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Rôle des dérivés réactifs de l'oxygène dans l'hypertension artérielle

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Résumé

Les dérivés réactifs de l'oxygène (DRO) sont des molécules de signalisation intracellulaire qui jouent un rôle important dans la fonction vasculaire. Les buts de notre étude ont été: 1) d'étudier les mécanismes moléculaires qui génèrent les DRO au niveau des cellules musculaires lisses vasculaires chez l'humain (CMLV) et 2) d'évaluer si les DRO contribuent aux changements fonctionnels et structuraux des vaisseaux en hypertension. Les études cellulaires ont été réalisées au niveau des CMLV humaines dans lesquelles nous avons analysé la présence d'un système NADPH actif et fonctionnel apparenté à celui des leucocytes. L'expression des sous-unités de la NADPH oxidase a été déterminée par immuno-buvargade. Les DRO induits par l'Ang II ont été mesurés en utilisant une sonde fluorescente; CM-H_2DCFDA. Toutes les cinq sous-unités de la NADPH oxidase leucocytaire sont exprimées dans les CMLV humaines. L'ARNm de Mox-1 est exprimé dans les cellules de rat et non pas dans les CMLV humaines. L'Ang II augmente significativement l'activité de la NADPH oxidase. Les études *in vivo* ont été réalisées chez les rats SHR-SP ayant subis une diète riche en sel; 4 groupes de rats ont été formés: contrôle (c) (n=6), vitamine C (n=7), vitamine E (n=8) et tempol (n=7). La pression artérielle est augmentée de 212±7 mm Hg à 265±6 mm Hg chez les rats témoins. Les traitements avec les différents antioxidants pendant 6 semaines ont prévenu la progression de l'hypertension. La vasodilatation induite par l'acéthylcholine et le ratio média/lumière vasculaire ont été améliorés. Le niveau d'O_2^- vasculaire a été plus bas chez les groupes traités. Nos résultats démontrent que les CMLV humaines expriment une NADPH oxidase fonctionnellement active régulée par l'Ang II et semblable à celle des leucocytes. Les études chez les animaux indiquent que les antioxidants préviennent la progression de l'hypertension, améliorent la fonction et la structure vasculaire et diminuent le niveau d'O_2^- vasculaire. Ces résultats suggèrent que les CMLV génèrent les DRO et que le stress oxydatif pourrait jouer un rôle physiopathologique dans le développement de l'hypertension causeé par le sel chez les rats génétiquement hypertendus.

Mots clés: NADPH oxydase, hypertension, antioxydant.

Abstract

Reactive oxygen species (ROS) are intracellular signaling molecules that play an important role in modulating vascular function. The aims of our studies were firstly to investigate molecular mechanisms that generate ROS in human vascular smooth muscle cells (VSMC) and secondly to evaluate whether ROS contribute to vascular functional and structural changes in hypertension.

Cellular studies were performed in human VSMCs in which we investigated whether a functionally active leucocyte-like NADPH oxidase is present. Expression of NADPH oxidase subunits was assessed by immunoblots. Ang II-induced generation of ROS was determined using the fluoroprobe CM-DCFDA. All five leukocyte NADPH oxidase subunits were expressed in human VSMCs. Mox-1 mRNA was expressed in rat cells but not in human VSMCs. Ang II significantly increased activity of NADPH oxidase. *In vivo* studies were performed in SHR-SP on a high salt diet, which were randomly divided into 4 groups: control (C) (n=6), vitamin C (n=7), vitamin E (n=8) and tempol (n=7). Blood pressure increased from 212±7 to 265±6 mmHg in controls. 6 week treatment with the various antioxidants prevented progression of hypertension. Acetylcholine-induced vasodilation and media to lumen ratio were improved. Vascular $\bullet O_2^-$ was lower in treated groups.

Our data show that human VSMCs express a functionally active leucocyte-like NADPH oxidase, which is regulated by Ang II. Results from the animal studies indicate that antioxidants prevent progression of hypertension, improve vascular function and structure and reduce vascular $\bullet O_2^-$. Taken together these findings suggest that VSMCs generate ROS and that oxidative stress may play a pathophysiological role in the development of salt-induced hypertension in genetically hypertensive rats.

Keywords: NADPH oxidase, hypertension, antioxidant.

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List of Abbreviations

ACE	acetylcholine
Ang II	angiotensin II
ASK-1	apoptosis signal-regulating kinase-1
DPI	diphenylene iodinium
EC	endothelial cell
EGF-R	epidermal growth factor receptor
Egr	early growth response
ERK	extracellular regulated protein kinase
ET-1	endothelin-1
FAK	focal adhesion kinase
GSH	glutathione
H_2O_2	hydrogen peroxide
5-HT	5-hydroxytryptamine
ICAM-1	intercellular adhesion molecule
IFN	interferon
IL	interleukin
IP3	Inositol 1,4,5-triphosphate
JNK:	c-Jun N-terminal Kinase (SAPK)
LDCL	lucigenin-derived chemiluminescence
LDL	low density lipoprotein
MAPK	mitogen activated protein kinase

MCP	monocyte chemotactic protein
M-CSF	monocyte colony-stimulating factor
mox-1	mitogenic oxidase
NAC	n-acetylcysteine , a free radical scavenger
NADH	nicotinamide adenine dinucleotide dehydrogenase
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
NF	nuclear factor
NO	nitric oxide
nox-1	NAD(P)H oxidase 1
$\cdot O_2^-$	superoxide anion
•OH	hydroxyl radical
$ONOO^-$	peroxynitrite
PDGF	platelet-derived growth factor
PGE_2	prostaglande E_2
phox	phagocyte oxidase
PI 3-K	phosphoinositide 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PLA_2	phospholipase A_2
PPAR	peroxisome proliferator-activated receptor
ROS	reactive oxygen species
RTK	Receptor Tyrosine Kinase
Ser	serine

Shc	src homology complex
SHR-SP	stroke prone spontaneously hypertensive rats
SMC	smooth muscle cells
SOD	superoxide dismutase
TAS	total antioxidant status
TNF-α	tumour necrosis factor
TRE	thyroid responsive element
TRX	thioredoxin
VCAM	vascular cell adhesion molecule
Vit C	vitamin C
Vit E	vitamin E
VSMC	vascular smooth muscle cells

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I. INTRODUCTION

1. Reactive Oxygen Species

Redox-sensitive processes modulate a wide range of cellular functions. Reduction-oxidation reactions generate Reactive Oxygen Species (ROS), which have important intracellular and intercellular signaling properties (1). ROS include both free radicals, which typically have an oxygen- or nitrogen-based unpaired electron, and other species, such as hydrogen peroxide (H_2O_2), which act as oxidants. For many years, these small diffusible molecules were thought to be unwanted and toxic by-products of living in an aerobic environment. But, it has now become clear that ROS act as second messengers in both normal and pathophysiologic conditions. Various agonists, acting through G-protein-coupled receptors and tyrosine kinase receptors induce formation of ROS in many cell types. Many enzyme systems generate ROS, of which NAD(P)H oxidase appears to be the primary source of vascular ROS involved in signaling (2).

Metabolism of oxygen by cells generates potentially deleterious ROS, including superoxide anion ($\cdot O_2^-$), H_2O_2, and hydroxyl radical ($\bullet OH$), as well as the potent vasodilator nitric oxide (NO). In the vasculature, ROS modulate vascular tone and structure. $\cdot O_2^-$ and H_2O_2 have been shown to induce vascular contraction and vascular smooth muscle cell growth, whereas NO plays a pivotal role in endothelium-dependent relaxation. Furthermore, oxygen free radicals are proinflammatory and stimulate monocyte migration and formation of oxidized

low-density lipoprotein. Consequently excessive ROS may underlie pathological processes associated with endothelial dysfunction and vascular remodeling, which are characteristic features of small vessels in hypertension (3).

1.1 Generation of ROS

ROS are generated as intermediates in redox processes in cell, leading from oxygen to water. The univalent reduction of oxygen yields $\cdot O_2^-$, H_2O_2 and •OH according to the following sequence:

$$O_2 \xrightarrow{e-} \cdot O_2^- \xrightarrow{e-} H_2O_2 \xrightarrow{e-} \bullet OH \xrightarrow{e-} H_2O$$

$\cdot O_2^-$ has an unpaired electron in its molecular orbit, spontaneously ejecting the electron to a greater degree of stability, and is therefore known as free oxygen radical. The unpaired electron imparts high reactivity and renders it unstable and short lived. At physiological pH, $\cdot O_2^-$ is the predominant species (4). $\cdot O_2^-$ is water soluble and can act either as an oxidizing agent, where it is reduced to H_2O_2, or as a reducing agent, where it donates its extra electron to form peroxynitrite (ONOO-) with NO. Under physiological conditions in aqueous solutions at a neutral pH, its preferred reaction is the dismutation reaction that yields H_2O_2. However, when NO is produced in excess, a significant amount of $\cdot O_2^-$ reacts with NO to produce ONOO- (5). $\cdot O_2^-$ is membrane impermeable, but can cross cell membranes via anion channels (3~5).

$\cdot O_2^-$ can be produced from numerous sources in the vessel wall (1,6), including the following. The mitochondrial respiratory chain of enzymes, such as nicotinamide adenine dinucleotide dehydrogenase (NADH) and ubiqinone Q-cytochrome B complex. Any electron-transferring protein or enzymatic system can result in the formation of ROS as "by-products" of electron transfer reactions. This "unintended" generation of ROS in mitochondria accounts for ~1-2% of total O_2 consumption under reducing conditions. Due to high concentrations of mitochondrial superoxide dismutase (SOD), the intramitochondrial concentrations of $\cdot O_2^-$ are maintained at very low steady-state levels. Thus unlike H_2O_2, which is capable of diffusing across the mitochondrial membrane into the cytoplasm, mitochondria-generated $\cdot O_2^-$ is unlikely to escape into the cytoplasm. Autooxidation of small molecules such as dopamine, epinephrine, flavins, and hydroquinones can be an important source of intracellular ROS production (7). In most cases, the direct product of such autooxidation reactions is $\cdot O_2^-$. Metabolic byproducts associated with metabolism of arachidonic acid, including cyclooxygenase, lipoxygenase and cytochrome P450 monooxygenase. Xanthine oxidase oxidizes xanthine and hypoxanthine to form $\cdot O_2^-$, H_2O_2 and uric acid. NAD(P)H oxidase catalyzes the production of $\cdot O_2^-$ by the one electron reduction of oxygen using NAD(P)H as the electron donor: $2O_2 + NAD(P)H \rightarrow 2O_2^- + NAD(P) + H^+$. Of the many sources of ROS, it appears that a non-mitochondrial membrane-associated NAD(P)H oxidase is the major source of $\cdot O_2^-$ in vascular cells (8).

Plasma membrane-associated oxidases have been implicated as the sources of most growth factor- and/or cytokine-stimulated oxidant production (9), although the precise enzymatic sources have yet to be fully characterized. The best characterized of the plasma membrane oxidases in general is the phagocytic NAD(P)H oxidase, which serves a specialized function in host defense against invading microorganisms. This multicomponent enzyme catalyzes the one-electron reduction of O_2 to $\cdot O_2^-$, with NAD(P)H as the electron donor through the transmembrane protein cytochrome b558 (a heterodimeric complex of gp91phox and p22phox protein subunits). The transfer of electrons occurs from NAD(P)H on the inner aspect of the plasma membrane to O_2 on the outside. Recent studies have suggested that functional components of the phagocytic NAD(P)H are present in nonphagocytic cells (6,9).

The prototypical and best characterized NAD(P)H oxidase is that found in leukocytes (figure 1.1). Leukocyte NAD(P)H oxidase comprises five components: p40phox (phox for PHagocyte OXidase), p47phox, p67phox, p22phox and gp91phox. In unstimulated cells, p40phox, p47phox and p67phox exist in the cytosol, whereas p22phox and gp91phox are located in the membranes, where they occur as a heterodimeric flavoprotein, cytochrome b558. Upon cell stimulation, p47phox becomes phosphorylated and the entire cytoplasmic complex migrates to the membrane where it associates with cytochrome b558 to assemble the active oxidase, which now transfers electrons from the substrate to O_2 leading to the generation of $\cdot O_2^-$. Activation also requires participation of two low-molecular weight guanine nucleotide-binding proteins, Rac 2 (Rac 1 in some

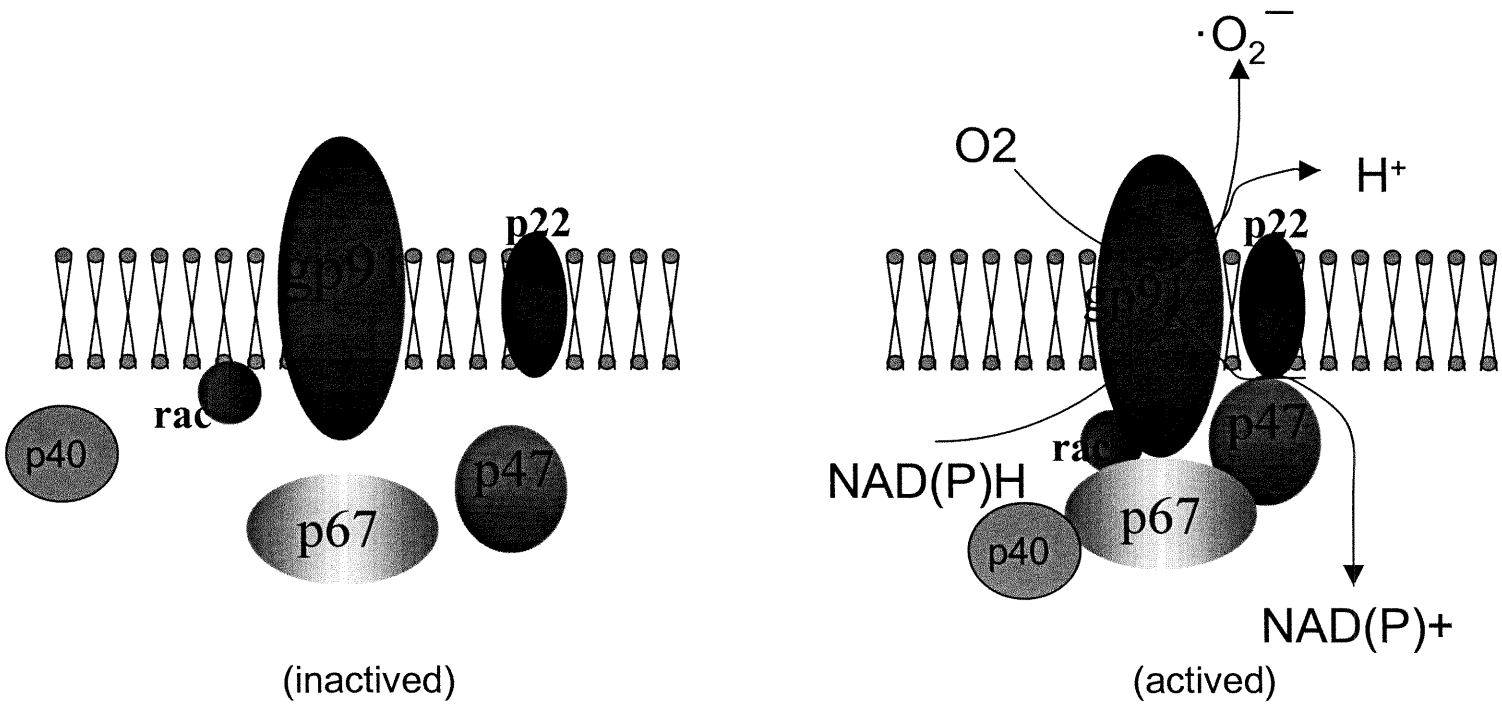

Figure 1.1: Structure of the NAD(P)H oxidase. Left, inactived structure of the neutrophil NAD(P)H oxidase. gp91phox and p22phox form the electron transfer component of the oxidase; p40phox, p47phox and p67phox are cytosolic components. The low molecular weight G protein rac also serves a regulatory function. Right, actived structure of the neutrophil NAD(P)H oxidase.

cells) and Rap 1A. Although it is evident that cells of the vasculature contain functionally active NAD(P)H oxidase, it is still unclear which of the leukocyte NAD(P)H oxidase subunits are present in vascular cells. In adventitial fibroblasts and endothelial cells, mRNAs for gp91phox, p22phox, p47phox and p67phox have been demonstrated (10). All phox subunits have been detected in rabbit aortic adventitia (11,12). Rat VSMCs express p22phox, p47phox and rac1, but have been reported to not express gp91phox (13,14). Whether a similar situation exists in human VSMCs is unclear. Since p22phox and gp91phox are essential for NAD(P)H oxidase activity, the possibility arose that there are gp91phox isoforms that are functionally active in VSMCs. Recently a homologue of gp91phox, nox-1 (for NADPH Oxidase, formerly termed mox-1 for Mitogenic OXidase), was cloned from rat aortic smooth muscle cells, which appears to be functionally important, as overexpression of nox-1 in fibroblasts results in increased $\cdot O_2^-$ production, whereas expression of nox-1 antisense in VSMCs leads to decreased agonist-stimulated NAD(P)H oxidase-induced generation of $\cdot O_2^-$ and attenuated cell growth. Thus nox-1, which generates ROS and participates in mitogenic responses to growth factors, may be the vascular counterpart of leukocyte gp91phox, at least in rat VMSCs (3) (figure 1.2). The above studies were performed in cells and experimental animal models. The status of gp91phox as well as the other major leukocyte NADPH oxidase subunits in human VSMCs has not been well characterized.

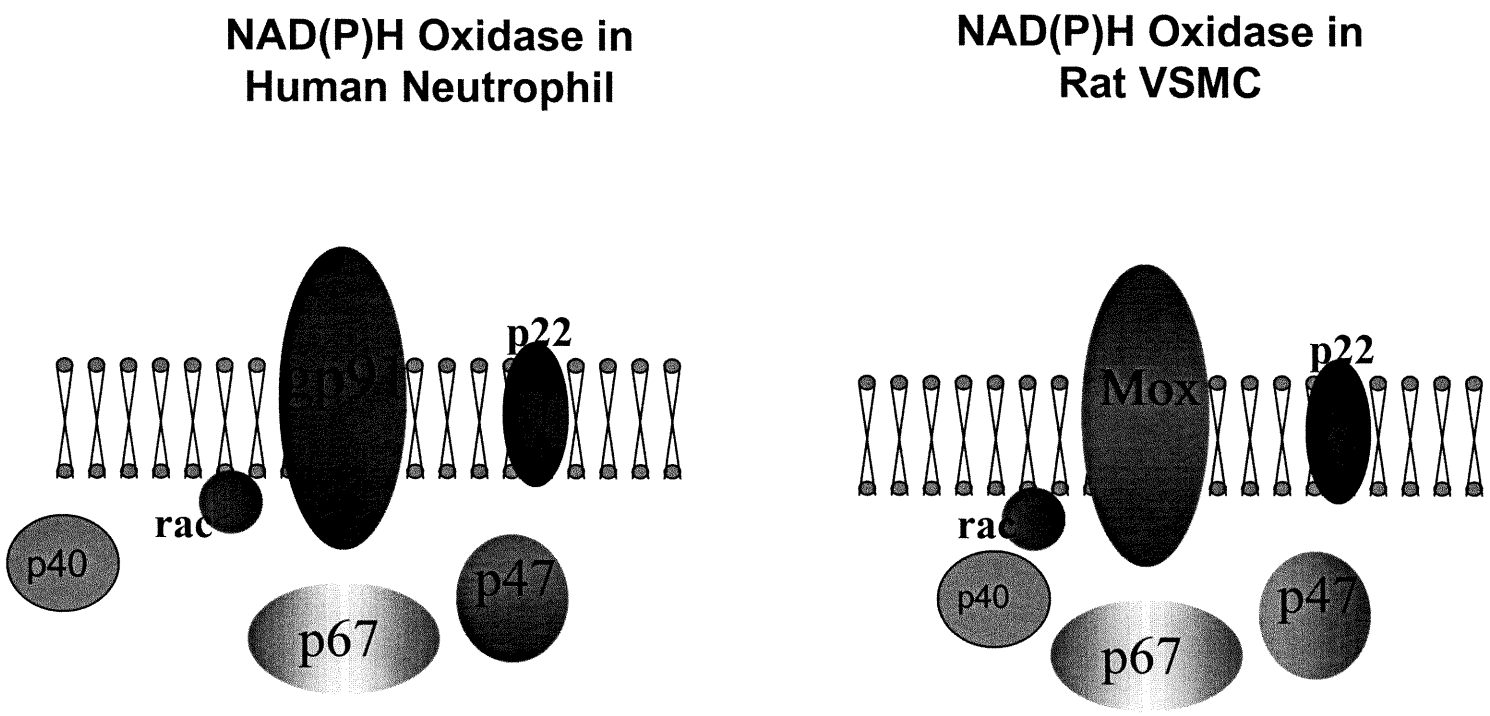

Figure 1.2: Differences of NAD(P)H oxidase between neutrophil and rat VSMCs. Left, the structure of neutrophil NAD(P)H oxidase. gp91phox and p22phox form the electron transfer component of the oxidase; p40phox, p47phox and p67phox are cytosolic components that interact with these 2 proteins to modulate its activity. Right, components of the neutrophil oxidase that have been identified in Rat VSMCs. Mox subunit, instead of gp91phox, is in rat VSMCs. The functional interaction among these subunits remains to be determined.

