decreased activity of SOD and GSH-Px was found in acute heart failure in rat due to 10 min hypoxia at 37°C (Dhaliwal *et al.*, 1991), associated with irreversible impaired function of the heart. However, this hypoxia injury was diminished by the addition of catalase in the medium, suggesting that the escaped or overproduced ROS are mainly responsible for the dysfunction of the heart in acute failure. Studies carried out on cardiac myocytes isolated from normal adult hearts also support this observation since isolated myocytes exposed to hypoxia and reoxygenation for 15 min, demonstrated a marked reduction of the activities of Mn-SOD from mitochondria and GSH-Px (Kirshenbaum & Singal, 1992). Although the exact mode of action of ROS in heart failure is not clear, the degree of the impaired cardiac function is tightly related to the decreased activity of the antioxidant system. In addition, the endogenous SOD activity was found to be significantly lower in the aorta homogenates of those two kidney-two clip hypertensive rats (Vega *et al.*, 1995). These results thus indicate the accumulation of a greater content of superoxide anion in the vessels of hypertensive rats that cannot be compensated by the lower SOD activity or concentration in the vascular wall of those animals. It has been reported that the levels of Vitamin E and SOD were lower in red blood cells of hypertension patients (Kumar & Das, 1993). The neutrophil SOD and GSH levels were also

shown to be reduced in hypertensive patients (Sagar *et al.*, 1992). In those patients, the blood pressure level and endogenous antioxidant level were closely but increasely correlated. More evidence that supports the impaired antioxidant system in hypertension comes from the recent studies. It has been reported that the supplement of vitamin E significantly decreased lipid peroxides in plasma and blood vessels and enhanced the total antioxidant status, including an increase in activity of SOD in SHR (Newaz & Nawal, 1998). In addition, the treatment with shichimotsu-koka-to that has been used to treat patients with essential and renal hypertension significantly decreased the activity of xanthine oxidase as well as increased the activity of SOD and reduced the histopathological damage in the kidney from stroke-prone SHR (Higuchi *et al.*, 1998). It was also observed, in aortic rings from spontaneously hypertensive rats (SHR) or renal and deoxycorticosterone salt hypertensive rats, that oxygen consumption was significantly increased (Seidel & Strong, 1986) and that there was an overproduction of superoxide anion in aortae of SHR (Mesaros *et al.*, 1995). All those studies suggest that the production of superoxide anion from SMCs is increased in hypertension.

5.3. Enhanced SMC sensitivity to ROS in hypertension.

A few studies have reported that the reactivity of SMCs to ROS is increased in hypertensive animals. Auch-Schwelk *et al.* (1989) observed that the ROS (generated from xanthine plus XO reaction)-induced contraction of endothelium-free aortic rings was twice as large in SHR than in WKY rats. Either SOD or catalase, or the combination of SOD with catalase reduced the contractions. Their results suggested an increased reactivity of vascular SMCs in hypertension to hydroxyl radicals. The sensitivity of a specific signalling pathway in

vascular SMCs in hypertension to ROS was recently addressed by Kojda *et al.* (1998). These researchers investigated the function of soluble guanylyl cyclase in blood vessels isolated from 15 month old SHR. The basal levels of superoxide anion in smooth muscle and the endothelial layer of aortae are greater in SHR than that in WKY. Endothelium-dependent relaxation by acetylcholine was markedly impaired in SHR as was the vasorelaxant activity of SNAP, pentaerythritol tetranitrate and GTN. Maximal cGMP production by sGC isolated from the lung and stimulated with SNAP was much lower in SHR than in non-hypertensive control rats. These results suggest that the excess superoxide anion production in hypertension may trigger a desensitization of vascular sGC. It is not clear, however, that whether the desensitization of sGC is due to the increased sensitivity of SMCs to superoxide anion. The reactivities of other signal transduction pathways including IP_3 pathway to superoxide anion in vascular SMCs in hypertension were not reported, to date.

5.4. ROS contribute to the α_1 adrenergic dominant sympathetic system in hypertension.

A hyperactive sympathetic system is an important component of the pathogenesis of hypertension in experimental animal and in human. This hyperactivity is largely manifested with the blunted β adrenergic function and increased α_1 adrenergic activity (de Champlain, 1990). Over-production of NE from adrenergic nerve endings in hypertension would generate superoxide anion either through the autooxidation of catecholamine (Cohen, 1984) or through the activation of dopamine-β-oxidase (White, 1991). Although the role of superoxide anion thus generated from the overproduced catecholamines in hypertension has been unclear, this free

radical has been at least linked to the myocardial damage during ischemia and reperfusion (Singal *et al.*, 1982).

The decreased β adrenergic responses of vascular SMCs (Kramer *et al.*, 1987; Engels *et al.*, 1985) and heart (Bast & Haenen, 1990) in hypertension could be linked to the ROS-induced membrane lipid peroxidation. The integrity and fluidity of cell membrane is essential for the signal to be promptly and correctly transmitted from extracellular space to cytosol. A rigid and leaky membrane due to the lipid peroxidation would severely disrupt the normal signalling pathways, leading to altered cellular function (Richter, 1987; Curtis *et al.*, 1984). Moreover, high oxidative stress in hypertension might change the affinity of β adrenoceptors (Kaneko *et al.*, 1991), depending on the severity of oxidative stress and tissue types, and enhance the desensitization of β adrenoceptors as well (Wong *et al.*, 1987).

The increased α_1 adrenergic activity is also under the influence of lipid peroxidation. Both the receptor affinity and the α_1 adrenergic responses could be reduced by oxidative stress (Bellomo *et al.*, 1987). The current hypothesis regarding the differential effects of oxidative stress on α_1 and β adrenergic activities speculates that the severe oxidative stress may affect more significanly α_1 adrenoceptors via membrane lipid peroxidation, whereas milder oxidative stress may mainly target on β adrenoceptors via the action of superoxide anion and hydrogen peroxide. To keep with this hypothesis, chronic diseases such as hypertension have been found to associate usually with milder but sustained oxidative stress. Therefore, the decreased β adrenergic activity may be more significant in hypertension, leaving relatively intact α_1 adrenergic activity becoming predominant.

6. SUMMARY OF THE LITERATURE REVIEW.

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ROS are generated under both physiological and pathological conditions. The presence of multiple cellular sources of ROS and the labile nature of ROS imply their important signalling role involved in various cellular signal transduction mechanisms. Although the four major signal transduction pathways, i.e. cAMP-, cGMP-, IP_3-, and tyrosine kinase-linked pathways, are controlled by their own regulating mechanism, it is obvious that they exert significant cross-talk among each other. For instance, the IP_3-linked signalling pathway can be regulated by the activities of cAMP- and cGMP-linked signalling pathways.

ROS may have different effects on these signalling pathways, altering both the function and structure of cells. With regard to the IP_3-linked signalling pathway as an example, it is likely that ROS exert significant influences on both the IP_3 formation and/or the function of IP_3 receptors.

It is well established that hypertension is characterized by abnormalities of many signalling pathways. It is also well known that the cellular metabolism and actions of ROS are significantly altered in hypertension. Either the abnormalities in the classical signalling pathways or alterations in ROS signalling process could play an important role in the development and/or maintenance of hypertension, but the combination of these two mechanisms may constitute novel mechanism for the pathogenesis of hypertension. A better understanding of the interaction of ROS and classical signal transduction pathways could pave the way for the improved treatments and prevention of hypertension and other cardiovascular diseases.

7. RATIONALES AND HYPOTHESES.

A careful analysis of the literature reveals that many important questions regarding the signalling role of superoxide anion remain unsolved. (1) The importance of the IP_3-linked

signalling pathway in the control of vascular SMC function has been acknowledged. The general effect of ROS on the intracellular calcium mobilization has been shown. However, it remains to be demonstrated whether superoxide anion or other ROS can regulate IP_3 pathways in specific types of vascular SMCs. (2) The activities of the cAMP- and cGMP-linked signalling pathways directly or indirectly through the cross-talk mechanisms affect the vascular contractility. The modulation of the cAMP- and cGMP-linked signalling pathways in vascular SMCs by superoxide anion is unknown and needs to be defined. (3) Although superoxide anion has been suggested to alter the activity of many individual signalling pathways, the effect of superoxide anion on the cross-talk among these signalling pathways has never been investigated. (4) The beneficial effects of many antioxidants are related to their scavenging capacity for specific types of ROS but the mechanisms of these antioxidants are still ill defined in term of signal transduction mechanisms and therefore need to be clarified. (5) The effects of superoxide anion on vascular SMCs and the corresponding signalling mechanisms have not been characterized or compared in normotensive and hypertensive tissues. To answer these interrogations could greatly improve our understanding of the pathological role of superoxide anion in hypertension.

Based on the aforementioned rationales, the following hypotheses have been elaborated to guide the present study:

(1) Superoxide anion may facilitate IP_3 formation in vascular SMCs.

(2) Superoxide anion may alter cGMP and/or cAMP metabolism in vascular SMCs.

(3) The action on the cross-talk mechanisms among different signalling pathways could underlie the effect of superoxide anion on IP_3 metabolism in vascular SMCs.

(4) Superoxide anion may have different effects on various isoforms of PLC in vascular SMCs.

(5) The increased IP_3 levels in vascular SMCs from SHR could result from the hypersensitivity of PLC and cGMP signalling pathways to superoxide anion in SMCs from SHR.

8. OBJECTIVES.

The general objective of this Ph.D. program was to characterize and evaluate the signalling role of superoxide anion in vascular smooth muscle cells under physiological condition and in hypertension.

The following specific objectives were undertaken.

(1) To determine whether superoxide anion affects IP_3 metabolism in different types of vascular smooth muscle cells.

(2) To determine whether superoxide anion affects the activities of cAMP signalling pathways in vascular smooth muscle cells.

(3) To determine whether superoxide anion affects the activities of cGMP signalling pathways in vascular smooth muscle cells.

(4) To determine whether superoxide anion affects the activities of the yrosine kinase-related signalling pathway in vascular smooth muscle cells.

(5) To determine the efficacy of various endogenous and exogenous antioxidants in opposing the effects of superoxide anions on signal transduction pathways in vascular smooth muscle cells.

(6) To characterize the altered signalling effects of superoxide anion in vascular smooth muscle cells from spontaneously hypertensive rats.

CHAPTER 2

GENERAL METHODOLOGY

MEASUREMENT OF VASCULAR TISSUE CONTRACTION

Thoracic aortae were isolated from rats and the connective tissues were cleaned under a dissecting microscope. The isolated aortic tissues were cut into rings of approximately 2 mm in width and mounted in a 10 ml organ bath chamber filled with a Krebs bicarbonate saline (bubbled with 95%O_2 / 5% CO_2). Aortic rings were stretched to a basal force of 2 g and equilibrated for 1 hour before the beginning of the experiments (Wang *et al.*, 1997). Indomethacin (1 μM) was added to the Krebs saline solution which was composed of (in mM): NaCl 115, KCl 5.4, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11, and $CaCl_2$ 1.8. The endothelium was removed with a rubbing procedure and the lack of endothelium was confirmed by the failure of acetylcholine (1 μM) to relax the vessel. The tension development was measured at 37°C with FT 03 force displacement transducers (Grass Ins. Co., Quincy). Data acquisition and analysis were accomplished using a Biopac system (Biopac Systems, Inc., Golata) including the MP100 WS acquisition units, TCI 100 amplifiers, an Acknowledge software (3.01), universal modules, and a Macintosh computer.

CULTURE OF VASCULAR SMOOTH MUSCLE CELLS

Single SMCs were isolated and identified as described previously (Wu & de Champlain, 1996; Wang *et al.*, 1989). To isolate single SMCs from rat aortae, male rats (12 weeks) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Rat aortae were isolated and immediately immersed into a cold Calcium-free Hank's buffered salt solution (HBSS, Gibco). After cleaning the connective tissues and washing off blood, aortae

were cut open. The cleaned tissues were washed twice with a sterilised calcium-free HBSS. Aorta strips with a length of 10-15 mm were incubated with E-I solution at 37°C for 60 minutes. E-I solution was composed of collagenase/dispase (1.5 mg/ml, Boehringer Mannheim GmbH-Co.), elastase (0.5 mg/ml, type II-a, Sigma), trypsin inhibitor (1 mg/ml, type I-S, Sigma), and bovine serum albumin (2 mg/ml, fatty acid-free, Sigma). The tissues were rinsed twice with cold sterilised calcium-free HBSS. Next, the advential layer of aorta was removed under microscope with tweezers. Thereafter, aortic tissues were cut into even small pieces and transferred into E-II solution, which was composed of collagenase (1 mg/ml, type II, Sigma), trypsin inhibitor (0.3 mg/ml, type I-S, Sigma), and bovine serum albumin (2 mg/ml. Sigma). Digestion took place at 37°C for about 40 minutes. Then, the tissues were transferred to a calcium-free HBSS, and triturated to release single SMCs. During the next one hour, calcium concentration of cell suspension was gradually increased to 0.7 mM at +4°C. Finally, dissociated SMCs were plated in the tissue culture flasks and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (final Ca^{2+} concentration of 1.8 mM) in a CO_2 incubator at 37°C. The cells were identified as SMCs by virtue of their ability to contract in the presence of 30 mM KCl, observed under phase-contrast optics (Olympus, Tokyo, Japan); and by the fact that their α-actin was positively stained with mouse anti-α-actin antibody and anti-mouse immunoglobulin G fluorescent isothiocyanate conjugate (Wu & de Champlain, 1996). Cultured SMCs were passed once a week by harvesting with trypsin-treatment and splitting at a ratio of 1:4. The medium was changed after attachment of the cells and twice weekly thereafter. Cells between passage 2-14 were seeded into 132-mm 6-well multidishes or 100-mm dishes and used after 4 to 6 days when they became confluent.

To isolate single SMCs from mesenteric arteries, male Sprague-Dawley rats (12 weeks) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Small mesenteric arteries below the second branch off the main mesenteric artery were dissected out. Only the small arteries and arterioles were kept in the ice-cold physiological salt solution (PSS) containing (in mM) NaCl 137, KCl 5.6, NaH_2PO_4 0.44, Na_2HPO_4 0.42, $NaHCO_3$ 4.17, $MgCl_2$ 1, $CaCl_2$ 2.6, HEPES 10, and glucose 5 with pH adjusted to 7.4 with NaOH. The vascular tissues were cut into 5 mm-long pieces, and incubated for 30 minutes at 37°C in a low-Ca^{2+} PSS (0.1 mM $CaCl_2$) containing 1 mg/ml albumin, 0.5 mg/ml papain, and 1.0 mg/ml dithioerythritol. The tissues were thereafter incubated for another 20 minutes in a Ca^{2+}-free PSS in which calcium was omitted and 1 mg/ml albumin, 0.8 mg/ml collagenase, and 0.8 mg/ml hyaluronidase were added. Single cells were released by gentle triturating through a Pasteur pipette. After gradually increasing calcium concentration of cell suspension to approximately 1 mM, dispersed cells were plated in 75 cm^2 flasks in DMEM containing 10% fetal calf serum in a CO_2 incubator at 37°C. Cells between passages 2 to 8 were seeded into 132-mm six well multidishes or 100-mm dishes and used for the measurements of IPs, cAMP and cGMP, or for the immunoblotting assays.

MEASUREMENT OF INOSITOL PHOSPHATE LEVELS

SMCs were incubated for 24 hours in a serum-free and inositol-free DMEM (2 ml/well) with addition of 5 μCi/ml of myo-[2-3H] inositol (Du Pont Canada Inc. Diagnosis and Biotechnology Systems, Ont, Canada). The culture medium containing unincorporated isotope was removed and the cells were rinsed three times with warm (37°C) Earle's balanced salt solution (EBSS) composed of (in mM): NaCl 117, KCl 5.3, $MgSO_4$ 0.8, $CaCl_2$ 1.8, NaH_2PO_4

1, D-glucose 5.6, $NaHCO_3$ 6, and HEPES 25. The cells were further incubated for 30 minutes in the same buffer containing 20 mM LiCl to inhibit the conversion of inositol phosphates to inositol so that the radiolabelled inositol phosphates could accumulate within the cells (Eid & de Champlain, 1988; Dean & Beaven, 1989). Agonists were applied for various periods and the reaction was terminated by adding 0.9 mL methanol: chloroform: HCl (40: 20: 1) (Gunther *et al.*, 1982). After the addition of 0.4 ml chloroform and 0.4 ml distilled water, the samples were centrifuged (International Equipment, Needham Heights, MA, USA) to separate lipid and aqueous phases. The aqueous phase was transferred to a column containing 0.8 ml resin AG 1-X8 (200-400 mesh, formate form, Bio-Rad, Richmond, CA, USA) from which IP_1, IP_2, and IP_3 were eluted sequentially with ammonium formate buffers of increasing molarity (Dean & Beaven, 1989). All of these sequentially eluted inositol phosphates were collected to give a total inositol phosphates (IPs). In some experiments, total IPs were directly eluted with a buffer containing 1 M ammonium formate (Dean & Beaven, 1989). Since those two methods did not yield different results for the total IP formation, the individual fractions of IP_1, IP_2 and IP_3 were pooled. 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 total inositol phosphates was expressed as a ratio of IPs over PIP [(IPs/PIP) x 10^3], and IP_3 level was expressed as a relative value of [(IP_3/PIP) x 10^3] (arbitrary units), to correct for the variation in the labeling of the lipid pool (Wu & de Champlain, 1996).

QUANTITATIVE DETERMINATION OF cAMP LEVELS

SMCs were incubated in a serum-free DMEM (2 ml/well) for 24 hours and then rinsed three times with a warm (37°C) EBSS. The cells were further incubated for 10 minutes in the same buffer containing 3-isobutyl-1-methylxanthine (IBMX, 100 μM), a phosphodiesterase inhibitor, before the beginning of the experiments. Agonists were applied for various periods and the reaction was terminated by scraping the cells off the dish with a Tris buffer containing 4 mM EDTA (1 ml/per well). The reason for including EDTA in Tris buffer was to prevent the enzymatic degradation of cAMP. The cell suspension was homogenized (Polytron homogenizer, PT2000, Brinkmann) and then heated for 3 minutes in a boiling water bath to coagulate proteins. After centrifugation of the homogenate, the supernatant was assayed freshly for cAMP content or stored at –80°C for future measurement. Cyclic AMP levels were determined using a standard [^{3}H] cAMP assay procedure. This assay involves the competition between unlabelled cAMP and a fixed quantity of [^{3}H] cAMP for binding to a protein that has a high specificity and affinity for cAMP ([^{3}H] cAMP assay system, Amersham Corp, Ontario, Canada). The protein concentration was determined using Lowry's method or Bradford assay with crystalline bovine serum albumin as standard (Lowry, *et al.*, 1951; Bradford, 1976).

QUANTITATIVE DETERMINATION OF cGMP LEVELS

SMCs were incubated in a serum-free DMEM (2 ml/well) for 24 hours and then rinsed three times with a warm (37°C) EBSS. The cells were further incubated for 10 minutes in the same buffer containing 100 μM IBMX before the beginning of the experiments. Agonists were

applied for various periods and the reaction was terminated by replacing the agonist-containing EBSS with 500 μl of the extraction solution (0.1 N HCl) per well, in which the cells were treated for 20 minutes on ice. Thereafter, the extraction solution was transferred to a tube containing 10 μl of sodium acetate (2.5 M) and 10 μl of NaOH (5 N), which was assayed freshly for cGMP content or stored at –80°C for future measurement. The quantitative determination of cGMP was achieved using a cGMP [^{125}I] assay system with Amerlex-M^{TM} magnetic separation. This system utilizes a high specific activity [^{125}I] 2'-O-succinyl-cGMP tyrosine methyl ester tracer, together with a highly specific and sensitive antiserum, thus allowing for a simple magnetic separation of the antibody bounded [^{125}I] cGMP from free the fraction (cGMP [^{125}I] assay system, Amersham Corp., Ont., Canada). The protein concentration was determined using Lowry's method or Bradford assay with crystalline bovine serum albumin as standard (Lowry, *et al.*, 1951; Bradford, 1976).

WESTERN IMMUNOBLOTTING ANALYSIS

Determination of the expression levels of PLCγ proteins

After SMCs grown on 100-mm dishes were incubated in a serum-free DMEM for 24 hours, the cells were stimulated with agonists at 37°C for various periods. Cells were washed twice with ice-cold phosphate buffered saline (PBS) containing (in mM) NaCl 140, KCl 2.7, Na_2HPO_4 10, and KH_2PO_4 1.7 and then were scraped off the dish with 400 μl of ice-cold lysis buffer containing (in mM) Tris-HCl 50, NaCl 50, HEPES 50, EDTA 5, Triton X-100 1%, Na-deoxycholate 0.25%, phenylmethylsulfonyl fluoride 1, NaF 1, Na_3VO_4 1, aprotinin 10 μg/ml,

pepstatin 10 μg/ml, and leupeptin 10 μg/ml. The suspension was gently rocked on a shaker at 4° C for 25 minutes to lyse cells. The lysates were centrifuged at x14,000 g at 4°C for 25 minutes and supernatants were used freshly or stored at –80°C. The samples containing 40 μg of proteins were separated on 5% and 10% polyacrylamide gels using a mini-vertical electrophoresis system (mini-Protein II cell, Bio-Rad, Richmond, CA, USA) and transferred to nitrocellulose membranes (Amersham Corp., Ont, Canada) overnight. The nitrocellulose membranes were blocked in PBS containing 0.1% Tween 20 and 3% dehydrated milk for 2 hours and then incubated with anti-PLC_{γ} monoclonal antibody (0.5 μg/ml, Upstate Biotechnology, Lake Placid, NY, USA) in PBS containing 0.1% Tween 20 and 1.5% dehydrated milk for 2 hours at room temperature. The membranes were washed 3 times with PBS containing 1.5% dehydrated milk and then incubated with the second antibody (goat anti-rabbit IgG, 1:4000, Bio-Rad Laboratories, Hercules, CA, USA) for 3 hours at room temperature. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, England). The autoradiograms were quantified using UN-SCAN-IT gel Automated Digitizing System (version 5.1, Silk Scientific Inc. Orem, Utah, USA).

Determination of the expression levels of G proteins

SMCs grown on 150 x 25 mm dishes were stimulated with different agonists at 37°C in a serum-free DMEM for various periods. The cells were washed twice with ice-cold Tris-EDTA buffer containing 1 mM EDTA and 20 mM Tris-HCl and then scraped off the dish. The cell suspension was homogenized in a Teflon / glass homogenizer with a Tris- HCl buffer. The homogenate was centrifuged at x16000g at 4°C for 15 minutes. The supernatant was discarded and the pellet was resuspended in the Tris-EDTA buffer and stored at –80°C. The samples

containing 50 μg of protein were separated on SDS-polyacrylamide gel using standard electrophoresis (Bio-Rad, Richmond, CA) and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) with a semi-dry transblot apparatus (Bio-Rad, Richmond, CA) at 15 V for 45 min. The nitrocellulose membranes were blocked in PBS containing 3% dehydrated milk and 0.1% Tween 20. Blots were then imunolabeled overnight at 4°C with antibodies AS/7 for $Gi_{\alpha-2}$, EC/2 for $Gi_{\alpha-3}$, QL for G_q/G_{11}, RM/1 for Gs_α, and SW/1 for $G_{\beta\gamma}$ (1: 4000, respectively) (NEN™ Life Science Products, Boston, MA, USA). Two hours after incubation with second antibody (goat anti-rabbit IgG, Bio-Rad, Laboratories, Hercules, CA) at room temperature, immunoreactive proteins were detected by enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, England). The autoradiograms were quantified using an enhanced laser densitometer (LKB Ultroscan XL, Pharmacia, PQ, Canada) and a gel scan XL evolution software (version 2.1, Pharmacia).

GENERATION OF ROS

In experiments performed on cultured SMCs, the hypoxanthine (HX) and xanthine oxidase (XO) reaction was used since oxidation of HX by XO leads to the generation of superoxide anions (Wolfgang *et al.*, 1989).

In *in vitro* cell-free experiments, ROS ($O_2^{\bullet-}$, $OH^{\bullet}$, $^1O_2$) and their by-products (H_2O_2, HOCl, OCl^-) were generated by electrolysis (Dumoulin *et al.*, 1996). Electrolysis was carried out by conducting 10 mA DC for 1 minute through 3 ml of Krebs-Henseleit buffer consisting of (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.25, $MgSO_4$ 0.86, KH_2PO_4 1.2, $NaHCO_3$ 25.4, glucose 11.1, EDTA 0.057. The amount of generated ROS was evaluated by the DPD (n,n-diethy-p-

phenylenediamine) colorimetric method (Dumoulin *et al.*, 1996). Briefly, 1 ml of electrolyzed Krebs-Henseleit buffer was mixed with 2 ml of DPD solution (25 mg/ml, dissolved in Krebs-Henseleit buffer). The reaction of oxidant species with DPD produces a visible red color instantly. This colorimetric change was detected at 515 nm using an UV spectrophotometer (UV160U, Shimazu Corp., Kyoto, Japan).

CHAPTER 3

SUPEROXIDE ANION-INDUCED FORMATION OF INOSITOL PHOSPHATES INVOLVES TYROSINE KINASE ACTIVATION IN SMOOTH MUSCLE CELLS FROM RAT MESENTERIC ARTERY

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ABSTRACT

Our previous studies have demonstrated an enhanced production of inositol phosphates (IPs) induced by superoxide in smooth muscle cells (SMCs). The mechanisms for this effect, however, remained largely unknown. In the present study, it was found that superoxide increased IP production in SMCs from rat mesenteric arteries in a time dependent manner. The effect of superoxide on IP formation was significantly inhibited by the antioxidants n-acetylcysteine or • -lipoic acid. Genistein and tyrphostin A25, two tyrosine kinase inhibitors, also inhibited the superoxide-induced IP formation. The application of monoclonal antibody for phospholipase C_γ (PLC_γ) significantly inhibited the superoxide-induced IP formation. Finally, the expression level of PLC_γ proteins was increased 6 hrs after exposing SMCs to superoxide. The present findings demonstrate that superoxide activate the tyrosine kinase pathway and suggest that the tyrosine kinase-mediated IP formation may represent a novel mechanism underlying the signalling role of superoxide in rat mesenteric artery SMCs.

INTRODUCTION

The metabolism of inositol phosphates (IPs) in smooth muscle cells (SMCs) is important for the regulation of cellular functions. The ligand-receptor interaction at the plasma membrane surface activates phospholipase C (PLC) that catalyzes the hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (1-4). IP_3 serves as an intracellular signal to release calcium from the IP_3-sensitive calcium pools, leading to an enhanced vascular contractility. Three PLC isoforms (β, γ, and δ) have been identified in mammalian tissues (5, 6), PLC_γ in rat vascular SMCs (7), PLC_β and PLC_γ in human and rat aortic SMCs (8, 9), and PLC_δ in rat aorta (10). The activation of Gq protein stimulates PLC_β (6, 11), whereas PLC_γ is activated when its tyrosine residues are phosphorylated by tyrosine kinase (6). The activation of either PLC_β or PLC_γ leads to the generation of IP_3 albeit involving different signal transduction pathways. Whether the activation of PLC_δ elicits IP_3 production is presently still unsettled.

Reactive oxygen species (ROS) classically have an oxygen-based unpaired electron, and are generated during oxidative metabolism (12, 13). Recent studies indicate that many extracellular ligands generate and/or require ROS to transmit biological signals to intracellular milieu. These observations suggest that ROS could serve as the second messengers under certain conditions (12, 14). Our previous studies have demonstrated that superoxide significantly increased IP formation in rat aortic SMCs (15). This effect was independent of the cross-talk between IPs and cAMP since the inhibition of adenylate cyclase with SQ 22536 did not alter the effect of superoxide on IP formation (16). However, the intermediate steps between the generation of superoxide and an increased IP formation have not been fully understood.

The present study was carried out to characterize the superoxide-induced increase in IP formation in rat mesenteric artery SMCs and to investigate the underlying signal transduction mechanisms. The interaction of superoxide and some potent antioxidants on IP formation was examined. The superoxide-induced IP formation was determined after blockade of tyrosine kinase-mediated signalling pathway by inhibition of the activities of tyrosine kinases and their down stream phospholipase C_γ (PLC_γ). The translational regulation of the expression of PLC_γ by superoxide was also explored.

MATERIALS AND METHODS

Cell culture.

Single SMCs from mesenteric arteries were prepared as described previously (17-19). Briefly, rat mesenteric arteries were isolated and connective tissues were removed. The vessels were enzymatically digested with collagenase/dispase, elastase and collagenase in a stepwise manner. Dispersed cells were plated in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum in a CO_2 incubator at 37°C. Cells between passages 2 to 8 were seeded into 132-mm six well multidishes or 100-mm dishes and used for both IPs and immunoblotting analysis. There was no significant difference in the superoxide-induced increase in the formation of inositol phosphates in cultured SMCs among passages 2-8.

Measurement of IP formation.

SMCs were incubated for 24 hrs in the serum-free and inositol-free DMEM to which 5 μCi/ml of myo-[2-^{3}H] inositol (Du Pont Canada Inc. Diagnosis and Biotechnology Systems, Ont,

Canada) were added (15, 17). Thereafter, the culture medium containing unincorporated isotope was removed and the cells were rinsed three times with an Earle's balanced salt solution (EBSS). In situation when the cell membrane needed to be permeabilized, the cells were then incubated for 4 min in a cytosolic buffer containing (in mM) NaCl 20, KCl 100, $NaHCO_3$ 25, $MgSO_4$ 5, $NaHPO_4$ 0.96, $CaCl_2$ 0.48, EGTA 1, ATP 1.5, creatine phosphate 5, creatine phosphokinase (10 U/ml), bovine serum albumin 2%, and saponin (75 μg/ml) (20,21). Within 4 min of exposure to saponin, more than 95% of cells were stained by 0.025% Azure A (Sigma Chemical Co. St. Louis, MO, USA), a blue DNA dye. This was the indication of the increased permeability of plasma membrane after saponin treatment. The cells were incubated for another 30 min in the same EBSS (if not treated with saponin) or the cytosolic buffer (if permeabilized) containing 20 mM LiCl. Finally, the cells were subjected to hypoxanthine-xanthine oxidase in the absence or presence of antioxidants or other treatments for various periods, and the reaction was terminated by adding 0.9 ml of methanol:chloroform:HCl (40:20:1). The tritiated IP pool of the aqueous phase composed of inositol 4-phosphate (IP_1), inositol 4,5-biphosphate (IP_2), and inositol 1,4,5-trisphosphate (IP_3) was eluted consecutively by ion-exchange chromatography (AG1-X8 resin, Bio-Rad Laboratories, Ont, Canada). The lipid phase was counted to measure the phosphatidylinositol lipid pool (PIP). IP_1, IP_2, IP_3 and IPs (IP_1+IP_2 +IP_3) were expressed as a relative value of [(IP/PIP) x 10^3] (arbitrary units) to correct for the variation in the labeling of the lipid pool.

Immunoblotting analysis.

SMCs grown in 100-mm dishes were stimulated with agonists at 37°C in a serum-free DMEM for various periods. Cells were washed twice with ice-cold PBS and then were scraped

off in 400 μl of ice-cold lysis buffer containing (in mM) Tris-HCl 50, NaCl 50, HEPES 50, EDTA 5, Triton X-100 1%, Na-deoxycholate 0.25%, phenylmethylsulfonyl fluoride 1, NaF 1, Na_3VO_4 1, aprotinin 10 μg/ml, pepstatin 10 μg/ml and leupeptin 10 μg/ml. The suspension was gently rocked on a shaker at 4° C for 25 min to lyse cells. The lysates were centrifuged at x14,000 g at 4°C for 25 min and supernatants were used freshly or stored at –80°C. The samples containing 40 μg of proteins were separated on 5% and 10% polyacrylamide gels using a mini-vertical electrophoresis system (mini-Protein II cell, Bio-Rad, Richmond, CA, USA) and transferred to nitrocellulose membranes (Amersham Corp., Ont, Canada) overnight. The nitrocellulose membranes were blocked in PBS containing 0.1% Tween 20 and 3% dehydrated milk for 2 hrs and then incubated with anti-PLC_γ monoclonal antibody (0.5 μg/ml, Upstate Biotechnology, Lake Placid, NY, USA) in PBS containing 0.1% Tween 20 and 1.5% dehydrated milk for 2 hrs at room temperature. The membranes were washed 3 times with PBS containing 1.5% dehydrated milk and then incubated with the second antibody (goat anti-rabbit IgG, 1:4000, Bio-Rad Laboratories, Hercules, CA, USA) for 3 hrs at room temperature. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, England). The autoradiograms were quantified using UN-SCAN-IT gel Automated Digitizing System (version 5.1, Silk Scientific Inc. Orem, Utah, USA).