1.2 Antioxidant Systems

To protect against the potentially damaging effects of ROS, cells possess several antioxidant enzymes, mainly metal-binding proteins and enzymes, such as SOD (which reduces $\cdot O_2^-$ to H_2O_2), catalase, and glutathion peroxidase (which reduces H_2O_2 to H_2O). The main source of H_2O_2 in vascular tissue is the dismutation of $\cdot O_2^-$:

$$2\,\cdot O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

This reaction can be spontaneous or it can be catalyzed by SOD. The SOD-catalyzed dismutation is favoured when the concentration of $\cdot O_2^-$ is low and when the concentration of SOD is high, which occurs in normal conditions. Three mammalian SODs have been identified and the genes cloned and characterized: copper/zinc SOD (SOD1), mitochondrial MnSOD (SOD2), and extracellular SOD (SOD3) (15). The concentration of SOD in the extracellular fluid is lower than in the intracellular fluid. Therefore $\cdot O_2^-$ can survive longer and travel furthers once it gains access to the extracellular space.

H_2O_2 is produced primarily from dismutation of $\cdot O_2^-$ typical human cell metabolizes about 10^{12} molecules of O_2 per day and generates approximately $3x10^9$ molecules of H_2O_2 per hour. H_2O_2 is lipid soluble, can cross cell membranes, and is stable under physiological conditions. In biologic systems, H_2O_2 is scavenged by catalase and by glutathione peroxidase. Glutathione

peroxidase utilizes reduced glutathione to convert H_2O_2 to water and catalase converts H_2O_2 to water without requiring cofactors. In the presence of iron-containing molecules such as Fe^{2+}, which acts as a redox catalyst, H_2O_2 can also be reduced to generate the highly reactive •OH (Haber-Weiss or Fenton reaction). •OH is a potent oxidant that can be produced directly from water or from H_2O_2. It is extremely reactive and therefore has a very short half-life and does not travel more than a few molecular diameters from its site of formation. •OH induces local damage where it is formed. Because of its extremely high reactivity, there are no special scavengers of •OH (1,3).

Antioxidant defenses also involve non-enzymatic systems, such as small molecules and antioxidant vitamins. Non-enzymatic antioxidants can be separated into water-soluble antioxidants and lipid-soluble or lipoprotein-associated antioxidants. The water-soluble antioxidants include ascorbic acid, glutathione, uric acid, and bilirubin. The most abundant lipid-soluble antioxidant in LDL is α-tocopherol (16), which is the chemically and biologically most active form of vitamin E. Other LDL-associated antioxidants, such as ubiquinol-10, ß-carotene, lycopene, and other carotenoids and oxycarotenoids, are present in much smaller amounts, usually less than 1/20th of the α-tocopherol concentration, or less than one molecule per LDL particle (16,17).

2. ROS-as-Intracellular Signaling Molecules

All multicellular organisms depend on highly complex networks of both extracellular and intracellular signals to orchestrate cell-cell communication in diverse physiological processes such as developmental organogenesis, maintenance of normal tissue homeostasis, and repair responses to tissue injury. Typically, extracellular signals are composed of growth factors, cytokines, hormones, and neurotransmitters that bind to specific cell surface receptors. These receptor-ligand interactions then generate various types of intracellular signals that may involve changes in ion concentrations (ion channel-linked receptors), activation of trimeric GTP-binding regulatory proteins (G protein-coupled receptors), and activation of receptor kinases (enzyme-linked receptors). Downstream signaling is then relayed by second messengers (such as cAMP, Ca^{2+}, and phospholipid metabolites) and by protein phosphorylation cascades. Ultimately, these intracellular signaling pathways lead to the activation of transcription factors that regulate the expression of specific sets of genes essential for diverse cellular functions. Accumulating data suggest that ROS play an important role in intracellular signaling (figure 1.3).

Although this role for ROS is a relatively novel concept in vertebrates, there is strong evidence of a physiological role for ROS in several non-mammalian systems. In bacteria, the OxyR protein functions as a transcriptional regulator of H_2O_2-inducible genes and has been shown to be directly activated by oxidation. A recent study has shown that H_2O_2 oxidizes two conserved cysteines

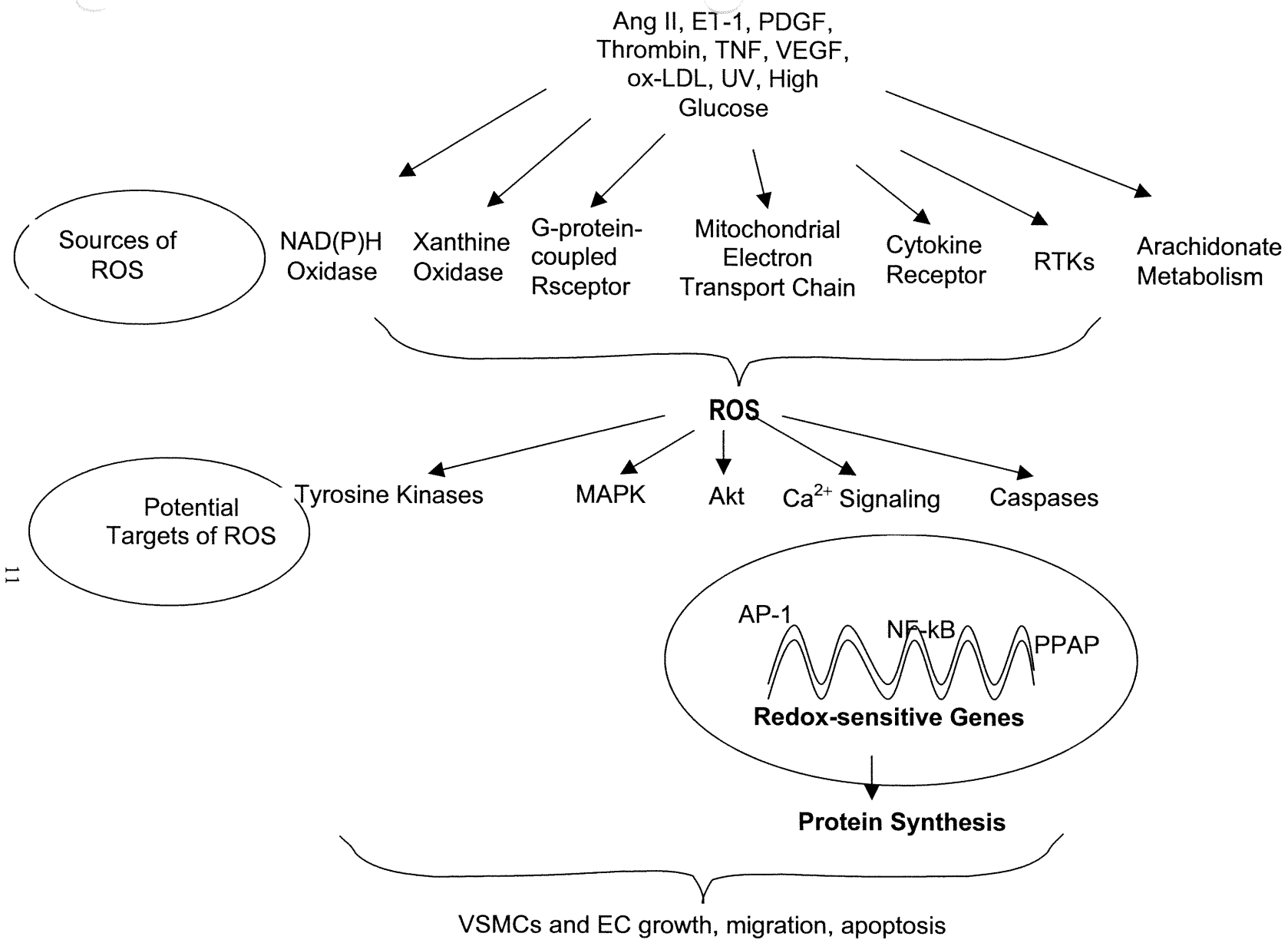

Figure 1.3: Schematic illustrating the potential sources and molecular targets of ROS in the VSMCs and EC. Interdependence and interactions between different ROS sources and signaling proteins are not shown.

in OxyR to form intramolecular disulfide linkages that trigger the activation of this transcription factor, presumably by changing its conformation. The Escherichia coli SoxR transcription factor is activated specifically in response to $\cdot O_2^-$ generating redox-cycling agents such as paraquat and menadione. Activated SoxR mediates SoxS gene transcription, resulting in an increase in SoxS protein that then activates the transcription of several other genes including superoxide dismutase (SOD). SoxR is a homodimer that contains two redox-active iron-sulfur [2Fe-2S] centers that are sensitive to oxidation by $\cdot O_2^-$. The iron-sulfur centers of SoxR must be in their oxidized state for them to be transcriptionally active, thus providing a plausible mechanism by which $\cdot O_2^-$ transmits its gene regulatory signal. In plant cells, generation of H_2O_2 in response to various pathogens elicits localized cell death to limit spread of the pathogen and a more systemic response involving the induction of defense genes regulating plant immunity (9).

It is becoming increasingly apparent that ROS, especially $\cdot O_2^-$ and H_2O_2, are important signaling molecules in cardiovascular cells. Their production is regulated by hormone-sensitive enzymes such as the vascular NAD(P)H oxidases, and their metabolism is coordinated by antioxidant enzymes such as SOD, catalase, and glutathione peroxidase. ROS serve as second messengers to activate multiple intracellular proteins and enzymes, including the epidermal growth factor receptor (EGF-R), c-Src, p38 mitogen-activated protein kinase (MAPK), Ras, and Akt (18,19). Activation of these signaling cascades and redox-sensitive transcription factors leads to induction of many genes with

important functional roles in the physiology and pathophysiology of vascular cells.

Thus, ROS participate in vascular smooth muscle cell growth, remodeling and migration; modulation of endothelial function, including endothelium-dependent relaxation and expression of a proinflammatory phenotype; and modification of the extracellular matrix. All of these events play important roles in vascular diseases such as hypertension and atherosclerosis, suggesting that the sources of ROS and the signaling pathways they modulate may represent important therapeutic targets.

ROS also influence signaling processes associated with vascular contraction, a major determinant of vascular tone. Oxygen radicals mobilize Ca^{2+} from sarcoplasmic and mitochondrial stores and activate the Na^{+}/H^{+} exchanger to promote intracellular alkalization. The deleterious effects of ROS are mainly due to the ability of ROS to produce changes in subcellular organelles, and induce intracellular Ca^{2+} overload (20). These signaling events are major determinants of vascular smooth muscle contraction and probably underlie mechanisms whereby $\cdot O_2^-$ and H_2O_2 directly stimulate contraction. ROS also regulate vascular tone by influencing endothelium-dependent relaxation. Heparin-binding SOD (1200 IU/day IV for 3 days) significantly improved relaxation to Ach (21). NO, as a free radical, is a endogenous vasodilator (22). The integrity of endothelium-dependent dilation depends on the balance between NO and $\cdot O_2^-$. Because NO can be scavenged by $\cdot O_2^-$ to form ONOO-, conditions associated with increased $\cdot O_2^-$, such as hypertension, can induce

vascular damage by reducing the beneficial effects of NO and by increasing the injurious effects of ONOO-, which can be protonated to peroxynitrous acid, the products of which are highly ROS (5). Under these conditions, acetylcholine (ACE)-induced dilation is impaired, vasoconstriction is increased, and lipid peroxidation is induced.

ROS are some of the newest additions to the family of second-messenger molecules. Although one ROS, nitric oxide (NO·), has been known for years to serve as a signaling molecule by activating guanylate cyclase, it has only recently become apparent that other ROS, including $\cdot O_2^-$ and H_2O_2, can alter the function of specific proteins and enzymes as well. In most cases, the mechanism by which these agents interact with their molecular targets is still unknown, but it is clear that they can mediate agonist-stimulated signaling.

2.1 Ligand-induced ROS Production

A variety of cytokines and growth factors that bind receptors of different classes have been reported to generate ROS in nonphagocytic cells.

2.1.1 Cytokine receptors

Cytokine receptors lack intrinsic kinase activity and are not directly linked to ion channels or G proteins. Cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interferon-γ (IFN-γ) were reported to generate ROS in nonphagocytic cells (23), (24). TNF-α induced generation of mitochondrial ROS has been implicated in apoptosis (25). Overexpression of Mn SOD, which has a

redox-active metal at the catalytic site, inhibits TNF-α induced apoptosis, supporting a role for mitochondrial $\cdot O_2^-$ production in mediating this effect (26). Gotoh and Cooper (27) showed that TNF-α, activates apoptosis signal-regulating kinase-1 (ASK-1), a member of the MAPK kinase kinase superfamily, by inducing oxidant-dependent dimerization of ASK-1. ROS-dependent mechanisms also appear to be involved in TNF-α induced expression of cell adhesion molecules (15), and induction of cardiac myocyte hypertrophy (28).

The effects of IL-1 on cell function appear to be similar to those of TNF-α. Both IL-1 and TNF-α have been implicated in playing a role in the cardioprotective effects of exercise by upregulating Mn SOD in a rat model of ischemia-reperfusion (29). These cytokines also upregulate heme oxygenase-1 in endothelial cells by redox-dependent mechanisms that involve protein kinase C (PKC), intracellular Ca^{2+}, and phospholipase A_2 (PLA_2) (30). Bonizzi et al. (31) recently demonstrated three different cell-specific pathways leading to NF-κB activation by IL-1: a pathway dependent on ROS production by 5-lipoxygenase in lymphoid cells, a ROS- and 5-lipoxygenase independent pathway in epithelial cells, and a pathway requiring ROS production by NADPH oxidase in monocytic cells. IL-1 may inhibit fibrinolysis and contribute to vascular injury by inducing plasminogen activator inhibitor type 1 and type 1 collagen expression in a ROS-dependent manner in rat cardiac microvascular endothelial cells (32).

2.1.2 Receptor Tyrosine Kinases (RTKs)

Growth factors that bind RTKs have been shown to generate intracellular ROS essential for mitogenic signaling. Among them, platelet-derived growth factor (PDGF) regulates gene expression by $\cdot O_2^-$ dependent pathways. PDGF stimulates flavoenzyme-dependent $\cdot O_2^-$ generation in human aortic smooth muscle cells by PKC and wortmannin-sensitive pathways (33). In this study, PDGF-induced $\cdot O_2^-$ production appears to participate in vascular lesion formation by activating NF-κB and inducing monocyte chemoattractant protein-1 expression. Human aortic smooth-muscle cells produced $\cdot O_2^-$ in response to PDGF. This $\cdot O_2^-$ production was inhibitable by diphenylene iodonium (DPI), a flavoprotein inhibitor that inhibits NAD(P)H oxidase, suggesting that a phagocyte-like NADPH oxidase was responsible for its production. PDGF-induced $\cdot O_2^-$ appears to be involved in its upregulation of inducible NOS^- and NO dependent release of prostaglande E_2 (PGE_2) in fibroblasts (34).

2.1.3 Receptor serine/threonine kinases.

All receptor serine/threonine kinases described to data in mammalian cells are members of the TGF-β superfamily. Unlike RTK(s)-linked growth factors, TGF-β1 typically inhibits growth of most target cells. TGF-β1 has been shown to stimulate ROS production in a variety of cell types (35~37).

TGF-β1 has also been shown to suppress the expression of antioxidant enzymes in some cells. Kayanoki et al. (35) showed that TGF-β1 inhibits the expression of Mn SOD, copper-zinc SOD, and catalase in rat hepatocytes, leading to increased cellular oxidative stress. Arsalane et al. (38) and White et al.

(39) have shown that TGF-β1 lowers cellular concentrations of intracellular glutathione (GSH) in lung-derives endothelial and epithelial cells. This appears to be mediated by an inhibitory effect of TGF-β1 on transcription of the rate-limiting enzyme involved in GSH synthesis, γ-glutamylcysteine synthetase (38). The reduction in cellular GSH levels by TGF-β1 in vascular endothelial cells is closely associated with its growth-inhibitory effect and appears to be modulated by thiol availability (40). A recent study (41) suggests that intracellular levels of GSH may be important in discriminating an oxidative stress from a "signaling" response to TGF-β1. Endogenously generated H_2O_2 appears to mediate TGF-β1 autoinduction but only under conditions in which intracellular GSH concentrations are high.

2.1.4 G protein-coupled receptors.

G protein-coupled receptors are the largest family of cell surface receptors, with >100 members characterized in mammals (42). A number of ligands that bind to these receptors have been shown to generate ROS in different cell systems. Examples of these ligands include angiotensin II (Ang II), endothelin-1 (ET-1), thrombin, serotonin [5-hydroxytryptamine (5-HT)], and bradykinin.

The octapeptide Ang II, the potent effector molecule of the renin-angiotensin system has been implicated in the pathology of hypertension. Ang II is a vasoactive peptide that, in addition to its effect on vasomotor tone, has hyperplastic and hypertrophic effects on vascular smooth muscle. Ang II (43,44)

has been shown to stimulate ROS production in cultured vascular smooth muscle cells (VSMC), glomerular mesangial cells (45), endothelial cells (EC) (46) and renal proximal tubular cells (47). Ang II activates both NADH- and NADPH-dependent $\cdot O_2^-$ production in VSMCs (43, 48). This activity requires the functional expression of p22phox (49). Physiological actions of Ang II that appear to be mediated by ROS include its vasoactive activity (50), smooth muscle cell growth as well as apoptosis (49).

Thrombin and ET-1 are potent vascular smooth muscle cell (co)mitogens that also signal via G protein-coupled receptor (42,182). Thrombin has been shown to generate ROS in both endothelial cells and smooth muscle cells (SMC) (28, 51). Patterson et al. (10) recently demonstrated the ability of thrombin to stimulate $\cdot O_2^-$ and H_2O_2 production in VSMCs that is associated with the NAD(P)H oxidase activity required for thrombin-induced mitogenesis. In this study, thrombin increased expression of both p47phox and Rac2 in these cells and stimulated their translocation to the membrane, suggesting that an oxidase similar to the phagocytic NADPH oxidase may be involved. Recently, ET-1 was also demonstrated to increase intracellular ROS production in cardiac myocytes by a p21Ras-dependent mechanism (52). In this study, the ET-1-induced ROS was essential for the induction of c-fos expression. In another study, ROS also appears to mediate the inotropic effects of ET-1 on the myocardium (53).

5-HT is a vasoactive substance that mediates both cellular hypertrophy and hyperplasia in VSMCs, while functioning as a neurotransmitter in neuronal cells. Intracellular transport of 5-HT induces tyrosine phosphorylation of GTPase-

activating protein (29) that is associated with the generation of $\cdot O_2^-$ by a NAD(P)H-dependent oxidase activity required for mitogenic signaling (54). Recent work (55) demonstrates that in both SMCs (via the 5-HT transporter mechanism) and Chinese hamster lung fibroblasts (via both 5-HT transporter and receptor activation), the generation of 5-HT-induced $\cdot O_2^-$ leads to the activation of the p42/p44 MAPK pathway and cellular proliferation. A study by Grewal et al. (56) in mesangial cells suggests that a similar signaling pathway via the 5-HT_2A receptor mediates PKC-dependent activation of a NAD(P)H oxidase that activates the p42/p44 MAPK pathway and TGF-β1 mRNA induction.

2.2 Signal Transduction Pathways Modulated by ROS

The downstream targets of ROS have remained largely unexplored. In order for ROS to modify the response of a cell to an agonist, it must affect specific signaling cascades. Over the past several years, many signal transduction pathways have been identified. The most-well studied classes are the tyrosine kinase/phosphatases and serine/threonine kinase/phosphatases. It has been estimated that there are upwards of 2000 distinct protein kinases and 1000 protein phosphatases in the mammalian genome (57). Therefore, it is not surprising that experimental evidence is beginning to emerge implicating multiple protein kinase/phosphatase pathways in redox-mediated signaling events.

2.2.1 Tyrosine Kinases

Stimulation of tyrosine kinase activity by oxidants or agents that induce oxidative stress has been observed by a number of laboratories in a variety of cell types. Growing evidence indicates that the epidermal growth factor receptor (EGF-R) is redox sensitive. In SMCs, H_2O_2 induces tyrosine phosphorylation of the EGF-R and stimulates its association with Shc (src homology complex)–Grb2 (growth factor receptor–bound protein 2) –Sos (son-of-sevenless) complex, to activate subsequent signaling cascades (58). Furthermore, Ang II–induced EGF-R transactivation is mediated through NAD(P)H oxidase–derived ROS because it is strongly inhibited by several antioxidants in SMCs and by n-acetylcysteine (NAC, a free radical scavenger) in cardiac fibroblasts (59). Heeneman et al (60) have most recently reported that Ang II–induced phosphorylation of the Shc/PDGFß-R complex is mediated by ROS. Fukai et al. demonstrated that c-Src is a more proximal kinase than the EGF-R (59). In mouse fibroblasts, H_2O_2 directly activates c-Src (61). Moreover, Ang II–induced c-Src phosphorylation at both the autophosphorylation site (Y418) and the SH2-domain (Y215) is inhibited by antioxidants, suggesting that in VSMCs, H_2O_2 is a proximal mediator of agonist-induced c-Src activation (59).

Another signaling molecule that is activated quite early after receptor stimulation is the low-molecular-weight GTP-binding protein Ras. Lander et al. demonstrated that Ras has a dual role in redox-sensitive signaling: it mediates

activation of NAD(P)H oxidase to generate intracellular ROS, and it is also activated by ROS (62). Moreover, ROS-triggered Ras activation induces recruitment of phosphatidylinositol 3'-kinase to Ras, an event that is required for activation of downstream signals such as Akt and mitogen-activated protein kinase (MAPK) (63).

Sundaresan et al. (64) demonstrated that stimulation of VSMCs by PDGF transiently increases intracellular H_2O_2 production, resulting in tyrosine phosphorylation, activation of MAPK activity, and chemotoxis. These effects were blocked by increasing the intracellular concentration of the free radical scavenging enzyme catalase or by NAC, suggesting a direct role for H_2O_2 as a signal-transducing molecule via modulation of a tyrosine phosphorylation event.

It is not yet clear whether ROS cause direct activation of tyrosine kinase activity or the observed increases in tyrosine phosphorylation are caused by inhibition of tyrosine phosphatase activity by oxidant-mediated signals. Because all tyrosine phosphatases have reactive cysteine residues in their active sites, it has been proposed that inhibition of tyrosine phosphatase activity by oxidants may account for the mechanisms of stimulation of tyrosine phosphorylation by oxidant stimuli (65). The activation of a signal transduction pathway in response to oxidative stress may be mediated by inhibitory activities of oxidants at the molecular level.

2.2.2 MAPKs

MAPKs play a role in relaying signals from extracellular stimuli to the cell nucleus, where they are often the ultimate regulatory proteins in a series of sequential kinase reactions that target transcription factor modification. MAPKs are serine/threonine kinases that transduce signals from the cell membrane to the nucleus in response to classical growth factors and G protein-coupled receptor agonists, as well as cellular stress. At least six groups of MAPKs have been identified in mammalian cells of which the best characterized include the extracellular signal-regulated kinases 1 and 2 (ERK1/2, also termed p42/44MAPK), the c-Jun NH2-terminal kinases (JNK, also termed stress-activated protein kinase, SAPK), p38MAPK, Big MAPK1 (BMK1, also termed ERK5) (66). Although the MAPK families are structurally related, they are generally activated by distinct extracellular stimuli and phosphorylate different molecular substrates. Considerable experimental evidence supports the notion that changes in the cellular redox state by either an induction of an oxidative stress or administration of antioxidants activate signaling pathways involving various members of the MAPK family.

Wilmer et al. (67) showed that IL-1-induced activation of ERK2 and JNK in human mesangial cells was inhibited by antioxidants, suggesting that ligand-stimulated ROS may be involved in mediating this effect. Several reports have begun to address the involvement of the MAPK signaling pathway in redox-mediated signaling in the vasculature. Initially, Baas and Berk (68) demonstrated

that addition of the superoxide-generating agent LY83583 to VSMCs resulted in a concentration-dependent increase in MAPK activity. Various physiological agents believed to play a role in vascular dysfunction have been shown to activate MAPK activity in VSMCs via the generation of intracellular ROS. In vascular ECs, cyclic strain-induced ROS can modulate early growth response (Egr-1) expression, at least partially, via the ERK signaling pathway (69). A study by Lo and Cruz (70) suggests that TNF-α and IL-1 induced activation of JNK may be mediated by intracellular H_2O_2. Ushio-Fukai et al. (71) demonstrated that Ang II elicited an increase in intracellular H_2O_2 and a rapid phosphorylation of both p42/44 MAPK and p38 MAPK. Inhibitors of NADPH oxidase and overexpression of catalase blocked Ang II–mediated phosphorylation of p38 MAPK. The increase in $\cdot O_2^-$ production resulted in activation of the MAPK pathway and ultimately induction of c-fos and cell proliferation.

Hypoxia and reoxygenation impose two extremes of redox stress on cardiac tissue and are principal components of myocardial ischemia and reperfusion. Reperfusion of ischemic tissue is associated with cell injury caused by ROS that are generated by reoxygenation. These ROS cause cell damage directly, by oxidation of cellular components, and also indirectly, by the activation of localized inflammation (72) likely via the action of redox-sensitive signaling pathways resulting in increased inflammatory gene expression. Although the signals and pathways that mediate the response of cardiac myocytes to this type of redox stress are unclear, data have shown that both p38 MAPK and

SAPK/JNK activity is strongly attenuated by preincubation of the cells with antioxidants and tyrosine kinase inhibitors (73). ROS provide a molecular link in MAPK signal pathways to physiological stimuli that alter intracellular ROS levels and changes in gene expression and cellular function.

2.2.3 Akt

The recently identified serine/threonine kinase Akt, which lies downstream of phosphoinositide 3-kinase (PI 3-K) plays a key role in cell survival and protein synthesis (74, 75). It appears that Akt lies in the crossroads of multiple cellular signaling pathways and acts as a transducer of many functions initiated by growth factor receptors (76).

Both Ang II and H_2O_2 stimulation of Akt are abrogated by the phosphatidylinositol 3-kinase (PI3-K) inhibitors wortmannin and LY294002 (2(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), suggesting that PI3-K is an upstream mediator of Akt activation in VSMCs (77). Wang et al. (78) showed that expression of a dominant negative mutant of p85 (regulatory component of PI3-K) and treatment with inhibitors of PI3-K (wortmannin and LY294002) prevented H_2O_2-induced Akt activation. Akt activation by H_2O_2 is also dependent on EGFR signaling; H_2O_2 treatment leads to EGFR phosphorylation, and inhibition of EGFR activation prevents Akt activation by H_2O_2 (79). H_2O_2 markedly induces tyrosine phosphorylation of focal adhesion kinase (FAK) and serine phosphorylation of Akt at 1 hour after stimulation. Concomitantly, the association

of FAK with PI3-K is also observed by the H_2O_2 stimulation (80). Stimulation of PI3-K and Akt is implicated in the regulation of protein synthesis in various cells. One mechanism involves PI3K/Akt-dependent phosphorylation of 4E-BP1, which dissociates from eIF4E, allowing initiation of translation from the 7-methylGTP cap of mRNAs (81).

Deora et al. (82) identified a signaling cascade triggered immediately upon redox activation of Ras. They examined two physiologically relevant models of redox signaling: 1) NO in human T cells, and 2) advanced glycation end product in rat pheochromocytoma cells. ROS by nitric oxide donors and the interaction of advanced glycation end product with its receptor led to the recruitment of p85/p110 PI3K to the plasma membrane, where it associated directly with the effector domain of Ras and became activated. Only the p110β and p110δ (but not p110α) catalytic subunits were recruited by redox-activated Ras. Activation of downstream targets of PI3-K such as Akt and MAPK was found to be PI3-K dependent. Their study demonstrated that nitrose-active and oxidative stressors trigger Ras-dependent and PI3K-regulated events in cells and define a biochemical pathway that is triggered by redox signaling.

2.2.4 Ca^{2+} Signaling

Ca^{2+} is a ubiquitous second messenger that regulates many biological processes, including gene expression, neurotransmission, cell motility, cell growth and contraction. In response to physiological stimuli at the cell surface,

the intracellular level of Ca^{2+} rises, and this elevation elicits the activation of Ca^{2+}-dependent proteins such as PKC, Ca^{2+}-calmodulin kinases, and calmodulin-dependent protein phosphatases (calcineurin). Oxidants have been shown to stimulate Ca^{2+} signaling by increasing cytosolic Ca^{2+} concentration (83), suggesting a possible physiological role of ROS and oxidative stress in the regulation of Ca^{2+}-induced signaling in the vasculature. Inositol 1,4,5-triphosphate (IP3)-induced Ca^{2+} release from VSMC can be selectively stimulated by ROS, which may enhance signal transduction for muscle contraction and gene expression. The subunit-subunit contact within the ryanodine receptor complex, as well as intermolecular interactions between the ryanodine receptor and triadin (an integral membrane protein), are redox sensitive, suggesting that ROS may regulate cardiac muscle Ca^{2+} -signaling events. The biochemistry of ROS and thiol regulation may allow for specific interactions between ROS and target molecules during redox regulation (84). Elliott and Koliwad (85) focus on two endothelial cell ion channels, the activities of which influence vascular cell signaling and the NO signaling pathway. The first channel is the GSSG (oxidized glutathione)-operated cation channel that depolarizes EC, leading to inhibition of capacitative Ca^{2+} entry. The second channel is the IP3-operated Ca^{2+} channel that is responsible for the agonist-stimulated release of Ca^{2+} from IP3-sensitive endoplasmic reticulum.

Increases in intracellular Ca^{2+} were detected in response to H_2O_2 treatment of VSMCs and H_2O_2 showed a transient release of Ca^{2+} from intracellular stores (86). Intracellular calcium homeostasis is regulated by the

redox state of cellular thiols, and it is evident that cell calcium may play a critical role in the activation of the redox-sensitive transcription factor NF-κB (87).

There seem two potential target of ROS-mediated Ca^{2+} flux in signaling pathways; direct modification of Ca^{2+} pumps by oxidants may be one mechanism of oxidant-mediated Ca^{2+} signaling and the other is enhanced Ca^{2+} transport through Ca^{2+} channels. However, the exact mechanisms remain to be elucidated.

2.2.5 Caspases

Caspases are cysteine proteases that execute the apoptotic message. Lieberthal et al. (88) examined the role of ROS in apoptosis induced by growth factor deprivation in primary cultures of mouse proximal tubular (MPT) cells. Growth factor deprivation results in an increase in the cellular levels of $\cdot O_2^-$ anion while apoptosis of MPT cells induced by growth factor withdrawal is inhibited by a number of antioxidants and scavengers of ROS. Growth factor deprivation also results in activation of caspase activity, which is inhibited by EGF and high-dose insulin as well as by the ROS scavengers and antioxidants that inhibit apoptosis. The cell-permeant caspase inhibitor, z-Val-Ala-Asp-CH2F (zVAD-fmk), prevents the increase in caspase activity and markedly inhibits apoptosis induced by growth factor deprivation. However, zVAD-fmk had no effect on the increased levels of $\cdot O_2^-$ associated with growth factor deprivation. Thus ROS play an important role in mediating apoptosis associated with growth factor deprivation.

ROS can induce proliferation and apoptosis, depending on concentrations. H_2O_2 has been shown to induce either apoptosis or features of senescence in different cultured cell lines (89). ROS appear to act upstream of caspases in the apoptotic pathway. It is hypothesized that oxidant stress represents a signaling mechanism for the default pathway of apoptosis (88). Li et al. (90) showed that coincident with EC detachment, there is a dramatic rise in the intracellular level of ROS. They demonstrated that the activities of both caspases and of the JNK are modulated by the rise in intracellular ROS levels. These results suggest that oxidants serve as signaling molecules and regulators of apoptose. Caspases are sensitive to redox changes in the cell. Specifically, in ECs, processing and activity of the downstream caspase-3 in response to cell detachment or TNF stimulation are regulated by ROS (18).

2.3 NO and ROS

NO has been known for many years to serve as a signaling molecule by activating guanylate cyclase, which increases the intracellular concentration of cyclic guanosine monophosphate, which in turn activates protein kinase G. Acting by this pathway, NO induces relaxation of vascular smooth muscle and inhibits platelet activation and aggregation (91). The nitric oxide-cGMP signal transduction pathway plays an important role in the regulation of vascular tone and resistance in hypertension.

More recently, a diminished bioavailability of NO has been identified as a mechanism responsible for endothelial dysfunction in hypertensive patients (92). Of particular importance to the subject of hypertension and vascular biology is the interaction between $\cdot O_2^-$ and NO. They undergo an extremely rapid, diffusion-limited radical/radical reaction, which occurs at a rate of 6.7×10^9 per mol/L per second. This rate is far faster than the reaction between $\cdot O_2^-$ and the SODs, $\cdot O_2^-$ and non-enzyme antioxidants, such as Vitamin C (Vit C) and Vitamin E (Vit E). A major product of this reaction is the peroxynitrite anion ($OONO^-$), which is a strong oxidant and involved in numerous pathophysiological processes. In the normal vessel, the balance between NO and $\cdot O_2^-$ favors the net production of NO and permits a state of basal vasodilatation and maintenance of normal blood pressure (5).

Vaziri et al. (93) hypothesize that oxidative stress may cause hypertension via (among other mechanisms) enhanced oxidation and inactivation of NO. To test this hypothesis, Sprague-Dawley rats were subjected to oxidative stress by GSH depletion by means of the GSH synthase inhibitor buthionine sulfoximine (BSO, in drinking water). The control group was given drug-free drinking water. In parallel experiments, subgroups of animals were provided Vit E-fortified chow and Vit C-supplemented drinking water. The BSO-treated group showed a 3-fold decrease in tissue GSH content, a marked elevation in blood pressure, and a significant reduction in the urinary excretion of the NO metabolite nitrate plus nitrite, which suggests depressed NO availability. These characteristics were

associated with a significant accumulation in various tissues of nitrotyrosine, which is the footprint of NO inactivation by ROS. Administration of Vit E plus Vit C ameliorated hypertension, improved urinary nitrate-plus-nitrite excretion, and mitigated nitrotyrosine accumulation (despite GSH depletion) in the BSO-treated animals but had no effect in the control group. They concluded that GSH depletion resulted in perturbation of the NO system and severe hypertension in normal animals and effects of BSO were mitigated by concomitant antioxidant therapy despite GSH depletion, which supported the notion that oxidative stress was involved in the pathogenesis of hypertension in this model.

Recently clinical studies also have been performed to restore normal endothelial function in patients, using interventions such as L-arginine (the NOS substrate) and Vit C (94,95).

Summarily, ROS are generated and regulated by a variety of cytokines and growth factors that bind different receptors; the production and metabolism of ROS, as intracellular signal molecule, modulate multiple intracellular transduction pathways.

3. Regulation of Gene Expression by ROS

Because hormones and growth factors alter tissue and intracellular levels of ROS and various critical signaling pathways are activated by ROS, specific DNA binding proteins, or transcription factors, will be the target of such a

signaling cascade. Transcription factors are central to gene regulation, as they are the nuclear components that are modulated by upstream signaling events. By virtue of their ability to interact with very specific DNA sequences (which are unique to each transcription factor) in the regulatory regions of genes, transcription factors serve to modulate not only the magnitude of gene expression, but also the specificity of the signal. This specificity is determined in part by the presence or absence of a binding site in the promoter region of the target gene. Many cardiovascular-related genes are redox sensitive. The redox-sensitive modulation of transcription factor activity can occur via (1) direct oxidative modification of the transcription factor itself by intracellular ROS or (2) posttranslational modifications (ie, phosphorylation/dephosphorylation), by the effects of redox-regulated intracellular signaling cascades. Either mechanism can potentially affect various aspects of transcription factor function, such as subcellular localization, DNA binding properties, and inherent transcriptional activity.

ROS regulate several classes of genes, including adhesion molecules and chemotactic factors, antioxidant enzymes, and vasoactive substances. Some of these are clearly an adaptive response, such as the induction of SOD and catalase by H_2O_2 (96). Most redox-sensitive genes have been identified because they are responsive to externally applied oxidant stress; only a few have been demonstrated to be downstream of an endogenous source of ROS, such as the NAD(P)H oxidase. These include TNF-α and lactosylceramide induction of

intercellular adhesion molecule (ICAM)-1 (97) and Ang II, PDGF, and TNF-α stimulation of monocyte chemotactic protein (MCP)-1 (98).

Induction of several genes by cytokines is inhibited by NO donors, including vascular cell adhesion molecule (VCAM)-1, ICAM-1, and monocyte colony-stimulating factor (M-CSF) (99). This is an interesting mechanism of regulation because NO· appears to act in a cGMP-independent manner to inhibit expression at the transcription level. Not only can NO· alter the activity and expression of transcription factors, but it also interacts with $\cdot O_2^-$ to form $ONOO^-$, thus modulating $\cdot O_2^-$-dependent transcription (5).

Regulation of gene expression by oxidant stress occurs at various levels. In some cases, regulation of the gene is redox sensitive owing to the susceptibility of upstream signaling pathways to ROS. For example, induction of Egr-1 by cyclic strain has been shown to depend on redox-sensitive activation of the Ras-Raf-ERK1/2 pathway (100). H_2O_2-induced AP-1 binding in porcine aortic endothelial cells requires activation of Src (65). In other cases, ROS mediate increased turnover, expression, or translocation of specific transcription factors, thus modifying their activity. This mechanism for redox regulation of many transcription factors has been shown to be effective for both the nuclear factor (NF)-κB and AP-1 transcription factors (18) (101). Recent evidence also implicates the potential role of the peroxisome proliferator-activated receptor (PPAR) family of transcriptional activators in oxidative stress (102).

3.1 Redox Regulation of AP-1

AP-1 and NF-κB are the most well-studied transcriptional factors influenced by the cellular redox state. They have been implicated in transcriptional regulation of many genes involved in cellular inflammatory responses, tissue destruction, and growth control. Homodimers and heterodimers of members of the c-jun and c-fos proto-oncogene families constitute the transcription factor AP-1. At least 3 mammalian Jun proteins (c-Jun, Jun B, and Jun D) and 4 Fos family members (c-Fos, Fra-1, Fra-2, and Fos B) have been identified. All of the Jun family proteins can form homo- and heterodimers that are capable of binding to AP-1 DNA binding sites. Fos proteins do not associate with each other but are capable of associating with any member of the Jun family to form stable heterodimers that have higher DNA binding activity than Jun-Jun homodimers (103). Both the Jun : Jun and Fos : Jun forms of AP-1 bind to a specific DNA sequence (thyroid responsive element, TRE) in promoters of many inducible genes. The promoter for the c-jun gene contains a TRE and is primarily activated by AP-1 in a positive autoregulatory fashion. The promoter for c-fos, however, does not contain a TRE and thus is not subject to autoregulation by AP-1.