Chemicals and data analysis.

Hypoxanthine (HX), xanthine oxidase (XO), platelet-derived growth factor (PDGF-BB), and angiotensin II were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Genistein, tyrphostin A25, n-acetylcysteine (NAC) and α-lipoic acid (LA) were purchased from Calbiochem (La Jolla, CA, USA). Data were expressed as means ± SEM and analyzed using

Student's *t*-test or analysis of variance in conjunction with the Newman-Keuls test where applicable. Differences between groups were considered statistically significant when $p < 0.05$.

RESULTS

Effect of superoxide on IP formation.

Superoxide generated from hypoxanthine (HX) and xanthine oxidase (XO) reaction significantly increased the formation of IP_1, IP_2, and IP_3 in a time-dependent manner (Fig.3-1). Sixty minutes treatment of SMCs with HX (100 μM) and XO (10 mU/ml) maximally increased the formation of IP_1, IP_2, and IP_3. Fig. 3-2 shows that n-acetylcysteine (NAC) or α-lipoic acid significantly attenuated the superoxide-induced increase in the formation of IP_1, IP_2, and IP_3. For instance, after the cells were pretreated with NAC (600 μM) for 20 min, the HX-XO-induced formation of IP_1, IP_2, and IP_3 was significantly reduced by 27 ± 9%, 40 ± 7% and 45 ± 7.3%, respectively ($p<0.05$, $n=6$). NAC (600 μM) or α-lipoic acid (600 μM) had no effect on the basal levels of IPs in SMCs from rat mesenteric arteries (data not shown).

The interaction of tyrosine kinase activity with the superoxide-induced IP formation.

To explore the possibility that the superoxide-induced IP formation may be secondary to the activation of tyrosine kinase pathway, we examined the effects of two tyrosine kinase inhibitors on the superoxide-induced IP formation in mesenteric artery SMCs. Genistein or tyrphostin A25 significantly inhibited the superoxide-induced formation of IPs in a concentration-dependent manner (1 to 50 μM) (Fig. 3-3). The inhibitory effect of tyrphostin A25 was much greater than that of genistein. At 50 μM, tyrphostin A25 completely inhibited the

superoxide-induced formation of IPs whereas genistein only inhibited the effect of superoxide by 52±8% ($p<0.05$, n=4-6 for each group). To confirm the specificity of the effects of genistein or tyrphostin A25 on tyrosine kinase pathway, the IP formation induced by platelet-derived growth factor (PDGF-BB) or angiotensin II in the presence of genistein or tyrphostin A25 was further tested. Fig. 3-4 shows that genistein (50 μM) significantly inhibited the formation of IPs induced by PDGF-BB (50 ng/ml)- or angiotensin II (1 μM) by 74±2.5% or 40±3% ($p<0.05$, n=4 for each group), respectively. Pretreatment of cells with tyrphostin A25 (50 μM) for 20 min significantly decreased the PDGF-BB (50 ng/ml)- or angiotensin II (1 μM)-induced IP formation by 88±3% or 50±5% ($p<0.05$, n=4 for each group), respectively.

The effects of superoxide on PLC$_\gamma$.

Whether the superoxide-induced IP formation was due to an increase in PLC$_\gamma$ was also investigated. Fig 3-5A shows that IP formation induced by HX (150 μM) and XO (15 mU/ml) was significantly inhibited by 26.3±5.2% one hour after these cells were incubated with anti-PLC$_\gamma$ monoclonal antibody (5 μg/ml) ($p<0.05$, n=4 for each group). There was no significant difference in the effect of HX (150 μM) and XO (15 mU/ml) on IP formation between the non-permeabilized and permeabilized cells (data not shown). Fig. 3-5B shows that the expression level of PLC$_\gamma$ was affected by HX-XO treatment in a time-dependent manner. Within 4 hrs of HX (150 • M)-XO (15 mU/ml) incubation, PLC$_\gamma$ protein contents of the rat mesenteric artery SMCs were not evidently changed. However, the relative level of PLC$_\gamma$ proteins was increased by 151.6 ± 6.7% 6 hrs after the HX-XO incubation of the cells ($p<0.05$, n=5). The elevated

PLC_γ protein level was maintained in the SMCs incubated with HX-XO for 12 hrs (160 ± 7.5% vs. control of 100%, n=5, $p<0.05$).

DISCUSSION

The cellular levels of IPs in SMCs are closely linked to calcium homeostasis via the modulation of intracellular calcium release from the IP_3-sensitive calcium pool (1, 22, 23). The observed increase in IP metabolism in SMCs following HX-XO reaction may constitute one of the mechanisms whereby superoxide participates in the functional regulation of vascular SMCs under either physiological or pathophysiological conditions (11,14,15). However, the mechanisms by which superoxide enhanced IP formation were still unknown. Binding of specific ligand, such as norepinephrine, to G protein-coupled receptor activates PLC_β. At variance, PLC_γ is directly activated by agonist stimulation of tyrosine kinase receptors which bind to *src* homology 2 (SH2) domains within PLC_γ molecules, leading to receptor-PLC_γ complex formation and PLC_γ activation (24, 25). Nevertheless, both PLC_β and PLC_γ act on the common target, i.e. PIP_2, to catalyze the formation of IP_3 (4). It is thus hypothesized that superoxide-induced IP formation in rat vascular SMCs may involve the activation of one or more isoforms of PLC. Our results, for the first time, demonstrated that the superoxide could stimulate tyrosine kinase *src* families, activate PLC_γ, and consequently increased IP formation in rat mesenteric artery SMCs. Several lines of evidence support the contribution of an activated tyrosine kinase-linked PLC_γ pathway to the superoxide-induced IP formation. (1) The stimulation of tyrosine kinases with PDGF-BB or angiotensin II increased the formation of IPs, which mimics the effect of HX-XO. (2) The inhibition of tyrosine kinases by genistein or

tyrphostine A25 attenuated the superoxide-induced IP formation (Fig. 4). (3) At the down-stream level of tyrosine kinase, the superoxide-induced IP formation was significantly inhibited by anti-PLC_γ monoclonal antibody treatment (Fig. 3-5A). (4) The expression levels of PLC_γ proteins were increased after the prolong treatment of cells with superoxide (Fig. 3-5B). Clearly, the regulation of tyrosine kinase- PLC_γ - IP_3 axis by superoxide represent a novel signal transduction mechanism not only because the effect of superoxide on PLC_γ had not been demonstrated before, but also because this mechanism can activate the tyrosine kinase-PLC_γ pathway in a membrane receptor-independent fashion. Future studies are needed however to clarify whether superoxide selectively acts on tyrosin kinase or on PLC_γ in vascular SMCs.

In our study, the superoxide-induced increase in IP production was completely inhibited by the tyrosine kinase inhibitor tyrphostin A25 in contract to a lesser inhibition with genistein (Fig. 3-3). Different potencies of genistein and tyrphostin A25 in inhibiting the superoxide-induced IP production in cultured SMCs from rat mesenteric arteries (Figs. 3-3 & 3-4) have also been observed in other studies (26). Hollenberg (26) found that the inhibitory effect of tyrphostin A25 was greater than that of genistein on the epidermal growth factor-triggered contraction of gastric longitudinal muscles. Since the relative inhibitory constant (Ki) of genistein and tyrphostin A25 were 1 and 3, respectively, the greater inhibitory potency of tyrphostin A25 on tyrosine kinases might be related to its greater competitive affinity to protein tyrosine kinases as well as to its greater membrane permeability (27, 28).

A superfamily of tyrosine kinases has been discovered since a transforming gene ($p60^{v\text{-}src}$) was found to encode a protein kinase that phosphorylated a wide variety of tyrosine substrates (29-31). Besides other cellular effects, tyrosine kinases are actively involved in the acute regulation of smooth muscle contractility (24). PDGF, for example, regulates vascular

tone by acting on specific membrane receptors, which possess intrinsic tyrosine kinase activity (32). Angiotensin II, on the other hand, regulates vascular smooth muscle contraction through two different signal transduction pathways. The activation of AT_1 receptors stimulates both G-protein-linked PLC_β and tyrosine kinase-linked PLC_γ (33). This dual signalling mechanism could be the reason why the tyrosine kinase inhibitors have greater inhibitory effects on the IP formation induced by PDGF than that induced by angiotensin II in mesenteric artery SMCs (Fig. 4). The residual effect of angiotensin II on the IP formation after the blockade of tyrosine kinases may result from the tyrosine kinase-independent, but G protein-coupled activation of PLC_β.

In conclusion, our results demonstrated that superoxide anions increased the formation of IPs in vascular SMCs. More importantly, our findings strongly suggest that the increased IP formation in SMCs induced by superoxide anions is largely due to the activation of the tyrosine kinase-linked PLC_γ signaling pathway. The superoxide anion-induced activation of tyrosine kinases may represent a novel mechanism by which ROS could be actively involved in the formation of phosphoinositide-derived second messengers to regulate the functions of vascular SMCs.

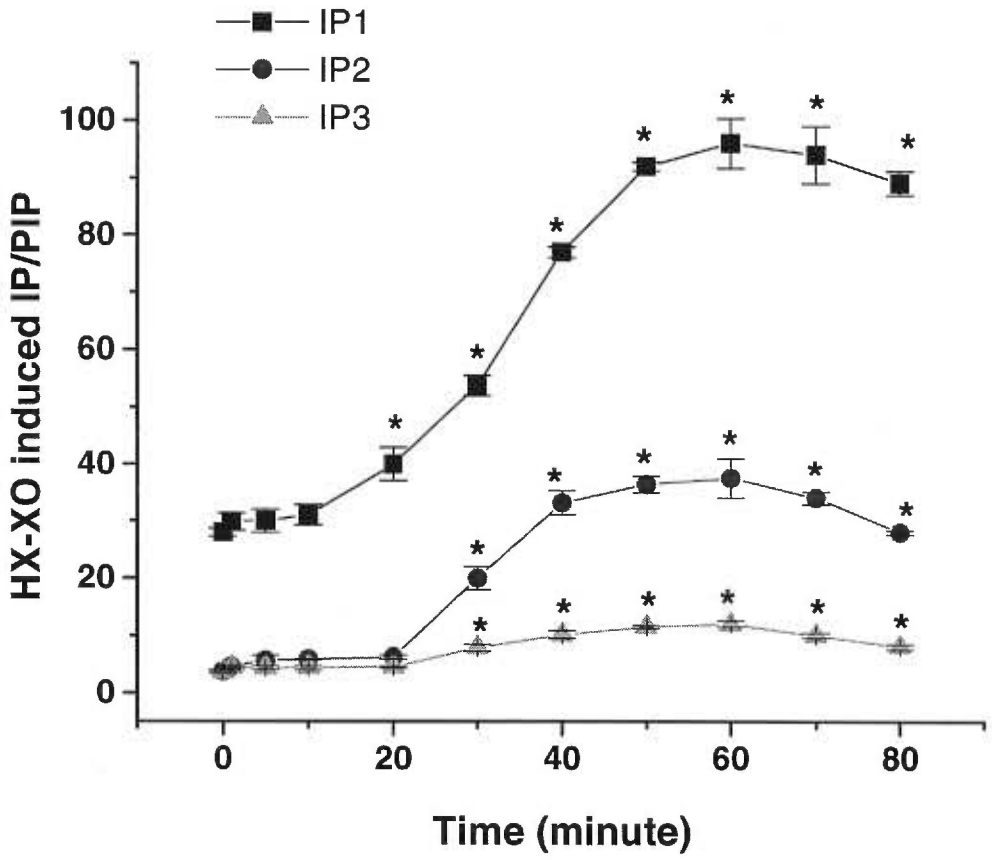


Fig. 3-1 The effect of superoxide anions on IP formation in rat mesenteric artery SMCs. Hypoxanthine (HX, 100 μM) and xanthine oxidase (XO, 10 mU/ml) increased the formation of IP_1, IP_2 and IP_3 in a time-dependent manner in SMCs. n = 5 per data point. * indicates $p<0.05$, compared to control group.

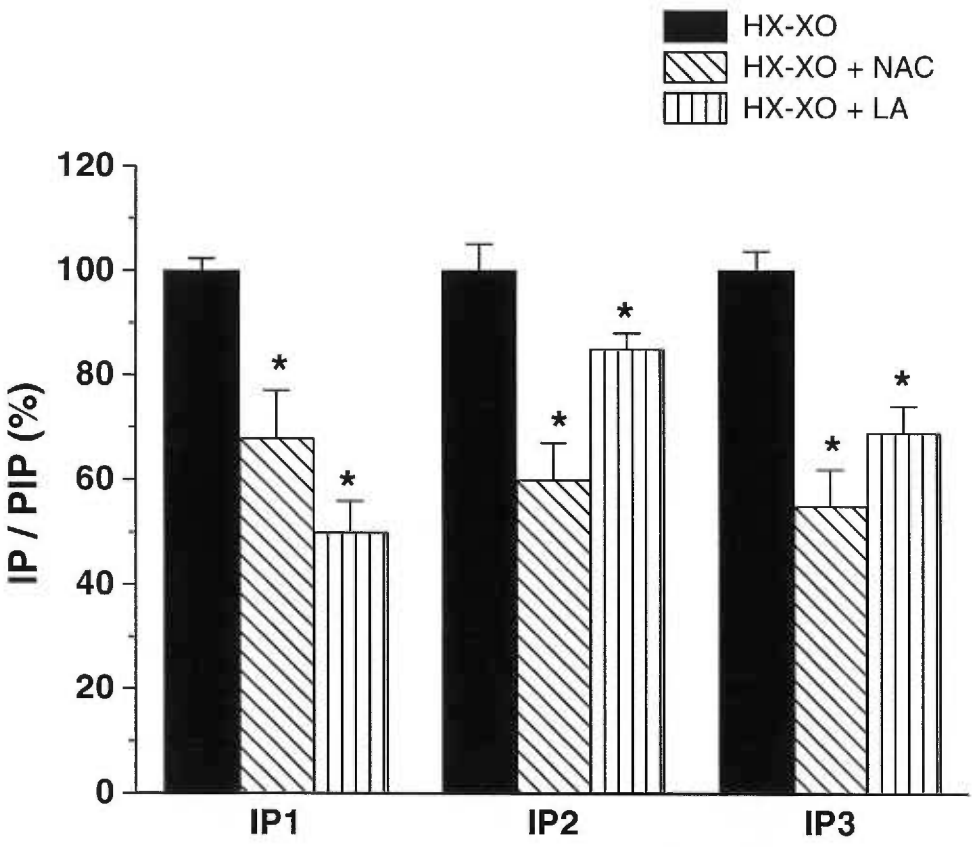


Fig. 3-2. Effects of antioxidants on the superoxide anion-induced IP formation in rat mesenteric artery SMCs. The HX (100 μM)-XO (10 mU/ml) induced IP formation was significantly inhibited by n-acetylcysteine (NAC, 600 μM), or α-lipoic acid (LA, 600 μM), respectively. * indicates $p<0.05$, compared to HX-XO group. n = 6 for each group.

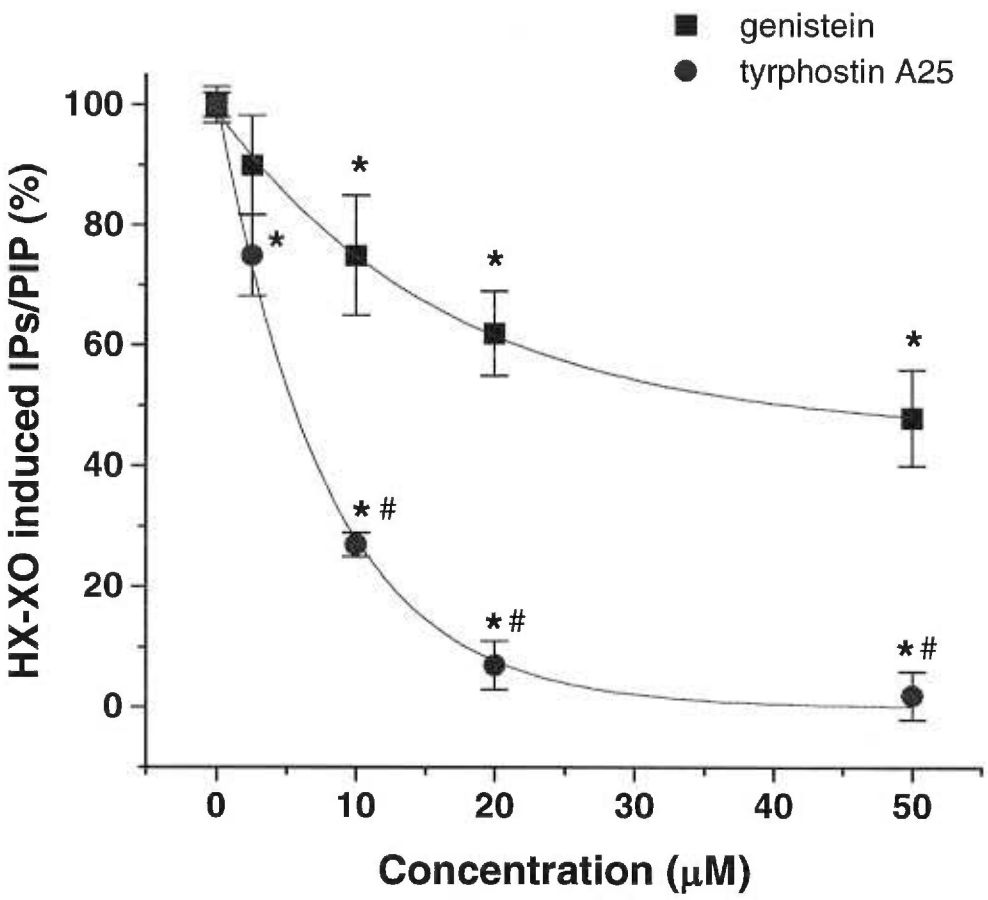


Fig. 3-3. The Superoxide anion-induced formation of IPs in SMCs after the inhibition of tyrosine kinases. Following pretreatment with genistein or tyrphostin A25 at different concentrations for 20 min, the HX-XO-induced IP formation was progressively and dose-dependently inhibited. n = 4-6 per data point. * indicates $p<0.05$, compared to HX-XO group. # indicates $p<0.05$, tyrphostin A25 group compared to genistein group.

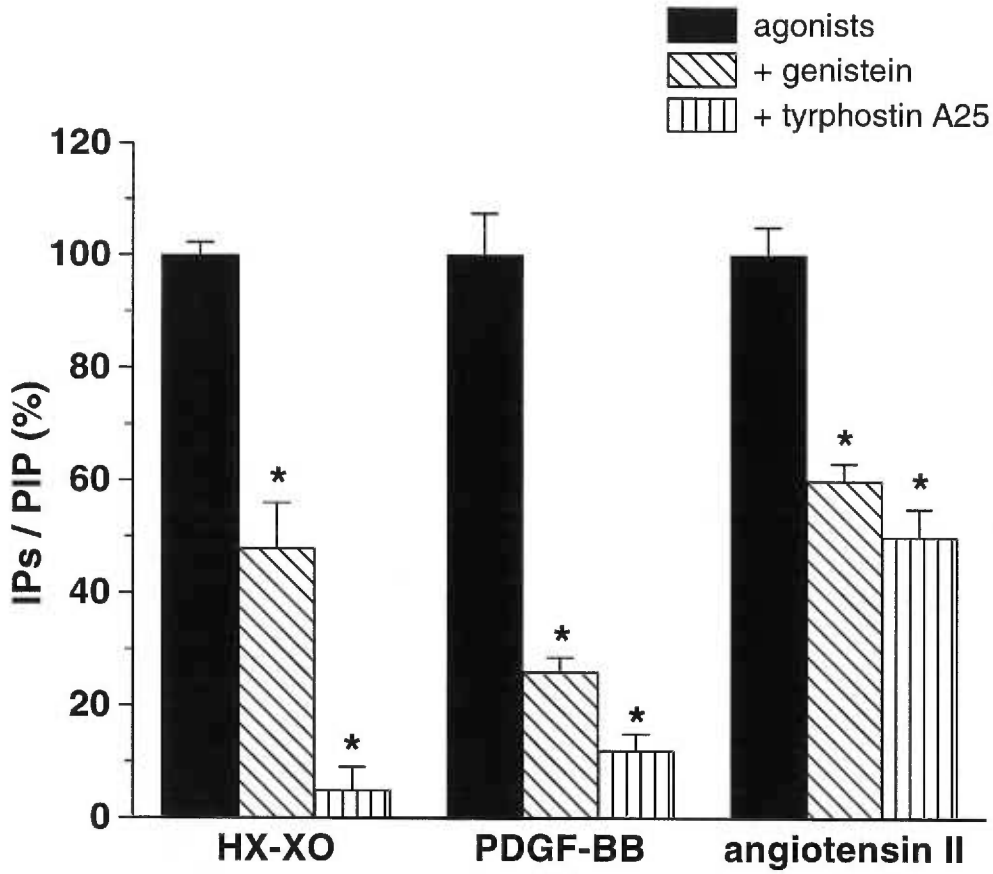
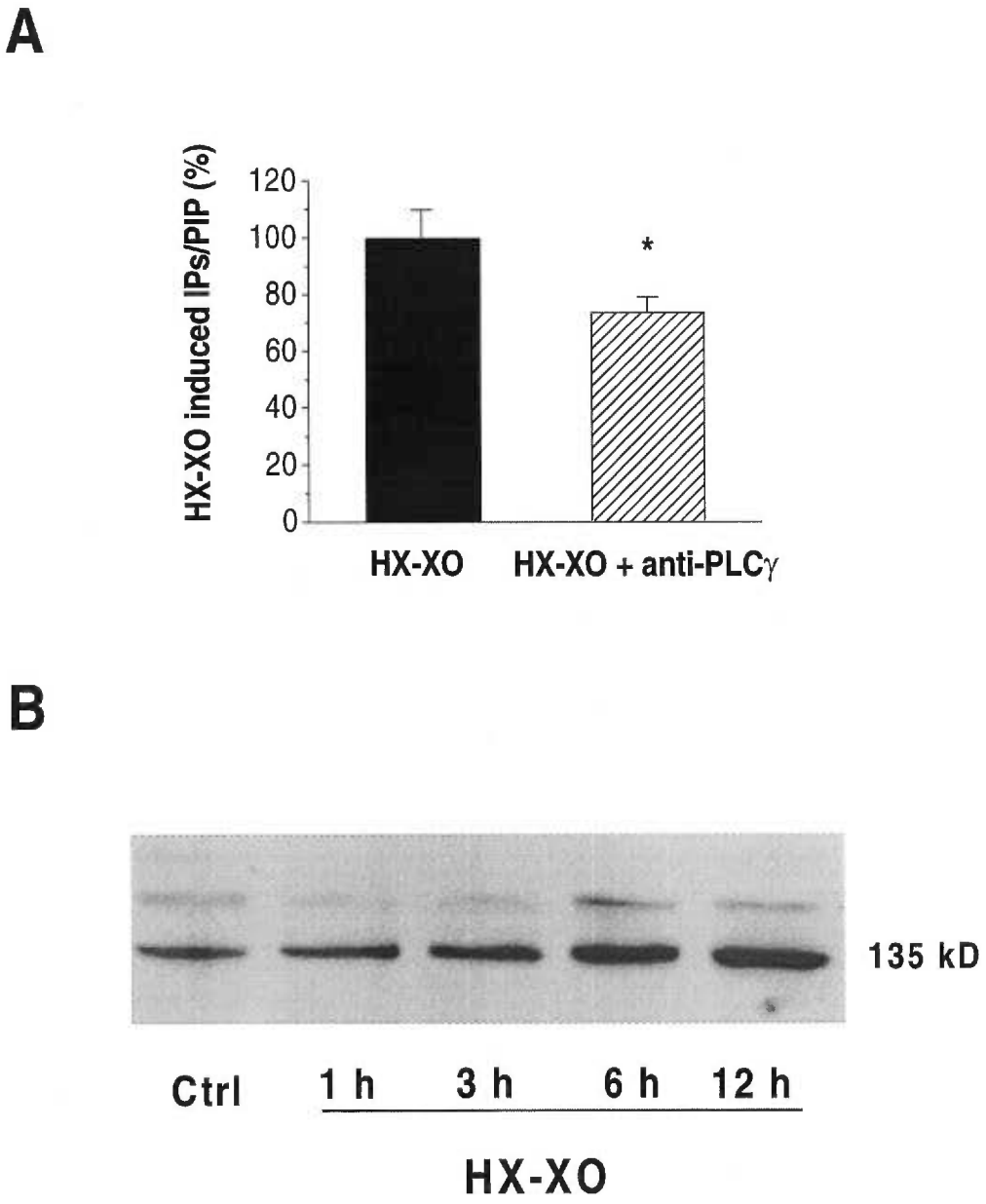


Fig. 3-4. The effects of tyrosine kinase inhibition on the formation of IPs in SMCs induced by superoxide anions, platelet-derived growth factor (PDGF-BB), and angiotensin II. The IP formation stimulated by HX (100 μM)-XO (10 mU/ml), angiotensin II (1 μM) or PDGF-BB (50 ng/ml) was examined in the absence or presence of genistein (50 μM) or tyrphostin A25 (50 μM), respectively. * indicates $p<0.05$ compared to agonist-treated control group. $n = 4$ for each group.

Fig. 3-5. The participation of PLC_γ in the superoxide anion-induce IP formation.



L. Wu, Superoxide anion as a signalling molecule.

Fig. 3-5. The participation of PLC_γ in the superoxide anion-induce IP formation. **A.** The IP formation stimulated by HX (150 • M)-XO (15 mU/ml) in the saponin-permibilized cells with or without pre-incubation with monoclonal antibody to PLC_γ (5 μg/ml) for 1 hr. **B.** The expression levels of PLC_γ proteins were determined 1, 3, 6 and 12 hrs after incubating the cells with HX (150 • M)-XO (15 mU/ml). The membrane proteins of SMCs (40 μg) were separated on SDS/PAGE and transferred to nitrocellulose, which was then immunoblotted using anti-PLC_γ monoclonal antibody as described in the Methods section. The positive bands at 135 kD represent PLCγ proteins. Similar results were obtained in 4 other experiments. * $p<0.05$.

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CHAPTER 4

THE DUAL EFFECTS OF SUPEROXIDE ANION ON THE INOSITOL 1,4,5-TRISPHOSPHATE AND THE CYCLIC GMP PATHWAYS:

A novel cross-talk mechanism in vascular smooth muscle cells.

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ABSTRACT

(1) Superoxide anion generated by hypoxanthine (HX)-xanthine oxidase (XO) reaction increased IP_3 production in smooth muscle cells from rat mesenteric arteries in a concentration-dependent manner. Superoxide dismutase or melatonin significantly inhibited the HX-XO-induced IP_3 formation. The inhibition of phospholipase C with U-73122 abolished the effect of superoxide on IP_3 formation

(2) The basal levels of cGMP were increased by sodium nitroprusside (SNP) and decreased by superoxide or by blockade of guanylyl cyclase with ODQ. The SNP induced increase in cGMP was markedly inhibited by superoxide.

(3) The basal levels of IP_3 were decreased by SNP or s-nitroso-n-acetylpenicillamine (SNAP), but were not affected by ODQ. The superoxide-increased IP_3 formation was significantly inhibited by SNP or SNAP. Moreover, ODQ or KT5823 (a cGMP-dependent protein kinase inhibitor) potentiated the superoxide-induced increase in IP_3 formation.

(4) The superoxide-enhanced IP_3 formation was not associated to simultaneous changes in cAMP pathway. Superoxide had no effect on the basal or the forskolin-induced cAMP production and the inhibition of adenylate cyclase or cAMP-dependent protein kinase did not affect the superoxide-enhanced IP_3 formation.

(5) Since cGMP inhibited the IP_3 formation, the elevated IP_3 levels in the presence of superoxide could result both from a direct stimulatory effect of superoxide on IP_3 formation and from an inhibitory effect of superoxide on cGMP metabolism. The cross-talk between cGMP and IP_3 pathways provides a novel mechanism for the signalling role of superoxide in vascular SMCs.

INTRODUCTION

The signal transduction pathway linked to inositol 1,4,5-trisphosphate (IP_3) plays an essential role in regulating the function and proliferation of cells (Berridge, 1993). IP_3 releases calcium from intracellular pools and facilitates, indirectly, the extracellular calcium entry (Somlyo & Somlyo, 1994). The resulting increase in intracellular free calcium concentration of vascular smooth muscle cells (SMCs) either triggers or enhances vascular contraction. The generation of IP_3 is usually initiated by the binding of hormonal ligands to their respective membrane receptors. The excitation of receptor-coupled G proteins or receptor tyrosine kinases activates phospholipase C (PLC) located on the inner surface of the membrane. Subsequent hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) releases IP_3 and diacylglycerol. Whether IP_3 can also be generated in a membrane receptor-independent manner has not been addressed. In this regard, reactive oxygen species (ROS) may represent a putative intracellular signalling system that could alter inositol phosphate (IP) metabolism and, thus, regulate vascular functions.

ROS generated during oxidative metabolism, have multiple detrimental effects including oxidative modification of membrane proteins (Götz *et al.*, 1994), and altered calcium homeostasis (Suzuki & Ford, 1992). An increased vascular reactivity to ROS has been reported in aorta from hypertensive rats (Wolfgang *et al.*, 1989). Recently, the role of ROS as endogenous signalling molecules has been suggested based on the common availability of ROS from biological metabolic sources and on the physiological effects of ROS. The IP_3-induced intracellular calcium release from bovine aortic smooth muscle sarcoplasmic reticulum, for instance, was facilitated by superoxide (Suzuki *et al.*, 1997). In human umbilical vein

endothelial cells, superoxide alone induced a transient rise in cytosolic calcium concentration, possibly due to calcium release from an IP_3-sensitive intracellular pool (Dreher *et al.*, 1995). These results can be explained either by a direct effect of superoxide on the function of the IP_3-sensitive calcium releasing channels, such as the phosphorylation of channel protein by the oxidant or by direct superoxide-induced changes in phosphoinositide metabolism. Studies on bovine aortic smooth muscles or pig coronary artery SMCs have suggested that the IP_3-sensitive calcium releasing channels were not affected by superoxide (Suzuki & Ford, 1992; Elmoselhi *et al.*, 1996), though the loading capacity of this IP_3-sensitive calcium pool might be impaired (Grover *et al.*, 1995). Our previous study demonstrated that the norepinephrine-induced vasoconstriction and IP formation in rat aortic SMCs were partially inhibited by superoxide dismutase, but not by catalase (Wu *et al.*, 1998). Accordingly, it was suggested that the norepinephrine-induced increase in IP formation might be partially related to the auto-oxidation of the catecholamine, leading to the production of superoxide. To our knowledge, there exists no evidence demonstrating an alteration in phosphoinositide metabolism in SMCs from peripheral resistance arteries following exposure to superoxide or to a superoxide generating system, such as the hypoxanthine (HX) - xanthine oxidase (XO) reaction.

An increase in either cAMP (Bennett & Waldman, 1995; Opie, 1998) or cGMP concentrations (Kawada *et al.*, 1997; Wang *et al.*, 1997) in vascular SMCs results in vasorelaxation. One possible mechanism underlying the same vascular response to these two different second messengers might be the cross-activation of cGMP-dependent protein kinase by both cGMP and cAMP (Lincoln *et al.*, 1991; Jiang *et al.*, 1992). The contractile states of vascular tissues may also be influenced by a cross-talk between cAMP and IP_3 signalling pathways or between GMP and IP_3 signalling pathways. It has been observed that 8-bromo-

cGMP or the guanylyl cyclase stimulator sodium nitroprusside significantly suppressed the IP_3 formation in arterial endothelial cells induced by thrombin (Lang & Lewis, 1991). The phenylephrine-induced IP formation in rat aortic SMCs was inhibited by 8-bromo-cAMP or by an adenylyl cyclase stimulator forskolin (Wu & de Champlain, 1996). Interestingly, an inhibition of the cAMP metabolism has recently been reported following superoxide exposure in mesangial cells and human fibroblast (Chini *et al.*, 1997; Raynaud *et al.*, 1997). These studies raised many intriguing and important issues concerning the cross interactions between the IP_3 pathway and other signalling pathways. Whether superoxide alters cGMP and/or cAMP metabolism in vascular SMCs and whether the effect of superoxide on IP_3 formation is modulated by simultaneous changes in cGMP or cAMP levels constitute a logical hypothesis which needed to be addressed. Therefore, in order to achieve a better understanding of the signalling role of superoxide in relation to the activities of different signal transduction pathways in vascular SMCs, the present study was undertaken. The cellular levels of IP_3 were measured in the absence or presence of superoxide in cultured rat mesenteric artery SMCs. Various putative mechanisms underlying the effect of superoxide on IP_3 formation were explored by evaluating the effects of superoxide on the basal level of cGMP or cAMP and by comparing the effects of specific modulators of cGMP or cAMP metabolism. Moreover, the superoxide-induced IP_3 formation was studied after the blockade of cAMP or cGMP pathways, allowing for the evaluation of the cross-talk interaction between the superoxide-induced IP_3 formation and other signal transduction pathways.