The activity of AP-1 is controlled by both transcriptional and posttranslational mechanisms in response to a variety of extracellular stimuli, including mitogens, phorbol esters, and differentiation signals. In addition, AP-1 behaves as a redox-sensitive transcription factor in several cell types including

EC and VSMCs and is activated, to different extents, under prooxidant conditions generated by treatment with agents such as O_2^-, H_2O_2, UV light, irradiation, and cytokines. Furthermore, regulation of the vascular inflammatory genes MCP-1 and ICAM-1 by H_2O_2 is mediated by AP-1 binding elements in the promoters of these genes. Little is known about the exact mechanisms underlying ROS-mediated AP-1 activation; however, phospholipase A_2, arachidonic acid, and PKC have been proposed for H_2O_2 induction of c-fos (104) and c-jun (105) expression in VSMCs. In addition, H_2O_2-induced AP-1 activation requires both tyrosine and serine/threonine phosphorylation, and it has been suggested that AP-1 activation under oxidative conditions may, at least in part, be mediated by phosphorylation of Jun proteins (106).

The nuclear redox factor Ref-1 was initially cloned as a molecule that stimulated DNA binding of AP-1 via reduction of the conserved cysteine residues. Initially identified in HeLa nuclear extracts, Ref-1 has subsequently been shown to be ubiquitous and can stimulate DNA binding of other eukaryotic transcription factors in addition to AP-1. Thioredoxin (TRX) is another pleiotropic cellular factor that has thiol-mediated redox activity and functions to facilitate protein-nucleic acid interactions (107). TRX has also been shown to enhance the DNA binding activity of Jun and Fos via direct interaction with Ref-1. Therefore, the involvement of TRX and Ref-1 in redox modulation of AP-1 activity represents an example of a cellular redox cascade modulating transcription factor activity.

3.2 Redox Regulation of NF-κB

NF-κB is an inducible transcription factor complex composed of homodimeric or heterodimeric complexes of the Rel family of transcriptional activators. The predominant form of NF-κB exists as a heterodimer of the p50 and p65 subunits. NF-κB is usually stored in the cytosol in its inactive form bound to the inhibitory unit IκBα, which prevents DNA binding and nuclear uptake of the factor. Degradation of IκBα is critical for NF-κB activation. Extracellular stimuli such as ROS signal the degradation and release of the inhibitory unit IκBα through a complex but rapid cascade of events resulting in a rapid translocation of active NF-κB to the nucleus (figure 1.4). NF-κB-inducing agents will initiate the phosphorylation of IκBα on its N-terminal serine residues (Ser32 and Ser36) (10). This phosphorylation of IκBα induces polyubiquination of IκBα at multiple sites, tagging the subunit for degradation by the 26S proteosome complex. The free NF-κB unit is now able to translocate into the nucleus and bind to consensus DNA binding sites in target genes (10).

The NF-κB transcription factor family controls the expression of many of genes involved in inflammation and proliferation. Unlike many other systems, NF-κB is already present in the cytosol; thus, no new protein synthesis is required for its activation. This unique activation system allows NF-κB to regulate immune and inflammatory processes in a rapid and very efficient manner.

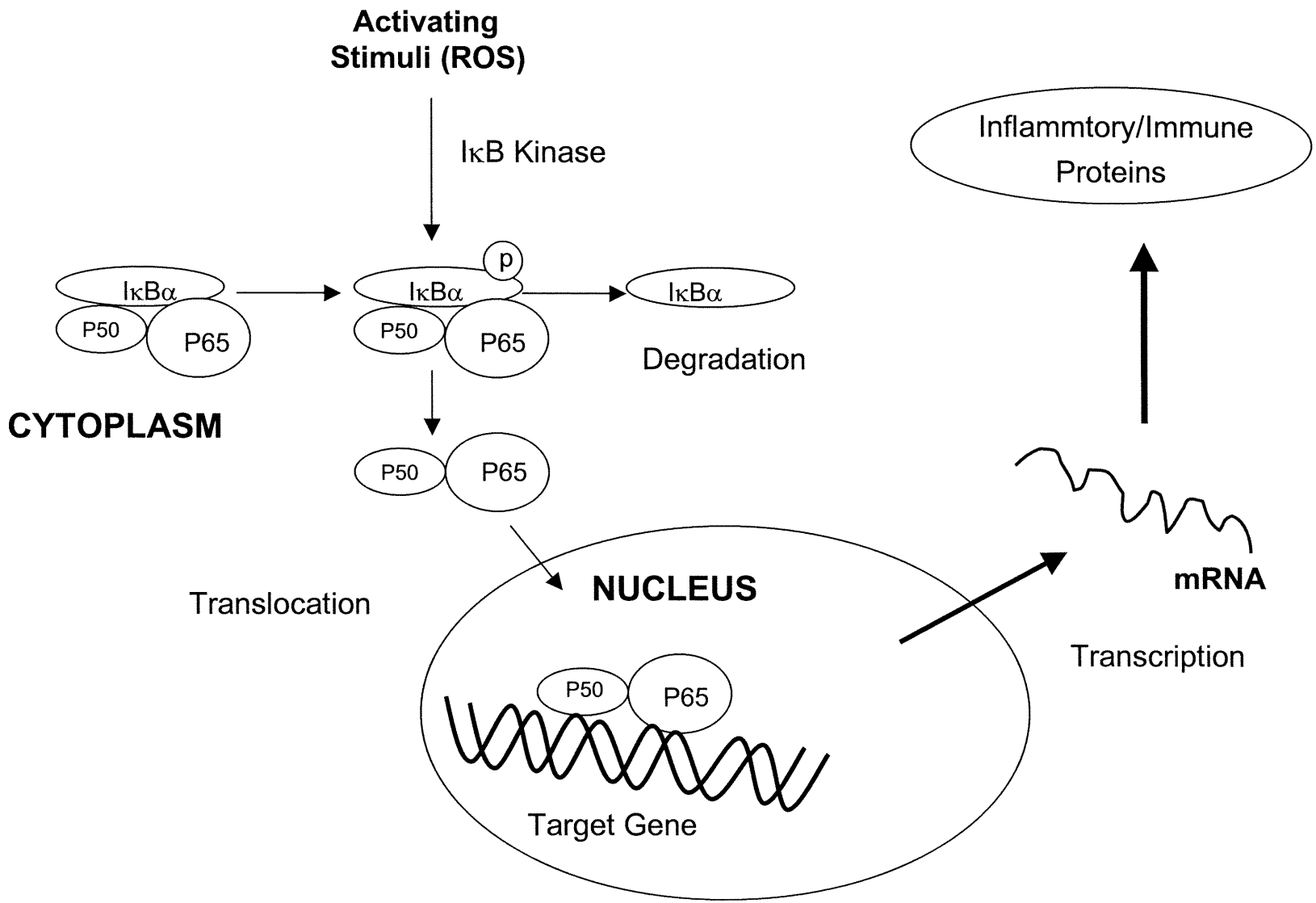

Figure 1.4: Schematic illustration of ROS-mediated NF-κB activation (47).

NF-κB was the first eukaryotic transcription factor shown to respond directly to oxidative stress. A common step in all of the activation mechanisms that lead to IκBα degradation and NF-κB nuclear translocation have been suggested to involve ROS (108~110). Many different agents can activate NF-κB, including phorbol esters, inflammatory cytokines, UV light, rays, viral and bacterial proteins, and lipopolysaccharide (10). All of these agents produce oxidative stress. Thus, despite the diverse stimuli, ROS appear to serve as the common intracellular agents involved in the activation of NF-κB (110). Antioxidants have also been known to have the ability to inhibit NF-κB activation, both in vitro and in vivo. NF-κB activation can be inhibited by addition of various antioxidants including Vit C (111), Vit E derivatives (112), and NAC (69).

Many reports have demonstrated inhibition of nuclear translocation by antioxidants, although the extent of this block appears to vary with the cell type and the nature of the signal. In some cells lines, H_2O_2 and peroxide-containing molecules result in a rapid activation of NF-κB. However, incubation with O_2^{-}, •OH, or NO-generating compounds fail to cause activation, suggesting that NF-κB activation is selectively mediated by peroxides. Conclusive support for a role of H_2O_2 in NF-κB activation came from studies in a catalase-overexpressing cell line that exhibited suppressed activation of NF-κB in response to TNF-α (113). Addition of a catalase inhibitor restored the NF-κB response. Also, overexpression of cytosolic SOD, which causes cytosolic H_2O_2 accumulation,

potentiated the NF-κB response. Together, these suggest that NF-κB activation is at least facilitated by some oxidative reactions.

The target molecules that are subject to redox regulation during NF-κB activation remain unknown. It is unlikely that the NF-κB subunits themselves are directly activated by oxidation, because only the reduced form of NF-κB binds to DNA in vitro (114) and attempts to activate isolated NF-κB by oxidation in vitro were unsuccessful (108). Direct oxidative inactivation of IκBα is also not likely to be involved in the redox regulation of NF-κB, given that treatment of isolated NF-κB/IκBα complexes with H_2O_2 in vitro failed to dissociate IκBα or lead to NF-κB DNA binding (108, 115). Most evidence suggests that oxidative stress induces, and antioxidants prevent, the cytoplasmic-nuclear translocation of NF-κB. Therefore, the most likely scenario is that the signaling cascade leading to the phosphorylation and subsequent degradation of IκBα is regulated by redox processes. Indeed, it has recently been demonstrated that antioxidants inhibit IκBα kinase activity and prevent the phosphorylation and subsequent degradation of IκBα (116, 117).

Activation of NF-κB by ROS may be important in hypertension. In SMCs, constitutive activation of NF-κB has been reported to be essential for proliferation. In addition, Ang II-induced effects on SMCs may also be mediated via NF-κB. In ECs, NF-κB is a prime target for ROS, and its activation has been linked to EC dysfunction and survival (18). Identifying the exact role of redox-

sensitive NF-κB may lead to a better understanding of pathogenic processes underlying hypertension.

3.3 Peroxisome Proliferator-Activated Receptors (PPAR)

PPARs are composed of members of the nuclear hormone receptor superfamily of transcription factors, a large and diverse group of proteins that mediate ligand-dependent transcriptional activation and repression. Peroxisome proliferators are a structurally diverse group of non-genotoxic chemicals that induce pleiotropic responses (98). Evidence derived from mice with PPAR-α gene disruption indicates that of the three PPAR isoforms (α, β/δ and γ), the isoform PPAR-α is essential for the pleiotropic responses induced by peroxisome proliferators. Peroxisome proliferator-induced activation of PPAR-α leads to transcriptional activation of genes encoding for the classical peroxisomal β-oxidation system and cytochrome P450 CYP 4A1 and CYP4A3, among others.

Poynter and Daynes (83) demonstrated that activation of PPAR-α in aged mice restored the cellular redox balance to that of young animals. This was evidenced by a lowering of tissue lipid peroxidation, elimination of constitutively active NF-κB, and a loss in spontaneous inflammatory cytokine production after administration of PPAR-α activators. These effects were not observed in animals bearing a null mutation in PPAR-α. Also, administration of the antioxidant, Vit E,

to aged mice (that contain reduced levels of PPAR-α mRNA) resulted in an elevated expression of PPAR-α to levels seen in younger mice. This observation suggests that balancing the cellular redox state may provide a level of transcriptional regulation for PPAR-α.

PPAR expression and functional activity have recently been observed in human vascular cell types such as EC (64) and SMC (118). Ricote et al. have reported that PPAR-γ is markedly upregulated in activated macrophages and inhibits the expression of the inducible NO synthase, gelatinase B and scavenger receptor A genes in response to 15d-PGJ2 and synthetic PPAR-γ ligands. H_2O_2 potently down-regulated PPAR-γ mRNA expression in rat dipocytes. TNF-α, which is considered to augment oxidative stress, also suppressed PPAR-γ expression. Thiazolidinediones, a new class of antidiabetic agents, dose-dependently recovered TNF-α-induced down-regulation of PPAR-γ mRNA expression (76). PPARs are key players in lipid and glucose metabolism and are implicated in metabolic disorders predisposing to atherosclerosis, such as dyslipidemia and diabetes. Recent reports suggest that PPARs may play a role in inflammatory processes involved in the pathogenesis of atherosclerosis and restenosis by their ability to modulate monocytic gene expression (119). These studies suggest that PPARs may be viewed as redox-sensitive transcription factors in the vasculature by their ability to be selectively activated by oxidatively modified fatty acids.

ROS have different effects on VSMC and EC growth, death and survival through activating various intracellular signaling pathways the targets of which are transcription factors. Transcription factors are central to gene regulation in pathophysiologic processes of many vascular diseases including hypertension.

4. ROS and Hypertension

Under physiological conditions, ROS produced in the course of normal metabolism are fully inactivated by intracellular and extracellular antioxidant defense systems. However, in certain pathological conditions, increased generation of ROS and/or depletion of antioxidant capacity leads to enhanced generation of ROS and consequent oxidative stress. By promoting lipid peroxidation, DNA damage, and protein modification, oxidative stress can cause cellular injury and tissue damage. These processes have been implicated in the pathogenesis of various pathological conditions including ischemia, inflammation, aging, degenerative diseases, as well as cardiovascular diseases such as hypertension (120).

4.1 ROS and Vascular Remodeling

Hypertension is associated with vascular structural changes, characterized by a reduced lumen diameter and an increase in the ratio of wall thickness (or media thickness) to lumen diameter (121). At the cellular level, these structural changes may be associated with VSMC hyperplasia, hypertrophy and/or apoptosis, all of which can be influenced by ROS. Some

studies have suggested that there is no net growth in vascular remodeling, whereas others indicate that there is an imbalance between VSMC growth and apoptosis (121). The net balance between proliferation and apoptosis determines the extent of SMC growth and remodeling. Normal arteries are characterized by a low turnover of EC and SMC. Different mechanisms protect the EC and SMC against apoptosis in the normal artery. In pathological conditions, such as in hypertension, SMC replication is increased but this may not be counterbalanced by increased apoptosis, resulting in thickening of the media (20).

SMCs respond to growth factor stimulation with intracellular production of ROS. This is particularly evident for Ang II, which stimulates $\cdot O_2^-$ formation by increasing the activity of NAD(P)H oxidase in cultured rat and human VSMCs, and in intact aortas of rats made hypertensive by Ang II infusion (50, 122). Ang II leads to the hypertrophic response in SMCs via the production of both $\cdot O_2^-$ and H_2O_2 and activation of p38 MAPK (3,66,123). ROS play a pivotal role in intracellular signal transduction. These signaling events mediate redox-sensitive growth in VSMCs and are particularly important in Ang II-stimulated proliferation and hypertrophy, which could contribute to vascular wall thickening and remodeling in hypertension (3). Structural changes of large and small arteries in hypertension contribute to elevation of blood pressure, and may participate in the complications of hypertension.

Media thickening itself also affects vessel redox state. Vascular wall thickening increases the distance required for diffusion of oxygen from the

lumen. A reduced pO_2, in turn, results in incomplete oxidation and increased concentrations of free radicals and abnormalities of the oxidant state. This $\cdot O_2^-$ formation further contributes to vascular smooth muscle cell growth, endothelial dysfunction and vascular damage in hypertension. Accordingly ROS influence multiple cellular processes underlying vascular structural changes associated with hypertension.

4.2 ROS and Endothelial Dysfunction

In addition to influencing morphological features of the arterial wall, ROS affect functional contractile and dilatory responses. Endothelial dysfuction was shown to be an important contributor to the responses. Increased $\cdot O_2^-$ in hypertension impairs endothelium-dependent vascular relaxation and increases vascular contractile reactivity. These effects may be mediated directly by increasing cytosolic Ca^{2+} concentration or indirectly by reducing concentrations of the vasodilator NO, because vascular endothelial cells are known to generate NO that mediates vasodilation. In the rat renal hypertension model, the elevation of $\cdot O_2^-$ was associated with a depression of the ACE-induced release of NO from aortic ring (124). Oxygen radicals also induce endothelial permeability, with extravasation of plasma proteins and other macromolecules, and recruitment of inflammatory proteins and cells, which could further impair endothelial function and aggravate vascular damage. Peripheral polymorphonuclear leukocytes, which generate $\cdot O_2^-$, participate in oxidative stress and inflammation in patients

with hypertension (44,136). The co-existence of an inflammatory reaction with oxidative stress probably contributes to further endothelial dysfunction. The antioxidant Vit C recovered endothelial function by restoring the NO -mediated vasodilation of the endothelium in hypertensive patients (125).

5. ROS, Antioxidants and Cardiovascular Diseases

Various studies reported that antioxidants decrease vascular $\cdot O_2^-$ generation and endothelial dysfunction, and reduce blood pressure in experimental and human hypertension. The prospect that vascular injury can be avoided or minimized by reducing oxidative stress through increased intake of antioxidants is appealing. A few studies reported that antioxidant vitamins, SOD mimetics and liposome-entrapped SOD normalize endothelial dysfunction and improve vascular remodeling in experimental hypertension (44,46). Other studies reported that antioxidants reduce blood pressure and improve antioxidant status in patients with essential hypertension suggesting that oxidative stress plays a pathophysiological role, at least in part, in human hypertension (126,127). The studies have focused mainly on the two vitamins with antioxidant properties, ascorbic acid (Vit C) and α–tocopherol (Vit E).

Numerous recent studies have reported beneficial effects of Vit C, administered either orally or by intra-arterial infusion, on vasodilation in various patient groups. In patients with hypertension (128,129), Vit C treatment

significantly improved vasodilation, which was comparable to that in healthy control subjects. Heitzer et al. (130) and Motoyama et al. (131) observed marked improvement in endothelial function in smokers given Vit C infusions. In addition, patients with non insulin-dependent and insulin-dependent diabetes mellitus demonstrated increased blood flow after Vit C infusion (132,133). Finally, healthy individuals given an oral dose of 1000 mg of Vit C in combination with 800 IU of Vit E exhibited normal vasodilation several hours after a single high fat meal, whereas control subjects not given the antioxidant combination showed impaired vasoreactivity (134).

Ascorbic acid is particularly important in inhibiting lipid peroxidation induced by oxidative stress (17). Under oxidizing conditions, ascorbic acid is the only antioxidant capable of completely preventing detectable lipid peroxidation in plasma; once ascorbic acid has been depleted, detectable amounts of various classes of lipid hydroperoxides are formed, despite the presence of other plasma antioxidants, including α-tocopherol and ß-carotene (17). Martin et al. reported that loading human aortic endothelial cells with ascorbic acid reduces production of ROS and inhibits cell-mediated oxidative modification of low-density lipoprotein (LDL) (135).

Although Vit E has been known as an essential nutrient for reproduction since 1922, the mechanisms of its physiological functions are far from understood. Vit E is the term for a group of tocopherols and tocotrienols, of which α-tocopherol has the highest biological activity. Due to the potent

antioxidant properties of tocopherols, the impact of α-tocopherol in the prevention of chronic diseases believed to be associated with oxidative stress has often been studied, and some benefic effects have been demonstrated.

Animal studies have provided consistent evidence for a beneficial effect of α-tocopherol on vasodilation, as well as insights into underlying mechanisms. Supplementation of cholesterol-fed rabbits with α-tocopherol increased both the resistance of LDL to oxidation and agonist-induced relaxation of thoracic aorta (136). These results suggest that α-tocopherol acts by increasing vascular antioxidant status.

Cell culture studies have demonstrated that pretreatment of endothelial cells with α-tocopherol inhibits cytokine or oxidized LDL–induced expression of ICAM-1, VCAM-1, or E-selectin and decreases adhesion of monocytes to these cells. Interestingly, Cominacini et al. found that both LDL-associated and cellular α-tocopherol are able to inhibit upregulation of ICAM-1 and VCAM-1 by endothelial cells exposed to oxidized LDL. α-Tocopherol also decreased stimulus-induced expression of ß1 and ß2 integrins on leukocytes and adhesion of these cells to cultured EC (137,138). Ex vivo studies in humans have shown an inverse correlation between serum α-tocopherol levels and ß1 integrin expression on monocytes, as well as decreased ex vivo monocyte-EC adhesion (139) after supplementation with α-tocopherol. α-Tocopherol could inhibit the expression of adhesion molecules and subsequent cell-cell interactions either by

directly scavenging ROS or by inhibiting PKC activation and associated ROS production (140).

Two recent studies reported that enrichment of monocytes with α-tocopherol caused decreased production of $\cdot O_2^-$ (138), and peripheral monocytes isolated from human subjects supplemented with α-tocopherol exhibited decreased $\cdot O_2^-$ production and IL-1ß release (139).

6. AIM

The overall aim of the present study was to investigate whether redox-sensitive processed contribute to vascular changes in hypertension. Studies were performed firstly at the cellular level to investigate mechanisms whereby ROS are generated in VSMCs, and secondly in whole animals, to assess whether ROS play a role in vascular changes in hypertensive rats.

1. Cellular Studies (Project 1)

The aims of these studies were to determine whether human VSMCs possess a functionally active NAD(P)H oxidase system and to determine the role of Ang II in processes involved in cellular generation of ROS.

2. Whole Animal Studies (Project 2)

The aims at these studies were to determine whether ROS play a role in endothelial dysfunction and vascular structural and functional changes in stroke prone spontaneously hypertensive rats (SHR-SP). We assessed the vascular redox state through production of $\cdot O_2^-$ changes in activation of NADPH oxidase, SOD and total plasma antioxidants concentrations in SHR-SP. Furthermore, we tested the possibility that antioxidants ameliorate vascular changes associated with hypertension.

II. MATERIALS AND METHODS

2.1 Materials

Du®-64 Spectrophotometer, Beckman company. AutoLumat LB953, EG &G Berthold company. Micro-capillary centrifuge, International IEC. TL-100 Ultracentrifuge, Beckman. Electrophoresis instrument, Biorad Lab (Hercules, CA). NADPH, SOD Assay Kit and Total Plasma Antioxidants Assay Kit were bought from Calbiochem® Inc (San Diaego, CA). All other chemicals were from Sigma Chemical, Fischer Scientific, and BDH Inc.

2.2 Project I. Mechanisms regulating generation of ROS in VSMCs

2.2.1 Human Cell culture

The study was approved by the Ethics Committee of the Clinical Research Institute of Montreal (IRCM). Written informed consent to participate in the study was obtained from each subject. Healthy volunteers (30-65 years) (n=6, 4 males) were recruited at the IRCM. These subjects were part of the control group in clinical studies performed by DR. EL Schiffrin. Gluteal biopsies of subcutaneous tissue of 1.0 x 0.5 x 0.5 cm^3 were obtained under local anaesthetic. Arteries were micro-dissected immediately after the biopsy was performed. Arteries with diameters less than 300 μm (corresponding to resistance arteries) were used for cell culture.

Arteries were placed in Ham's F-12 culture medium containing 1% gentamicin, 0.2% collagenase (type 1), 0.012% elastase, 0.036% soybean trypsin inhibitor, and 0.2% BSA and vortexed in an incubator for 1 hour at 37°C. The digested tissue was further dissociated by repeated aspiration through a syringe (needle gauge, 25). The cell suspension was centrifuged (200g, 5 minutes) and the cell pellet resuspended in Ham's F-12 culture medium containing 10% heat-inactivated fetal calf serum (FCS). Cells were seeded onto round glass coverslips (25 mm in diameter) that had been coated with Matrigel basement membrane matrix (Becton Dickinson Labware, CA), which is a cell culture preparation optimized for contractile phenotypic states. Matrigel was diluted 1:3 and prepared according to the manufacturer's instructions. For the first 48 hours, cells were incubated in Ham's F-12 culture medium (141). Purity of cell culture was confirmed immunocytochemically with monoclonal anti-α-smooth muscle actin. Low passaged cells (passages 2-6) were studied.

2.2.2 Rat Cell Culture

VSMCs derived from mesenteric arteries of 16-week-old WKY were studied. Mesenteric arteries were cleaned, smooth muscle cells were dissociated by digestion, the tissue was filtered, and the cell suspension was centrifuged and resuspended in DMEM that contained 10% FCS, 0.029% L-glutamine, 0.01% penicillin, and 0.01% streptomycin (142). VSMCs were grown on round glass cover slips and maintained at 37°C in a humidified incubator.

Primary and first-passage cells were studied at confluence. Cells were rendered quiescent by serum deprivation for 30 hours before experimentation.

2.2.3 Cell fractionation

Since p22phox and gp9lphox are membrane-associated subunits, whereas the other subunits are located in the cytoplasm, the membrane and cytosolic fractions were prepared by differential centrifugation. Control VSMCs or cells exposed to Ang II for the indicated times were washed in ice-cold phosphate-buffered saline (PBS), scraped and transferred to Eppendorf tubes, and then centrifuged at 750 g at 4°C for 5 min. The supernatant was discarded and the pellet resuspended in 500 μl lysis buffer containing protease inhibitors (sodium pyrophosphate 50 mmol/L, NaF 50 mmol/L, NaCl 50 mmol/L, EDTA 5 mmol/L, EGTA 5 mmol/L, Na_3VO_4 2 mmol/L, HEPES 10 mmol/L, and phenylmethylsulfonyl fluoride 50 mmol/L, and 0.1% Triton X-100 (pH 7.4)), and sonicated for 5 seconds. Cell homogenates were centrifuged at 50,000 rpm for 20 min at 4°C. The supernatant (cytosolic fraction) was removed and the pellet, containing the plasma membrane, was resuspended in lysis buffer. Protein content was measured in aliquots of cytoplasmic and membrane fractions using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA) and then prepared for Western blot analysis or NADPH oxidase assay.

2.2.4 Immunoblotting

Membrane preparations were used to detect gp9lphox and p22phox, whereas cytoplasmic fractions were used to detect p40phox, p47phox and p67phox. Equal amounts of proteins (15 μg) were loaded on a SDS-polyacrylamide gel (8% for gp9lphox, 10% for p40phox, p47phox and p67phox, and 12% for p22phox) and transferred to polyvinylidene difluoride membrane (Boehinger Mannheim, Quebec, Canada) for 1 hour at 100 V. Membranes were blocked with blocking buffer containing Tris-buffered saline (Tris 20 mmol/L, NaCl 140 mmol/L, pH 7.6) and 0.1% Tween-20 with 5% wt/vol non fat dry milk and incubated for 24 hours at 4°C. Membranes were incubated with mouse monoclonal antibodies diluted 1:500 or rabbit polyclonal antibodies diluted 1:1000 for 1 hour. They were then washed, incubated with a goat anti-mouse or anti-rabbit HRP conjugated antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:7000 for 1 hour and washed extensively. Protein blots were developed by ECL reagent (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, England) and then immediately exposed to film. The film was scanned by Arcus 1200 scanner (AGFA, Belgium), and the images were saved on computer. Band intensity was measured by computer analysis, using the Image Quant program (Amersham Biosciences, Piscataway, NJ). Previously characterized monoclonal and polyclonal antibodies specifically recognizing p22phox (clone 44.1 and R3179, respectively), gp9l phox (clone 54.1 and R2085, respectively), p40phox (clone 1.9), p47phox (clone 43.27 and R360, respectively), p67phox (clone 81.1

and R1497, respectively). NADPH oxidase antibodies and protein positive controls were kindly provided by M.T. Quinn (Montana State University, Montana).

2.2.5 Determination of $\cdot O_2^-$ yield from xanthine/xanthine oxidase reactions

$\cdot O_2^-$ production in nonphagocytic cells is often measured using lucigenin, an acridylium dinitrite compound that emits light on reduction and interaction with $\cdot O_2^-$. To detect $\cdot O_2^-$, lucigenin must first be reduced by one electron to produce the lucigenin cation radical. The biological system that

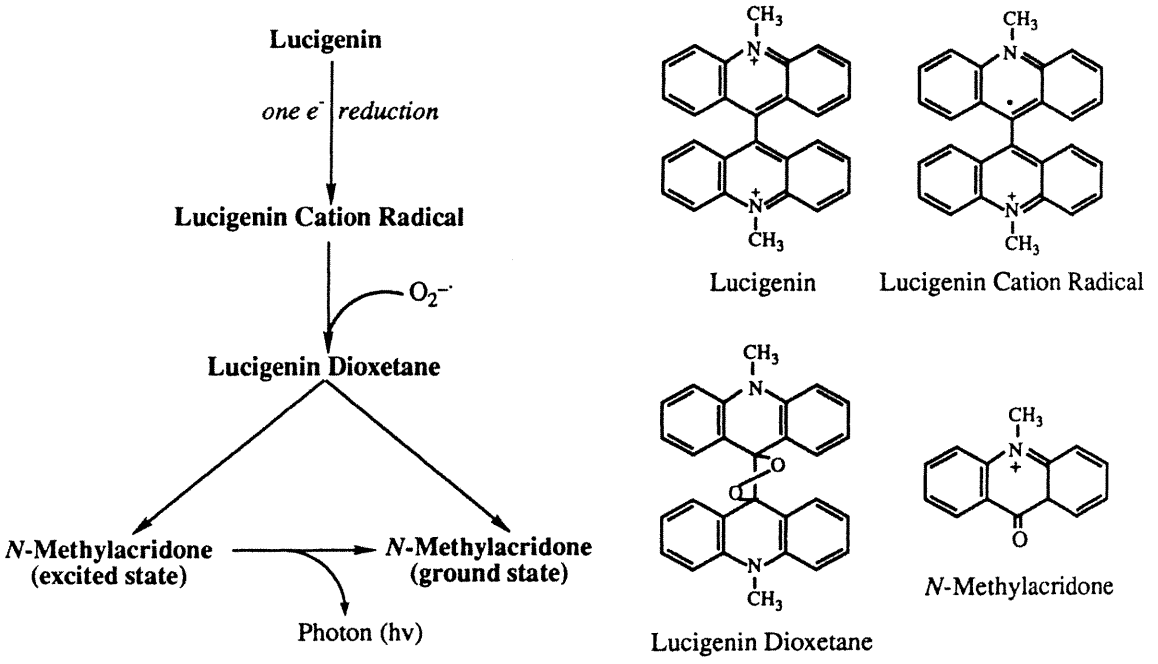

Figure 2.1: Schematic illustration of the reaction pathway leading to lucigenin-derived chemiluminescence (LDCL).

reduces lucigenin may also be the same one that produces the $\cdot O_2^-$. The lucigenin cation radical then reacts with the biologically derived $\cdot O_2^-$ to yield an unstable dioxetane intermediate. The lucigenin dioxetane decomposes to produce two molecules of N-methylacridone, one of which is in an electronically excited state, which upon relaxation to the ground state emits a photon. Through sensitive measurement of the photon emission, the biological production of $\cdot O_2^-$ can be monitored (74) (figure 2.1).

The reduction of oxygen by xanthine oxidase occurs via both univalent and divalent pathways. To determine the degree of univalent reduction of oxygen by xanthine oxidase in our experimental conditions, we examined the reduction of ferricytochrome *c* spectrophotometrically and determined the amount of $\cdot O_2^-$ produced from of xanthine. The absorbance of the rate of reduction of ferricytochrome *c* in response to different xanthine concentrations (10^{-9} ~ 5×10^{-6} mol/L) and the counts of lucigenin-mediated chemiluminescence in response to respectively different xanthine concentrations were obtained. To measure ferricytochrome *c* reduction, 10μl xanthine oxidase was added to the solution containing different xanthine concentrations and by gently mixed by inversion. The augmentation in absorbance of the solution at 550 nm for 3 min was read (read the absorbance at 10 second intervals). The rate of $\cdot O_2^-$ production was then calculated on the basis of the molar extinction coefficient of reduced ferricytochrome *c* and and the portion that is inhibited by SOD (7U/μl), according

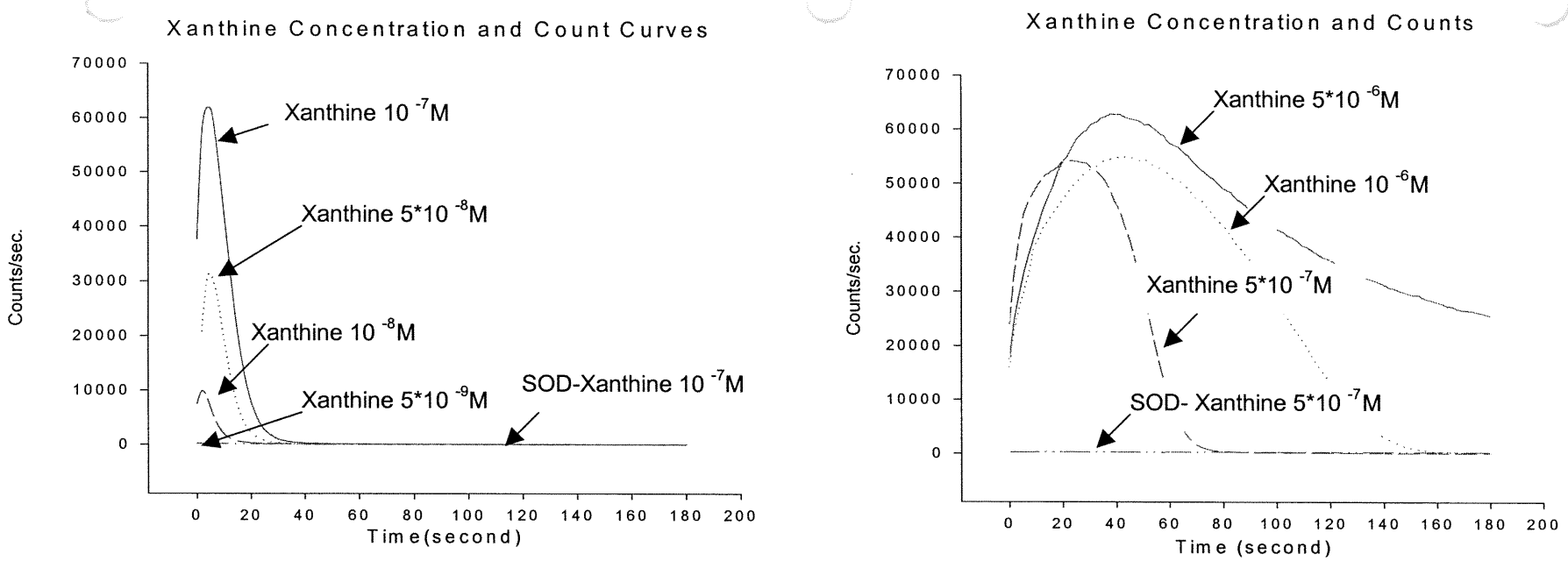

Figure 2.2: Line graphs demonstrate the chemiluminescence as counts/sec in response to difference xanthine concentration.

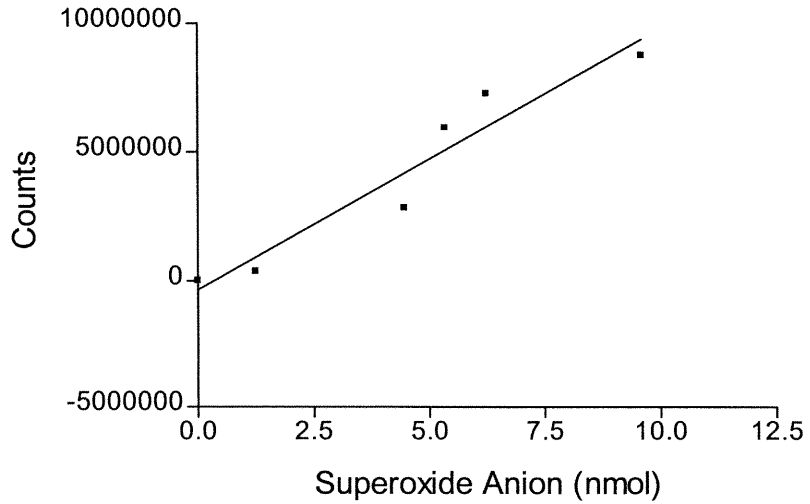

Figure 2.3: Line graph demonstrates the correlations between $\cdot O_2^-$ release by xanthine/xanthine oxidase and lucigenin chemiluminescence was $r = 0.95$.

to the formula: OD_{550nm} without SOD for 3 min - OD_{550nm} with SOD for 3min. For the lucigenin assay xanthine oxidase 10μl was added to the solutions containing different xanthine concentrations in the presence of 5 μmol/L lucigenin. Lucigenin chemiluminescence was then recorded every 1.8 seconds for 3 minutes to obtain 100 points data (AutoLumat LB953, EG&G Berthold, Munich, Germany)(figure 2.2). Chemiluminescence was recorded as counts/sec. A buffer blank containing xanthine, SOD (7U/μl) and lucigenin (without xanthine oxidase) was subtracted before transformation of the data. Using the same xanthine and xanthine oxidase concentration during 3 min, the counts of lucigenin-mediated chemiluminescence in response to different xanthine concentrations were converted to the absorbance of the rate of reduction of ferricytochrome C in response to the respective xanthine concentration. All units of luminescence without SOD (3 min) – all units luminescence with SOD (3 min) = $1000/\epsilon/2.5 \times$ (OD_{550nm} without adding SOD (3 min) - OD_{550nm} with SOD (3min)), where ϵ = 21.6 $mmol/L^{-1}cm^{-1}$ (143). The correlation between $\cdot O_2^-$ release by xanthine/xanthine oxidase and lucigenin chemiluminescence was r = 0.95 (figure 2.3).

2.2.6 Cell NADPH oxidase assay

NADPH oxidase activity was measured by a luminescence assay in a 50-mmol/L phosphrate buffer, pH 7.0, containing 1mmol/L EGTA, 150mmol/L sucrose, 5μmol/L lucigenin was used as the electron acceptor, and 100 μmol/L NADPH as the substrate. This concentration fell within the linear range of the

assay (1μmol/L to 10 mmol/L for NADPH) (155). The counts of test tube containing lucigenin and NADPH were recorded as blank. The reaction was started by the addition of 100 μl of homogenate or cell fraction. Luminescence was recorded every 1.8 second for 3 minutes in a luminometer. A buffer blank was subtracted from each reading before transformation of the data. The amount of $\cdot O_2^-$ produced at each sample was calculated by comparison with a standard curve generated using xanthine/xanthine oxidase. The amount of $\cdot O_2^-$ produced expresses the NADPH oxidase activity. Chemiluminescence was recorded as counts/sec. The protein content was measured in aliquots of cytoplasmic and membrane fractions using the Bio-Rad Protein Assay reagent. The activity of NADPH oxidase was expressed as nmol $\bullet O_2^-$/min/mg protein.

2.3 Project II. Role of ROS in the development of hypertension in SHR-SP

2.3.1 Animal experiments

The study was conducted according to recommendations from the Animal Care Committee of the Clinical Research Institute of Montreal (IRCM) and the Canadian Council of Animal Care. Male SHRSP were studied. Rats were bred at the IRCM, with the original breeding pair obtained from the National Institutes of Health (Bethesda, Maryland). They were housed at 22°C and 60% humidity under a 12-hour light/dark cycle and were maintained on standard chow. At 16 weeks of age, rats were divided into four groups: control group (n=6), Vit C-

treated group (n=7) (ascorbic acid 1000 IU/day), Vit E-treated group (n=8) (α-tocopherol 1000 IU/day) and tempol-treated group (n=6)(tempol 10^{-3} mol/L/day). Ascorbic acid and tempol were added to drinking water. α-Tocopherol was mixed in sesame oil and added to the chow. All rats were placed on a high salt diet by adding 4% NaCl to the food to accelerate the progression and severity of hypertension. Rats were studied for 6 weeks. Systolic blood pressure (SBP) was measured weekly by the tail-cuff method and recorded on a model 7 polygraph fitted with a 7-P8 preamplifier and PCPB photoelectric pulse sensor (Grass Instruments Co, Cambridge, MA). The average of 3 pressure readings was obtained. Rats were killed by decapitation at 22 weeks of age.