MATERIALS AND METHODS

Smooth muscle cell culture.

Single SMCs from mesenteric arteries were prepared as previously described (Ullian & Linas, 1990; Wu & de Champlain, 1996). Briefly, rat mesenteric arteries were isolated and connective tissues were removed. The vessels were enzymatically digested with collagenase/dispase, elastase and collagenase in a stepwise manner. Dispersed cells were plated in 75 cm^2 flasks in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in a CO_2 incubator at 37°C. Cells between passages 2 to 8 were seeded into 132-mm six-well multi-dishes, and used for the measurements of IP_3, cAMP, and cGMP. There was no significant difference in the superoxide-induced increase in the formation of IP_3 in cultured SMCs among passages 2-8.

Measurement of IP_3 formation.

SMCs were incubated for 24 hours in a serum-free and inositol-free Dulbecco's modified Eagle's medium to which 5 μCi/ml of myo-[2-^{3}H] inositol (Du Pont Canada Inc. Diagnosis and Biotechnology Systems, Ont., Canada) were added. As described in our previous publications (Wu & de Champlain, 1996; Wu *et al.*, 1998), the culture medium containing unincorporated isotope was removed and the cells were rinsed three times with an Earle's balanced salt solution. The cells were further incubated for 30 minutes in the same buffer containing 20 mM LiCl. Treatments with hypoxanthine-xanthine oxidase alone or combined with other agents were carried out for various durations, and the reaction was terminated by adding 0.9 ml of methanol: chloroform: HCl (40:20:1). The tritiated IP pool of the aqueous phase constituted by inositol 4-

phosphate (IP_1), inositol 4,5-biphosphate (IP_2), and inositol 1,4,5-trisphosphate (IP_3) was eluted consecutively by ion-exchange chromatography (AG1-X8 resin, Bio-Rad Laboratories, Ont., Canada). The lipid phase was counted to measure the phosphatidylinositol lipid pool (PIP). IP_3 was expressed as a relative value of [(IP_3/PIP) x 10^3] (arbitrary units) to correct for the variation in the labeling of the lipid pool.

Quantitative determination of cAMP level and cGMP level.

The level of cAMP in cultured SMCs was determined by a protein-binding assay. This assay involves the competition between unlabeled cAMP and a fixed quantity of [^{3}H] cAMP for the binding to a protein that has a high specificity and affinity for cAMP (cAMP ([^{3}H] assay system, Amersham Corp., Ont., Canada). The quantitative determination of cGMP was achieved with a cGMP [^{125}I] assay system with Amerlex-M^{TM} magnetic separation. This system utilizes a high specific activity [^{125}I] 2'-O-succinyl-cGMP tyrosine methyl ester tracer, together with a highly specific and sensitive antiserum, thus allowing for a simple magnetic separation of the antibody bounded [^{125}I] cGMP from free fraction (cGMP [^{125}I] assay system, Amersham Corp., Ont., Canada).

Chemicals and data analysis.

Hypoxanthine (HX), xanthine oxidase (XO), melatonin, superoxide dismutase (SOD), catalase, forskolin (FSK), (-)isoproterenol (ISO) and methylene blue (MB) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). SQ 22536, n-acetylcysteine (NAC), U-73122, KT5720, KT5823, sodium nitroprusside (SNP) and s-nitroso-n-acetylpenicillamine (SNAP) were purchased from Calbiochem (La Jolla, California, USA). ODQ was purchased from Tocris

Cookson Inc. (Ballwin, MO, USA). Data were expressed as means ± SEM and were analyzed using the Student's *t*-test or the analysis of variance in conjunction with the Newman-Keuls test where applicable. Differences between groups were considered statistically significant when the p value was <0.05.

RESULTS

Effect of superoxide anions on IP_3 formation.

The HX-XO reaction significantly increased IP_3 formation in rat mesenteric artery SMCs in a concentration-dependent manner (Fig. 4-1A). For instance, after treatment of SMCs with HX (100 μM) and XO (10 mU/ml) for 1 hour, the formation of IP_3 was 6-fold greater than the basal level ($p<0.05$, $n=10$). A near saturated formation of IP_3 was achieved with HX at 150 μM and XO at 15 mU/ml. A further increase in concentrations of HX (200 μM) and XO (20 mU/ml) decreased the viability of the cells as they detached from the culture dishes and became fragmented (not shown).

The HX-XO reaction has been commonly used to generate OFR, especially superoxide (Wolfgang *et al.*, 1989). A further transfer of one electron to superoxide generated from the HX-XO reaction can produce hydrogen peroxide. Thus, both superoxide and hydrogen peroxide could theoretically be responsible for the HX-XO induced IP_3 formation. To confirm that the HX-XO reaction-generated superoxide was the effective ROS in our experiments, the effects of different antioxidants were examined. Melatonin (Wu *et al.*, 1998) and SOD (Suzuki and Ford, 1992) were used for their selective scavenging effect against superoxide in vascular SMCs. Catalase was used for its selective scavenging effect against hydrogen peroxide (Suzuki *et al.*,

1997). After the cells were pretreated with melatonin (600 μM) or SOD (120 U/ml) for 20 minutes, the HX (100 μM) and XO (10 mU/ml)-induced IP_3 formation was significantly reduced by 44±4% ($p<0.05$, n=6) or 34±3% ($p<0.05$, n=6), respectively. However, catalase (120 U/ml) had no significantly inhibitory effect on the HX-XO-induced increase in IP_3 formation ($p>0.05$, n=6). In addition, melatonin, SOD, and catalase had no effect on the basal IP_3 formation in cultured SMCs ($p>0.05$, n=4 for each group) (data not shown). To clarify whether hydrogen peroxide affected IP_3 formation in rat mesenteric artery SMCs similarly to superoxide, the direct effect of hydrogen peroxide was further tested. It was found that hydrogen peroxide at 10 μM, which is within the physiological concentration range (Suzuki *et al.*, 1997), had no effect on IP_3 formation ($p>0.05$, n=4). However, at a high concentration (1 mM), hydrogen peroxide slightly increased the IP_3 formation by 30% ($p<0.05$, n=4), and this increase was completely inhibited by catalase (data not shown).

To examine the effect of superoxide on the upstream mechanisms of IP_3 formation, the effect of U-73122, a PLC inhibitor (Smith *et al.*, 1996; Tatrai *et al.*, 1994) was tested. Fig. 4-2 shows that the blockade of PLC with U-73122 completely inhibited the effect of HX-XO-generated superoxide on IP_3 formation ($p<0.05$, n=4). In addition, the treatment with U-73122 significantly reduced basal control IP_3 levels when administered in the absence of HX-XO.

Effect of superoxide anions on cGMP and cAMP levels.

Ten minutes incubation of rat mesenteric artery SMCs with SNP (10 μM) significantly elevated cellular cGMP level ($p<0.05$, n=5) (Fig. 4-3). An 80±2.2% decrease in cGMP from the basal level was observed after the cells were treated with HX (150 μM)-XO (15 mU/ml) for 1 hour ($p<0.05$, n=5). In addition, the SNP-induced increase in cGMP level was markedly

inhibited by the pretreatment of the cells with HX (150 μM)-XO (15 mU/ml) ($p<0.05$, n=5). The basal level of cGMP was also significantly decreased by 36±3.1% after the application of ODQ (100 μM) for 80 minutes, a selective inhibitor of soluble guanylyl cyclase (Fedele *et al.*, 1996) ($p<0.05$, n=5). The inhibitory effect of HX (150 μM)-XO (15 mU/ml) on cGMP levels was not further potentiated when combined with ODQ (100 μM) treatment (Fig. 4-3), indicating that the target affected by both superoxide and ODQ might have been maximally inhibited by superoxide.

Fig. 4-4 shows that in SMCs treated with forskolin (10 μM) for 1 hour or isoproterenol (100 μM) for 10 minutes, a significant increase in cAMP above the basal level occurred ($p<0.05$, n=8 for each group). Unlike the finding on the cGMP response to superoxide, HX (150 μM)-XO (15 mU/ml) had no effect on the basal level or on the elevated cAMP level induced by forskolin or isoproterenol.

The interaction of cGMP and the superoxide anion-induced IP_3 formation.

Since superoxide simultaneously increased the IP_3 formation and decreased cGMP formation, it was postulated that the superoxide-enhanced IP_3 formation in vascular SMCs might be regulated by cGMP. First, the effects of guanylyl cyclase stimulators SNP and SNAP were evaluated on the basal level of IP_3 and it was found that those agents decreased the basal IP_3 formation by 18±2.6% or 30.4±2.7% ($p<0.05$, n=4 for each group), respectively. Thereafter, the superoxide-induced IP_3 formation was evaluated after stimulating or inhibiting the soluble guanylyl cyclase. Fig. 4-5A shows that the HX (100 μM)-XO (10 mU/ml)-induced IP_3 formation was significantly inhibited by 25±2.8% or 30±2.6% after the cells were pretreated either 10 minutes with SNP (10 μM) or 20 minutes with SNAP (100 μM) ($p<0.05$, n=4 for each

group), respectively. In contrast, the superoxide-induced IP_3 formation was significantly facilitated by 60±4.7% or 46±2.8% ($p<0.05$, n=4 for each group) after the inhibition of guanylyl cyclase by pre-incubating the cells 20 minutes either with ODQ (100 μM) or methylene blue (10 μM), respectively (Fig. 4-5B).

The interaction of cAMP and the superoxide anion-induced IP_3 formation.

Although the exposure of SMCs to superoxide *per se* did not alter cAMP levels, fluctuation in cAMP level induced by other factors might still modulate the superoxide-induced IP_3 formation. This hypothesis was tested by inhibiting cAMP formation first and evaluating the effects of superoxide on cAMP or IP_3 formation thereafter. In these experiments, the cells were pre-incubated with SQ 22536 (500 μM), an inhibitor of adenylyl cyclase (Talpain *et al.*, 1995), for 20 minutes and then exposed to HX (100 μM)-XO (10 mU/ml) for 1 hour. Fig. 4-6A shows that the superoxide-induced increase in IP_3 formation was not significantly modified by SQ 22536. In addition, similar basal levels of cAMP were observed in untreated (control) or in cells treated with SQ 22536, although the forskolin-induced cAMP formation was significantly inhibited by this adenylyl cyclase inhibitor ($p<0.05$, n=4 for each group) (Fig. 4-6B).

The modulation of the superoxide anion-induced IP_3 formation by cGMP-dependent protein kinase (PKG) or cAMP-dependent protein kinase (PKA).

To further explore the interaction of cGMP or cAMP pathway with the superoxide-induced IP_3 formation on the downstream mechanisms related to cGMP or cAMP levels, the effects of PKG or PKA inhibitors were examined on the superoxide-induced increase in IP_3 formation in mesenteric artery SMCs. Fig. 4-7 shows that the superoxide-induced increase in IP_3

formation was not altered by a 20 minute pretreatment of the cells with PKA inhibitor KT5720 ($p>0.05$, n=5 for each group). However, KT5823, a PKG inhibitor, significantly potentiated the superoxide-induced increase in IP_3 formation. At the concentration of 1 μM, KT5823 increased the HX (100 μM)-XO (10 mU/ml)-induced IP_3 formation by 50±11% ($p<0.05$, n=5).

DISCUSSION

An overproduction of superoxide anion may constitute an important factor contributing to the pathogenesis of various cardiovascular diseases. Nevertheless, at subtoxic levels this species of ROS was postulated to serve as a physiological signalling messenger (Suzuki *et al.*, 1997), participating in the regulation of the contraction and growth of vascular SMCs (Griendling *et al.*, 1994). Therefore, a better knowledge on the effects of superoxide on different signalling pathways may provide a better understanding of the modulating role of superoxide in vascular SMCs under both physiological and pathological conditions.

Superoxide anion has been postulated to be involved in IP_3 signal transduction pathway. The experimental evidence showed that superoxide transiently increased the IP_3-mediated intracellular calcium release from the fractionated sarcoplasmic reticulum of SMCs (Suzuki *et al.*, 1997) or vascular endothelial cells (Dreher *et al.*, 1995). These results could be explained either by an enhanced IP_3 formation or by an enhanced sensitivity of IP_3 receptors induced by superoxide. In the present study, the direct effect of superoxide was investigated on IP_3 formation in cultured SMCs from rat mesenteric artery. Our results clearly demonstrated that the HX-XO-generated superoxide increased IP_3 formation in vascular SMCs and that superoxide stimulated IP_3 formation by acting on the upstream mechanism of IP_3 formation, i.e. at the site of

PLC or above since the inhibition of PLC with U-73122 significantly abolished the effect of superoxide on IP_3 formation (Fig. 4-2).

The interaction between PLC signalling pathway and cGMP pathway has been reported previously (Lang & Lewis, 1991). It is known that elevated cGMP, through activation of PKG, inhibits PLC, thus decreasing the basal levels of IP_3 or attenuating the agonist-stimulated IP_3 formation. It can therefore be hypothesized that the superoxide-induced IP_3 formation is modulated by the activity of cGMP signalling pathway. Our observations demonstrated, for the first time, that superoxide inhibits cGMP formation, and that a cross-talk between cGMP and IP_3 pathways may be involved in the superoxide-induced increase in IP_3 formation in rat mesenteric artery SMCs. The following lines of evidence support this conclusion. (1) Superoxide anion generated through HX-XO reaction significantly decreased the basal level of cGMP and the SNP-stimulated cGMP formation. (2) The blockade of guanylyl cyclase by ODQ decreased the basal level of cGMP, but failed to further decrease the superoxide-reduced cGMP level (Fig. 4-3). Since the cGMP generation or guanylyl cyclase activity is probably maximally inhibited by high level of superoxide (HX 150 μM plus XO 15 mU/ml), the present results suggest that guanylyl cyclase is most likely the common target of ODQ and superoxide. (3) The superoxide-induced increase in IP_3 formation was significantly inhibited by the activation of soluble guanylyl cyclase with SNP or SNAP, but was potentiated by the inhibition of guanylyl cyclase with ODQ or methylene blue (Fig. 4-5). At basal level, the IP_3 pathway may be relatively insensitive to low concentration of cGMP, thus explaining why increasing markedly the basal cGMP level with SNP inhibited the basal IP_3 formation, whereas decreasing the basal cGMP level with ODQ did not increase basal IP_3 formation. Moreover, in addition to enhancing the IP_3 formation, superoxide at a sub-maximal concentration (HX 100 μM plus XO 10 mU/ml) could

increase the sensitivity of IP_3 pathway to cGMP regulation, thus explaining why the IP_3 formation can be further activated by the decrease in cGMP formation following the treatment with ODQ. (4) The superoxide-induced increase in IP_3 formation was significantly potentiated by a PKG inhibitor KT5823 (Fig. 4-7). Overall, Our data, therefore, suggest that the inhibitory effect of superoxide on cGMP formation probably contribute to the activation of IP_3 formation induced by superoxide by lifting the negative feedback of cGMP on the IP_3 pathway(s).

Previous studies from our laboratory as well as those of others have shown the existence of a complex cross-talk interaction between IP_3 and cAMP pathways. An activation of the cAMP pathway was shown to inhibit the phenylephrine-induced IP formation in rat aortic SMCs (Wu & de Champlain, 1996; Monolopoulos *et al.*, 1991). In addition, downstream the site of IP_3 formation, it was postulated that the IP_3 receptor might be phosphorylated and desensitized by an elevated cAMP level (Komalavilas & Loncoln, 1996). Since PKG which inhibit the IP_3 formation, can be cross-activated by an increase in cAMP (Jiang *et al.*, 1992), it is thus possible that cAMP could also participate indirectly in the regulation of IP_3 levels by the activation of PKG. The hypothesis that superoxide would inhibit the cAMP formation while simultaneously stimulating IP_3 formation was postulated on the findings of previous studies. For instance, Chini *et al.* (1997) reported that PKA exerted an inhibitory effect on ROS generation in rat mesangial cells and Raynaud *et al.* (1997) demonstrated that the exposure of cultured human skin fibroblasts to hydrogen peroxide and other types of ROS decreased PKA activity as well as the binding affinity of cAMP. Our results, however, do not confirm the original hypothesis involving the implication of a cross reaction with cAMP in the superoxide-induced increase in IP_3 formation in rat mesenteric artery SMCs. Instead, our findings demonstrated the selectivity

of the effects of superoxide on different signal pathways involving cGMP and IP_3 pathways whereas the cAMP pathway was not affected by exposure to superoxide.

In conclusion, the present study demonstrated that (1) superoxide anion directly stimulates the IP_3 formation in vascular SMCs by stimulating on the PLC-linked signal transduction pathway; (2) superoxide anion decreases intracellular cGMP levels as well as PKG activation thus decreasing an inhibitory influence on the IP_3 pathway which could facilitate the superoxide-induced IP_3 formation; and (3) the effect of superoxide on IP_3 formation did not involve reciprocal changes in the activity of cAMP pathway. It therefore appears that the inhibition of IP_3 pathway by the cGMP pathway may represent an important functional interaction in vascular SMCs. In the absence of superoxide, this cross-talk mechanism mediates a negative feedback exerted by cGMP, favoring a decrease in IP_3 formation. By inhibiting the cGMP pathway and stimulating the IP_3 pathway simultaneously, superoxide anion potentiates its stimulating effect on IP_3 formation by suppressing the inhibitory influence of cGMP. It is thus logical to postulate that alteration in the subtle equilibrium between oxidative stress, antioxidant buffering properties of the cells of the cardiovascular system and the sensitivity of the various signal transduction pathways could contribute to the development of cardiovascular diseases.

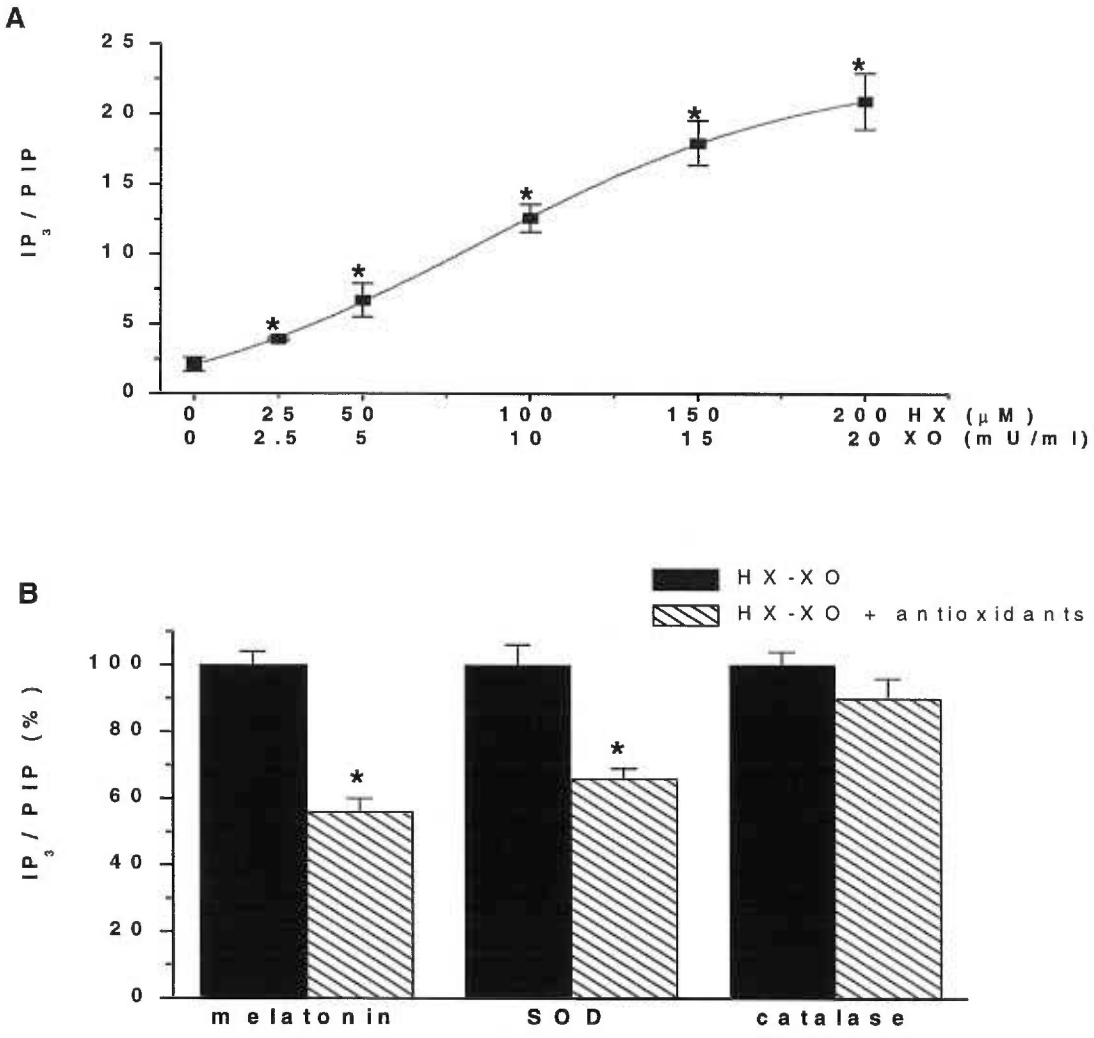

Fig. 4-1. The effect of superoxide anion on IP_3 formation in rat mesenteric artery SMCs. **A**. Dose response increase of IP_3 formation in SMCs after incubation with increasing concentrations of hypoxanthine (HX) and xanthine oxidase (XO) for 1 hour. * indicates $p<0.05$, compared to basal IP_3 level; n = 4-10 per data point. **B**. HX (100 μM)-XO (10 mU/ml)-induced IP_3 formation in cultured SMCs in the presence of melatonin (600 μM), superoxide dismutase (SOD, 120 U/ml) or catalase (120 U/ml), respectively. * indicates $p<0.05$, compared to HX-XO group; n = 6 for each group.

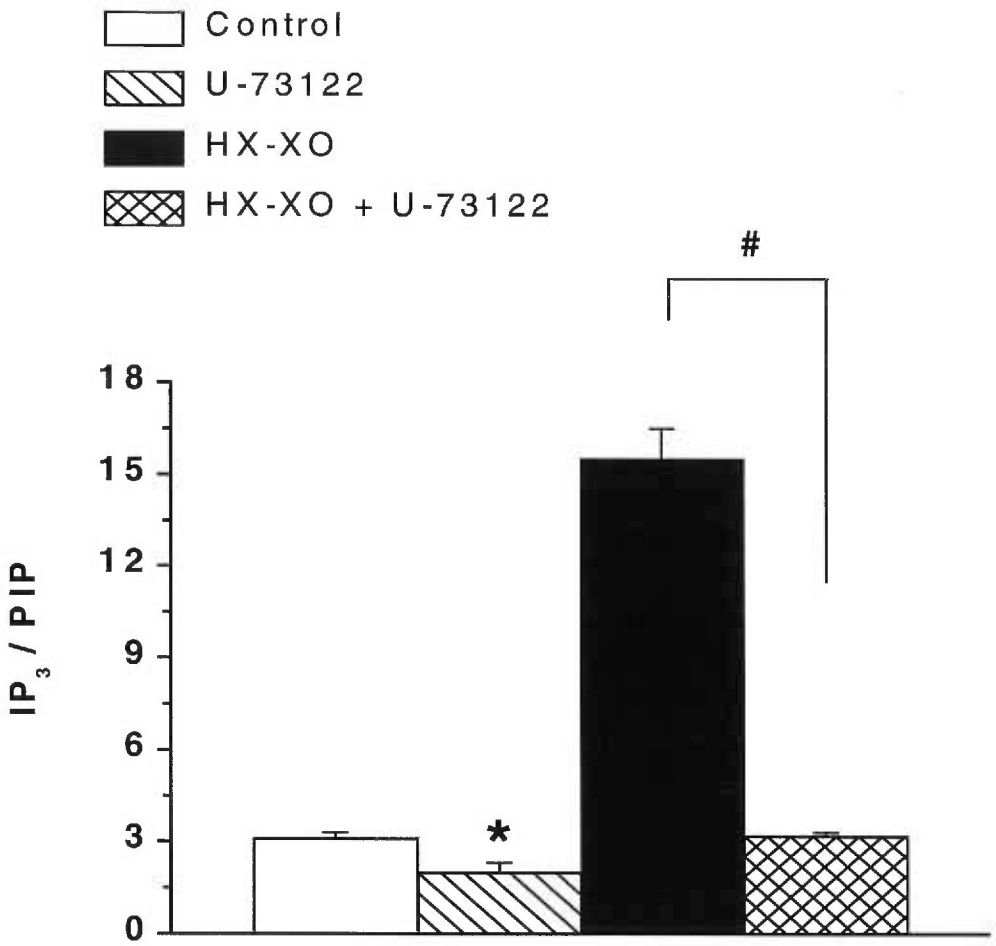

Fig. 4-2. The effect of PLC inhibition on the basal IP_3 level and the superoxide anion-induced IP_3 formation in rat mesenteric artery SMCs. The IP_3 levels were measured in the absence or presence of U-73122 (10 μM), HX (100 μM)-XO (10 mU/ml) as well as following the combination of both treatments. * indicates $p<0.05$, compared to control group; # indicates $p<0.05$ between the related groups. n = 4 for each group.

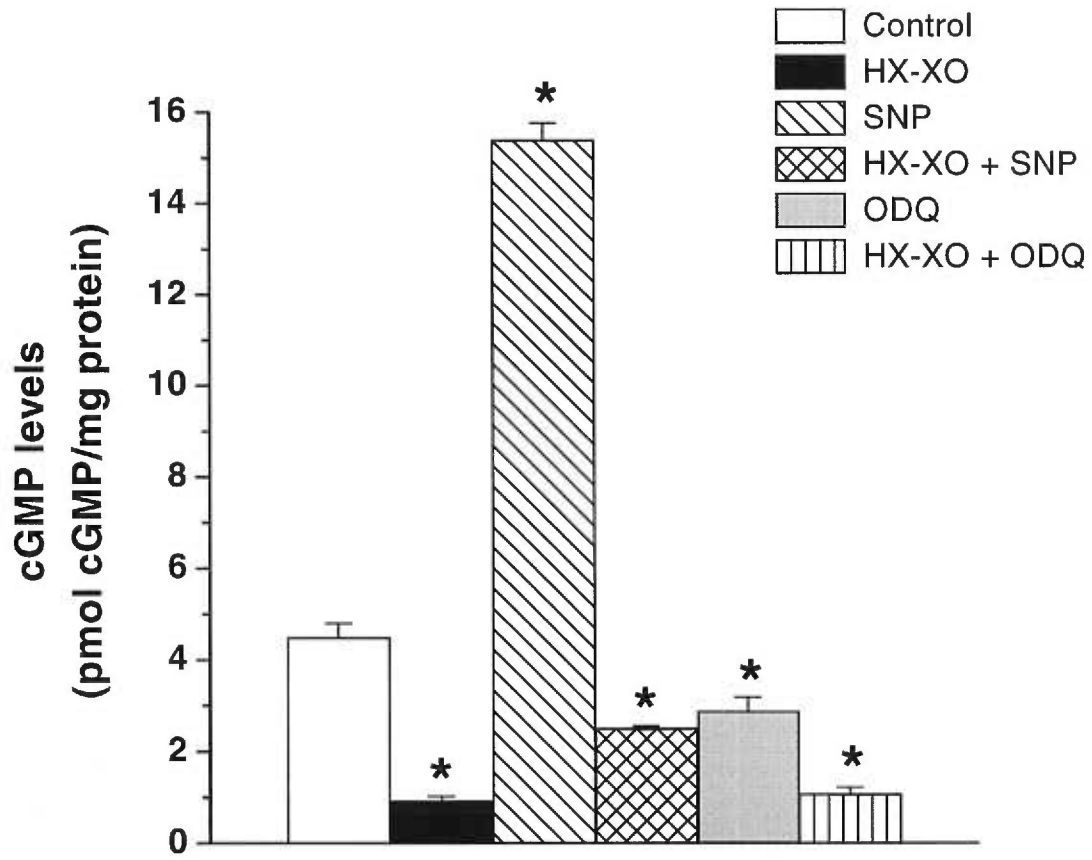

Fig. 4-3. The effects of superoxide anion on the basal cGMP level and the stimulated cGMP formation in rat mesenteric artery SMCs. The levels of cGMP were determined in the absence or presence of HX (150 μM)-XO (15 mU/ml), under basal condition or after treatment with SNP (10 μM) or ODQ (100 μM). * indicates $p<0.05$, compared to control group. n = 5 for each group.

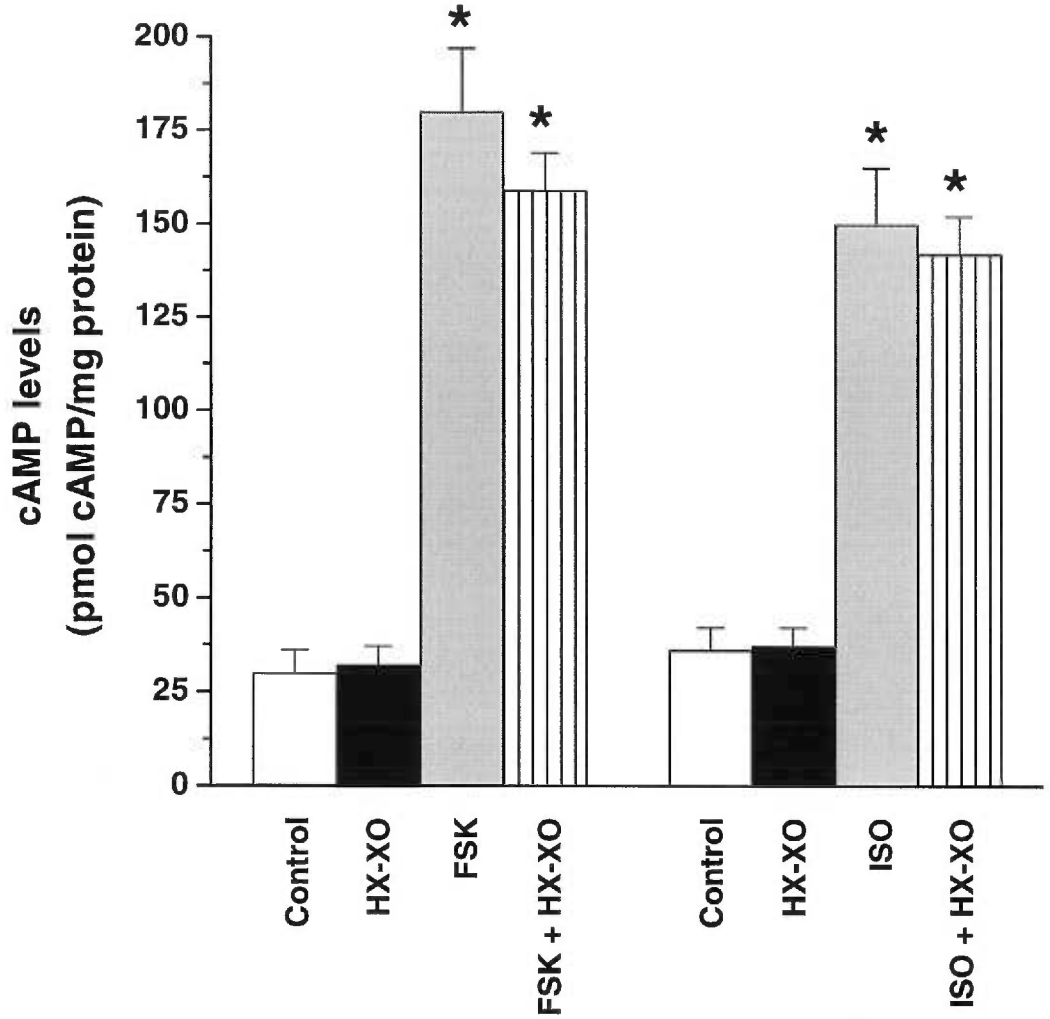

Fig. 4-4. The effect of superoxide anion on the basal cAMP level and the stimulated cAMP formation in rat mesenteric artery SMCs. The levels of cAMP were measured in the absence or presence of HX (150 μM)-XO (15 mU/ml), under basal condition or after stimulation with forskolin (FSK, 10 μM) as well as with (-)isoproterenol (ISO, 100 μM). * indicates $p<0.05$, compared to control group or HX-XO treated cells. N=8 for each group.