2.3.2 Study of small arteries

Superior mesenteric arteries were taken from the part of the mesenteric vascular bed that feeds the jejunum 8 to 10 cm distal to the pylorus and placed in cold physiological salt solution (PSS) of the following composition: NaCl 120 mmol/L $NaHCO_3$ 25 mmol/L, KCl 4.7 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4$ 1.2 mmol/L, $CaCl_2$ 2.5 mmol/L, ethylenediaminetetraacetic acid (EDTA) 0.026 mmol/L, and glucose 5.5 mmol/L. A third-order branch of the mesenteric arterial tree (approximately 2 mm in length) was carefully dissected 1 mm from the intestine and cleaned of all adherent connective tissue under a dissecting microscope. The arterial segments were mounted in a pressure myograph chamber and slipped onto two glass microcannulae. The axial length of the

arterial segment was adjusted by carefully positioning the cannula until vascular walls were parallel without any stretch. Intraluminal pressure was set to 45 mm Hg with a servocontrolled pump. Vessels were then equilibrated for 1 hour with PSS which was bubbled with 95% air (21% O_2) and 5% CO_2 to give a pH of 7.4-7.45 and incubated at 37°C.

Endothelium-dependent and -independent relaxation were assessed by measuring the dilatory response of small arteries pre-contracted with norepinephrine ($5x10^{-5}$ mol/L) to cumulative doses of acetylcholine (Ach) (10^{-7} to 10^{-5} mol/L) and sodium nitroprusside (10^{-7} to 10^{-4} mol/L). Thereafter, mesenteric arteries were deactivated with Ca^{2+}-free PSS containing 10^{-3}mol/L EGTA for 30 minutes to eliminate tone. Lumen and media dimensions were measured with the intraluminal pressure maintained at 45 mmHg. Cross-Sectional Area was calculated as $(\pi/4) \cdot (D_e^2-D_i^2)$, where D_e and D_i were external and lumen diameters, respectively.

2.3.3 Detection of vascular $\bullet O_2^-$ by lucigenin chemiluminescence

The method for measuring lucigenin chemiluminescence was based on that described by Harrison's group (48). The descending thoracic aorta was cleaned of adherent adipose tissue and 5 mm long rings were cut and equilibrated in PSS at 37°C for 30 min. The solution contained rings were bubbled with 95%-air 5%-CO_2 to maintain pH 7.4. Aortic superoxide anion concentration was measured by luminometry (AutoLumat) with lucigenin (5 μmol/L). After equilibrating, rings were gently transferred to test tubes containing

warmed HEPES buffer (NaCl 119 mmol/L, HEPES 20 mmol/L, KCl 4.6 mmol/L, $MgSO_4$ 1.0 mmol/L, Na_2HPO_4 0.15 mmol/L, KH_2PO_4 0.4 mmol/L, $NaHCO_3$ 5 mmol/L, $CaCl_2$ 1.2 mmol/L, glucose 5.5 mmol/L), pH 7.4. The rings were placed in warm 37°C HEPES buffer containing lucigenin (5 μmol/L). Lucigenin chemiluminescence was then recorded (AutoLumat LB953, EG&G Berthold, Munich, Germany). Chemiluminescence was recorded as counts/sec. A buffer blank containing lucigenin was subtracted from reading before transformation of the data. The amount of superoxide produced at each sample was calculated by comparison with a standard curve generated using xanthine/xanthine oxidase as described above. The aorta was dried at 50°C, for 24 hours in oven. Tissue $\cdot O_2^-$ formation was expressed as nmol/min/g dry tissue weight.

2.3.4 Assay of Aortic Superoxide dismutase (SOD) activity

Aortic SOD activity was assayed by Superoxide Dismutase (SOD) Assay Kit by spectrophotometry. It utilizes 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo© fluorene. This reagent undergoes alkaline autoxidation, which is accelerated by superoxide dismutase. Autoxidation of the reagent yields a chromophore which absorbs maximally at 525 nm. The kinetic measurement of the 525 nm absorbance change is recorded after the addition of the reagent. The SOD activity is determined from the ratio of the autoxidation rates measured in the presence (Vs) and in the absence (Vc) of SOD. This method also utilizes 1,4,6-trimethyl-2-vinylpyridinium to eliminates major interferences normally

caused by mercaptans (RSH), which traps mercaptans by the means of a rapid alkylation reaction.

Rat aorta was washed with 0.9% NaCl containing 0.016% to remove red blood cells, immediately placed in liquid N_2 before extraction, then kept in -80°C. The frozen aorta was homogenized in 10% NaCl and centrifuged at 3000 *g* for 10 min. The supernatant (250 μl) was aspirated into a glass tube and extracted by the extraction solution (absolute ethanol/chloroform 62.5/37.5 (V/V)). It was then vortexed and centrifuged at 3000 *g* for 5 min. The upper aqueous layer undergone alkaline autoxidation, which was accelerated by SOD. Autoxidation of the reagent yielded a chromophore, which absorbed maximally at 525 nm. The assay was performed according to manufacturer's instructions. Activity of superoxide dismutase was expressed as U/mg protein. The lower limit of detection (LLD) is 0.1 SOD_{-525} units per ml assay volume. The protein quantity was determined by the method of Bradford. SOD activity was expressed relative to protein (U/mg protein).

2.3.5 Measurement of Total Antioxidant Status

The plasma antioxidant status was measured using the Total Antioxidant Status (TAS) Assay Kit. The assay principle relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{®\cdot+}$ by metmyoglobin (a peroxidase). The amount of $ABTS^{®\cdot+}$ produced can be monitored by reading the absorbance at 600 nm. Under the

reaction conditions used, the antioxidants in the sample cause suppression of the absorbance at 600 nm to a degree which is proportional to their concentration.

Venous blood was obtained from a tail vein and centrifuged for 6 min using a micro-capillary centrifuge at room temperature. The assay was performed in 37°C according to manufacturer's instructions. The spectrophotometer was set to zero at 600 nm against air. Water was used as blank, 1.5 mmol/L 6-hydroxy-2,5,7,8-tetrametylchroman-2-carboxylic acid as standard, stabilized H_2O_2 as substrate, and metmyoglobin and ABTS® as chromogen. 20 µl sample were mixed well with chromogen and the sample absorbance read. The final plasma antioxidant concentration was obtained using the following formula: antioxidant concentration (mmol/L) = 1.5 mmol×[(absorbance of H_2O added – initial blank-H_2O absorbance) – (absorbance of sample added – initial blank-sample absorbance)] / [(absorbance of H_2O added – initial blank-H_2O absorbance) – (absorbance of standard added – initial blank-standard absorbance)].

2.3.6 Measurement of vascular NADPH oxidase activity

Aortic segments were prepared as described above for measurement of $\cdot O_2^-$. The method for measuring activity of NADPH oxidase based on that described by Katty Griendling's group (49). Activity of NADPH oxidase was measured in a luminescence assay with 5 µmol/L lucigenin as the electron

acceptor and 100 μmol/L NADPH as the substrate. This concentration fell well within the linear range of the assay (1 μmol/L to 10 mmol/L for NADPH). The reaction was started by the addition of 100 μL of sample. Luminescence was measured as described above. A buffer blank was subtracted from each reading. The amount of $\cdot O_2^-$. generated was calculated as described above. The amount of $\cdot O_2^-$ produced expresses the NADPH oxidase activity. The activity of NADPH oxidase was expressed as nmol $\cdot O_2^-$/min/g dry tissue weight.

III. RESULTS (PUBLICATIONS)

Project I: Mechanisms Regulation Generation of ROS in VSMCs

3.1

Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries – regulation by angiotensin II.

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Circ Res 2001 (re-submitted)

My contribution to this study:

Protein extraction and cell fractionation,

Immunoblotting,

Immunoprecipitation and serine phosphorylation of p47phox,

Measurement of NADPH oxidase activity.

ABSTRACT

We assessed the presence of a functionally active NAD(P)H oxidase similar to the neutrophil oxidase ie containing subunits p22phox, gp91phox, p40phox, p47phox and p67phox, in smooth muscle cells from human resistance arteries (HVSMC). The regulatory role of angiotensin II (Ang II) was also assessed. Furthermore we examined mRNA expression of the gp91phox homologues, nox1 and nox4 in HVSMC, human aortic smooth muscle cells (ASMC) and rat vascular smooth muscle cells (RVSMC). HVSMC were derived from small arteries from gluteal subcutaneous biopsies of healthy subjects. gp91phox mRNA and nox4 but not nox1 were detected in HVSMCs. gp91phox mRNA was not detected in ASMC or RVSMC, but nox1 and nox4 were present. All five leukocyte NAD(P)H oxidase subunits were expressed in HVSMCs as detected by immunoblotting. Long-term Ang II stimulation (2-24hours) increased oxidase subunit abundance. Cycloheximide, but not actinomycin D, inhibited these effects. Short-term Ang II stimulation (10-15minutes) increased p47phox serine phosphorylation and induced cytosolic subunit translocation. This was associated with activation of NADPH oxidase. gp91ds-tat and apocynin, which block p47phox association with gp91phox, inhibited Ang II-induced NADPH oxidase activation. Our results suggest that HVSMCs express a functionally active gp91phox-containing neutrophil-like NAD(P)H oxidase. Furthermore Ang II regulates NAD(P)H oxidase by inducing serine phosphorylation of p47phox, translocation of cytosolic subunits and de novo protein synthesis at the post-transcriptional level. These novel findings provide insights into the molecular

regulation of NAD(P)H oxidase by Ang II in HVSMCs. Furthermore we identify differences in gp91phox homologue expression in VSMCs from small and large human arteries and rat VSMCs.

Key words: oxygen free radicals, superoxide, renin-angiotensin system, cultured cells.

INTRODUCTION

Reactive oxygen species (ROS), including superoxide ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), nitric oxide ($NO\bullet$) and peroxynitrite ($ONOO^-$) are important intracellular and intercellular signaling molecules that regulate vascular tone and structure. Emerging evidence supports a critical role for these free radicals in pathological processes underlying cardiovascular diseases, such as hypertension, atherosclerosis and restenosis (1-3). In the normal vascular wall, $\bullet O_2^-$ is produced primarily in vascular smooth muscle cells (VSMC) and fibroblasts (3). The major enzyme responsible for vascular $\bullet O_2^-$ appears to be NAD(P)H oxidase (4), which catalyzes the production of $\bullet O_2^-$ by the one electron reduction of oxygen using NAD(P)H as the electron donor: $2O_2 + NAD(P)H \rightarrow 2O_2^- + NAD(P) + H^+$.

The prototypical and best characterized NAD(P)H oxidase is that found in neutrophils, which is comprised of five components: p40phox (phox for PHagocyte OXidase), p47phox, p67phox, p22phox and gp91phox (5). In unstimulated cells p40phox, p47phox and p67phox exist in the cytosol whereas p22phox and gp91phox are located in the membranes where they occur as a heterodimeric flavoprotein, cytochrome b558 (5). Upon cell stimulation, p47phox becomes phosphorylated and the cytoplasmic complex migrates to the membrane where it associates with cytochrome b558 to assemble the active oxidase, which now transfers electrons from the substrate to O_2 leading to generation of $\bullet O_2^-$ (5,6). Activation also requires participation of two low-

molecular weight guanine nucleotide-binding proteins, Rac 2 (or Rac 1) and Rap1A (3,5). Although it is evident that cells of the vasculature contain functionally active NAD(P)H oxidase, it is still unclear which of the neutrophil NADPH oxidase subunits are present in vascular cells. In adventitial fibroblasts and endothelial cells, mRNAs for gp91phox, p22phox, p47phox and p67phox have been demonstrated (7-12). mRNA for gp91phox is barely detectable in rat aortic VSMCs (13). All phox subunits have been identified in rabbit aortic adventitia (7,11). Rat aortic VSMCs express p22phox, p47phox and rac1, but not gp91phox (10,14-16). Whether a similar situation exists in human VSMCs is unclear. Gorlach et al. (10) demonstrated a gp91phox containing NADPH oxidase in human umbilical endothelial cells (HUVEC) but not in human aortic smooth muscle cells. Since p22phox and gp91phox are essential for NAD(P)H oxidase activity, the possibility arose that there are gp91phox isoforms which are functionally active in VSMCs. Homologues of gp91phox, nox1 (for NADPH Oxidase, formerly termed mox-1 for Mitogenic Oxidase) and nox-4 have recently been identified in rat aortic smooth muscle cells and found to be functionally important (13,17). The above studies were performed in cells from large arteries from experimental animal models. The status of gp91phox as well as the other major leukocyte NAD(P)H oxidase subunits in human VSMCs, particularly from peripheral resistance arteries, the vessels important in blood pressure regulation, has not been fully investigated.

The aims of the present study were to determine whether smooth muscle cells from human small arteries (HVSMC) express gp91phox, nox1 and/or nox4

and to investigate whether p22phox and the cytoplasmic subunits of neutrophil NAD(P)H oxidase are present. Since angiotensin II (Ang II) has been implicated as a major mediator of vascular oxidative stress, we investigated the regulatory role of Ang II on expression of NAD(P)H oxidase subunits, and assessed whether Ang II induces translocation of cytoplasmic subunits to the cell membrane. p47phox is the subunit chiefly responsible for transporting the cytosolic complex to the membrane during oxidase activation. However, before the cytosolic oxidase components can be translocated, p47phox must be phosphorylated. Accordingly, we also determined whether Ang II stimulation influences the phosphorylation status of p47phox in VSMCs from human resistance arteries.

MATERIALS AND METHODS

Cell culture

The study was approved by the Ethics Committee of the Clinical Research Institute of Montreal (IRCM). Healthy volunteers (30-65 years) (n=7, 4 males) were recruited at the IRCM Hypertension Clinic. Gluteal biopsies of subcutaneous tissue of 1.0x0.5x0.5 cm^3 were obtained under local anaesthetic and vessels dissected as described previously (18). Arteries with diameters <200 μm (corresponding to resistance arteries) were used for culture. VSMCs were isolated and cultured as we described (19,20). Human aortic smooth muscle cells (ASMC) were purchsed from Clonetics (San Diego). Cells were grown in DMEM containing 10% fetal bovine serum (FBS). At subconfluence cells were

placed in quiescent culture medium (serum-free DMEM containing selenite (5 ng/mL), transferrin (5 μg/mL) and insulin (5μg/mL) for 30 hours prior to experimentation. Low passaged cells (passages 2-6, HVSMC, passages 3-7, ASMC) were studied.

Wistar Kyoto rats (WKY) were killed by decapitation. VSMCs from mesenteric arteries were isolated as we described (19,20). Low passaged cells (passages 3-6) were investigated. Cells were rendered quiescent as described above.

Human colon carcinoma cells (CaCo2) were purchased from the American Type Culture Collection (Rockville, MD) and maintained in DMEM containing 10% FBS. These cells are known to express nox1 (16), and were used as positive controls.

Confocal immunofluorescence microscopy.

To characterize cells as VSMCs and not contaminating fibroblasts or endothelial cells, immunofluorescence studies were performed. Cells were labeled with smooth muscle-specific monoclonal antibodies (anti-α-smooth muscle actin, anti-smooth muscle myosin, and anti-calponin), fibroblast-specific antibody (monoclonal anti-human fibroblast surface protein, clone 1B10) and endothelial cell-specific antibody (anti-Von Willebrand factor). All antibodies were from Sigma. Cells were identified as VSMCs if they labeled positively with antibodies to α-smooth muscle actin, heavy chain myosin and calponin, and negatively with anti-fibroblast and Von Willebrand antibodies. In some

experiments cells were duel-labeled with phalloidin-TRITC (labels actin) and anti-gp91phox antibody. VSMCs grown on coverslips were fixed with methanol (-20°C for 10 minutes), washed with PBS and exposed to blocking buffer (PBS containing 10% FBS and 0.2% triton). Washed slides were incubated (37°C, 60 mins) with different primary antibodies: anti-α-smooth muscle actin (1:100), anti-smooth muscle myosin (1:100), anti-calponin (1:100), anti-human fibroblast surface protein (1:100) anti-Von Willebrand factor (1:100) anti- gp91phox (1:200). Slides were incubated (37°C, 60 mins) with secondary antibodies: anti-mouse rhodamine conjugate or anti mouse-fluorescein conjugate (Chemicon International Inc.). Slides were fixed with 90% glycerol. Confocal microscopy was performed with a Zeiss LSM 510 system (Zeiss, Germany). Two lasers were used: an argon laser (488 nm) for the FITC conjugate and a helium neon laser (543 nm) for the rhodamine-conjugate. There was no overlap in fluorescence emission between these probes. The following settings were used in our studies: x63 or x100 objective, stack size: 1024 x 1024 pixels, pixel time: 2.24 μs, pinhole: channel 1, 106 μm; channel 2, 94 μm and 4-line averaging. In order to obtain an image of the cell as a whole, the specimen was scanned in a point-by-point, line-by-line system by means of an x-y light deflection system. Using the Z-sectioning optical device, we obtained a stack of slice images. Images were assessed at the midplane level.

Cell fractionation

Quiescent HVSMCs were stimulated with Ang II for various times. Cells were washed in ice-cold phosphate-buffered saline (PBS), scraped and transferred to eppendorf tubes, and then centrifuged at 750 g at 4°C for 5 min. The supernatant was discarded and the pellet resuspended in 500 μl lysis buffer containing protease inhibitors (sodium pyrophosphate 50 mM, NaF 50 mM, NaCl 50 mM, EDTA 5 mM, EGTA 5 mM, Na_3VO_4 2 mM, HEPES 10 mM, and phenylmethylsulfonyl fluoride 50 mM, and 0.1% Triton X-100 (pH 7.4)), and sonicated for 5 sec. Cell homogenates were centrifuged at 50,000 rpm for 20 min at 4°C. The supernatant (cytosolic fraction) was removed and the pellet, containing the plasma membrane was resuspended in lysis buffer. Protein content was measured in aliquots of cytoplasmic and membrane fractions using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA) and then prepared for Western blot analysis or NADPH oxidase assay.

Preparation of human and rat neutrophils:

Since neutrophils possess all NAD(P)H oxidase subunits, these cells were used as positive controls. Neutrophils were isolated from human and rat blood using dextran sedimentation and Ficoll-Hypaque gradient centrifugation were homogenized by N_2 cavitation, and membrane and cytosolic fractions were prepared from the cavitate by sequential centrifugation as described previously (22).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from human VSMCs and rat VSMCs using Trizol Reagent (Gibco BRL) and reverse transcription was performed as we described (23). After first-strand synthesis of DNA, 2 μL cDNA was amplified using specific primers selected on the bases of published sequences. lable online : human gp91phox, sense 5'-TCACTTCCTCCACCAAAACC-3', antisense 5'-CACCTTCTGTTGAGATCGCC-3', rat gp91phox, sense 5'-TCACATCCTCCACCAAAACC-3', antisense 5'- GTATTGTCCCACCTCCATCC-3', human nox1, sense 5'-TAACAGCACGCTGATCCTG-3', antisense 5'-CTGGAGAGAATGGAGGCAAG-3', rat nox1, sense 5'-CACCTGCTCATTTTGCAACCACAC-3', antisense 5'-CAACTCCTTTCATACTTATCCCACTC-3', human nox4, sense 5' GTACAAATTCCAGTGTGCAGACCAC-3', antisense 5'-cagactggaatatcggtgacagca-3'. For amplification of gp91phox the protocol involved denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min for 35 cycles. For amplification of nox1, the protocol involved denaturation at 94°C for 30 secs, annealing at 59°C for 30 secs and extension at 72°C for 30 secs for 35 cycles. For amplification of nox4, the protocol involved denaturation at 95°C for 30 secs, annealing at 64°C for 20 secs and extension at 72°C for 30 secs for 35 cycles. PCR products were electrophoresed on a 1.5% agarose gel for 60 mins at 9V/cm gel. Bands corresponding to RT-PCR products were visualized by UV light.

Sequencing of PCR products

Amplified PCR products were gel purified, cloned with Topo cloning Kit according to manufaturer's instructions (Clontech Palo Alto). Selected colonies were cultured and mini-prepped using QIA prep Spin kit (Qiagen, Ontario). DNA samples were sequenced automatically by Dye Terminator Cycle Sequencer using a CEO 2000 XL DNA analysis system (Beckman Coulter Fullerton, CA). Sequencing reactions were performed at least three times for each insert. Sequence comparisons were made with the NCBI web server (Blastn 2.1.2) available on the Internet.

Immunoblotting

Western blotting was performed as we described (19,20). Membrane preparations were used to detect gp91phox and p22phox, whereas cytoplasmic fractions were used for p40phox, p47phox and p67phox. To determine whether cytoplasmic subunits are translocated to the membrane following Ang II stimulation, expression of p40phox, p47phox and p67phox was also assessed in membrane fractions. Equal amounts of proteins (15 μg) were loaded on a SDS-PAGE and transferred to PVDF membrane (Boehinger Mannheim, Quebec) for 1 hour at 100 V. Membranes were blocked in 5% non-fat milk and incubated with mouse monoclonal or rabbit polyclonal antibodies diluted 1:1000 for 1 hour. They were then washed, incubated with a goat anti-mouse or anti-rabbit HRP conjugated antibody (1:5000) (Santa Cruz Biotechnology, CA) for 1 hour and washed. Membranes were incubated with Blotting Substrate (POD) (Boehinger Mannheim), exposed to film and developed. The film was scanned and band

intensity measured densitometrically. Previously characterized antibodies specifically recognizing p22phox (monoclonal, clone 44.1) (24,25), gp91phox (monoclonal, clone 54.1) (24,26), p47phox (polyclonal, clone R360), p67phox (polyclonal, clone R1497) (27), and p40phox (monoclonal, clone 1.9) were used for immunoblotting.

Immunoprecipitation and serine phosphorylation of p47phox.

For immunoprecipitation of p47phox, the cytosolic fraction (100 μg protein) from unstimulated and Ang II-stimulated cells were transferred to microcentrifuge tubes and anti-p47phox antibody (12 μg) added and incubated for 60 mins at 4°C. 20μl agarose conjugate (Protein G PLUS-Agarose, Santa Cruz Biotechnology, CA) was then added and incubated for 60 mins at 4°C. The sample was centrifuged at 12 000rpm for 30 secs and the supernatant subjected to immunoblotting as described above. Membranes were probed with rabbit polyclonal antibody to phosphoserine (1:1000) (Zymed Labs Inc. CA) and immunoreactive proteins detected by chemiluminescence.

Measurement of NADPH oxidase activity

Quiescent HVSMc were stimulated with Ang II for 10-15 minutes. In some experiments, HVSMCs were pre-exposed for 30 minutes to gp91ds-tat, a novel competitive inhibitor of NADPH oxidase assembly (pagano) ($5x10^{-6}$ mol/L), scrambled gp91-tat (negative control) or apocynin, a methoxy-substituted catechol that inhibits association of p47phox and p67phox with gp91phox (stolk), prior to Ang II addition. Cells were fractionated as described above.

The lucigenin-derived chemiluminescence assay was used to determine NADPH oxidase activity in cell homogenates and membrane and cytosolic fractions (21). NADPH (10^{-4} mol/L) was added to the cell/membrane/cytosolic suspension (400 μl) containing lucigenin (5 μmol/L). This concentration of lucigenin is not involved in redox cycling and specifically detects $\bullet O_2^-$ (30). Luminescence was measured every 18 seconds for 3 mins in a luminometer (AutoLumat LB 953, Berthold). A buffer blank was subtracted from each reading. The amount of $\bullet O_2^-$ generated was calculated by comparison with a standard curve using xanthine/xanthine oxidase (21). Activity is expressed as nmol $\bullet O_2^-$ /min/mg protein. To verify the specificity of the lucigenin assay for $\bullet O_2^-$ in our models, we examined effects of superoxide dismutase (SOD) (120 U/ml) (enzymatic scavengers of $\bullet O_2^-$) and tiron (10 mmol/L) (non-enzymatic scavengers of $\bullet O_2$) on Ang II-stimulated activation of NADPH oxidase.

Measurement of reactive oxygen species (ROS) in intact cells.

Ang II-induced generation of ROS was measured with the fluoroprobe CM-H_2DCFDA (Molecular Probes, OR) (31). Generation of ROS was measured in unstimulated cells and in cells exposed to Ang II in the absence and presence of 10^{-5} mol/L diphenylene iodonium (DPI), a flavoprotein inhibitor that inhibits NAD(P)H oxidase (32). Cells were pretreated with DPI for 20 mins.

Statistics.

Experiments were repeated 3-6 times in duplicate or triplicate. Results are presented as means±SEM and compared by ANOVA or by Student's t test

where appropriate. Tukey-Kramer's correction was used to compensate for multiple testing. $p<0.05$ was significant.

RESULTS

Immunofluorescence studies.

As shown in figure 1, cells labeled positively with anti- α-smooth muscle actin, anti-smooth muscle myosin, anti-calponin and phalloidin, but did not label with anti-human fibroblast surface protein or anti-Von Willebrand factor antibodies. These results indicate that the cells under investigation are indeed VSMCs and not contaminating fibroblasts or endothelial cells. In further support of this, cells exhibited the typical "hill and valley" growth characteristics of VSMCs.

VSMCs from human small arteries contain gp91phox mRNA.

gp91phox mRNA was detected by RT-PCR in HVSMCs, but not in ASMS or RVSMC (figure 2). Neutrophils, which characteristically express gp91phox, were used as positive controls. Sequence analysis of cloned cDNA demonstrated that the obtained sequence of our samples was 99% identical to human CYBB (human gp91phox gene) cDNA (583-899).

We detected nox1 mRNA in ASMC and RVSMC but not in HVSMC (figure 2). The human CaCo2 cell line was used as a positive control for human nox1. Nox4 was present in HVSMC, ASMC and RVSMC. Negative controls performed with RNA without RT did not yield any PCR products, indicating an absence of genomic DNA contamination.

Expression of NADPH oxidase subunits in HVSMCs.

Western blotting demonstrated the presence of gp91phox and p22phox protein in membrane fractions (figure 3) prepared from HVSMC. gp91phox protein was not expressed in ASMC or RVSMCs (data not shown). p40phox, p47phox and p67phox were detected in the cytosolic fractions of HVSMCs (figure 2). Long-term exposure (4-24 hours) of cells to Ang II significantly increased expression of NAD(P)H oxidase subunits.

To further characterize the presence of gp91phox in HVSMCs, confocal immunofluorescence microscopy was performed. Figure 1F shows x-y sections of HVSMCs dual-labeled with phalloidin (red fluorescence) and anti-gp91phox antibody (green fluorescence). Gp91phox-labeling had a reticular staining extending toward the cell membrane.

Regulation of NAD(P)H oxidase subunits by Ang II.

To evaluate in greater detail the regulatory role of Ang II on NAD(P)H oxidase, we determined whether Ang II stimulation causes translocation of cytoplasmic subunits to the cell membrane and whether Ang II influences expression of NAD(P)H oxidase subunits by regulating synthesis of these proteins. Western blotting demonstrated weak presence of the NAD(P)H oxidase cytoplasmic subunits in membrane fractions in the basal state (figure 4). Ang II stimulation (10-15 mins) significantly decreased abundance of cytosolic p47phox and p67phox, whereas membrane abundance of these subunits was increased following stimulation (figure 4).

To determine whether Ang II mediates effects by stimulating protein synthesis, cells were exposed to actinomycin D (inhibitor of translation) and

cycloheximide (inhibits protein synthesis by interfering with translocation) (figure 5). Cycloheximide, but not actinomycin D, significantly decreased ($p<0.01$) Ang II-induced expression of gp91phox, p22phox, p47phox and p67phox. These data suggest that Ang II influences expression of NAD(P)H oxidase subunits by de novo protein synthesis, probably at the post-transcriptional level.

Ang II stimulates serine-phosphorylation of p47phox.

Phosphorylation of p47phox is critical for cytoplasmic complex formation and activation of NAD(P)H oxidase in neutrophils (5,6). To determine whether a similar situation exists for Ang II-stimulated VSMCs, we immunporecipitated p47phox from cytoplasmic fractions and then probed with an anti-phospho-serine antibody. As demonstrated in figure 6, Ang II stimulation (15 mins) increased serine-phosphorylation of p47phox 2-3 fold.

Activation of NADPH oxidase by Ang II in HVSMCs.

Having demonstrated the presence of all NAD(P)H oxidase subunits in HVSMCs, and that Ang II induces serine phosphorylation of p47phox and translocation of cytoplasmic subunits, we sought to investigate whether the enzyme was functionally active in response to Ang II. Unstimulated cells exhibited some basal NADPH oxidase activity (figure 7a). Exposure of cells to Ang II increased activation of NADPH oxidase. Assessment of the different cell fractions demonstrated that activity was located primarily in the membrane component with little activity in the cytoplasmic fraction, suggesting that upon stimulation the subunits become membrane-associated to activate the oxidase. To confirm that chemiluminescence measurements were not an artifact of the

lucigenin assay, effects of SOD and tiron ($\bullet O_2^-$ scavengers) were determined. SOD and tiron inhibited the lucigenin signal in Ang II-stimulated cells by 87% and 91% respectively.

Ang II stimulates generation of ROS in HVSMCs.

To evaluate the functional significance of Ang II-activated NAD(P)H oxidase, we measured the capacity of HVSMCs to generate ROS in the absence and presence of DPI, a flavoprotein inhibitor that reduces NAD(P)H oxidase activity (31,32). Ang II dose-dependently increased DCFDA fluorescence indicating intracellular generation of ROS (figure 7b). DPI significantly attenuated these effects, suggesting that Ang II-induced production of ROS is mediated via DPI-inhibitable enzymes, including NAD(P)H oxidase.

Association between gp91phox and cytoplasmic subunits is essential for functionally active NADPH oxidase in HVSMCs.

To demonstrate that gp91phox is necessary for activation of NADPH oxidase, HVSMCs were exposed to two different experimental conditions whereby gp91phox association to cytoplasmic subunits was blocked. Firstly, cells were pre-treated with a chimeric peptide that inhibits p47phox complex formation with gp91phox (gp91ds-tat) and secondly cells were exposed to apocynin, a methoxy-substituted catechol, which inhibits NAD(P)H oxidase activation by preventing association between gp91phox and the cytoplasmic subunits (p47phox and p67phox). Gp91ds-tat, but not the control peptide,

scrambled-tat, abrogated Ang II-induced activation of NADPH oxidase (figure 8). Apocynin also abolished Ang II-mediated actions.

DISCUSSION

The major findings of our study demonstrate that VSMCs derived from human resistance arteries, vessels important in blood pressure regulation, express gp91phox both at the mRNA and protein levels. The gp91phox homologue nox1 is expressed in human aortic smooth muscle cells and rat VSMCs but not in VSMCs from human small arteries. Nox4, another gp91phox homologue, appears to be present in HVSMC, ASMS and RVSMC. We also demonstrate that the other major neutrophil NAD(P)H oxidase subunits, p22phox, p40phox, p47phox and p67phox, are present in VSMCs from human small arteries. Furthermore we show that Ang II increases expression of gp91phox, p22phox, p47phox and p67phox by stimulating de-novo protein synthesis at the post-transcriptional level. Acute Ang II stimulation resulted in translocation of cytoplasmic subunits, activation of NADPH oxidase and production of reactive oxygen species. Inhibition of the interaction of gp91phox with p47phox and p67phox abrogated Ang II-induced activation of NADPH oxidase. These novel findings suggest that VSMCs from human small arteries possess a functionally active gp91phox-containing NAD(P)H oxidase that generates free radicals in response to Ang II. In addition, we demonstrate differential mRNA expression of gp91phox, nox1 and nox4 in VSMCs from human small and large arteries. Whereas HVSMC possess gp91phox, ASMC

and RVSMC possess nox1. Nox4 appears to be expressed in VSMCs from both small and large human arteries as well as in RVSMCs.

Although there is strong evidence that NAD(P)H oxidase is a major source of $\bullet O_2^-$ in VSMCs, the enzyme has not been fully characterized in vascular cells, and there is much controversy whether the classical neutrophil subunits, and particularly gp91phox, are present and functional in these cells (3,13,17,33). Using molecular biological techniques and knockout models of gp91phox and p47phox mice, recent studies demonstrated that p22phox, p47phox, gp91phox and rac are important in vascular NAD(P)H oxidase activity and in Ang II-mediated generation of free radicals (7-15,34). gp91phox and p22phox are essential for NAD(P)H oxidase activation (5). Therefore it is intriguing that VSMCs should not contain gp91phox. Using monoclonal and polyclonal antibodies (against human neutrophil NAD(P)H oxidase) and carefully prepared membrane fractions, our data clearly show that gp91phox is expressed in VSMCs from human small arteries These findings were confirmed by confocal immunocytochemical microscopy, which demonstrated a reticular staining extending to the cell membrane. This pattern of labeling suggests that in the basal state, gp91phox is located in the cell membrane as well as intracellularly. Since gp91phox is membrane-associated, the intracellular component that we detected may reflect gp91phox bound to intra-organelle membranes or associated granules. In addition it is possible that cytoplasmic labeling indicates

newly synthesized gp91phox. Bayraktutan et al. (35) also demonstrated intracellular localization of gp91phox in endothelial cells.

Although our gp91phox findings are in contrast to those of Gorlach et al. (10) who did not detect gp91phox in human VSMCs, our nox1 results in aortic cells confirm their results (10). Taken together, these data suggest that gp91phox, but not nox1 is present in VSMCs from human small arteries, whereas nox1 but not gp91phox is present in VSMCs from human large arteries. Reasons for the differential expression of gp91phox homologues between VSMCs from small and large arteries is unclear, but may indicate heterogeneity of VSMCs derived from different vascular beds. Nox1 is a gp91phox homologue that has 56% identity with human gp91phox (17), and together with p22phox, is probably the functionally active component of cytochrome b558 in ASMC and RVSMC (13). Unlike gp91phox and nox1, which were differentially expressed, nox4 was ubiquitously expressed in all VSMCs studied. The exact function of this gp91phox homologue is unclear, but it may be antagonistic to nox. Lassègue et al. (13) recently demonstrated in rat aortic smooth muscle cells that Ang II upregulates nox1, but downregulates nox4. The exact role of nox4, particularly in human VSMCs, awaits further clarification.

Western blotting revealed that p40phox, p47phox and p67phox are abundantly expressed in cytoplasmic fractions of HVSMCs. In addition, these subunits were detectable in membrane fractions in unstimulated cells, suggesting some translocation of the subunits and activation of the oxidase in the basal state. This was confirmed by our chemiluminescence studies

demonstrating that NADPH oxidase is partially activated in unstimulated VSMCs. These findings are in contrast to leukocytes, which do not exhibit basal activation of NAD(P)H oxidase (5). Within a few minutes of Ang II stimulation, the abundance of p47phox and p67phox in the cytoplasmic fraction decreased whereas the content of these cytoplasmic subunits increased in the membrane fraction. These findings indicate rapid translocation of p47phox and p67phox by Ang II. The physiological role of each subunit remains unclear, but p47phox phosphorylation seems to be pivotal and the presence of p67phox is obligatory for NAD(P)H oxidase activation (6,34). p40phox may be an inhibitory oxidase subunit (5).

Possible mechanisms whereby Ang II regulates NAD(P)H oxidase could be via its effects on abundance of the oxidase subunits and/or by modulating phosphorylation of the proteins. In our study, long-term Ang II stimulation (hours) increased NAD(P)H oxidase content. To evaluate whether this was due to de-novo protein synthesis, effects of actinomycin D and cycloheximide were assessed. Ang II-induced expression of NAD(P)H oxidase subunits was significantly inhibited by cycloheximide, but not by actinomycin D, suggesting that Ang II regulates synthesis of NAD(P)H oxidase subunits at the post-transcriptional level. Pagano et al. reported that in rabbit aortic adventitial fibroblasts, p67phox is regulated by Ang II both at the level of transcription and translation (7,11). Similar findings have been shown for p22phox in rat aortic cells (36). Taken together, it is evident that Ang II influences synthesis of NAD(P)H oxidase subunits at multiple levels.

It is also possible that Ang II regulates NAD(P)H oxidase activity by stimulating subunit phosphorylation. Serine-phosphorylation of p47phox seems to be critical for cytoplasmic subunit complex formation and translocation to the cell membrane. During oxidase activation, serines S359 and/or S370 have to be phosphorylated first, then S379, allowing the cytsolic complex to translocate to cytochrome b558 (gp91phox and p22 phox). Finally, S303 and/or S304 are phosphorylated, endowing the oxidase with full catalytic activity (37,38). We demonstrate for the first time that Ang II induces phosphorylation of serine residues of p47phox. These effects were evident within 10-15 mins of stimulation, suggesting that p47phox phosphorylation is a rapid event in the activation of NAD(P)H oxidase. Interestingly, PLA_2, ERK1/2, p38MAPK and phosphatidic acid, all downstream signaling molecules of Ang II (39), have been implicated in phosphorylation of p47phox and in activation of the oxidase (40-42).

To demonstrate the functional significance of NADPH oxidase, we determined whether Ang II activates the enzyme and whether it generates free radicals in HVSMCs. Furthermore, the importance of gp91phox in these processes was assessed by exposing cells to gp91ds-tat and apocynin, both of which inhibit association of gp91phox with p47phox (28,29). Ang II increased activity of NADPH oxidase and dose-dependently stimulated production of oxygen free radicals. Ang II actions were abrogated by gp91ds-tat and apocynin and generation of free radicals was reduced by DPI. These phenomena indicate that NADPH oxidase is actived by Ang II and that complex formation between

gp91phox and cytoplasmic subunits is critical for a functionally active oxidase in VSMCs from human small arteries.

In conclusion data from the present study demonstrate that VSMCs from human resistance arteries express gp91phox and nox4 but not nox1. In contrast, nox1 but not gp91phox is present in human aortic cells and rat VSMCs. VSMCs from human small arteries also express the other major neutrophil NAD(P)H oxidase subunits, p22phox, p40phox, p47phox and p67phox. Ang II increases expression of NAD(P)H oxidase subunits by stimulating de-novo protein synthesis at the post-transcriptional level. Moreover, we demonstrate that Ang II induces phosphorylation of p47phox and translocation of cytoplasmic subunits with subsequent activation of NADPH oxidase and generation of oxygen free radicals. These novel findings suggest that VSMCs from human resistance arteries possess a functionally active Ang II-regulated gp91phox-containing neutrophil-like NAD(P)H oxidase, which is a major source of vascular-derived reactive oxygen species.