Fig. 4-5. The interaction of cGMP with the superoxide anion-induced IP_3 formation in rat mesenteric artery SMCs.

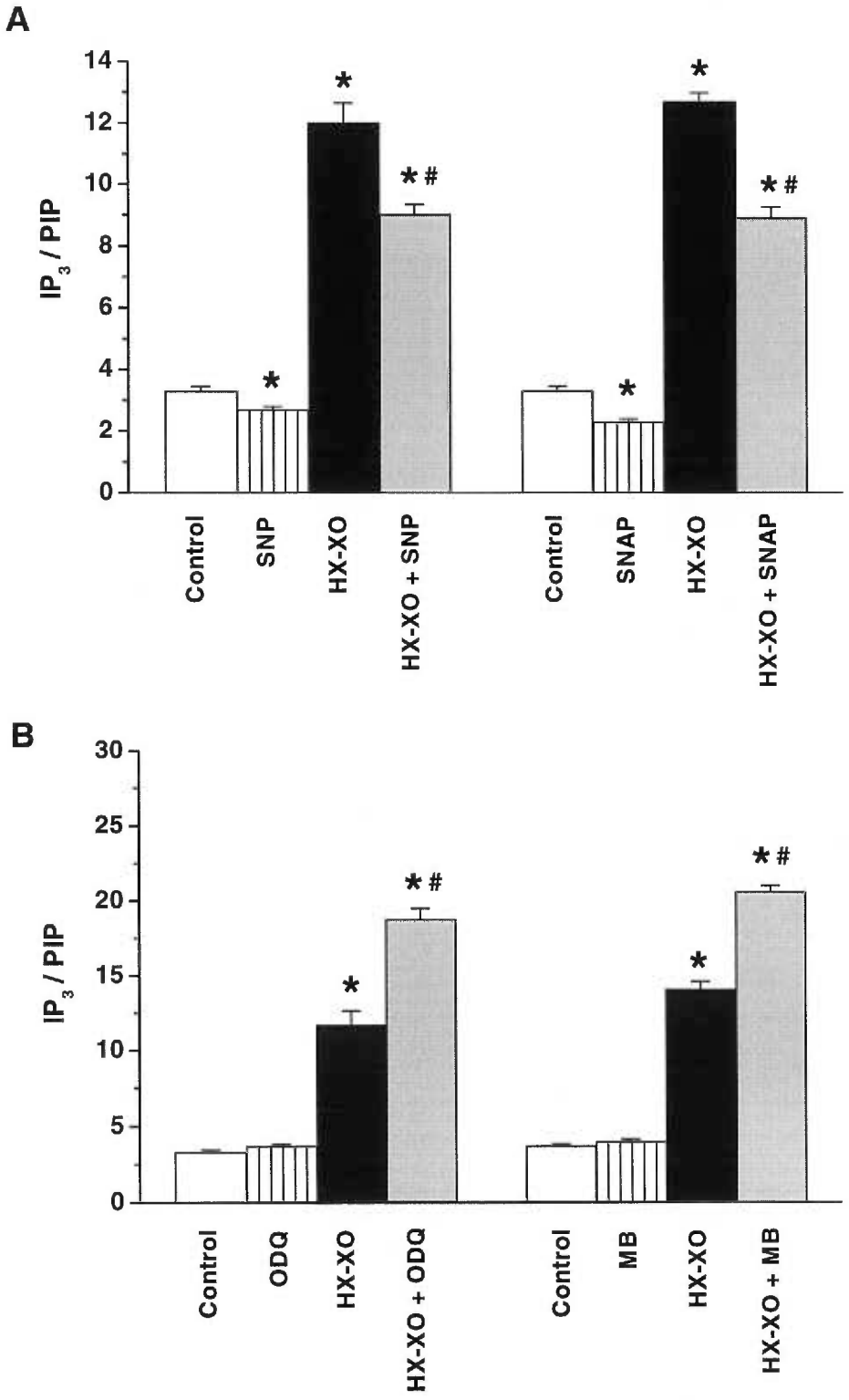

Fig. 4-5. The interaction of cGMP with the superoxide anion-induced IP_3 formation in rat mesenteric artery SMCs. **A**. The HX (100 μM)-XO (10 mU/ml)-induced IP_3 formation was determined in the absence or presence of SNP (10 μM) or SNAP (100 μM). **B**. The HX (100 μM)-XO (10 mU/ml)-induced IP_3 formation was measured in the absence or presence of ODQ (100 μM) or methylene blue (MB, 10 μM). * indicates $p<0.05$ compared to control group. # indicates $p<0.05$ compared to the HX-XO alone treated group. n = 4 for each group in **A** and **B**.

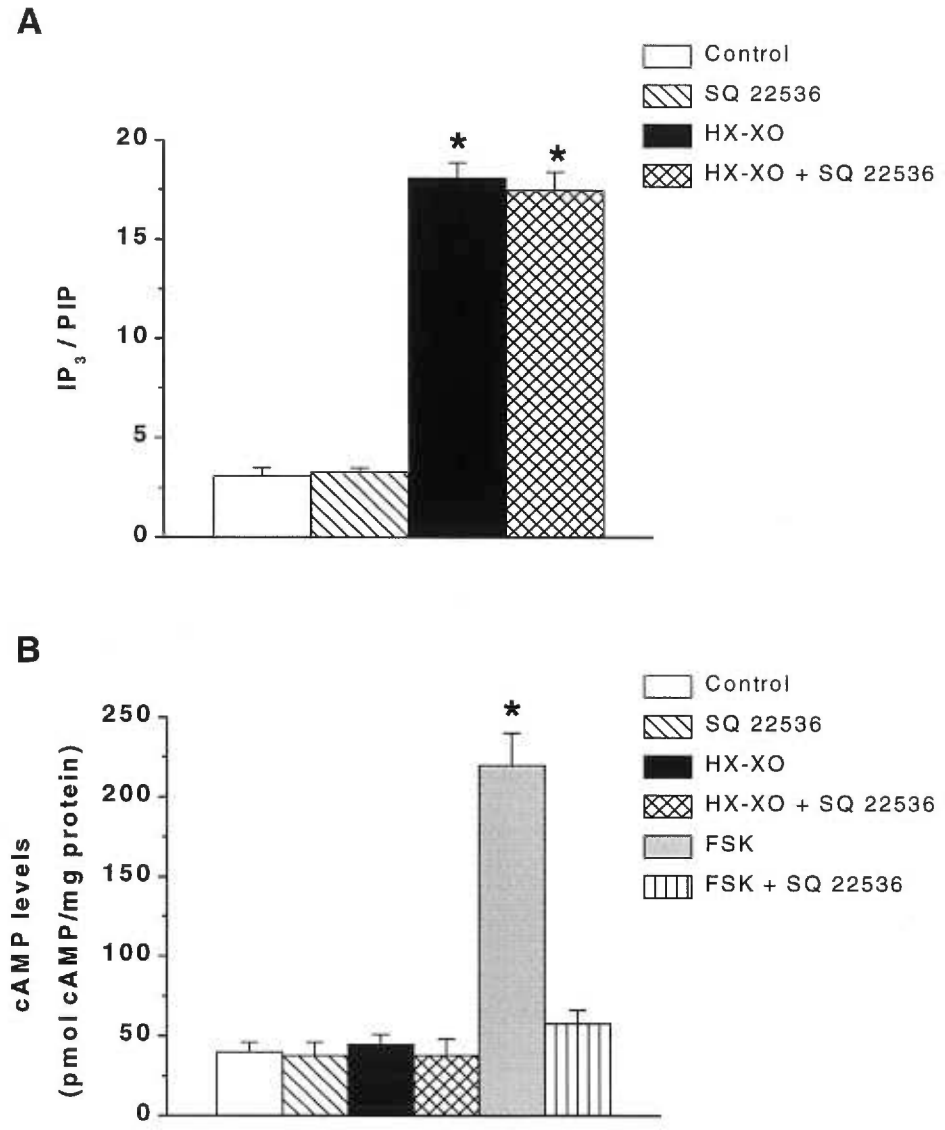

Fig. 4-6. Altered cAMP level and IP_3 level by different adenylyl cyclase modulators in rat mesenteric artery SMCs. **A**. The basal levels and the HX (100 μM)-XO (10 mU/ml)-activated IP_3 formation were measured in the absence or presence of SQ 22536 (500 μM), respectively. **B**. The basal levels and the stimulated cAMP formation by HX (100 μM)-XO (10 mU/ml) or foskolin (FSK, 10 μM) were measured in the absence or presence of SQ 22536 (500 μM), respectively. * indicates $p<0.05$, compared to control group; n = 4 for each group in **A** and **B**.

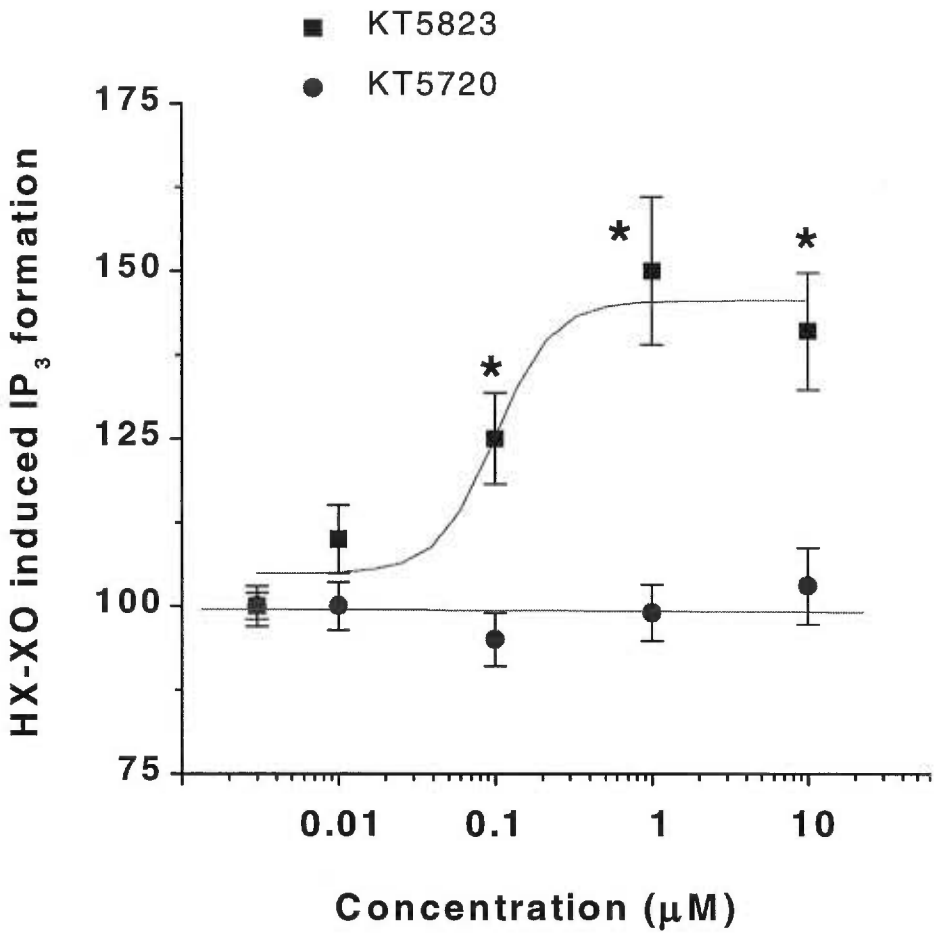

Fig. 4-7. The interaction of PKG or PKA with the superoxide anion-induced IP_3 formation in rat mesenteric artery SMCs. The IP_3 formation induced by HX (100 μM)-XO (10 mU/ml) in the absence of inhibitors of PKG or PKA was determined as control. The percentage changes in the HX-XO-induced IP_3 formation [IP_3/PIP (%)] were then measured after the cells were pretreated with KT5720 (PKA inhibitor) or KT5823 (PKG inhibitor), respectively. * indicates $p<0.05$, compared to HX-XO control

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CHAPTER 5

ENHANCED INHIBITION BY MELATONIN OF α-ADRENOCEPTOR-INDUCED AORTIC CONTRACTION AND INOSITOL PHOSPHATE PRODUCTION IN VASCULAR SMOOTH MUSCLE CELLS FROM SPONTANEOUSLY HYPERTENSIVE RATS

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ABSTRACT

Objective: To test the hypothesis that the enhanced inhibition by melatonin of the norepinephrine (NE)-induced vasoconstriction and inositol phosphate (IP) formation in spontaneously hypertensive rats (SHR) may be mediated by its antioxidant effect.

Methods: Aortic rings from SHR and age-matched Wistar-kyoto (WKY) rats were used for measuring vascular contraction forces. Cultured aortic smooth muscle cells (SMCs) were prelabelled with myo-[2-^{3}H]-inositol for evaluation of IP formation after exposure to agonist or antagonist. Basal or forskolin-induced cAMP formation was evaluated using a [^{3}H] cAMP assay system. Oxygen-derived free radicals were generated with a hypoxanthine (HX) and xanthine oxidase (XO) system.

Results: The inhibition of the NE-induced aortic contraction by melatonin was more potent in SHR than in WKY rats. The inhibition of the NE-induced IP formation by melatonin (0.3 to 300 μmol/L) was also greater in SMCs from SHR than in cells from WKY rats. In contrast, the inhibition of the NE-induced IP formation in SMCs from SHR and WKY rats by 2-iodomelatonin, an agonist of melatonin receptors, was not different. Prazosin, but not yohimbine eliminated or partially inhibited the NE-induced IP formation in SMCs from WKY rats or from SHR, respectively. In the presence of both prazosin and melatonin, the NE-induced IP production was abolished in SMCs from SHR. Furthermore, superoxide dismutase (SOD) significantly inhibited the NE-induced aortic contraction or IP formation in SMCs from SHR, but not in WKY rats. In contrast, catalase had no effect on the NE-induced IP formation or vascular

contraction in either SHR or WKY rats. HX-XO induced a greater IP formation in SMCs from SHR than in those from WKY rats. Melatonin and SOD similarly inhibited the HX-XO induced IP formation with a greater inhibitory effect in SHR than in WKY rats. However, melatonin had no effect on basal or the forskolin-induced cAMP formation in SMCs from SHR or WKY rats.

Conclusion: The enhanced inhibitory effect of melatonin on the NE-induced cellular IP production in SHR was not mediated by melatonin receptors or α-adrenoceptors. Rather, the antioxidant effect of melatonin may become important in SHR where a reduced cellular content of antioxidants and/or a greater sensitivity to superoxide anions of the vascular tissue may be suspected.

INTRODUCTION

Melatonin, a hormone mainly produced in the pineal gland, has been known for decades to regulate circadian rhythms. This hormone may also participate in the regulation of cardiovascular functions. Zanoboni and Zanoboni-Muciaccia (1967) (1) showed that pinealectomy led to the development of hypertension in the rats. The specific involvement of melatonin in the pathogenesis of hypertension was supported by the observation that the administration of melatonin normalized the blood pressure in pinealectomized rats (2). In addition, lower circulating melatonin levels have been reported in an experimental model of hypertension, the spontaneously hypertensive rat (SHR) (3), as well as in human hypertension (4). Since melatonin receptors were demonstrated in vascular tissues, such as cerebral and caudal arteries (5,6), and since intravenous or intraperitoneal injection of melatonin reduced arterial pressure (3,4) without affecting cardiac output in normotensive rats or SHR (7,8), the antihypertensive effect of melatonin was postulated to result from the modulation of vascular tone rather than cardiac functions. Although a central effect of melatonin could not be excluded in earlier studies (1-4), the local effect of melatonin on vascular tissues has been demonstrated. Melatonin inhibits α-adrenergic stimulation (8) or 5-hydroxytryptamine- (9) and KCl (10)-induced contraction of vascular tissues from rat, rabbit, or cow. However, conflicting results have been reported in rat tail artery where melatonin had no effect on the phenylephrine-induced contraction (11), or even potentiated the norepinephrine-induced contraction (12). These discrepancies might be explained by the differences in animal species, in tissues, and in the concentrations of melatonin used. Nevertheless, the intracellular mechanisms whereby melatonin could modulate vascular tone still remain largely unknown.

An overproduction of oxygen-derived free radicals (OFR) and a simultaneous decrease of antioxidants, such as superoxide dismutase (SOD) and vitamin E, have been reported in human essential hypertension (13) and in SHR (14). OFR could play a critical role in the pathogenesis of hypertension and in the development of cardiovascular injuries associated with hypertension and diabetes (15, 16). In aorta of SHR, the production of superoxide anions or the OFR-induced vasoconstriction was shown to be significantly enhanced (17, 18), suggesting that the vascular smooth muscle may be a target for oxidative damage in this experimental model (16). Interestingly, melatonin has also been suggested to be a potent antioxidant (19-21) with the property to reduce OFR production or, once produced, to neutralize them. Melatonin was demonstrated to be more effective in neutralizing hydroxyl radicals than the well known antioxidant, glutathione (22, 23). When compared with vitamin E, melatonin proved to be two folds more potent in inactivating peroxyl radicals generated during the lipid peroxidation (21). Whether the antioxidant effect of melatonin contributes to its antihypertensive effect deserves further investigation.

In the present study, we examined the effects of melatonin on the aortic contraction force and the production of IPs in cultured aortic SMCs from SHR or WKY rats under resting condition or during stimulation by norepinephrine (NE), a natural adrenergic agonist (24). The involvement of melatonin receptors in the vascular effects of melatonin was examined using 2-iodomelatonin, a melatonin receptor agonist. Hypoxanthine/xanthine oxidase (HX-XO) system was used to directly generate superoxide anions (18,25). The antioxidant effect of melatonin was indicated by the inhibition of the HX-XO-induced IP formation in cultured SMCs from SHR or WKY rats, and this effect of melatonin was further compared to that of SOD. Our results suggest that, although melatonin inhibits the NE-induced IP formation mainly through the

stimulation of melatonin receptors, the antioxidant effect of melatonin may explain the enhanced inhibitory effect of this hormone on the NE-induced IP formation in SHR.

MATERIALS AND METHODS

Measurement of vascular tissue contraction

Aortae were isolated from age-matched rats, WKY or SHR (12 weeks old, male). Aortic rings of approximately 2 mm in width were mounted in a 10 mL organ bath chamber filled with a Krebs' bicarbonate saline (bubbled with 95%O_2/ 5% CO_2). Tissues were stretched to a basal force of 2 g and equilibrated for 1 hour before the beginning of the experiments, as described previously (26). Indomethacin (1 μmol/L) was added to the Krebs' saline which was composed of (in mmol/L) : NaCl 115, KCl 5.4, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11, and $CaCl_2$ 1.8. The endothelium was removed with a rubbing procedure and the lack of endothelium was confirmed by the failure of acetylcholine (1 μmol/L) to relax the tissue. The tension development was measured at 37 °C with FT 03 force displacement transducers (Grass Ins. Co., Quincy). Data acquisition and analysis were accomplished using a Biopac system (Biopac Systems, Inc., Golata) including the MP100 WS acquisition units, TCI 100 amplifiers, an acknowledge software (3.01), universal modules, and a Macintosh computer.

Culture of aortic smooth muscle cells (SMCs)

Single SMCs were isolated and identified as described previously (27, 28). Briefly, rat aortae were isolated and connective tissues were removed. The vessel was cut open longitudinally and enzymatically digested with collagenase/dispase, elastase and collagenase in a

stepwise manner. The tissue was triturated, and dispersed cells were plated in the tissue culture flasks and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum in a CO_2 incubator at 37°C. Cultured SMCs were passed once a week by harvesting with trypsin-treatment and splitting at a ratio of 1:4. The medium was changed after attachment of the cells and twice weekly thereafter. Cells between passage 6-14 were seeded into 132 mm 6 well multidishes and used after 4 to 6 days when they became confluent. There was no significant difference in the NE-induced IP formation in cultured SMCs between passage 6 to 14 from either SHR or WKY rats.

Measurement of IP formation

SMCs were incubated for 24 hours in a serum-free and inositol-free DMEM (2 mL/well) with addition of 5μCi/mL of myo-[$^{2-3}$H] inositol (Du Pont Canada Inc. Diagnosis and Biotechnology Systems, Ont, Canada). The culture medium containing unincorporated isotope was removed and the cells were rinsed with warm (37°C) Earle's balanced salt solution (Gibco) composed of (in mmol/L): NaCl 117, KCl 5.3, $MgSO_4$ 0.8, CaCl2 1.8, NaH_2PO_4 1, D-glucose 5.6, $NaHCO_3$ 25. The cells were further incubated for 30 min in the same buffer containing 20 mmol/L LiCl to inhibit the conversion of inositol phosphates to inositol so that the radiolabelled inositol phosphates could accumulate within the cells (24, 29). Agonists were applied for various periods and the reaction was terminated by adding 0.9 mL methanol: chloroform: HCl (40: 20: 1) (30). After the addition of 0.4 mL chloroform and 0.4 mL distilled water, the samples were centrifuged (International Equipment, Needham Heights, MA, USA) to separate lipid and aqueous phases. The aqueous phase was transferred to a column containing 0.8 mL resin AG 1-X8 (200-400 mesh, formate form, Bio-Rad, Richmond, CA, USA) from which IP1, IP2, and IP3

were eluted sequentially with ammonium formate buffers of increasing molarity (29). All of these sequentially eluted inositol phosphates were collected to give a total inositol phosphates (IPs). In some experiments, total IPs were directly eluted with a buffer containing 1 M ammonium formate (29). Since these two methods did not yield different results of the total IP formation, the obtained data were pooled. 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 phosphates was expressed as a ratio of IPs over PIP (IPs / PIP x 10^{-3}) to correct the variations in the labeling of the lipid pool.

Determination of cAMP levels

Cyclic AMP levels in vascular SMCs were determined by a protein-binding assay as described previously (31). This assay involves the competition between unlabelled cAMP and a fixed quantity of [^{3}H] cAMP for binding to a protein that has a high specificity and affinity for cAMP ([3H] cAMP assay system from Amersham Corp, Ontario, Canada).

Chemicals

Norepinephrine (NE), melatonin (MT), superoxide dismutase (SOD), catalase, hypoxanthine (HX), xanthine oxidase (XO), prazosin, phenylephrine, and yohimbine were purchased from Sigma (Chemical Company, St. Louis, MO, USA). 2-iodomelatonin was purchased from Research Biochemicals International (Natick, MA, USA). Melatonin was dissolved in ethanol and then diluted in distilled water. The final concentration of ethanol in the

stimulating solution was less than 0.3%, which had no effect on the IP formation in SMCs from both SHR and WKY rats.

Statistical analysis

Unless otherwise specified, all the data were expressed as the mean ñ SEM of the ratio of IPs/PIP for the IP measurement. Comparison between hypertensive (SHR) and control rats (WKY) were performed using Student's t-test. Analysis of variance in conjunction with the Newman-Keuls test was used in multiple-group data comparison. Values of $p<0.05$ were considered statistically significant.

RESULTS

Effect of melatonin on the NE-induced vasoconstriction

Melatonin inhibited the NE-induced contraction of aortic tissues from both SHR and WKY rats. Fig. 5-1A showed one typical record where melatonin (1 mmol/L) was applied to the aortic tissue from SHR after the NE-induced contraction reached the plateau phase. Fig. 5-1B showed that NE (10 nmol/L) induced a greater contraction of aortic rings from SHR than tissues from WKY rats ($p<0.05$). In the presence of melatonin (1 mmol/L), the NE-induced contraction force was significantly decreased by 64±2% (from 1350±50 to 490±30 mg) in SHR (n=8) or by 52±1% (1120±80 to 530±20 mg) in WKY rats (n=8), respectively. The inhibitory effect of melatonin was significantly greater in aortic tissues from SHR than that from WKY rats ($p<0.05$). However, melatonin at 300 μmol/L significantly inhibited aortic contraction only in SHR (24±1%, n=8, $p<0.05$) but not in WKY rats (10±1.2%, n=8, $p>0.05$).

Effect of melatonin on basal and the NE-induced IP formation

Basal IP formation was greater in SMCs from SHR. However, melatonin (300 μmol/L) had no effect on basal levels of IP production in cultured aortic SMCs from either SHR or WKY rats ($p>0.05$) (Fig. 5-2A). The NE-induced IP formation was also greater in SMCs from SHR. Melatonin significantly inhibited the NE (1 μmol/L)-induced IP formation in SMCs from both SHR and WKY rats in a concentration-dependent manner (0.3 to 300 μmol/L), as shown in Fig. 5-2B. The inhibitory effect of melatonin on the NE-induced IP formation was enhanced in SHR, compared to WKY rats. For instance, at the concentration of 300 μmol/L, melatonin inhibited IP formation by 56±1% (from 108±4 to 48±2) in SMCs from SHR (n=6) whereas only a 44±2% (from 80±4 to 45±2) inhibition was observed in WKY rats (n=4) ($p<0.05$).

To test whether the inhibitory effect of melatonin on the NE-induced IP formation was mediated by melatonin receptors, 2-iodomelatonin was used to stimulate specific melatonin receptors. It was found that 2-iodomelatonin induced a significant inhibition of the NE-stimulated IP production in both SHR and WKY rats (n=4, respectively). In the presence of 2-iodomelatonin (300 μmol/L or 600 μmol/L), the inhibitory effect of melatonin on the NE-induced IP formation was negligible in SMCs from WKY rats (Fig.5-3). Interestingly, melatonin further inhibited the NE-induced IP formation in SMCs from SHR in the presence of 2-iodomelatonin (Fig. 5-3).

In another group of experiments, we tested the effect of prazosin on the NE-induced IP formation in SHR and WKY rats. At 1 μmol/L, prazosin eliminated the NE-induced IP formation in SMCs from WKY rats (n=4) but did not completely inhibit the NE-induced IP production in the cells from SHR (n=4). In the presence of both prazosin (1 μmol/L) and

melatonin (300 μmol/L), the NE-induced IP production was completely inhibited (Fig. 5-4A). However, yohimbine (1 μmol/L) had no effect on the NE-induced IP formation in SMCs from either WKY rats or SHR (Fig. 5-4B). Additionally, in the presence of prazosin (1 μmol/L), the phenylephrine (100 μmol/L)-induced IP production was completely blocked in SMCs from both SHR and WKY rats (data not shown).

Effect of antioxidants on the NE-induced vasoconstriction and IP formation

To further investigate the interaction between OFR and IP formation, the effects of melatonin, SOD, and catalase were examined on the NE-induced vasoconstriction and IP production in SHR or WKY rats. SOD at a concentration of 120 U/mL had no effect on the NE (10 nmol/L)-induced contraction of aortic rings (Fig 5-5A) or the NE (1 μmol/L)-stimulated IP formation in SMCs from WKY rats. However, SOD significantly inhibited the NE-induced vasoconstriction by 26±2% (n=8, $p<0.05$) and the NE-stimulated IP formation by 15±0.9% in SMCs from SHR (n=4, $p<0.05$), respectively. In contrast, catalase (80 U/mL) had no effect on the NE-induced contraction of aortic rings (n=8) or IP formation in SMCs (n=4) from both SHR and WKY rats ($p>0.05$, respectively). Neither SOD nor catalase had effect on basal IP levels in cultured SMCs from WKY rats or SHR (data not shown).

Effects of melatonin and SOD on the superoxide anion--induced IP formation

To evaluate the effect of OFR on IP formation, superoxide anions were generated by HX-XO reaction (18,25,32,33). HX (100 μmol/L) and XO (10 mU/mL) induced a greater production of IP formation in SHR than in WKY rats (n=4, respectively, $p<0.05$) (Fig. 5-6). When the cells were pretreated with melatonin (300 μmol/L) for 20 min, the HX-XO-induced IP formation was

inhibited by 75±0.6% (from 167±3 to 43±1) in SHR (n=4) and by 68±1.4% (from 131±8 to 43±1.9) in WKY rats (n=4, $p<0.05$). In the presence of SOD (120 U/mL), the HX-XO-induced IP formation was also decreased by 67±4.7% (from 167±3 to 56±8) in SHR (n=4), compared to a lesser inhibition of 34±1.7% (from 131±8 to 87±2.2) in WKY rats (n=4, $p<0.05$). At the concentration range tested, the inhibitory effect of melatonin on the superoxide anion-induced IP formation was greater than that of SOD in WKY rats ($p<0.05$), but both were similarly efficacious in blocking the effect of superoxide anions on IP formation in SMCs from SHR.

Effect of melatonin on the cyclic AMP formation

The effect of melatonin (300 μmol/L) on basal or the forskolin-induced cAMP formation was investigated in cultured SMCs from SHR and WKY rats. It was found that melatonin had no effect on basal or the forskolin-induced cAMP formation in SMCs from both SHR and WKY rats (n=4, respectively, $p>0.05$) (Table 5-1).

DISCUSSION

The vasorelaxing effects of melatonin have been demonstrated in rabbit aorta (9), rat aorta (8), and bovine pulmonary artery and vein (10). The inhibition of protein kinase C (8) and increases in cGMP (9, 24) or cAMP levels (8) are few examples of many plausible mechanisms for the vascular effects of melatonin. In the present study, the effects of melatonin were evaluated on the vascular contraction and on the IP production in SMCs. Several mechanisms through which melatonin may act on vascular tone and/or IP formation in SMCs were explored,

including 1) the activation of cAMP by melatonin, 2) the activation of melatonin receptors, and 3) the scavenging of OFR by melatonin.

In a previous study, we have demonstrated a cross-talk between cAMP and IP signaling pathways (27) by showing that cAMP decreased the phenylephrine-induced IP formation in cultured vascular SMCs from SHR in a dose-dependent manner. Accordingly, the melatonin-induced inhibition of the NE-induced IP production in vascular SMCs could be mediated through an increase in cellular cAMP level. However, this hypothesis is not supported by the present data since melatonin had no effect on basal or the forskolin-induced cAMP level in vascular SMCs from SHR or WKY rats.

The activation of melatonin receptors may underlie many vascular effects of melatonin, such as the inhibition of vascular contractions induced by various stimuli (8, 9,10). The existence of melatonin receptors in vascular tissues from both SHR and WKY rats (6) supports the above hypothesis. Our data also suggest that in WKY rats the inhibition of the NE-induced IP formation by melatonin might be fully mediated by a membrane-receptor related mechanism. Several lines of evidence were provided to strengthen this view: (1) SOD and catalase had no effect on the NE-induced vasoconstriction or IP formation in SMCs from WKY rats, indicating that OFR was not involved under these circumstances; (2) The inhibition of α-adrenoceptors by prazosin completely abolished the NE-induced IP formation, speaking against the involvement of OFR in the NE effects. The possibility that the NE-generated OFR might also stimulate α-adrenoceptors is remote since SOD and catalase did not affect the NE-induced IP formation; (3) The stimulation of melatonin receptors with 2-iodomelatonin, which has a higher binding affinity to the melatonin receptors than melatonin (6, 34), also inhibited the NE-induced IP formation in SMCs from WKY rats (Fig. 5-3). 2-iodomelatonin is a specific and potent melatonin receptor

agonist and whether this melatonin receptor agonist interacts with OFR is not known yet. The mechanisms underlying the interaction between melatonin receptor activation and IP formation are not clear to date. It has been suggested that the activation of high-affinity melatonin receptors could inhibit adenylyl cyclase (34). Such an inhibition in cAMP production would lead to an increase in IP formation because of an inhibitory cross-talk between those two signaling pathway (27). However, our study demonstrated that melatonin did not affect cellular cAMP level and it actually decreased the NE-induced IP formation.

The melatonin-induced inhibition of the NE effects on vasoconstriction and IP formation in SHR is also largely mediated by a receptor-related mechanism. However, an additional receptor-independent mechanism, i.e. the scavenging of OFR by melatonin, comes into play in this genetic hypertensive animal model. This notion is supported by the following observations. (1) SOD significantly inhibited the vascular and cellular effects of NE in SHR, suggesting an increased oxidative stress upon NE stimulation. This oxidative stress may result from the generation of superoxide anions in SHR since catalase failed to inhibit NE effects. (2) Melatonin further inhibited the NE-induced IP formation in SHR in the presence of 2-iodomelatonin (at saturated concentrations), indicating that this additional effect of melatonin is not mediated by melatonin receptors. (3) The blockade of α-adrenoceptors did not fully eliminate the NE-induced IP formation in SHR, and the residual NE effect was abolished by melatonin. Both α_1 and α_2 subtypes of α- adrenoceptors have been found in arterial SMCs. The predominant subtype is believed to be α_1- adrenoceptors (35). In our study, yohimbine had no effects on the NE-induced IP formation in SMCs from either WKY rats or SHR, thus excluding the involvement of α_2-adrenoceptors in the NE effect (36). Prazosin has been shown to mainly inhibit α_1-adrenoceptors. In cells containing both α_1 and α_2-adrenoceptors, prazosin at an

appropriate concentration (equal or greater than 1 μmol/L) can block both subtypes (37, 38). The effect of melatonin on the prazosin-resistant NE effect as well as the lack of α_2-adrenoceptors in these SMCs strongly suggest that an α-adrenoceptor-independent mechanism was responsible for the enhanced effect of NE on IP formation in SHR. Additionally, NE was used in the present study to induce vasoconstriction and IP formation. Mainly because the major objective of this study was to examine how the antioxidant effect of melatonin contributes to the enhanced inhibition of IP formation induced by the natural neurotransmitter in SHR.

To establish an antioxidant effect of melatonin, it was necessary to show that melatonin could directly scavenge OFR in a receptor-independent way. It is known that melatonin is highly lipophilic. This hormone can easily diffuse into cells despite the presence of specific melatonin receptors (19), effectively scavenging OFR intracellularly or extracellularly. When OFR was generated in our study with a HX-XO system, both melatonin and SOD significantly inhibited the effect of OFR on IP production in SMCs. Our observation, together with other reports showing that melatonin is an efficient neutralizer of hydroxyl radicals and peroxyl radicals (19-21, 39-40), strongly support an antioxidant effect of melatonin.

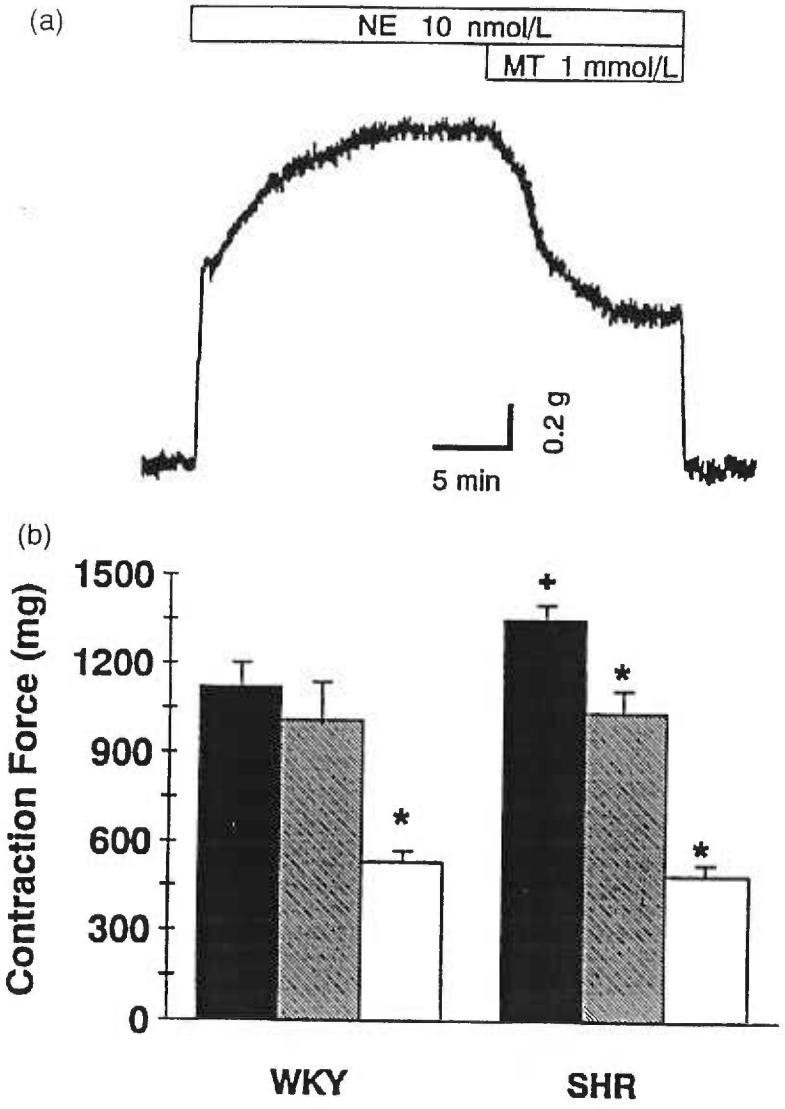
In the presence of oxygen, HX and XO react to generate $O_2^{\bullet-}$ and hydrogen peroxide and eventually hydroxyl radicals, through the Haber-Weiss reaction (39). The stimulation of the intracellular calcium release by superoxide anions has been reported (25). However, whether $O_2^{\bullet-}$ can directly affect IP formation and whether the interaction between $O_2^{\bullet-}$ and IP is altered in hypertension are still unknown. In the present study, the exposure of vascular SMCs to HX-XO significantly increased the production of IPs. A significant portion of the effect of HX-XO was blocked (34%-66.5%) in SMCs by SOD, suggesting that superoxide anions are implicated in the HX-XO-induced IP production. Since melatonin and SOD inhibited the IP formation induced by

HX-XO to a similar degree in cultured SMCs from SHR, it appears, at least in vascular SMCs, that the antioxidant effect of melatonin is mainly targeted at scavenging $O_2^{\bullet-}$. That HX-XO reaction induced a greater production of IPs in SMCs from SHR can be explained either by the possibility that SMCs from SHR may be more sensitive to superoxide anions, or by impaired endogenous antioxidant mechanisms in SMCs from SHR. The latter explanation is in line with the reported reduction of SOD at tissue level associated with an increase in XO activities in vessels from SHR (15). In SHR myocardium, an increase in superoxide anions was also suggested by morphological visualization of increased formazan deposits (14). More recently, measurement of superoxide anions with the use of lucigenin directly demonstrated an overproduction of free radicals in aortic tissues of SHRSP (17, 39). Although the exact mechanisms whereby $O_2^{\bullet-}$ induces IP formation in vascular SMCs can not be elucidated from the present study, its enhanced effect on IP formation in SMCs of SHR would result in an increase in intracellular calcium concentration and thus facilitate vascular contraction. An overproduction of OFR and IPs have been observed in hypertension, and either OFR or IPs can induce vascular contraction (19, 25). It may be postulated, therefore, that an interaction between OFR and IP production could be involved in the pathogenesis of hypertension. Since superoxide anions are overproduced in SHR, the $O_2^{\bullet-}$ scavenging effect of melatonin in those SMCs could explain a greater vasorelaxing effect of melatonin on aortic tissues from SHR (Figs. 5-3, 5-4, 5-5). It should be mentioned that our study emphasized the role of superoxide anions in the antioxidant effects of melatonin in SMCs from SHR. This by no means excludes the possibility that SMCs from SHR may also be subject to the action of other species of OFR.

In conclusion, our results show that the oxidative stress induced by superoxide anions may be greater at the level of SMCs from SHR due either to a reduced cellular content of

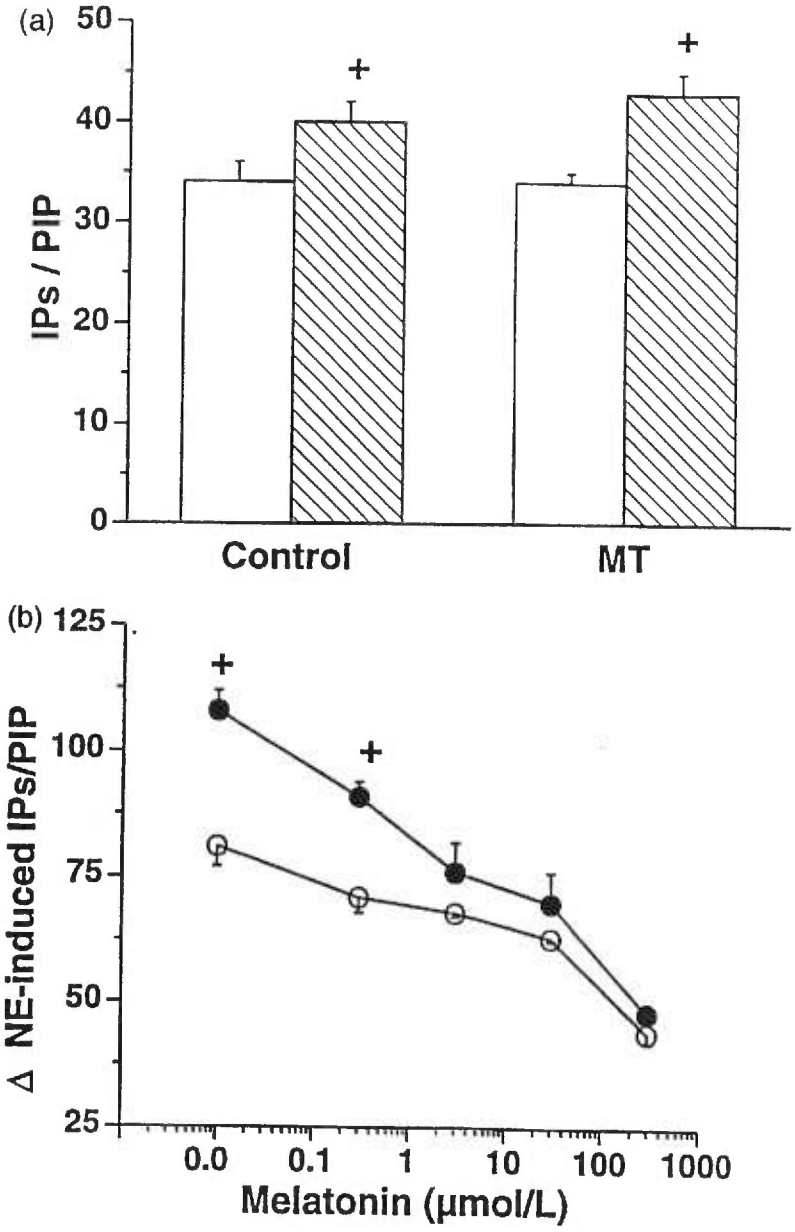
antioxidants and/or to a greater sensitivity to superoxide anions of the vascular tissues in SHR. Thus, the altered α-adrenoceptor-mediated signal transduction and vascular contraction could be partly associated to the production of superoxide anions in SHR. Although the inhibition by melatonin of the NE-induced vasoconstriction and IP formation are mainly mediated by a receptor-dependent mechanism, a receptor-independent OFR scavenging effect of melatonin becomes significant in SMCs from SHR. It is this antioxidant property of melatonin that accounts for the enhanced inhibition by melatonin of the α-adrenergic-induced activation of the phosphoinositide pathway in SHR.

Fig. 5-1. Effect of melatonin (MT) on the norepinephrine (NE)-induced vasocontraction.



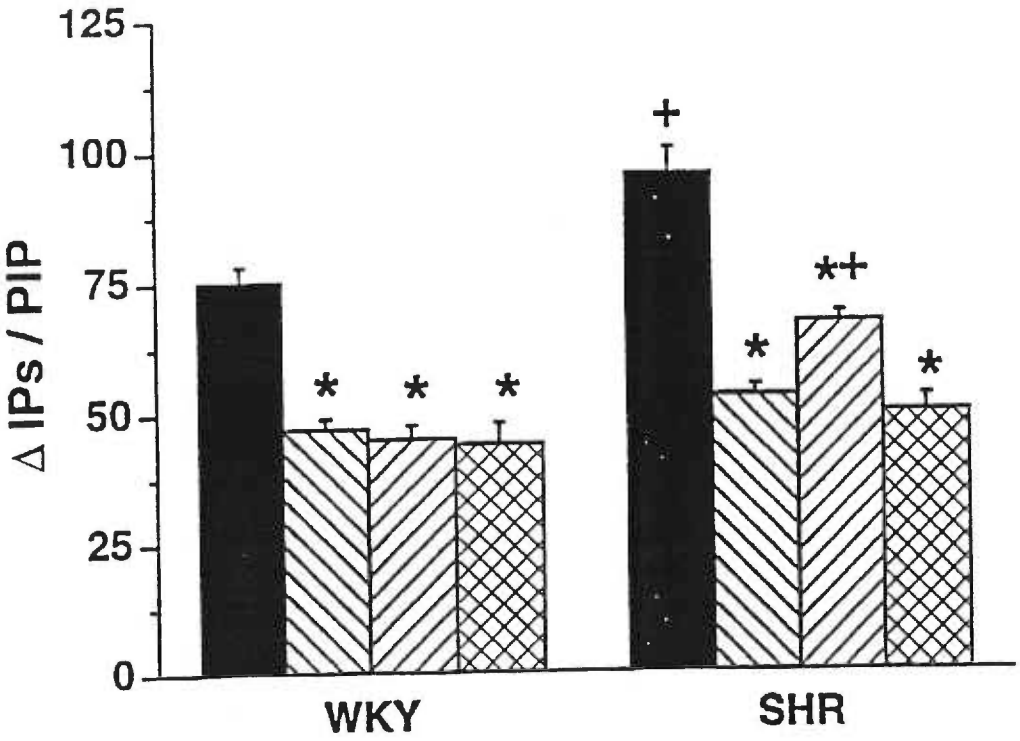
Effect of melatonin (MT) on the norepinephrine (NE)-induced vasocontraction. (a) One original record illustrating the inhibition of the 10 nmol/l norepinephrine-induced contraction of aortic rings from spontaneously hypertensive rats (SHR) by 1 mmol/l MT. (b) Two different concentrations [300 μmol/l (▨) and 1 mmol/l (□)] of MT inhibited the NE-induced contraction of aortic rings both from SHR and from Wistar–Kyoto (WKY) rats. Eight experiments were performed in each group on tissues from SHR and WKY rats with 10 nmol/l NE (■). $^{*}P < 0.05$, versus NE group; $^{+}P < 0.05$, versus WKY rat tissues

Fig. 5-2. Effect of melatonin (MT) on the NE-induced IP formation.



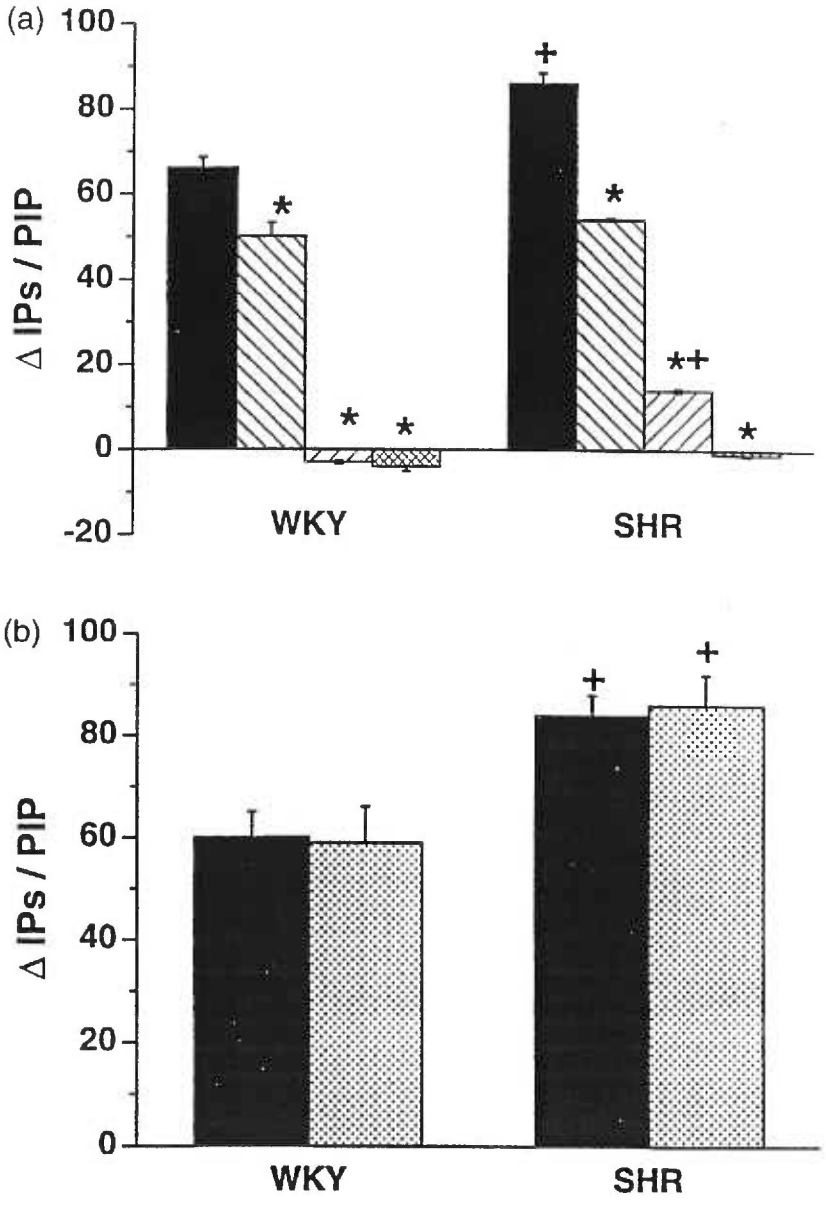
Effect of melatonin (MT) on the norepinephrine (NE)-induced formation of inositol phosphates (IPs). (a) Basal formation of IPs in cultured smooth muscle cells with and without pretreatment with 300 μmol/l MT for 1 h. Five experiments were performed in each group for tissues from Wistar–Kyoto (WKY) rats (□) and seven experiments in each group for tissues from spontaneously hypertensive rats (▨). (b) The 1 μmol/l NE-induced (Δ) formation of IP in smooth muscle cells that had been treated beforehand with MT at various concentrations for 20 min. Four experiments were performed in each group for tissues from WKY rats (○) and six experiments in each group for tissues from spontaneously hypertensive rats (●). Δ indicates the absolute change in IPs/phosphatidylinositol pool (PIP) after subtraction of basal values. $^{+}P < 0.05$, versus tissues from WKY rats.

Fig. 5-3. Effect of 2-iodomelatonin on the NE-induced IP formation.



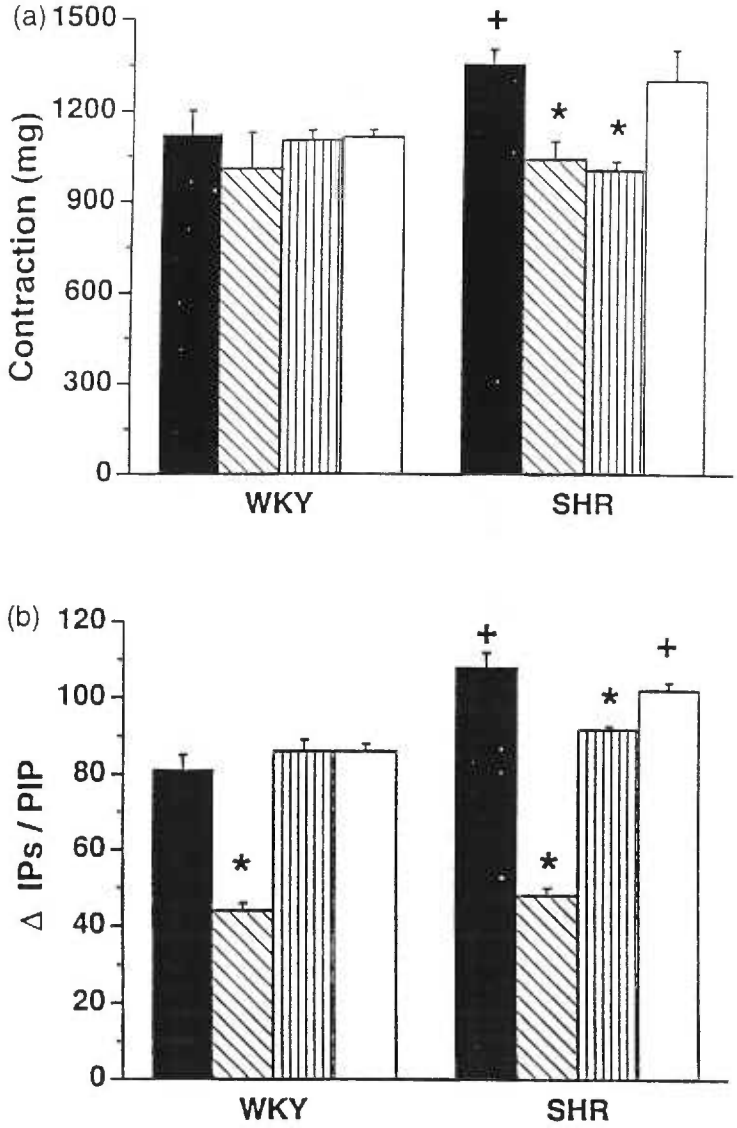
Effect of 2-iodomelatonin on the norepinephrine-induced formation of inositol phosphate. The 1 μmol/l norepinephrine-induced formation of inositol phosphate in smooth muscle cells that had been treated beforehand with 300 μmol/l 2-iodomelatonin (▨) or with 300 μmol/l 2-iodomelatonin plus 300 μmol/l melatonin (▩) for 20 min. Four experiments were performed in each group for tissues from spontaneously hypertensive rats (SHR) and from Wistar–Kyoto (WKY) rats. ▧ , Norepinephrine plus melatonin. Δ indicates the absolute change in inositol phosphates/phosphatidylinositol pool (IPs/PIP) after subtraction of basal values. $^{*}P < 0.05$, versus norepinephrine (■) group; $^{+}P < 0.05$, versus tissues from WKY rats.

Fig. 5-4. Effect of prazosin and yohimbine on the NE-induced IP formation.



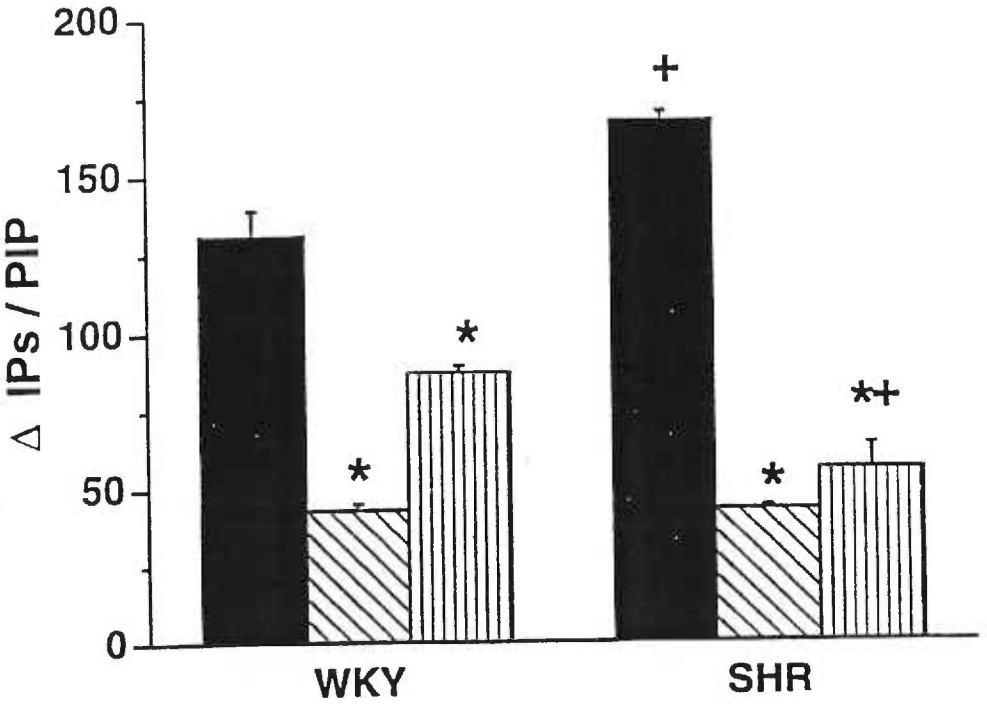
Effects of prazosin and yohimbine on the norepinephrine-induced formation of inositol phosphate. (a) The 1 μmol/l norepinephrine-induced formation of inositol phosphate in the presence of 1 μmol/l prazosin (▨) or 1 μmol/l prazosin plus 300 μmol/l melatonin (▩) in smooth muscle cells from spontaneously hypertensive rats (SHR) and from Wistar–Kyoto (WKY) rats for 1 h. ■, Norepinephrine; ▧, norepinephrine plus melatonin. (b) The 1 μmol/l norepinephrine-induced formation of inositol phosphate in the presence (▩) and in the absence (■) of 1 μmol/l yohimbine in smooth muscle cells for 1 h. Four experiments were performed in each group for tissues from SHR and from WKY rats. Δ indicates the absolute change in inositol phosphates/phosphatidylinositol pool (IPs/PIP) after subtraction of basal values. $^{*}P < 0.05$, versus norepinephrine group; $^{+}P < 0.05$, versus tissues from WKY rats.

Fig. 5-5. Effect of melatonin (MT), SOD and catalase on the NE-induced vasoconstriction and IP formation.



Effects of melatonin (▨), superoxide dismutase (SOD, ▥), and catalase (□) on the norepinephrine-induced (■) vasoconstriction and formation of inositol phosphate. (a) The 10 nmol/l norepinephrine-induced contraction of aortic rings in the absence and presence of 300 μmol/l melatonin, 120 U/ml SOD, and 80 U/ml catalase, from spontaneously hypertensive rats (SHR) and from Wistar–Kyoto (WKY) rats. Eight experiments were performed in each group for tissues from SHR and from WKY rats. (b) Smooth muscle cells were treated beforehand with 300 μmol/l melatonin, 120 U/ml SOD, and 80 U/ml catalase, for 20 min. Four experiments were performed in each group for tissues from SHR and from WKY rats. Δ indicates the absolute change in inositol phosphates (IPs)/phosphatidylinositol pool (PIP) after subtraction of basal values. $^{*}P < 0.05$, versus norepinephrine group; $^{+}P < 0.05$, versus tissues from WKY rats. (For the purpose of comparison, related data presented in Figures 1 and 2 have been extracted and summarized in this figure.)

Fig. 5-6. Effect of melatonin (MT) on the superoxide-induced IP formation.



Effect of melatonin on the O_2^--induced formation of inositol phosphate (IP). Formation of IP was studied in smooth muscle cells in the presence of 100 μmol/l hypoxanthine and 10 mU/ml xanthine oxidase for 1 h (■). The hypoxanthine–xanthine oxidase-induced formation of IP was evaluated after cells had been treated with 300 μmol/l melatonin (▨) and 120 U/ml superoxide dismutase (▥) for 20 min. Four experiments were performed in each group for tissues from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. Δ indicates the absolute change in IPs/phosphatidylinositol pool (PIP) after subtraction of basal values. $^*P < 0.05$, versus hypoxanthine–xanthine oxidase group; $^+P < 0.05$, versus tissues from WKY rats.

Table 5-1. Cyclic AMP levels (pmol cyclic AMP/mg proteins)

Strain	Basal	Forskolin (10 μmol/L)	Melatonin (300 μmol/L)	Forskolin + melatonin
WKY	18 ± 4	540 ± 50	16.8 ± 4	520 ± 40
SHR	14 ± 4	520 ± 70	17 ± 5	510 ± 40

WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rat.

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CHAPTER 6

HYPERSENSITIVITY OF PHOSPHOLIPASE C AND CYCLIC GMP-MEDIATED SIGNALLING PATHWAYS TO SUPEROXIDE ANION IN AORTIC SMOOTH MUSCLE CELLS FROM SPONTANEOUSLY HYPERTENSIVE RATS

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ABSTRACT

The effects of hypoxanthine and xanthine oxidase-induced superoxide anion were evaluated on various signal transduction pathways in aortic smooth muscle cells (SMCs) from spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). Superoxide increased IP_3 formation in a concentration- and time-dependent manner in both strains but more importantly in SMCs from SHR. Various antioxidants significantly decreased the superoxide-induced IP_3 formation in both strains. In addition, tyrosine kinase inhibitors, genistein or tyrphostin A25, inhibited the superoxide-induced IP_3 formation more markedly in SHR than in WKY. The pretreatment of SMCs with antibodies to phospholipase Cγ also inhibited the superoxide-induced IP_3 formation in both strains. Moreover, superoxide decreased the basal level of cGMP to a greater extent in SHR and also suppressed the rise in cGMP induced by s-nitroso-n-acetylpenicillamine. In addition, the superoxide-induced increase in IP_3 formation was significantly inhibited by guanylyl cyclase stimulator s-nitroso-n-acetylpenicillamine, but was potentiated by guanylyl cyclase inhibitor ODQ and KT5823 (a cGMP-dependent protein kinase inhibitor) with a greater effect in SHR. Finally, the superoxide-enhanced IP_3 formation was not accompanied by simultaneous changes in cAMP levels and the inhibition of adenylyl cyclase pathway did not modify the superoxide-induced IP_3 formation. Our results thus demonstrate a stimulatory effect of superoxide on IP_3 formation, mediated by the tyrosine kinase-coupled PLCγ activity, and an inhibitory effect of superoxide on cGMP formation in vascular SMCs. The increased reactivity of PLC pathway and the decreased cross-inhibition of IP_3 pathway by cGMP in the presence of superoxide may underly the altered functions of vascular SMCs in SHR.

INTRODUCTION

The signal transduction pathway linked to inositol 1,4,5-triphosphate (IP_3) plays an important role in the regulation of cardiovascular functions under physiological and pathological conditions. The specific ligand-receptor interaction at the plasma membrane surface activates phospholipase C (PLC) that catalyzes the hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP_2) to form IP_3 and diacylglycerol (DAG). Three PLC isoforms (β, γ, and δ) have been identified in mammalian tissues [1], PLCγ in rat vascular smooth muscle cells (SMCs) [2], PLCγ and PLCβ in human aortic tissue [3], and PLCδ in rat aorta [4]. PLCβ is activated by the receptor-coupled Gq, G11 [5], or Go proteins [6], whereas PLCγ is activated when its tyrosine residues are phosphorylated by tyrosine kinase [5]. It is clear that the activation of either PLCβ or PLCγ leads to the generation of IP_3, although the relationship between the activation of PLCδ and the IP_3 production is still unclear.

The possibility that IP_3 can be generated in a membrane receptor-independent manner is presently unsettled. Like nitric oxide, reactive oxygen species (ROS) exert their effects without specific requirement of membrane receptors. Our previous study as well as that of others have shown that certain oxidants, or oxidant-derived products, could result in the hydrolysis of phosphatidylinositol [7,8]. Since it was found in our study that the norepinephrine-induced vasoconstriction and formation of inositol phosphates (IP) in rat aortic SMCs were partially inhibited by superoxide dismutase (SOD), but not by catalase [8], it therefore suggested that the norepinephrine-induced increase in IP formation might be partially related to the auto-oxidation of catecholamine, leading to the generation of superoxide. As mentioned above, IP_3 formation could result from the activation of either Gq proteins or tyrosine kinases or both, thus ROS could

activate IP_3 formation through any of those mechanisms. Studies carried out in liver cells showed that orthovanadate-generated ROS activated the tyrosine kinase-phosphatidyinositol kinase coupled system [9-11]. A stimulation of insulin receptor tyrosine kinase activity by hydrogen peroxide was also reported [12-13]. However, whether the activation of tyrosine kinase pathway is a common mechanism for the cellular effect of ROS is presently unsettled. At present, there is no evidence that tyrosine kinase-linked signalling pathway in vascular SMCs is modulated by superoxide.

Previous studies suggest that the metabolism of IP_3 could be modulated by the cGMP or cAMP pathways. It has been reported that IP_3 response of arterial endothelial cells to thrombin can be inhibited by cGMP or by the guanylyl cyclase stimulator sodium nitroprusside [14]. We have also reported that the phenylephrine-induced IP formation in rat aortic SMCs is inhibited by cAMP or by the adenylyl cyclase stimulator forskolin [15]. Interestingly, recent studies have provided some evidence that hydrogen peroxide either stimulated or had no effect on soluble guanylyl cyclase in different cell preparations, whereas superoxide either inhibited or had no effect on soluble guanylyl cyclase[16-17]. In addition, the inhibition by superoxide of the cAMP metabolism has recently been reported in mesangial cells and human fibroblast [18-19]. These studies raised many intriguing and important issues concerning the cross interactions between the PLC pathway and other signalling pathways. Whether superoxide alters cGMP and/or cAMP metabolism in vascular SMCs and whether the effect of superoxide on IP_3 formation is modulated by simultaneous changes in cGMP or cAMP levels have constituted pertinent interrogations which needed to be addressed.

In aortic rings from spontaneously hypertensive rats (SHR) or renal and deoxycorticosterone salt hypertensive rats, oxygen consumption was found to be significantly

increased [20]. An overproduction of superoxide was also observed in aortae of SHR [21]. In addition, xanthine-xanthine oxidase reaction-induced contraction of endothelium-free aortic rings was reported to be twice greater in SHR than in WKY [22]. All those studies suggest that the production of superoxide and the reactivity of SMCs to superoxide are increased in hypertension. Therefore, a better knowledge on the effects of superoxide on different signalling pathways may provide a better understanding of the mechanisms responsible for the abnormal functions of vascular SMCs in hypertension. The purpose of this study was to determine the modulatory role of superoxide on the activities of different signalling pathways in vascular SMCs and to investigate whether the reactivities of different signalling pathways to superoxide are altered in hypertension. The cellular levels of IP_3 were measured in the absence or presence of superoxide in SMCs from SHR and WKY and various putative mechanisms underlying the effect of superoxide on IP_3 formation were explored in both strains. More specifically, the superoxide-induced IP_3 formation was determined after blockade of tyrosine kinase-mediated signalling pathway either by inhibiting directly tyrosine kinase or its associated phospholipase Cγ (PLCγ). The effects of superoxide on the basal level of cGMP or cAMP were also studied and compared with the effects of specific modulators of cGMP or cAMP metabolism. Moreover, the superoxide-induced IP_3 formation was determined after the blockade of cAMP or cGMP pathways in order to evaluate the cross-talk interactions between the superoxide-induced IP_3 formation and other signal transduction pathways in both strains.

MATERIALS AND METHODS

Cell culture.

Single aortic SMCs were isolated and identified as described previously [15]. Briefly, aortae from 12-week SHR or WKY were isolated and connective tissues were removed. The vessels were enzymatically digested with collagenase/dispase, elastase and collagenase in a stepwise manner. Dispersed cells were plated in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum in a CO_2 incubator at 37°C. Cells between passages 2 to 10 were seeded into 132-mm six-well multi-dishes and used for the measurements of IP_3, cGMP, and cAMP. There was no significant difference in the superoxide-induced increase in IP_3 formation in cultured SMCs among passages 2 to10.

Measurement of IP_3 formation.

SMCs were incubated for 24 hours in the serum-free and inositol-free DMEM, to which 5 μCi/ml of myo-[2-^{3}H] inositol (Du Pont Canada Inc. Diagnosis and Biotechnology Systems, Ont, Canada) were added [8,15]. Thereafter, the culture medium containing unincorporated isotope was removed and the cells were rinsed three times with an Earle's balanced salt solution (EBSS). In situation when the cell membrane needed to be permeabilized, the cells were then incubated for 4 minutes in a cytosolic buffer containing (in mmol/L) NaCl 20, KCl 100, $NaHCO_3$ 25, $MgSO_4$ 5, $NaHPO_4$ 0.96, $CaCl_2$ 0.48, EGTA 1, ATP 1.5, creatine phosphate 5, creatine phosphokinase (10 U/ml), bovine serum albumin 2%, and saponin (75 μg/ml) [23]. Within 4 minutes of exposure to saponin, more than 95% of cells were stained by 0.025% Azure A (Sigma Chemical Co. St. Louis, MO, USA), a blue DNA dye. This was the indication of the increased permeability of plasma membrane after saponin treatment. The cells were incubated for another 30 minutes in the same EBSS (if not treated with saponin) or the cytosolic buffer (if permeabilized) containing 20 mmol/L LiCl. Finally, the cells were subjected to hypoxanthine-xanthine oxidase in the

absence or presence of antioxidants or other treatments for various periods, and the reaction was terminated by adding 0.9 ml of methanol:chloroform:HCl (40:20:1). The tritiated IP pool of the aqueous phase composed of inositol 4-phosphate (IP_1), inositol 4,5-biphosphate (IP_2), and IP_3 was eluted consecutively by ion-exchange chromatography (AG1-X8 resin, Bio-Rad Laboratories, Ont, Canada). The lipid phase was counted to measure the phosphatidylinositol lipid pool (PIP). IP_3 was expressed as a relative value of [(IP_3/PIP) x 10^3] (arbitrary units) to correct for the variation in the labeling of the lipid pool.

Quantitative determination of cAMP level and cGMP level.

The level of cAMP in cultured SMCs was determined by a protein-binding assay. This assay involves the competition between unlabeled cAMP and a fixed quantity of [^{3}H] cAMP for binding to a protein that has a high specificity and affinity for cAMP (cAMP [^{3}H] assay system, Amersham Corp., Ont., Canada). The quantitative determination of cGMP was performed with a cGMP [^{125}I] assay system with Amerlex-M^{TM} magnetic separation. This system utilizes a high specific activity [^{125}I]-2'-O-succinyl-cGMP tyrosine methyl ester tracer together with a highly specific and sensitive antiserum, thus allowing for a simple magnetic separation of the antibody bounded [^{125}I] cGMP from free fraction (cGMP [^{125}I] assay system, Amersham Corp., Ont., Canada).

Generation of superoxide anion.

In experiments performed on cultured SMCs, the hypoxanthine (HX) and xanthine oxidase (XO) reaction was used since oxidation of HX by XO leads to the generation of superoxide [24].

In *in vitro* cell-free experiments, ROS ($O_2^{\bullet-}$, $OH^{\bullet}$, $^1O_2$) and their by-products (H_2O_2, HOCl, OCl^-) were generated by electrolysis [25]. Electrolysis was carried out by conducting 10 mA DC for 1 minute through 3 ml of Krebs-Henseleit buffer consisting of (in mmol/L): NaCl 118, KCl 4.8, $CaCl_2$ 1.25, $MgSO_4$ 0.86, KH_2PO_4 1.2, $NaHCO_3$ 25.4, glucose 11.1, EDTA 0.057. The amount of generated ROS was evaluated by the DPD (n,n-diethy-p-phenylenediamine) colorimetric method [25]. Briefly, 1 ml of electrolyzed Krebs-Henseleit buffer was mixed with 2 ml of DPD solution (25 mg/ml, dissolved in Krebs-Henseleit buffer). The reaction of oxidant species with DPD produces a visible red color instantly. This colorimetric change was detected at 515 nm using an UV spectrophotometer (UV160U, Shimadzu Corp., Kyoto, Japan).

Chemicals and data analysis.

Hypoxanthine (HX), xanthine oxidase (XO), superoxide dismutase (SOD) and forskolin (FSK) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Genistein, tyrphostin A25, n-acetylcysteine (NAC), α-lipoic acid (LA), KT5720, KT5823, s-nitroso-n-acetylpenicillamine (SNAP) and SQ 22536 were purchased from Calbiochem (La Jolla, California, USA). ODQ was purchased from Tocris Cookson Inc. (Ballwin, MO, USA). Unless otherwise specified, the HX-XO treatment indicated that 150 μmol/L of HX and 15 mU/ml of XO were applied to the cells. Data were expressed as means±SEM and were analyzed using Student's *t*-test or analysis of variance in conjunction with the Newman-Keuls test where applicable. Differences between groups were considered statistically significant when $p < 0.05$.

RESULTS

Effect of superoxide anion on IP_3 formation

Superoxide generated from HX-XO reaction significantly increased the IP_3 formation in a concentration-dependent manner in SMCs from both strains. The increases in IP_3 formation were significantly greater at all concentrations tested in SHR than in WKY (Fig. 6-1A), suggesting an enhanced sensitivity of IP_3 pathway to superoxide in SMCs from SHR. The effect of superoxide on IP_3 formation was also time-dependent and reached a plateau at sixty minutes in SMCs from both strains (Fig. 6-1B). After the cells were pre-incubated with an antioxidant NAC (0.3 to 10 mmol/L) for 20 minutes, HX-XO-induced IP_3 formation was significantly inhibited in both strains (Fig. 6-2A). In *in vitro* cell-free studies, it was found that ROS generated by electrolysis of Krebs-Henseleit buffer were significantly scavenged by NAC (Fig. 6-2B), indicating that NAC is an effective and potent antioxidant. In addition, the antioxidants SOD and α-lipoic acid had a greater inhibitory effect on superoxide-induced IP_3 formation in SMCs from SHR than those from WKY (table 6-1). As shown in table 6-1, the increased IP_3 formation induced by HX-XO was significantly inhibited by 22±3% in WKY and 45±4.5% in SHR ($p<0.05$, as compared to WKY) after 20 minutes pretreatment of cells with SOD (120 U/ml), whereas IP_3 formation was inhibited by 36±4.5% in WKY and 61±4.0% in SHR following 20 minutes pretreatment with α-lipoic acid ($p<0.05$). To clarify whether hydrogen peroxide affected IP_3 formation similarly to superoxide, the direct effect of hydrogen peroxide was further tested. It was found that hydrogen peroxide at 10 μmol/L, which is within the physiological concentration range [9], had no effect on IP_3 formation in both strains ($p>0.05$, n=4 for each group). A similar result was also observed in mesenteric artery SMCs from Sprague-

Dawley rats and only at a high concentration (1 mmol/L), hydrogen peroxide slightly increased the IP_3 formation by 30% in those cells ($p<0.05$, n=4), and this increase was completely inhibited by catalase (unpublished observations).

Effect of superoxide anion on tyrosine kinase-PLCγ pathway.

To explore the possibility that the superoxide-induced IP_3 formation may be secondary to the activation of tyrosine kinase pathway, we examined the effects of two tyrosine kinase inhibitors on the superoxide-induced IP_3 formation in SMCs from both strains. Fig. 6-3 shows that the HX-XO-induced IP_3 formation was significantly inhibited by genistein or tyrphostin A25 (20 minutes exposure to the cells) in a concentration-dependent manner (5 to 50 μmol/L). The inhibitory effects of both tyrosine kinase inhibitors in SMCs from SHR were more potent than in SMCs from WKY. These data suggest that superoxide-induced IP_3 formation is at least in part mediated by an increase in the activity of tyrosine kinase signalling pathway.

Since PLCγ could be directly activated by tyrosine kinase, whether the superoxide-induced IP_3 formation was due to an enhanced PLCγ activity was investigated in SMCs from both strains. In these experiments, the saponin-permeabilized cells were pre-incubated with anti-PLCγ monoclonal antibody (5 μg/ml) for one hour and then were treated with HX-XO. The increased IP_3 formation induced by HX-XO in the permeabilized SMCs was significantly inhibited by 14±3% in WKY ($p<0.05$, n=5) and by 27±4.2% in SHR ($p<0.05$, n=5) (Fig. 6-4). The inhibition by anti-PLCγ monoclonal antibody of the HX-XO-induced IP_3 formation was significantly greater in SMCs from SHR than in SMCs from WKY ($p<0.05$). In addition, the effect of HX-XO on IP_3 formation between the non-permeabilized and permeabilized cells was not significantly different (data not shown).

Effect of superoxide anion on cGMP pathway.

The basal levels of cGMP were significantly higher in SMCs from SHR than that from WKY ($p<0.05$, n=5 for each group). The cGMP levels were significantly increased 30 minutes after incubation of SMCs with SNAP (100 μmol/L), a stimulator of soluble guanylyl cyclase (Fig. 6-5). This effect of SNAP was greater in SHR than in WKY ($p<0.05$). The treatment of the cells for 1 hour with HX-XO significantly decreased basal cGMP levels by 58±2.5% in WKY ($p<0.05$, n=5) and 73±2.7% in SHR ($p<0.05$, n=5), respectively, thus suggesting a greater sensitivity of cGMP pathway to superoxide in SHR. In addition, the SNAP-induced increase in cGMP level was significantly inhibited by the pretreatment of the cells with HX-XO in both strains ($p<0.05$, n =5 for each group), suggesting that the guanylyl cyclase is most likely the common target of SNAP and superoxide.

Effect of superoxide anion on the cross-inhibition of IP_3 formation by cGMP.

Since superoxide simultaneously increased IP_3 formation and decreased cGMP formation, it was speculated that the superoxide-increased IP_3 formation in vascular SMCs might also be under the influence of the activity of cGMP pathway. Therefore, the superoxide-induced IP_3 formation was evaluated after stimulating or inhibiting the soluble guanylyl cyclase. Fig. 6-6A shows that the HX-XO-induced IP_3 formation was significantly inhibited by 15±2% in WKY ($p< 0.05$, n=4) or 27±3% in SHR ($p<0.05$, n=4) after pretreatment of cells with SNAP (100 μmol/L) for 20 minutes. In contrast, the superoxide-induced increase in IP_3 formation was significantly potentiated by 20±2.1% in WKY ($p<0.05$, n=4) or 75±9.4% in SHR ($p<0.05$, n=4) by a soluble guanylyl cyclase inhibitor ODQ (20 minutes pretreatment at 100 μmol/L). Both the

inhibitory effect of SNAP and the stimulatory effect of ODQ on the superoxide-induced IP_3 formation were significantly greater in the cells from SHR than that from WKY ($p<0.05$). In addition, SNAP (100 μmol/L) decreased the basal IP_3 formation by 16±4% in WKY ($p<0.05$, n=4) and by 20±3.5% in SHR (n=4 for each group) but ODQ (100 μmol/L) had no significant effects on basal levels of IP_3 in both strains ($p>0.05$, n=4 for each group) (Fig. 6-6B).

We further examined the interaction of the superoxide-induced IP_3 formation with the cGMP-dependent protein kinases (PKG). It was observed that the superoxide-induced increase in IP_3 formation was significantly potentiated by a PKG inhibitor, KT5823 (Fig. 6-7). Also, the effect of KT5823 was dose-dependent (0.1 to 30 μmol/L) and was more potent in SMCs from SHR than in SMCs from WKY.

Effect of superoxide anion on cAMP pathway.

Similar concentrations of basal cAMP levels were observed in SMCs from SHR and WKY. In addition, forskolin treatment (1 hour at 10 μmol/L) also induced similar increases in cAMP formation in both strains (n = 8 for each group) (Fig. 6-8). Unlike the cGMP response to superoxide, HX-XO had no effect on the basal levels of cAMP. The forskolin-enhanced cAMP levels were also unaffected by the HX-XO treatment. Moreover, the HX-XO-induced increase in IP_3 formation was not altered either by 20 minute pretreatments of cells with an adenylyl cyclase inhibitor SQ 22536 (500 μM) or with a selective cAMP-dependent protein kinase (PKA) inhibitor KT5720 (10 μmol/L) in both SHR and WKY (Fig. 6-9A). In addition, both SQ 22536 and KT5720 had no effect on the basal levels of IP_3 in SMCs from both strains (Fig. 6-9B). In agreement with our previous findings [15], foskolin (10 μmol/L) induced a similar inhibition on the basal IP_3 levels or on the HX-XO-stimulated IP_3 formation in SMCs from both strains ($p>0.05$,

n=4 for each group). Overall, these data suggest that superoxide-induced increase in IP_3 formation is not associated with the simultaneous changes in the activity of cAMP signalling pathway.

DISCUSSION

Growing evidence are suggesting the importance of superoxide anion in the regulation of cardiovascular functions. In hypertension, an abnormal production of superoxide in vascular SMCs and an altered responsiveness of SMCs to superoxide have been postulated [26]. An overproduction of superoxide was shown in aortae of SHR [21] and the intravenous administration of SOD was found to reduce arterial blood pressure in SHR. However, the cellular responsiveness to superoxide has not yet been explored in SHR regarding the effects of superoxide on different signal transduction pathways, including IP_3, cGMP, and cAMP pathways, in vascular SMCs.

Auch-Schwelk & Katus (1989) [22] reported that the contraction of endothelium-free aortic rings induced by xanthine plus xanthine oxidase reaction, presumably due to superoxide production, was twice greater in SHR than in WKY. Our previous studies also demonstrated that both the vasoconstriction and IP formation induced by norepinephine were greater in aortic SMCs from SHR than that from WKY [8] and that those enhanced responses were partially inhibited by SOD, but not by catalase [8]. In addition, we also observed the stimulatory effect of superoxide on IP formation in aortic SMCs [8] which confirmed the earlier observations that some oxidants or oxidant-derived products contribute the hydrolysis of phospholipids such as phosphatidylinositol [7]. The present studies have permitted to improve our understanding on the

signalling role of superoxide under physiological conditions and in hypertension in the following aspects. The superoxide-induced increase in IP_3 formation in aortic SMCs resulted from the activation of PLCγ since the application of anti-PLCγ monoclonal antibody significantly inhibited the superoxide-induced IP_3 formation in both strains with a greater effect on SHR (Fig. 6-4). The latter finding thus suggested an increased sensitivity of the PLCγ pathway in SHR. Our study also suggests that tyrosine kinases are the most likely targets of superoxide in aortic SMCs, whereas the activation of PLCβ by superoxide is less likely. This conclusion is based on the facts that the application of tyrosine kinase inhibitors (genistein or tyrphostine A25) significantly reduced or even completely abolished the superoxide-induced IP_3 formation in aortic SMCs in both strains with a more potent inhibition observed in SHR (Fig. 6-2). The specificity of the inhibitory effects of genistein or tyrphostine A25 on tyrosine kinases has been demonstrated with platelet-derived growth factor (PDGF) stimulation of rat vascular SMCs. PDGF is well known for its stimulatory effect on tyrosine kinases [27] in vascular SMCs [28] and we have found that the PDGF-induced increase in IP_3 formation in rat vascular SMCs is significantly inhibited by tyrphostin A25 or genistein [29]. Therefore, the regulation of tyrosine kinase- PLCγ - IP_3 axis by superoxide may represent a novel signal transduction mechanism. Through this mechanism superoxide can activate the tyrosine kinase-PLCγ pathway in a membrane receptor-independent fashion.

The interaction between the IP_3 signalling pathway and the cGMP pathway has been reported previously [18]. It was also shown that elevated cGMP levels, through the activation of PKG, can inhibit PLC and lower the basal levels of IP_3 or attenuate the agonist-stimulated IP_3 formation [30]. It may thus be hypothesized that the superoxide-induced IP_3 formation is in part modulated by the activity of cGMP signalling pathway. The present findings show that

superoxide inhibits cGMP formation and this effect is greater in aortic SMCs from SHR. Moreover, it was found that superoxide not only decreased the basal cGMP levels but also suppressed the cGMP response to SNAP stimulation in aortic SMCs (Fig. 6-5). In addition, the superoxide-induced increase in IP_3 formation was significantly inhibited by a soluble guanylyl cyclase stimulator SNAP and was markedly potentiated by a soluble guanylyl cyclase inhibitor ODQ (Fig. 6-6) or a PKG inhibitor KT5823 in aortic SMCs (Fig. 6-7). Our data, therefore, suggest that the inhibitory effect of superoxide on cGMP formation probably contributes to the activation of IP_3 formation induced by superoxide by lifting the negative feedback exerted by cGMP on the PLC pathway(s). Since the basal levels of cGMP in aortic SMCs are higher in SHR than in WKY (Fig. 6-5), it may thus be postulated that the interaction of cGMP and IP_3 is more important in SHR due to the fact that higher cGMP levels, by exerting a greater inhibitory effect on the increased IP_3 formation, would tend to counteract the increased vascular tone in SHR. However, this buffering mechanism of cGMP may be impaired by an overproduction of superoxide in SHR. First, the increase or decrease in cGMP levels induced by SNAP or superoxide was greater in SMCs from SHR than in SMCs from WKY. Our findings are agreement with the previous observation that the sodium nitroprusside-stimulated cGMP levels are higher in aortic SMCs from SHR than that from WKY [31]. Those observations thus suggest that the existence of a hypersensitivity of cGMP pathway to superoxide or NO-related stimulation in SMCs from SHR. Second, the IP_3 responsiveness to superoxide was markedly decreased in the presence of SNAP in SMCs from SHR and WKY, but significantly more enhanced in the presence of ODQ or the PKG inhibitor KT5823 in aortic SMCs from SHR than in those from WKY (Fig. 6-7). These findings indicate that the inhibition of cGMP-mediated pathway by superoxide contributes to the stimulatory effect of superoxide on IP_3 pathway and

that the cross-inhibition of IP_3 pathway by cGMP pathway is more suppressed by superoxide in SHR than in WKY.

An increase in either cGMP [32-33] or cAMP concentrations [34-35] in vascular SMCs results in vasorelaxation. This phenomenon has been partially interpreted as the consequence of the cross-activation of PKG by both nucleotides. Since PKG which inhibits the IP_3 formation, can be cross-activated by an increase in cAMP [36], it is thus possible that cAMP could also participate indirectly in the regulation of IP_3 levels by the activation of PKG. Previous studies from our laboratory as well as those of others have shown the existence of a complex cross-talk interaction between IP_3 and cAMP pathways whereby an activation of the cAMP pathway resulted in an inhibition of the phenylephrine-induced IP formation in aortic SMCs from SHR and WKY [15]. The present data confirm our previous observation by showing that the elevated IP_3 level induced by superoxide is reduced by forskolin treatment (Fig. 6-9). However, it was clearly demonstrated from our results that superoxide did not cause any changes in cAMP levels in vascular SMCs from SHR or WKY, while it significantly affected IP_3 and cGMP levels. This conclusion is further supported by the finding that FSK increased cAMP concentration in vascular SMCs to similar levels in the absence or presence of superoxide (Fig. 6-8). Thus, the superoxide-induced increase in IP_3 formation in SMCs from both SHR and WKY is not under the influence of postulated simultaneous changes in cAMP levels. Importantly, these results also demonstrated the selectivity of the effects of superoxide on different signal transduction pathways. Although cGMP and IP_3 pathways were differentially affected, cAMP pathway was not subjected to an acute modulation by superoxide.

In conclusion, our results demonstrated that superoxide increased IP_3 formation in aortic SMCs mainly through the activation of the tyrosine kinase-linked PLCγ pathway. The

superoxide-induced decrease in intracellular cGMP levels and its associated reduced activation of PKG could also facilitate the superoxide-induced IP_3 formation by lifting an inhibitory feedback on the PLC pathway. This selective modulation of superoxide on IP_3 and cGMP signal transduction pathways may represent a novel mechanism by which superoxide could be actively involved in the functional regulation of vascular SMCs. More importantly, the simultaneous increase in IP_3 formation and the decrease in cGMP level induced by superoxide were significantly enhanced in vascular SMCs from SHR as compared to WKY. Therefore, the increased IP_3 levels in vascular SMCs from SHR could result from both a direct stimulatory effect of the overproduction of superoxide on a hypersensitive tyrosine kinase-PLCγ pathway and from an indirect inhibitory effect of superoxide on a hypersensitive cGMP pathway. Our results not only emphasize the complexity of interactions among different signal transduction pathways but also reveal an important signalling role of superoxide in vascular SMCs in SHR. This study may unveil new mechanism to explain the development of alterations in vascular function in the genesis or maintenance of hypertension. Moreover, the finding of novel signalling effects of superoxide in vascular SMCs and their alterations in hypertension may provide avenues for the new strategies in the prevention and treatment of hypertension.

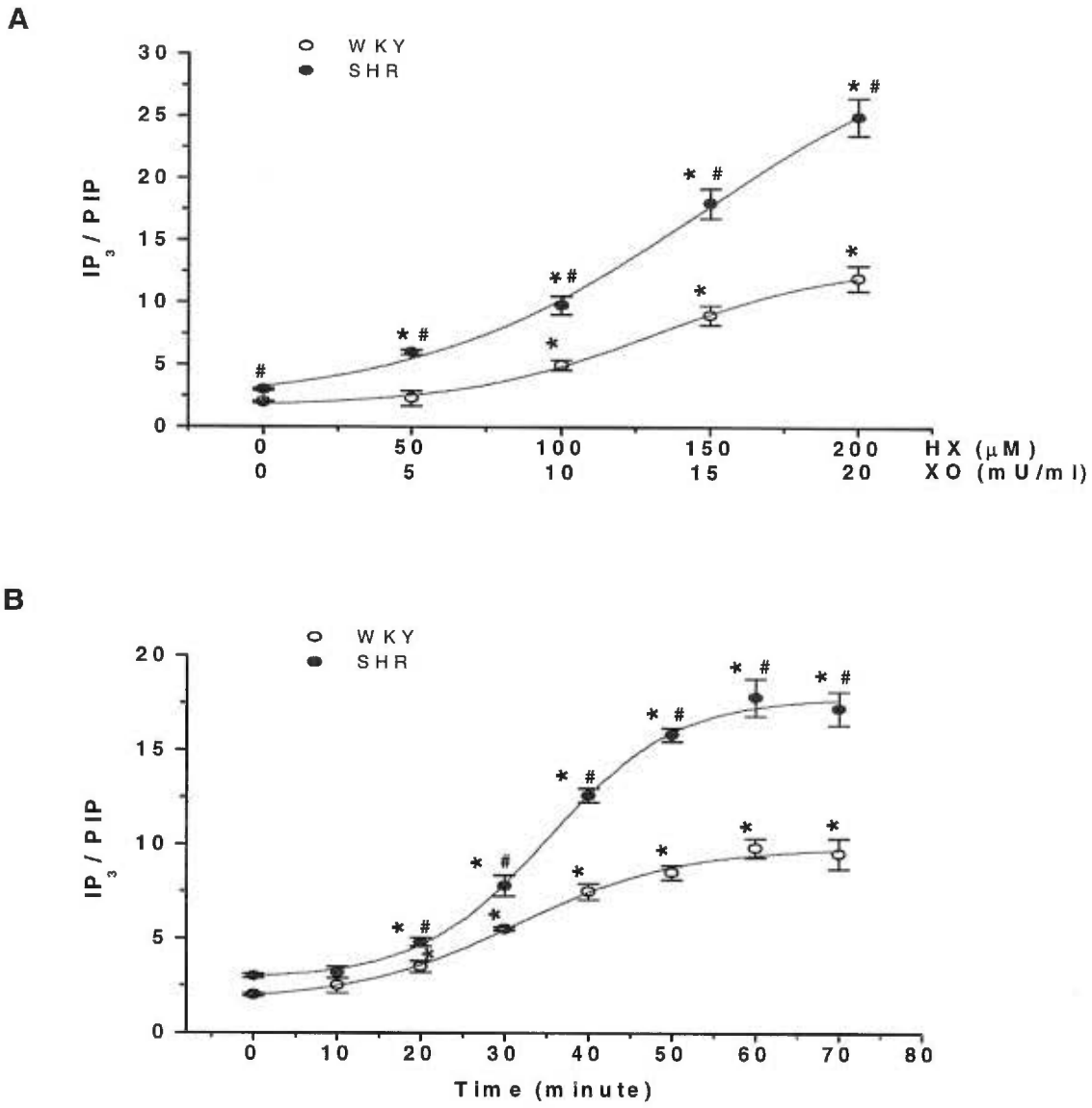

Fig. 6-1. The effect of superoxide on IP_3 formation in aortic SMCs from SHR and WKY. **A**. The increased IP_3 formation in SMCs after 1 hour incubation of the cells with hypoxanthine (HX) and xanthine oxidase (XO). The increase in superoxide-induced IP_3 formation was greater in SHR (filled circle) than in WKY (open circle). n = 5-9 per data point. **B**. HX-XO-induced IP_3 formation in a time-dependent manner and was greater in SHR (filled circle) than in WKY (open circle). * indicates $p<0.05$ as compared to basal IP_3 levels; # indicates $p<0.05$ significant difference between SHR and WKY. n= 3-6 per data point.

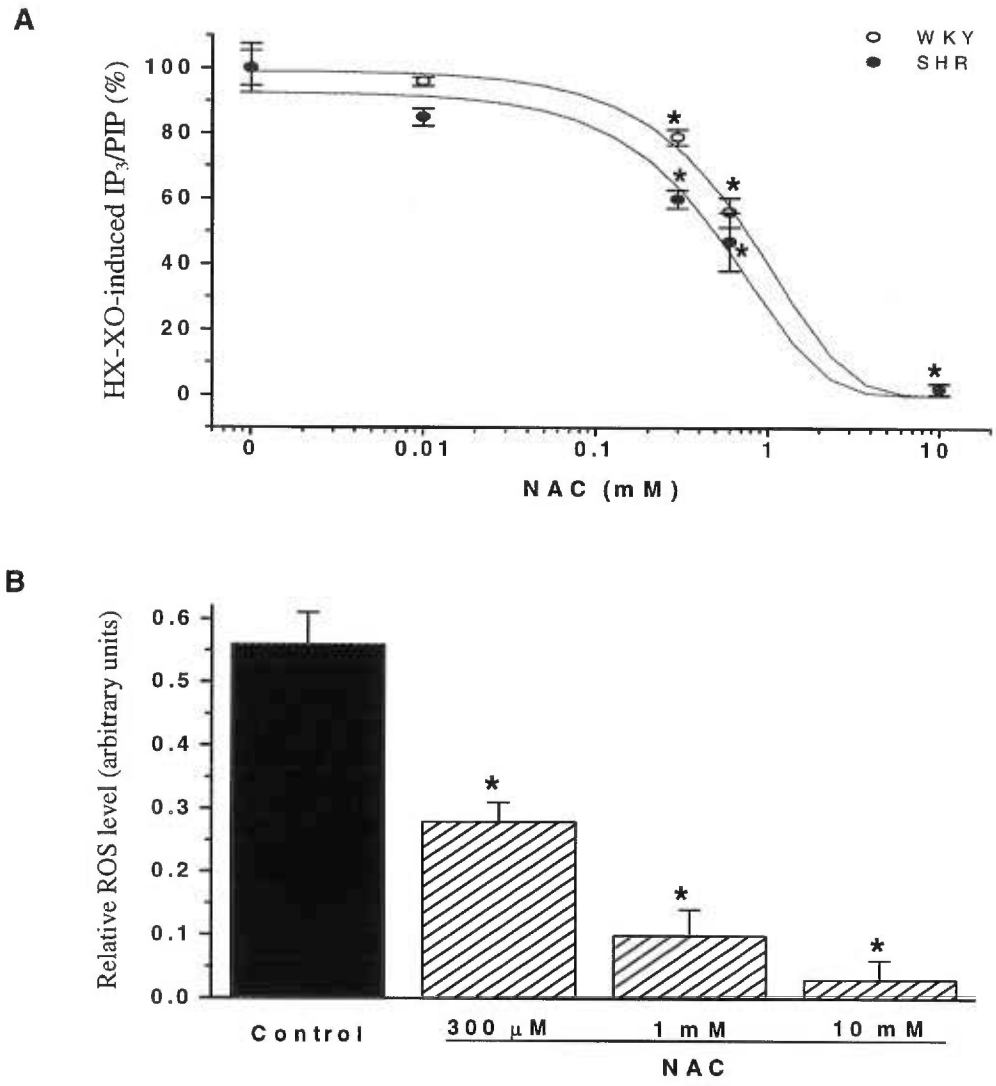

Fig. 6-2. The inhibitory effect of antioxidant on the superoxide-induced IP_3 formation in aortic SMCs from SHR and WKY. **A**. The HX-XO-induced IP_3 formation was significantly inhibited by n-acetylcysteine (NAC) in a concentration-dependent manner in SHR (filled circle) and in WKY (open circle). * indicates $p<0.05$ as compared to HX-XO groups. n=4-6 per data point. **B**. The scavenging effect of NAC on the electrolysis-generated ROS in a cell-free system. * indicates $p<0.05$ as compared to control. n = 4 for each group.

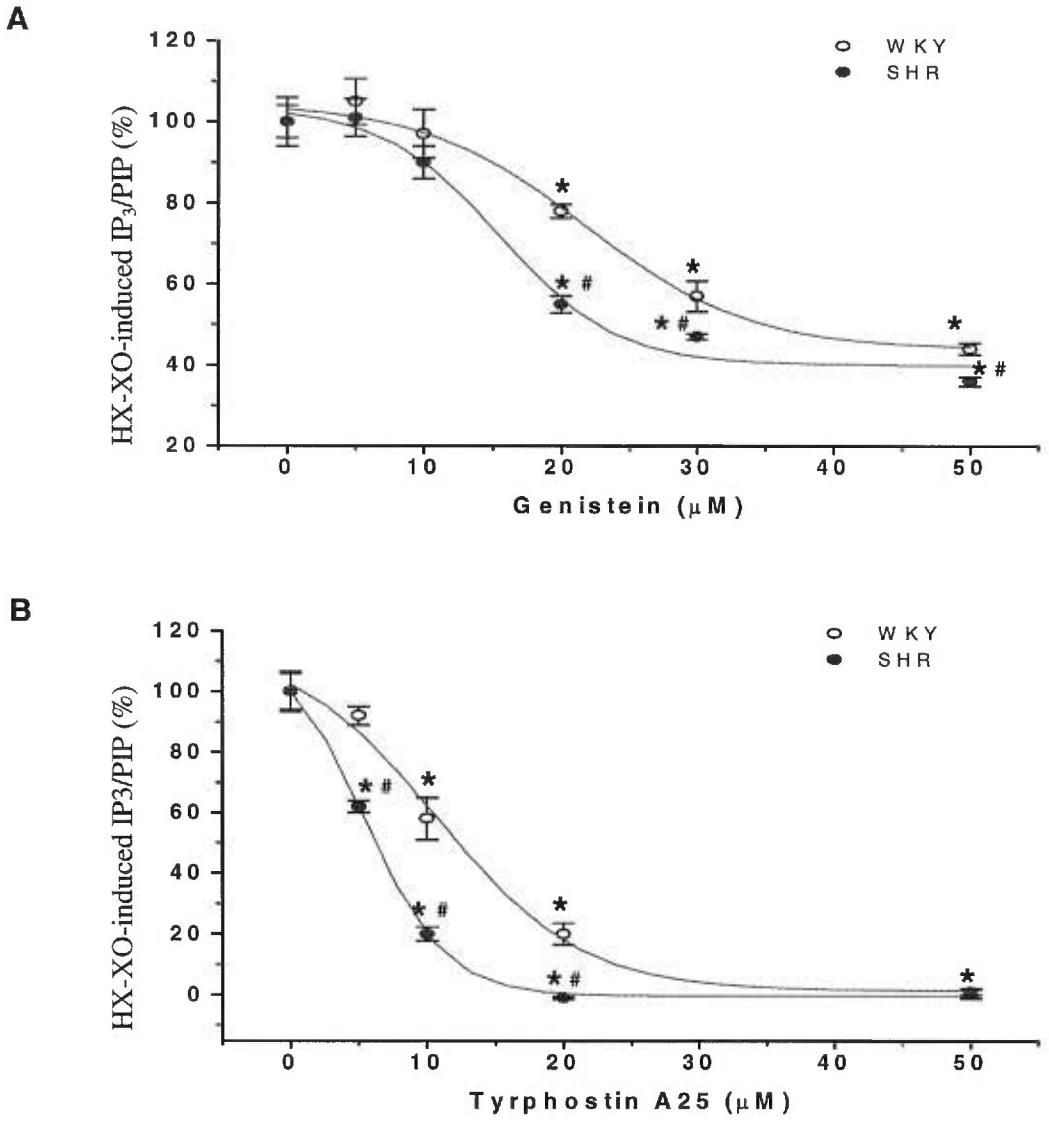

Fig. 6-3. The superoxide-induced IP_3 formation in aortic SMCs from SHR and WKY after the inhibition of tyrosine kinases. Following the pretreatment with genistein (**A**) or tyrphostin A25 (**B**) at different concentrations, the HX-XO-induced IP_3 formation was inhibited dose-dependently. There was a greater inhibition on the HX-XO-induced IP_3 formation in SMCs from SHR (filled circle) than those from WKY (open circle). * indicates $p<0.05$ as compared to the results obtained in the absence of tyrosine kinase inhibitors; # indicates $p<0.05$ significant difference between SHR and WKY. n=4-6 per data point in A and in B.

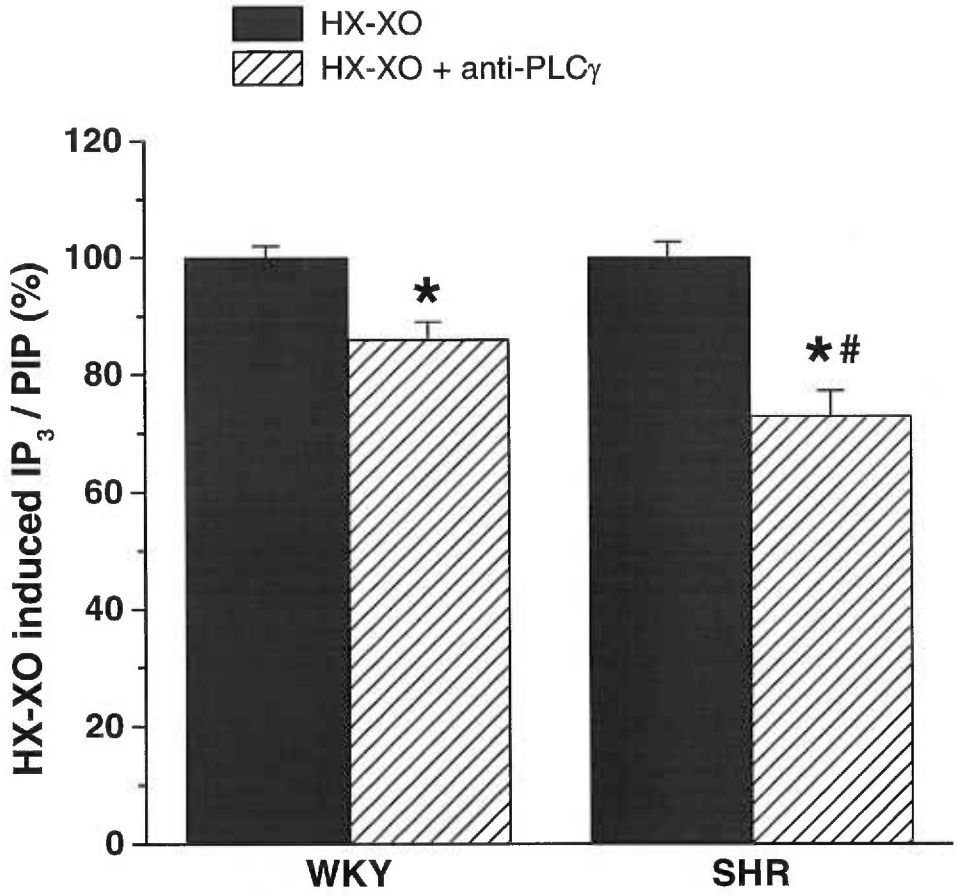

Fig. 6-4. The role of PLCγ in the superoxide-induce IP_3 formation in SMCs from SHR and WKY. The IP_3 formation stimulated by HX-XO in the saponin-permibilized cells with or without pre-incubation with anti-PLCγ (5 μg/ml) for 1 hour. There was a greater inhibition of anti-PLCγ monoclonal antibody on the HX-XO-induced IP_3 formation in SMCs from SHR than that from WKY. * indicates $p<0.05$ as compared to HX-XO groups; # indicates $p<0.05$ significant difference between SHR and WKY. n=5 for each group.

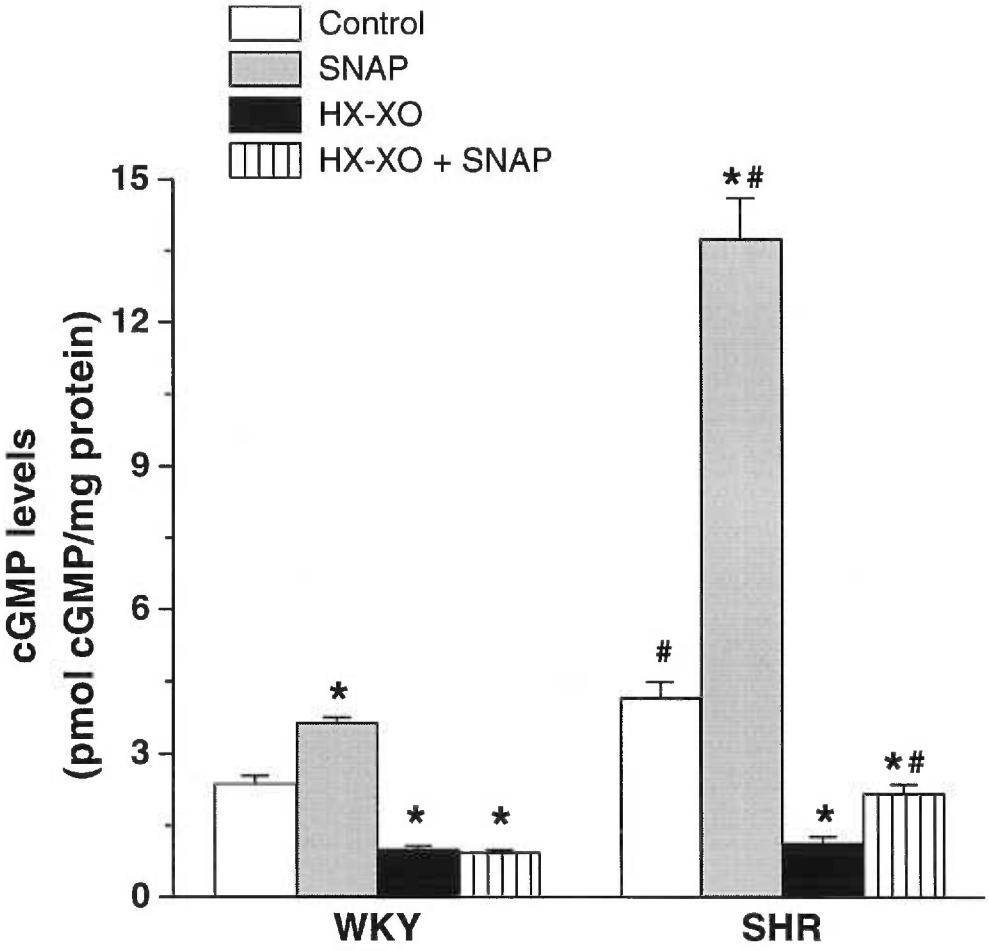

Fig. 6-5. The effects of superoxide on basal cGMP level and the stimulated cGMP formation in aortic SMCs from SHR and WKY. The basal level of cGMP (control) and the levels of cGMP in SMCs treated with SNAP (100 μmol/L), HX-XO or HX-XO plus SNP were measured. * indicates $p<0.05$ as compared to control; # indicates $p<0.05$ significant difference between SHR and WKY. n=5 for each group.

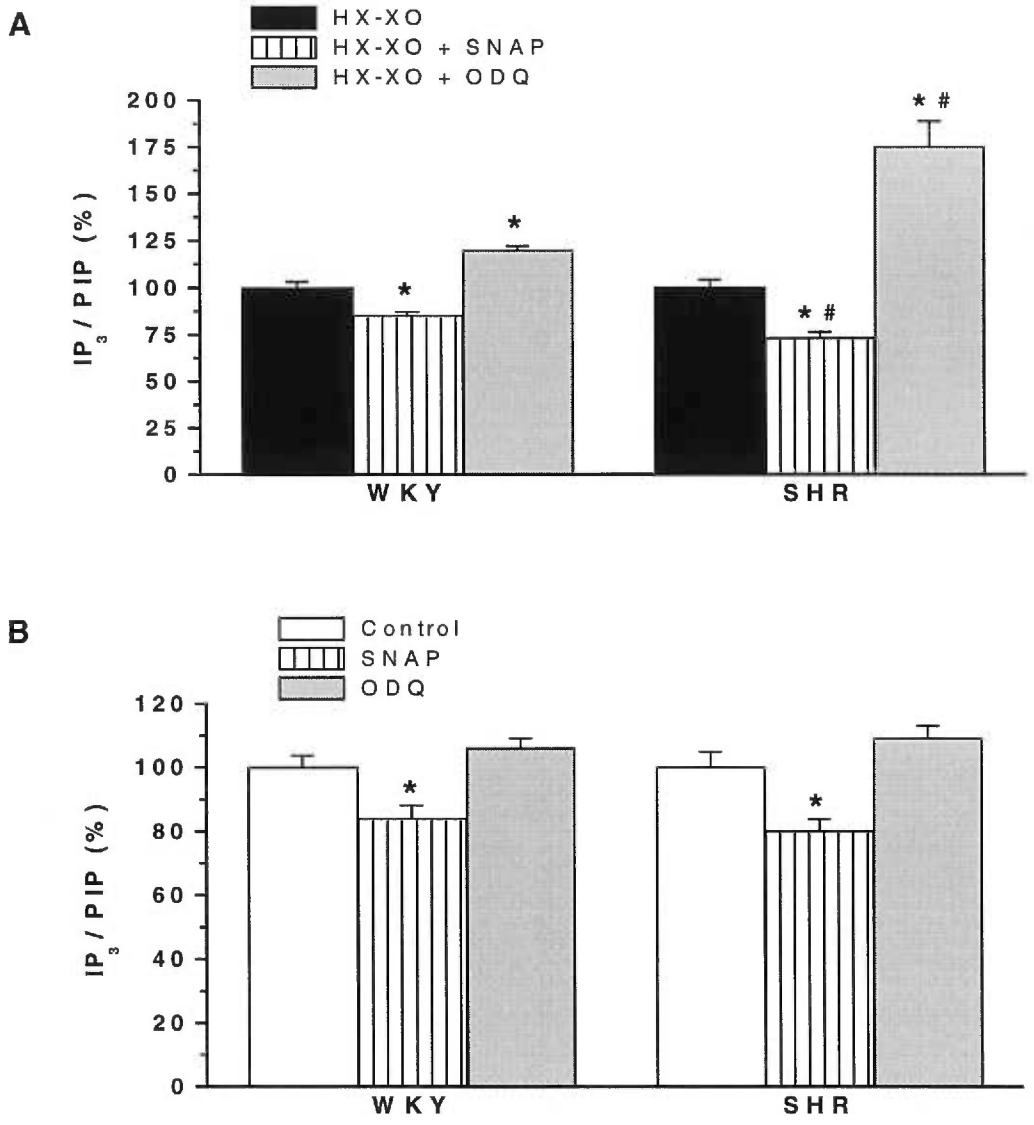

Fig. 6-6. The interaction of cGMP with the superoxide-induced IP_3 formation in aortic SMCs from SHR and WKY. **A**. The HX-XO-induced IP_3 formation was measured in the absence or presence of SNAP (100 μmol/L) or ODQ (100 μmol/L), respectively. * indicates $p<0.05$ as compared to HX-XO groups; # indicates $p<0.05$ significant difference between SHR and WKY. **B**. The IP_3 formation was measured in the untreated SMCs (control), and in the cells treated with SNAP (100 μmol/L) or ODQ (100 μmol/L). * indicates $p<0.05$ as compared to control group. n = 4 for each group.

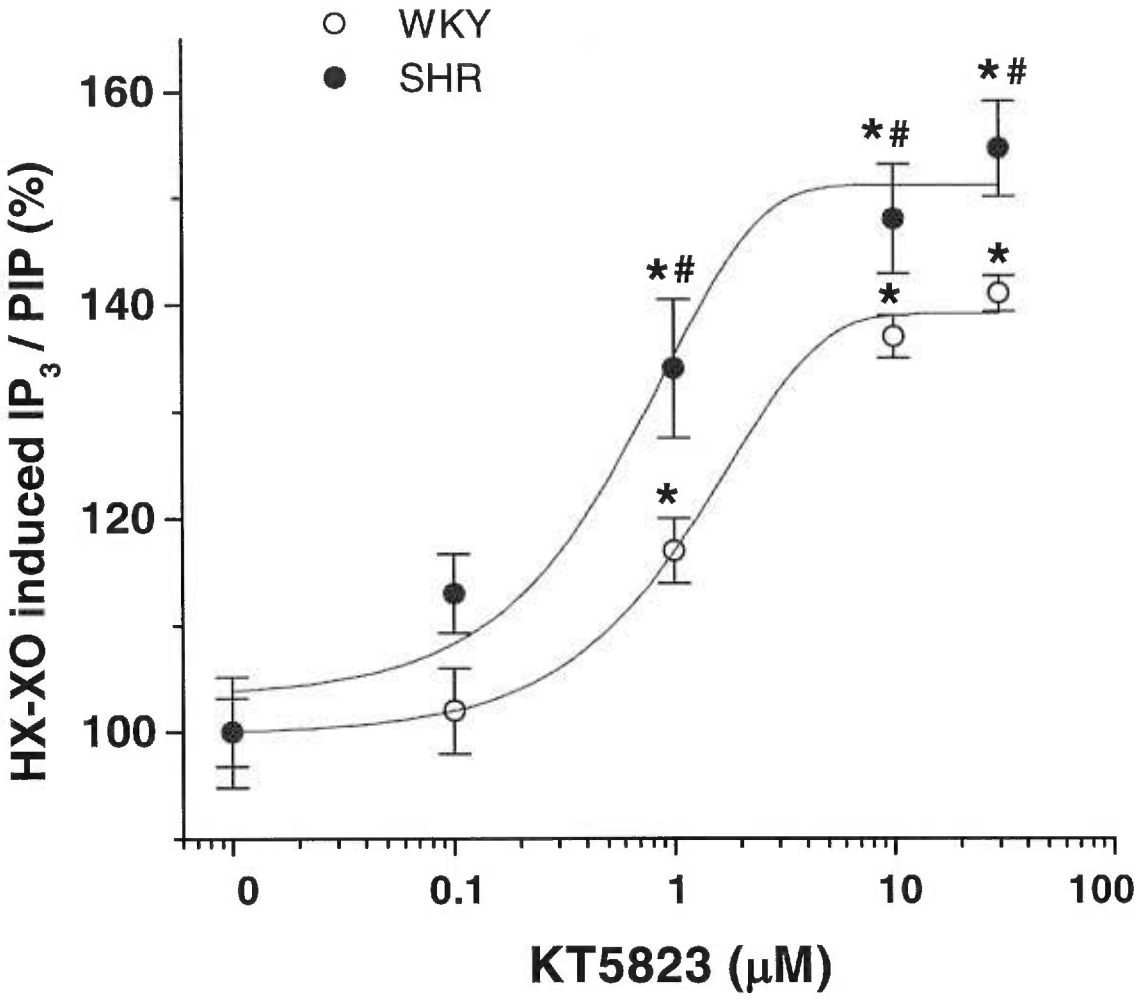

Fig. 6-7. The interaction of PKG with the superoxide-induced IP_3 formation in aortic SMCs from SHR and WKY. The IP_3 formation induced by HX-XO was significantly potentiated by a PKG inhibitor KT5823 in a concentration-dependent manner with a greater increase in the cells from SHR (filled circle) than in those from WKY (open circle). * indicates $p<0.05$ as compared to HX-XO groups; # indicates $p<0.05$ significant differences between SHR and WKY. n=5 per data point.

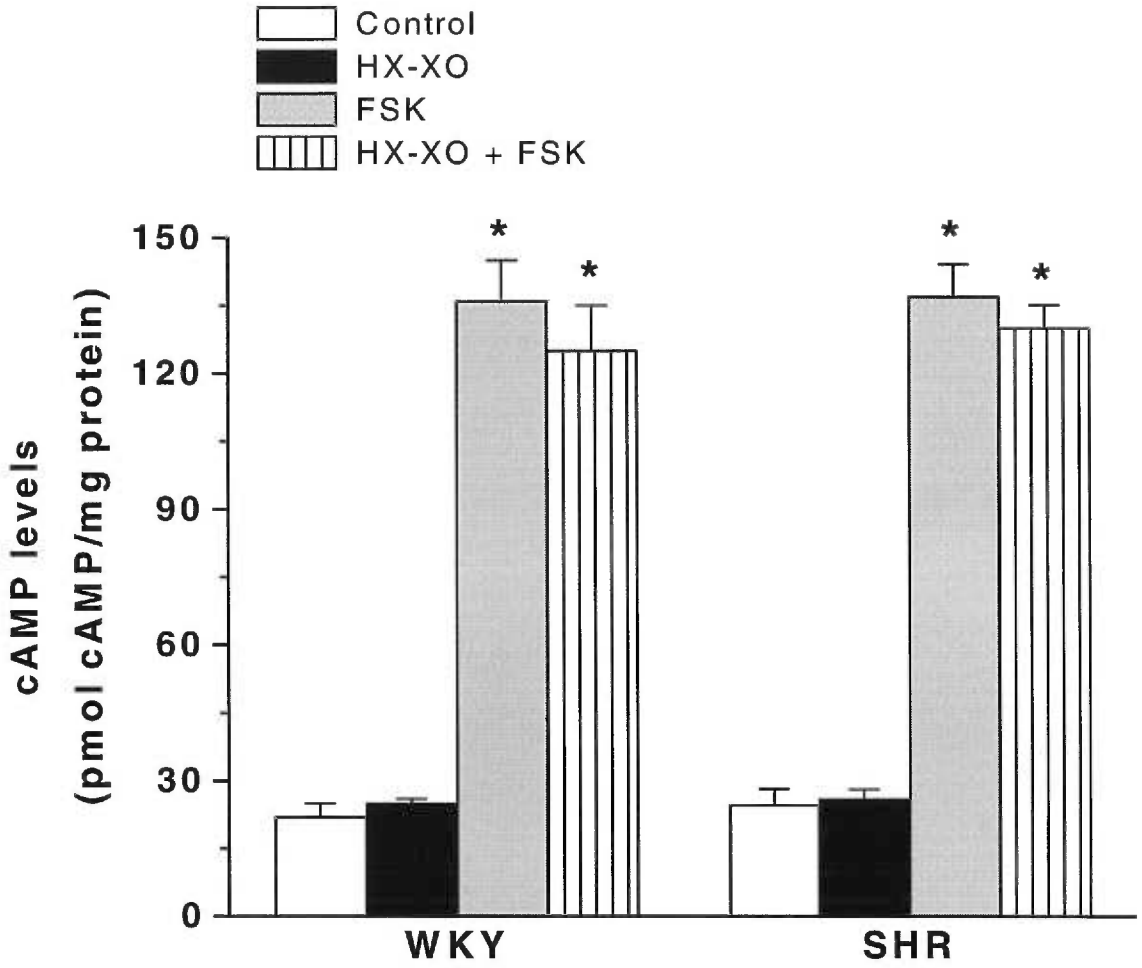

Fig. 6-8. The effect of superoxide on basal cAMP level and the stimulated cAMP formation in aortic SMCs from SHR and WKY. The basal levels of cAMP (control) and the stimulated levels of cAMP in cells treated with HX-XO, forskolin (FSK, 10 μmol/L) or HX-XO plus FSK were measured. * indicates $p<0.05$ as compared to control group or HX-XO treated cells. n = 8 for each group.

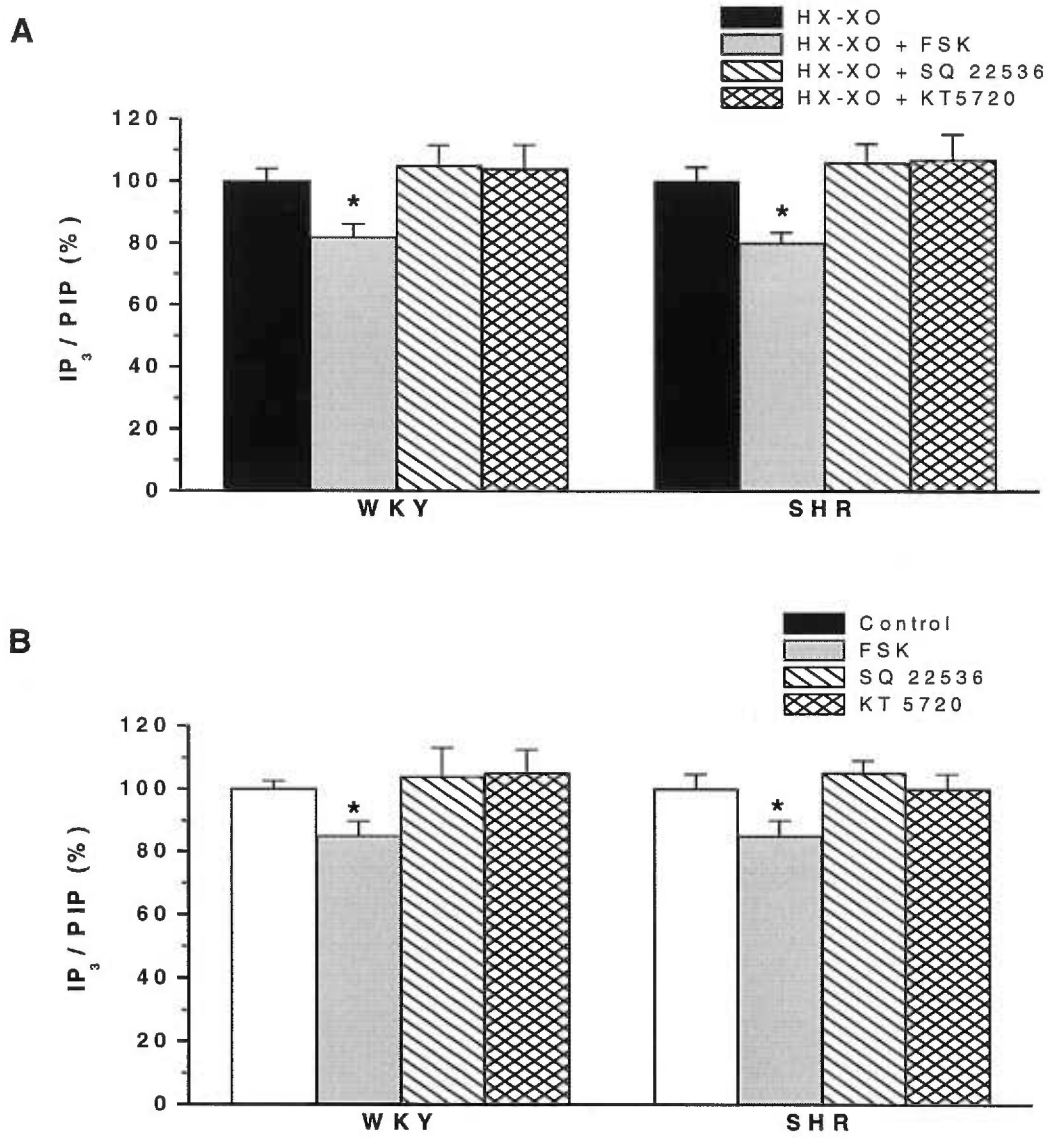

Fig. 6-9. The interaction of cAMP or PKA with the superoxide-induced IP_3 formation in aortic SMCs from SHR and WKY. **A**. The HX-XO-induced IP_3 formation was measured in the absence or presence of foskolin (FSK, 10 μmol/L), SQ 22536 (500 μmol/L) or KT5720 (10 μmol/L), respectively. * indicates $p<0.05$ as compared to HX-XO groups. **B**. The IP_3 formation was measured in untreated cell (control) and in cells treated with foskolin (FSK, 10 μmol/L), SQ 22536 (500 μmol/L) or KT5720 (1 μmol/L), respectively. * indicates $p<0.05$ as compared to control groups. n = 4 for each group.

Table 6-1. The inhibitory effects of antioxidants on HX-XO-induced IP_3 formation in aortic SMCs.

Strain	HX-XO (n = 6)	HX-XO + SOD (n=8)	HX-XO + NAC (n = 4)	HX-XO + LA (n = 8)
WKY	100 ± 3.4%	78 ± 3.0% *	56 ± 4.5% *	64 ± 4.5% *
SHR	100 ± 6.5%	55 ± 4.5% *#	47 ± 8.1% *	39 ± 4.0% *#

The HX-XO-induced IP_3 formation was measured in the absence or presence of SOD (120 U/ml), NAC (600 μmol/L) or α-lipoic acid (LA, 600 μmol/L). * indicates $p<0.05$ as compared to HX-XO groups. # indicates $p<0.05$ difference between SHR and WKY.

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CHAPTER 7

DISCUSSION AND CONCLUSION

GENERAL DISCUSSION

Since most discussions related to specific results have been given in Chapters 3-6, only certain issues not discussed previously or the general association between results presented in individual chapters will be further discussed in this chapter.

The novel signalling role of superoxide anion in vascular SMCs is summarized in Fig. 7-1. In particular, the inhibitory effect on cGMP formation and the stimulatory effect on IP_3 formation of superoxide anion are illustrated. Clearly, these effects of superoxide anion do not appear to involve the cAMP pathway in vascular SMCs.

The effect of superoxide anion on IP_3 formation seems solely or at least mainly mediated by tyrosine kinase-PLCγ system since a non-selective PLC inhibitor U-73122 was found to block the effect of superoxide anion on IP_3 formation in vascular SMCs (Fig. 4-2). Therefore, this result alone suggested that either PLC$_\beta$ or PLCγ or both were involved in the effect of superoxide anion on IP_3 formation. However, the potent inhibitory effects of genistein and tyrphostin A25 on the superoxide-induced IP_3 formation suggested the involvement of the tyrosine kinase pathway in this phenomenon. In addition, specificity of the action of superoxide anion on PLCγ was supported by the finding that the application of a specific monoclonal antibody against PLCγ significantly reduced the superoxide-induced IP_3 formation. It is worth to notice that, at the concentration used, anti-PLCγ antibody did not completely block the superoxide-induced IP_3 formation in vascular SMCs. This observation might be explained either by the concentration of antibody which might not have been sufficiently high or else by the duration of the treatment of the cells with antibody which might not have been long enough. A longer incubation time and higher antibody concentrations may show a greater inhibitory effect. It should be re-emphasized

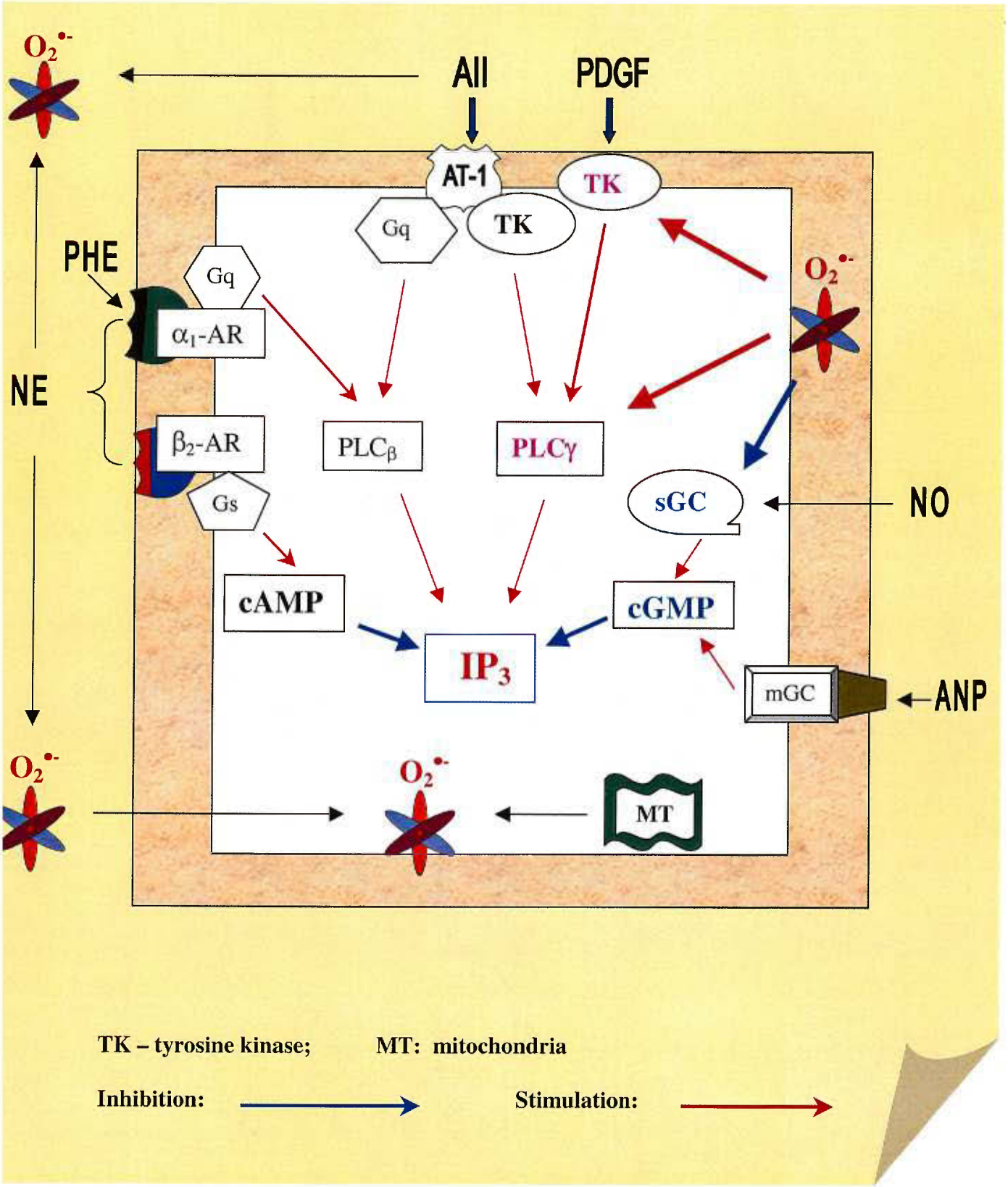

Fig. 7-1. Effects of superoxide anion on signal transduction pathways in vascular SMCs.

that the permeabilized cells in our study were functionally normal since (1) superoxide anion still stimulated the formation of IP_3; (2) the saponin-treated SMCs did not show morphological abnormalities under microscope as compared to the control SMCs; and (3) the treated SMCs were positively stained with Azure A which is absent in non-permeablized cells, but present in the nuclei of permeablized cells.

Not only the activity of PLCγ was stimulated by superoxide anion, but the expression of PLCγ protein was also enhanced. It has been shown in cultured rat fetal distal lung epithelial cells that superoxide anion was in part responsible for the activation of nuclear factor-kappa B, which was associated with increased mRNA levels of epithelial Na^+ channel and amiloride-sensitive Na^+ transporter (Rafii *et al.*, 1998). In the present studies, superoxide anion might have activated transcription factors, which are responsible for the increase in the synthesis of new PLCγ protein. Our results have shown that the expression of PLCγ protein induced by superoxide anion is time-dependent (Fig. 3-5). It is likely that PLCγ protein is relatively stable with a long half-life. As long as its synthesis surpasses its degradation, which can occur 6 hours after the beginning of superoxide anion treatment, PLCγ proteins will be maintained at an elevated level that could exert an inhibitory feedback control mechanisms on the synthesis of new PLCγ protein. This may explain the plateau phase of the PLCγ protein level observed between 6 hours to 12 hours after superoxide anion treatment.

Our study has also shown that the expression of PLCγ proteins induced by superoxide anion is specific. Indeed, we have examined the possibility that the superoxide-induced IP_3 formation might be related to an increased expression of Gq or G11 proteins that activate PLCβ. Incubating rat mesenteric artery SMCs with HX-XO for 4 hours (Fig. 7-2 and Table 7-1), 6 hours

or 12 hours (data not shown) did not change the expression levels of Giα-2 and Gq/G11 ($p>0.05$, $n=4$ for each group). There were also no significant changes in the expression levels of Giα, Gsα, and Gβγ 4 hours (Table 7-1) or 6 hours (data not shown) after incubating rat mesenteric artery SMCs with HX-XO in association or not with NAC. Similar results were obtained in aortic SMCs from SHR or WKY during incubation with HX-XO with or without various antioxidants (Fig. 7-3).

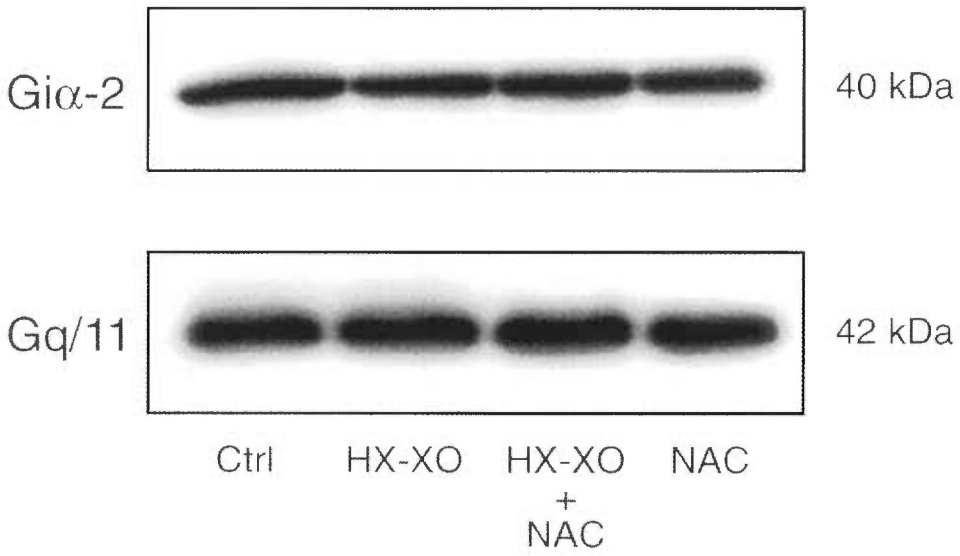

Fig. 7-2. The effects of superoxide anion and n-acetylcysteine on the expression levels of G-proteins in rat mesenteric artery SMCs. The expression of G proteins (Gi_{α_2} and G_q/G_{11}) was examined 4 hours after incubating the cells with HX (100 μM)-XO (10 mU/ml) with or without n-acetylcysteine (NAC, 600 μM), or NAC alone. The membrane proteins of SMCs (50 μg) were separated on SDS/PAGE and transferred to nitrocellulose, which were then immunoblotted using antibody As/7 for Gi_{α_2} and antibody QL for G_q/G_{11}. Similar results were obtained in 4 other experiments.

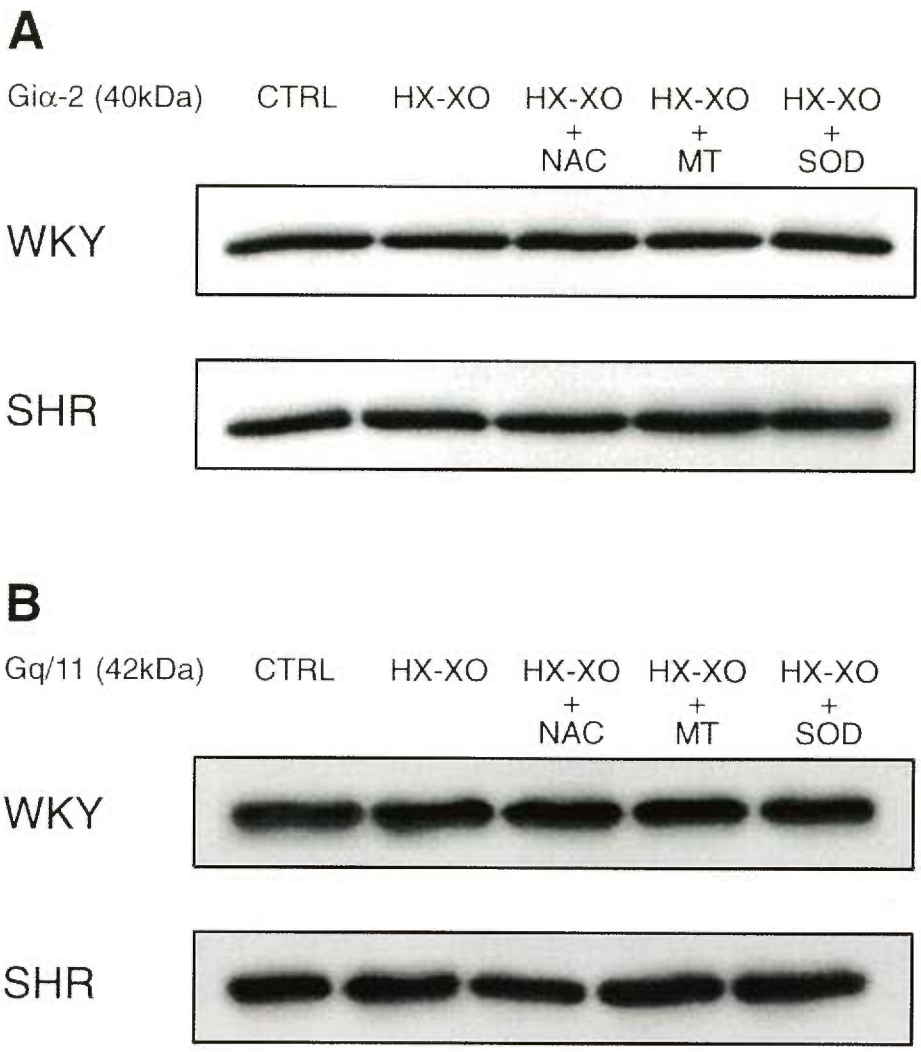

Fig. 7-3. The effect of superoxide anion on the expression levels of G-proteins in aortic SMCs from SHR and WKY. The expression of G proteins ($Gi_{\alpha\text{-}2}$ and G_q/G_{11}) was determined 4 hrs after the treatment of SMCs with HX (100 μM)-XO (10 mU/ml) in the absence or presence of acetylcysteine (NAC, 600 μM), melatonin (MT, 600 μM) or SOD (120 U/ml). The membrane proteins of SMCs (50 μg) were separated on SDS/PAGE and transferred to nitrocellulose, which were then immunoblotted using antibody As/7 for $Gi_{\alpha\text{-}2}$ and antibody QL for G_q/G_{11}. Similar results were obtained in 4 other experiments.

Table 7-1. Quantitative analysis of G-protein levels in cultured SMCs from mesenteric arteries.

	G protein levels (arbitrary units)				
Treatment	$Gs_{\alpha 45}$ (n=3)	$Gi_{\alpha-2}$ (n=4)	$Gi_{\alpha-3}$ (n=3)	G_q/G_{11} (n=4)	$G_{\beta\gamma}$ (n=3)
Control	1.26 ± 0.09	3.54 ± 0.11	2.24 ± 0.13	3.78 ± 0.16	1.55± 0.12
HX-XO	1.11 ± 0.10	3.24 ± 0.23	2.45 ± 0.21	4.08 ± 0.10	1.65 ± 0.10
HX-XO + NAC	1.01± 0.23	3.57 ± 0.20	2.27 ± 0.12	3.76 ± 0.14	1.49 ± 0.21
NAC	1.23 ± 0.21	3.48 ± 0.16	2.36 ± 0.26	3.71 ± 0.10	1.60 ± 0.08

Note: The expression of G proteins were carried out 4 hours after incubating the cells with HX (100 μM)-XO (10 mU/ml) , HX-XO plus n-acetylcysteine (NAC, 600 μM), or NAC alone. The autoradiograms were quantified by scanning with an enhanced laser densitometer.

The inhibition of tyrosine kinase activity with two selective inhibitors, genistein and tyrphostin A25, markedly inhibited (genistein) or completely inhibited (tyrphostin A25) the effect of superoxide anion on IP_3 formation. This finding and the above observations strongly suggest that the superoxide-induced IP_3 formation in vascular SMCs might be mainly mediated by tyrosine kinase–PLCγ system without involvement of the PLCβ pathway.

The existence of the cross–talk interaction between IP_3 and cGMP pathways may represent an important homeostatic control mechanism for the IP_3 metabolism through the negative feedback regulation of the IP_3 formation by intracellular cGMP levels. Applying this concept to vascular SMCs, one can easily imagine that the IP_3-coupled vascular contraction would be at least partially counteracted through the inhibitory effect of cGMP on IP_3 formation. In the presence of superoxide anion, this homeostatic mechanism is altered since superoxide anion, on one hand, increased IP_3 formation, and on the other hand, decreased cGMP formation. The decrease in cGMP formation could facilitate the IP formation by lifting the negative feedback on IP_3 formation, and consequently, the vasorelaxing effect of cGMP is weakened and the IP_3-coupled vasoconstriction becomes dominant. This novel observation raises several new interrogations but does not provide all the answers. For instance, whether the superoxide-activated IP_3 pathway could directly affect cGMP pathway has not been studied. Whether superoxide anion exerts its effects on soluble GC and/or membrane GC needs to be further explored. Moreover, the exact sites of the IP_3 pathway where cGMP acts, either on tyrosine kinase or on PLCγ, should be clarified. Finally, the cross-talk interaction between cAMP and the superoxide-induced IP_3 formation cannot be ruled out as a possible interacting mechanism. Although superoxide anion alone had no effect on cAMP production in vascular SMCs under our experimental conditions, a cross-talk interaction between cAMP and the NE-induced IP_3 formation had been shown in SMCs (Wu & de Champlain, 1996), and cAMP has been found to cross-activate cGMP pathway (Jiang *et al.*, 1992).

In the present study, the effects of several antioxidants on the superoxide-induced IP_3 formation have been investigated (Fig. 7-4). Among those antioxidants, SOD is a well-known specific scavenger for superoxide anion and catalase is a well-known specific scavenger for

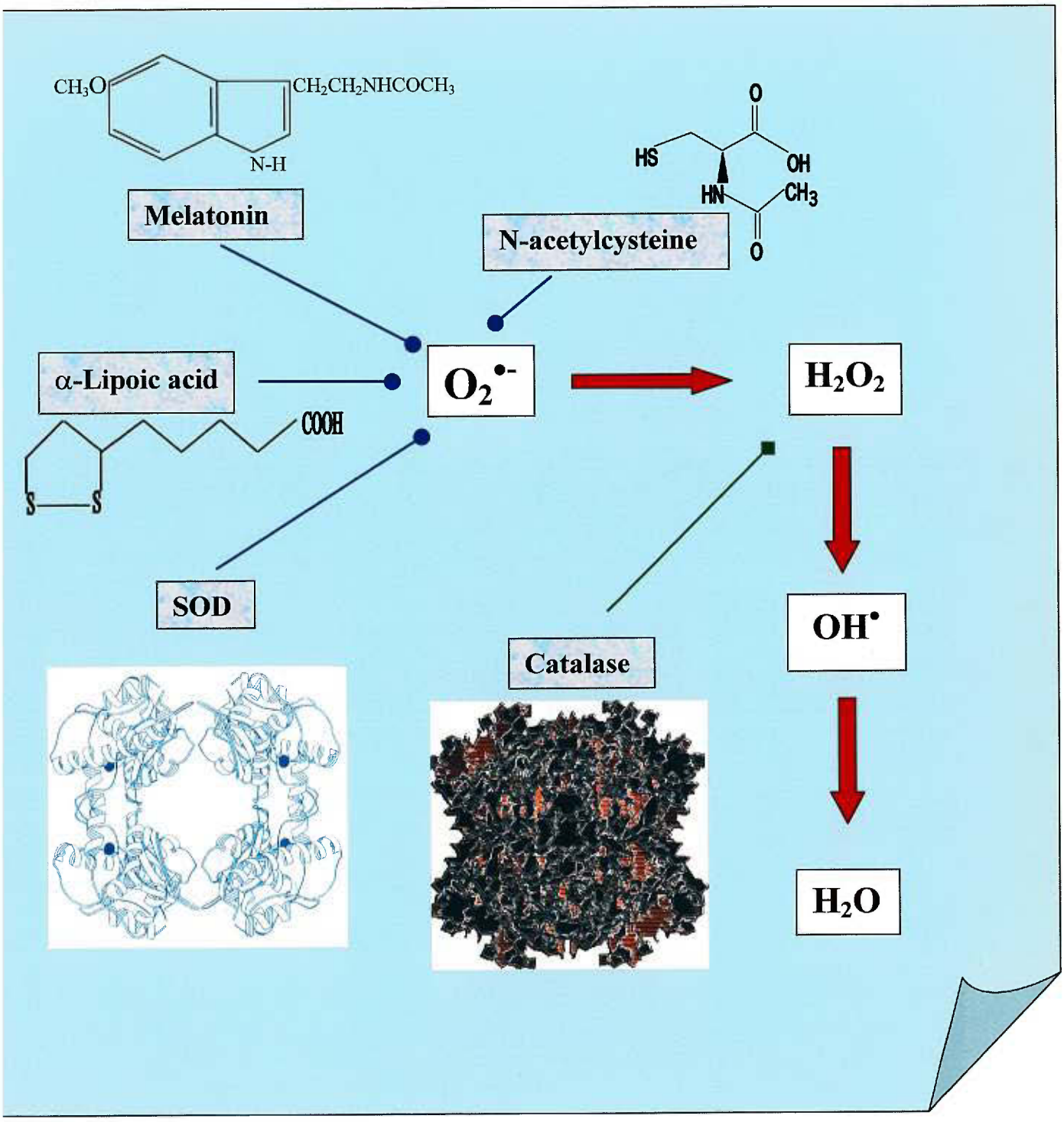

Fig. 7-4. Selective scavenging effects of several antioxidants in vascular SMCs.

L. Wu, Superoxide anion as a signalling molecule.

hydrogen peroxide (H_2O_2). Using these two antioxidants has permitted to characterize the involvement of either superoxide anion or H_2O_2 in our different experiment protocols and to conclude that superoxide anion is the major ROS responsible for the present observations. However, the selectivity of other antioxidants for different species of ROS has been questioned. For example, NAC is converted to the ROS scavenger GSH in the cell and it also prevents the induction of c-jun and the activation of the stress-activated protein kinase in response to reoxygenation (Laderoute and Webster, 1997). NAC also inhibits apoptosis or programmed cell death (Chang *et al.*, 1999). It has been demonstrated from the present study that NAC can effectively scavenge superoxide anion, both *in vivo* and *in vitro*, decreasing the superoxide-induced IP_3 formation in vascular SMCs. In the case of melatonin and α-lipoic acid, they were also proven to be effective, if not selective, in scavenging superoxide anion in vascular SMCs. These results may help to understand the tissue-specific pharmacological profiles of these antioxidants.

Alterations in the signalling pathways of SMCs from SHR have been reported previously. For example, in cultured aortic SMCs from SHR, SNP was found to induce an enhanced cGMP formation as compared to WKY (Papapetropoulos *et al.*, 1994). Moreover, alterations in the IP_3 pathway in vascular SMCs from hypertensive rats have been shown by the enhanced IP_3-dependent vasoconstriction induced by NE (Wu and de Champlain, 1996) and by an increased IP formation following α_1 adrenoceptor activation in cardiovascular tissue (Turla and Webb, 1990; Eid and de Champlain, 1988). Our results reported in Chapter 5 and Chapter 6 have demonstrated the alterations in signalling pathways following exposure of vascular SMCs from SHR to superoxide anion. Since these results have been discussed at length in the respective

chapters, we have summarized those alterations in the signalling role of superoxide anion in SMCs from SHR in comparison to the observations made in SMCs from WKY (Fig. 7-5).

In the present study, the effects of superoxide anion on different signal transduction pathways have been investigated in SMCs from mesenteric artery of Sprague-Dawley rats and in aortic SMCs from normotensive (WKY) or hypertensive rats (SHR). Generally speaking, our results show that the effects of superoxide anion on IP_3 pathway, cGMP pathway, cAMP pathway or the cross-talk interactions among these pathways are similar in mesenteric artery SMCs and in aortic SMCs. These observations not only confirm our observation in two different types of vascular SMCs but also demonstrate the ubiquitous signalling role of superoxide anion in different types of vascular SMCs. On the other hand, we acknowledge the possibility that tissue-specific differences may exist between SMCs from conduit artery and resistant artery, such as the distribution of receptor and ion channel subtype, calcium handling and signal transduction pathways. In our future studies, the signalling role of superoxide anion in mesenteric artery SMCs from SHR and WKY needs to be further characterised and compared to the effects of superoxide anion on aortic SMCs from both strains.

Superoxide anion has been reported to play a number of paradoxical physiological and pathological roles. The variable properties of superoxide anion can be exemplified by the mediation of both normal cell division and the malignant cell transformation and apoptosis as well as by the regulation of both the initiation and the termination of lipid peroxidation (McCord, 1995). Whether this species of ROS functions as a toxic substance or as a regulatory signalling molecule depends on its concentration, on targeted cell types, and on the experimental paradigm used. In principle, superoxide anion constitutes a good candidate for a signalling molecule due to the fact that it is small, rapidly diffusible, highly reactive and rapidly produced in a signal-

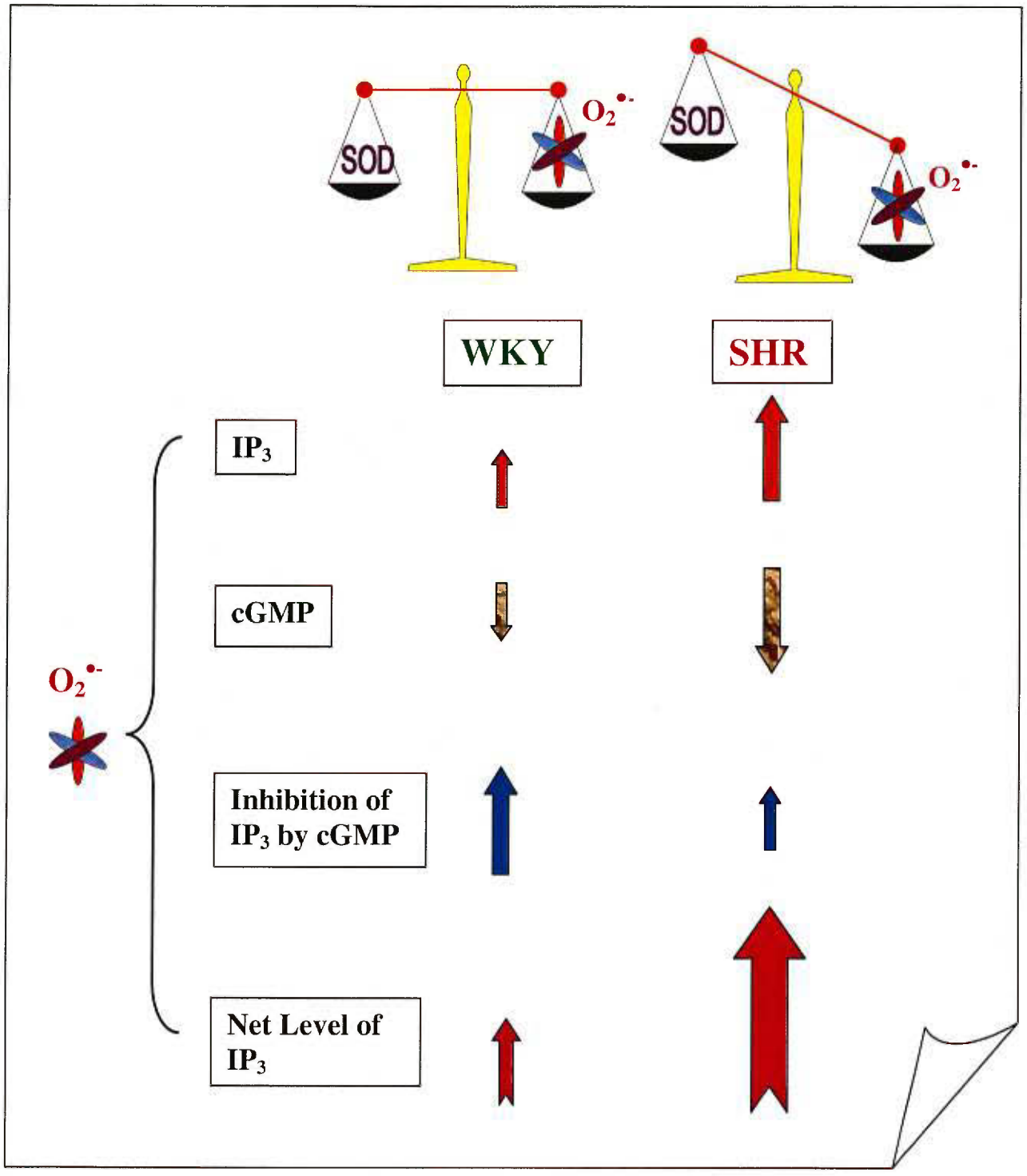

Fig. 7-5. Altered effects of superoxide anion in hypertensive vascular SMCs. The increased IP_3 formation and the decreased cross-inhibition of IP_3 formation by cGMP in the presence of superoxide anion contribute to the final cellular levels of IP_3, which is designated as "Net Level of IP_3".

dependent fashion (Baeyerke *et al.*, 1996; Brumel *et al.*, 1996). The regulation of PKC, protein tyrosine kinase, and MAP kinase activation by ROS has been demonstrated previously (Whisler *et al.*, 1995). Our present study, for the first time, demonstrated the signalling role of superoxide anion in the modulation of IP_3 and cGMP formation as well as in the cross-talk interaction between IP_3 and cGMP pathways in vascular SMCs. The identification of the specific targets of superoxide anion in vascular SMCs, i.e. tyrosine kinases and soluble guanylyl cyclase which are important links in different signal transduction cascades, has permitted to characterise the specificity of the signalling role of this species of ROS. Similar signalling roles have been ascribed to nitric oxide and carbon monoxide. These two gases are freely permeable to plasma membrane, and directly interact with guanylyl cyclase and/or K channels (Wang, 1998). The novel finding of our study is also in that superoxide anion stimulates the formation of IP_3 through activation of the PLCγ pathway, thus inducing the contraction of SMCs in contrast to NO or CO, which induced a relaxation of SMCs. It is likely that the integrated effect of endogenous vasoconstrictors and vasodilators provides a balanced fine-tuning of the vascular tone. Any alterations in this delicate balance such as an increase in superoxide anion and a reduction in NO in SHR might explain the increase in vasoconstriction and elevated blood pressure in this hypertension model.

SIGNIFICANCE OF THE PRESENT STUDY

Although the signalling role of ROS has been postulated, the modulation of the IP_3 signalling pathway activity by superoxide anion in vascular SMCs had not been previously investigated. The aim of this study was to evaluate the effects of superoxide anion on various signal transduction pathways in vascular SMCs under physiological and pathological conditions

and to explore the possible mechanisms whereby superoxide anion may contribute to the pathogenesis of hypertension. The results of the present study may help to better understand the regulation of the cellular signal transduction under physiological and pathophysiological conditions. The finding of the altered signalling role of superoxide anion in hypertension may also pave the way for unveiling novel mechanisms which might explain the abnormal functions of vascular SMCs in hypertension. Moreover, our results may provide new avenues for the development of improved strategies for the prevention and treatment of hypertension.

SUMMARY AND CONCLUSIONS

The following original major discoveries have been obtained from my Ph.D. studies.

1. Superoxide anion, generated either by the auto-oxidation of catecholamines or by the HX-XO reaction, stimulated IP_3 production in the cultured SMCs from rat aorta or mesenteric artery. The effect of superoxide anion on IP_3 production in vascular SMCs was concentration- and time-dependent.

2. The superoxide-induced increase in IP_3 formation was significantly inhibited by antioxidants including SOD, n-acetylcysteine, melatonin, and α-lipoic acid in SMCs from rat aorta or mesenteric artery. However, catalase had no effect on the superoxide-induced IP_3 formation in SMCs.

3. The superoxide-induced IP_3 formation was mainly mediated by the activation of tyrosine kinase pathway. The inhibition of PLC abolished the effect of superoxide anion on IP_3 formation. Genistein and tyrphostin A25, two tyrosine kinase inhibitors, also significantly inhibited the superoxide-induced IP_3 formation. The application of monoclonal antibody against PLC_γ significantly inhibited the superoxide-induced IP_3 formation. In addition, the expression of PLC_γ protein levels was increased after exposing SMCs for 6 hours to superoxide anion.

4. The cellular prodution of cGMP in vascular SMCs was inhibited by superoxide anion. The basal levels of cGMP were increased by SNP, but decreased by superoxide anion or by the guanylyl cyclase inhibitor ODQ. The superoxide-suppressed cGMP levels were increased following stimulation with SNP.

5. The IP_3 pathway was cross-inactivated by the cGMP pathway. The basal levels of IP_3 were decreased by guanylyl cyclase stimulators (SNP or SNAP), but were not affected by ODQ. The superoxide-induced increase in IP_3 formation was significantly inhibited by SNP or SNAP. Moreover, ODQ or KT5823 (a cGMP-dependent protein kinase inhibitor) potentiated the superoxide-induced IP_3 formation.

6. A reduced cellular content of antioxidants and/or a greater sensitivity to superoxide anion of the vascular tissue might occur in SHR. Superoxide anion generated by auto-oxidation of NE induced a greater increase in both the vascular contraction force and the IP_3 formation in SHR. SOD significantly inhibited the NE-induced aortic contraction or IP

formation in aortic SMCs from SHR, but not in those from WKY. In contrast, catalase had no effect on the NE-induced IP formation or vascular contraction in either SHR or WKY.

7. Melatonin was a potent antioxidant against superoxide anion. The inhibitory effect of melatonin on the NE-induced aortic contraction was greater in SHR than in WKY. The inhibition of the NE-induced IP formation by melatonin was also greater in aortic SMCs from SHR than in cells from WKY. Melatonin receptors or α-adrenoceptors did not appear to mediate the antioxidant effect of melatonin.

8. Superoxide anion, generated from the HX-XO reaction, induced a greater IP_3 formation in SMCs from SHR than in SMCs from WKY. Tyrosine kinase inhibitors, genistein or tyrphostin A25, inhibited the superoxide-induced IP_3 formation more significantly in SHR than in WKY.

9. The inhibitory effect of superoxide anion on cGMP pathway was greater in vascular SMCs from SHR than in SMCs from WKY.

10. The superoxide-induced increase in IP_3 formation was significantly more inhibited by SNAP, and more potentiated by ODQ and KT5823 in SMCs from SHR than in SMCs from WKY.

11. The superoxide-enhanced IP_3 formation was not accompanied by simultaneous changes in basal or stimulated cAMP levels in vascular SMCs from SHR or WKY. Moreover, the inhibition of adenylyl cyclase or cAMP-dependent protein kinase did not modify the superoxide-induced IP_3 formation. However, the stimulation of cAMP pathway with forskolin inhibited the basal activity of IP_3 pathway.

In conclusion, these results have permitted to uncover new signalling mechanisms for the actions of superoxide anion in vascular SMCs. For the first time, it was demonstrated that IP_3 levels in vascular SMCs are elevated by superoxide anion mainly due to the stimulation of the tyrosine kinase pathway. It was also demonstrated for the first time that cGMP levels are decreased by superoxide anion presumably due to the inhibition of soluble guanylyl cyclase, thus lifting the cGMP inhibition on the IP_3 formation. The direct effects of superoxide anion on cGMP and IP_3 pathways as well as the existence of a cross-talk interaction between these two pathways not only emphasize the complexity of interactions among different signal transduction pathways but also reveal an important role of superoxide anion on the regulation of those pathways in vascular SMCs. Therefore, the altered signalling effects of superoxide anion on IP_3 and cGMP pathways, which were demonstrated for the first time in vascular SMCs from SHR in this study, may unveil new mechanisms to explain the development of alterations in vascular functions in the genesis or maintenance of hypertension. Moreover, the finding of novel signalling effects of superoxide anion in vascular SMCs and their alterations in hypertension may provide novel avenues for the development of new strategies in the prevention and treatment of hypertension.

PERSPECTIVES

To extend and expand our findings reported in this thesis, we are planning to carry out the following experiments in the future.

1. To study whether the altered effects of superoxide anion on IP_3 metabolism in vascular SMCs from SHR also occur in other types of hypertension, such as DOCA-Salt or NOS inhibition-induced or angiotensin-induced hypertension models.

2. To explore and develop more potent superoxide-scavenging antioxidants and to evaluate their anti-hypertensive effects.

3. To correlate our findings in rat vascular SMCs to the responses in cultured human vascular SMCs.

4. To examine the effects of superoxide anion on IP_3 and cGMP pathways in the presence of NO, a condition encountered *in vivo*.

5. To determine the effects of superoxide anion on proliferation and apoptosis of vascular smooth muscle cells from normotensive and hypertensive rats.

6. To correlate the modulation by superoxide anion of IP_3 and cGMP pathways to the functions of vascular SMCs or vascular smooth muscle tissues.

7. To study the effects of superoxide anion on intracellular calcium concentration changes and on calcium mobilizing mechanisms in vascular SMCs.

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