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Figure 3.1.1

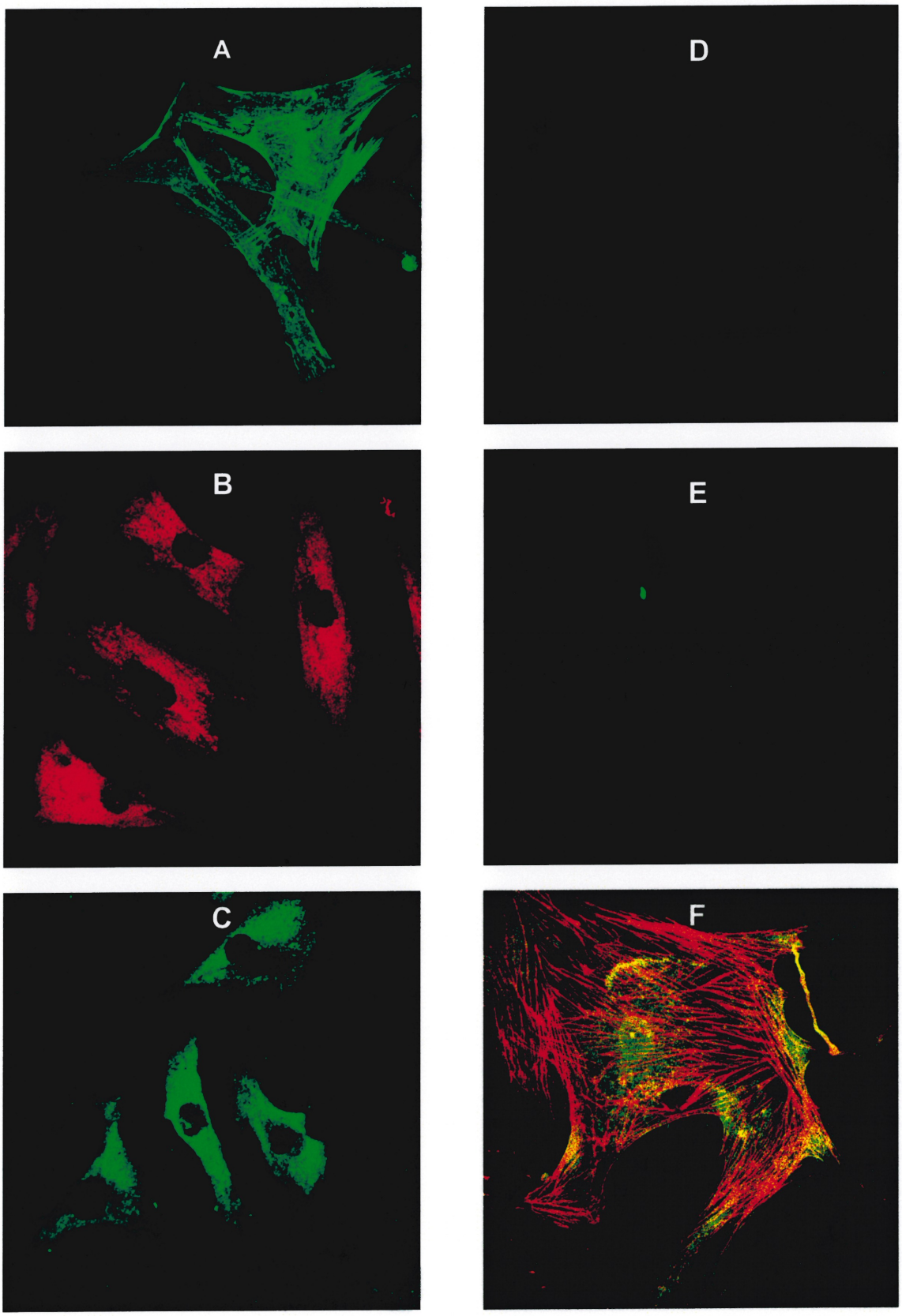


Figure 3.1.2

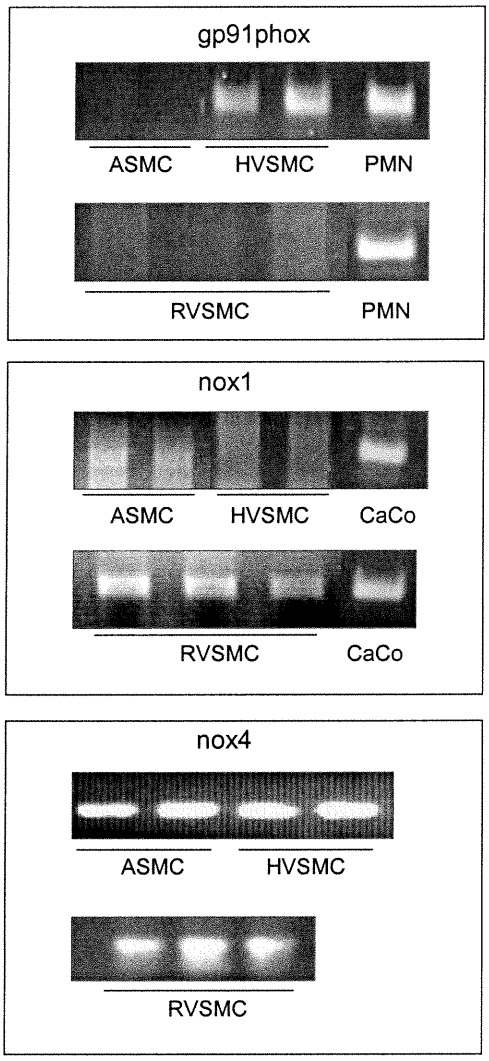

gp91phox
ASMC
HVSMC
PMN
RVSMC
PMN
nox1
ASMC
HVSMC
CaCo
RVSMC
CaCo
nox4
ASMC
HVSMC
RVSMC

Figure 3.1.3

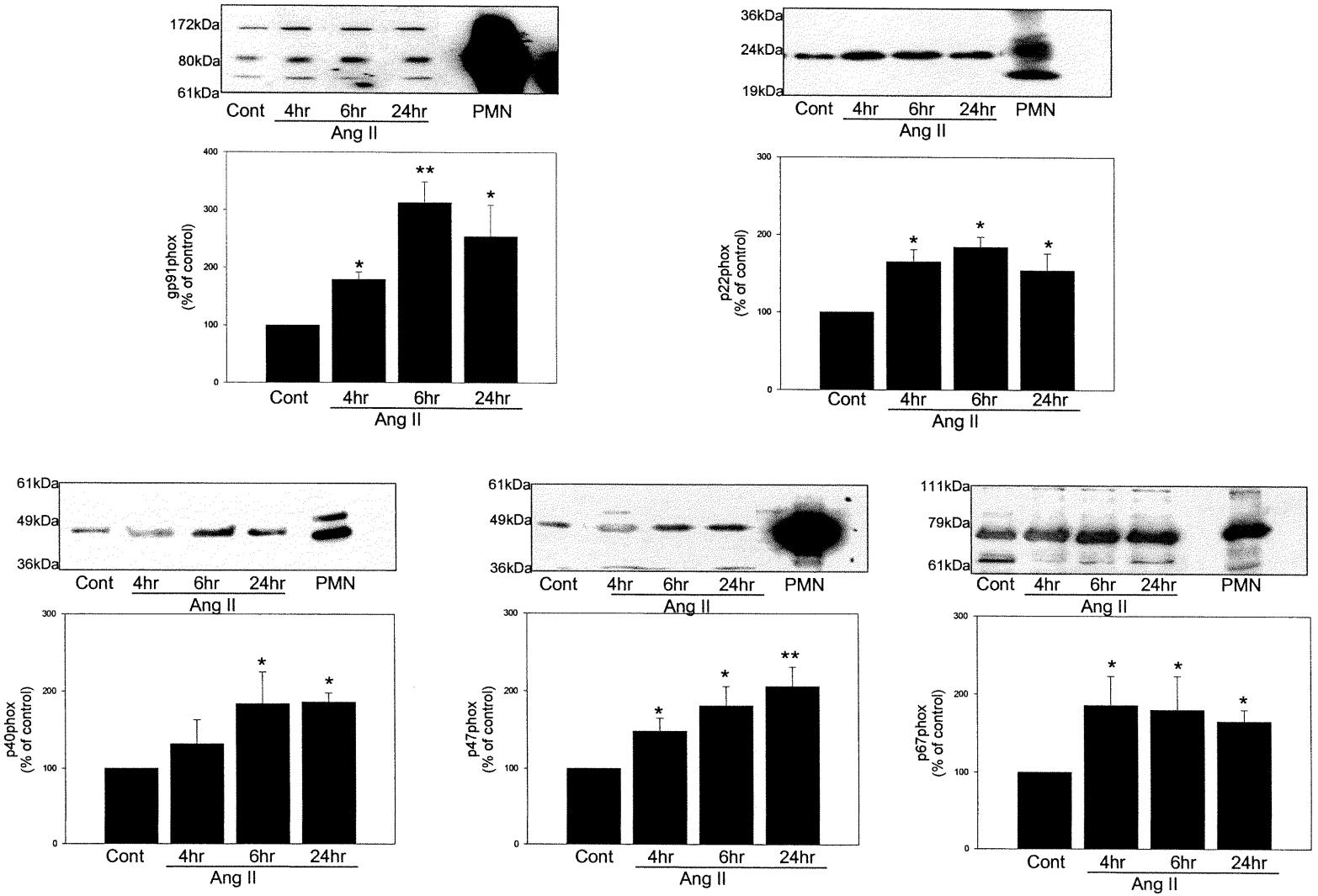

172kDa
80kDa
61kDa
Cont 4hr 6hr 24hr PMN
Ang II
gp91phox (% of control)
36kDa
24kDa
19kDa
p22phox (% of control)
61kDa
49kDa
36kDa
p40phox (% of control)
p47phox (% of control)
111kDa
79kDa
61kDa
p67phox (% of control)

Figure 3.1.4

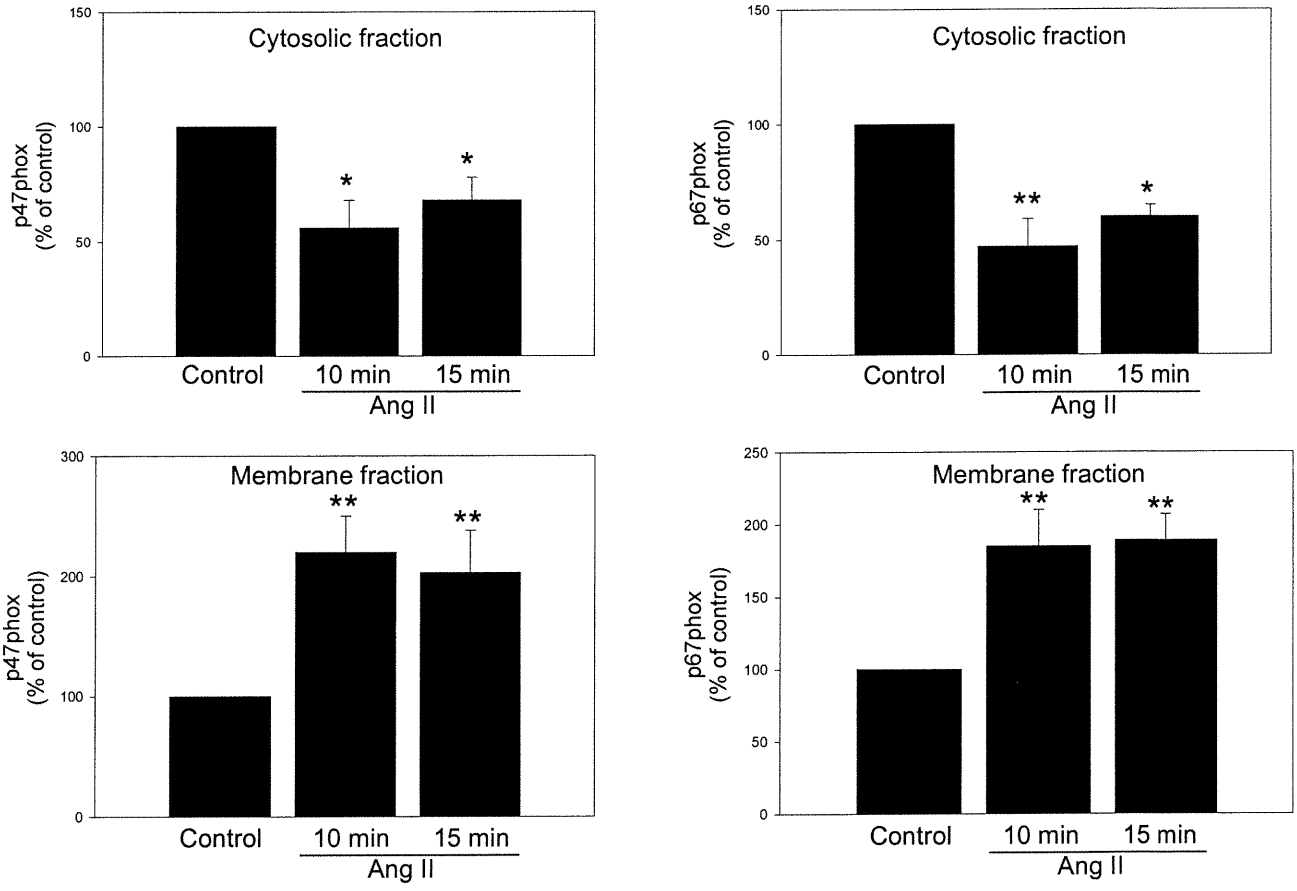

Cytosolic fraction
p47phox (% of control)
150
100
50
0
*
*
Control
10 min
15 min
Ang II
Cytosolic fraction
p67phox (% of control)
150
100
50
0
**
*
Control
10 min
15 min
Ang II
Membrane fraction
p47phox (% of control)
300
200
100
0
**
**
Control
10 min
15 min
Ang II
Membrane fraction
p67phox (% of control)
250
200
150
100
50
0
**
**
Control
10 min
15 min
Ang II

Figure 3.1.5

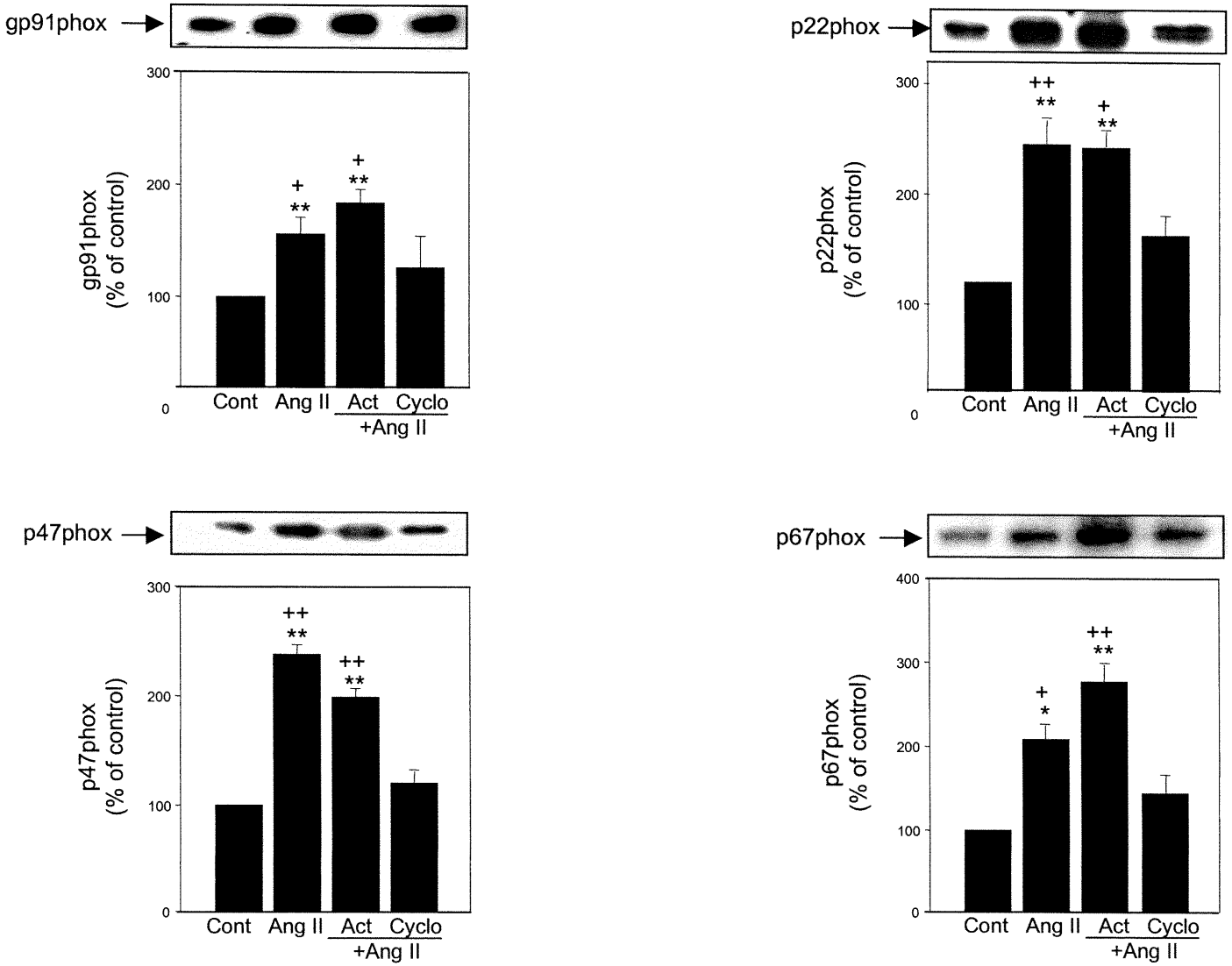

gp91phox
gp91phox (% of control)
300
200
100
0
+ **
+ **
Cont
Ang II
Act
Cyclo
+Ang II
p22phox
p22phox (% of control)
++ **
+ **
p47phox
p47phox (% of control)
++ **
++ **
p67phox
p67phox (% of control)
400
+ *
++ **

Figure 3.1.6

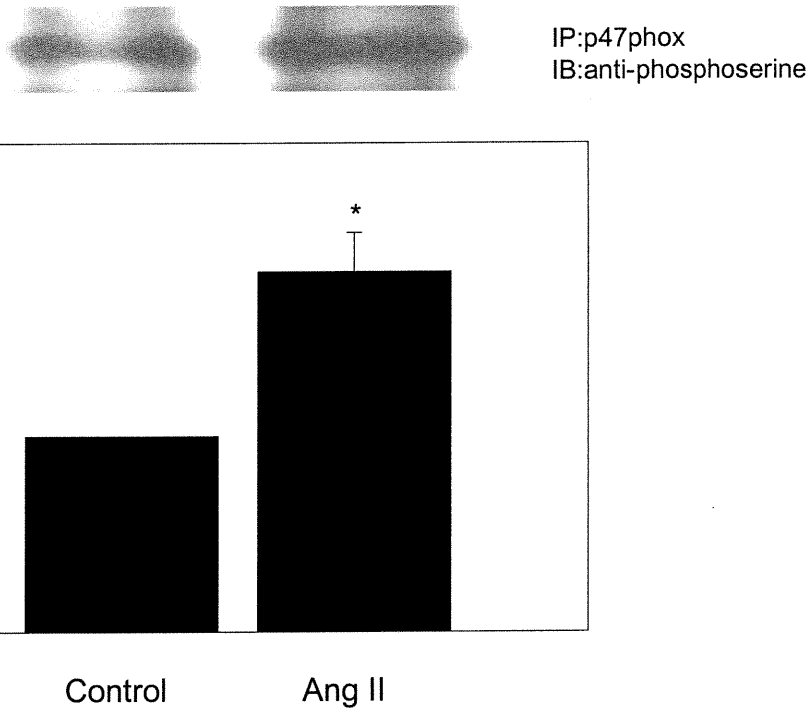
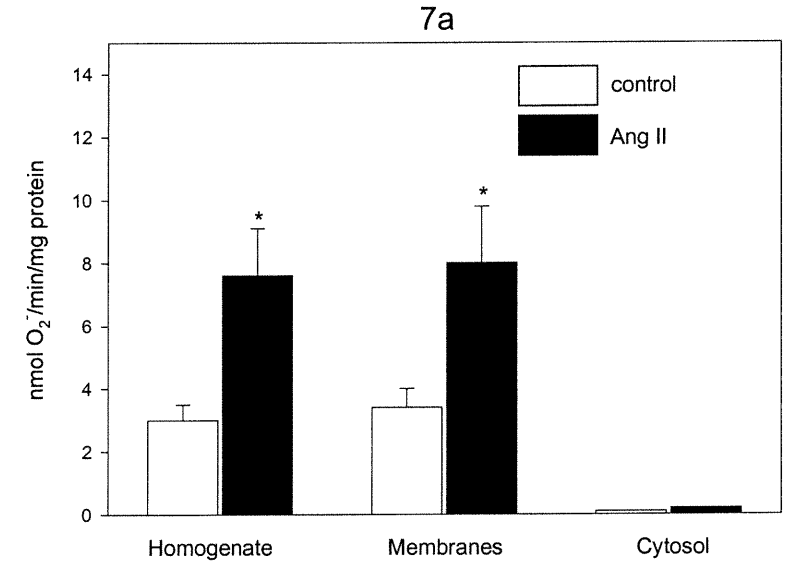

IP:p47phox
IB:anti-phosphoserine
p47phox phosphorylation
(% of control)
250
200
150
100
50
0
*
Control
Ang II

Figure 3.1.7


7a
control
Ang II
nmol O_2^-/min/mg protein
0
2
4
6
8
10
12
14
*
*
Homogenate
Membranes
Cytosol

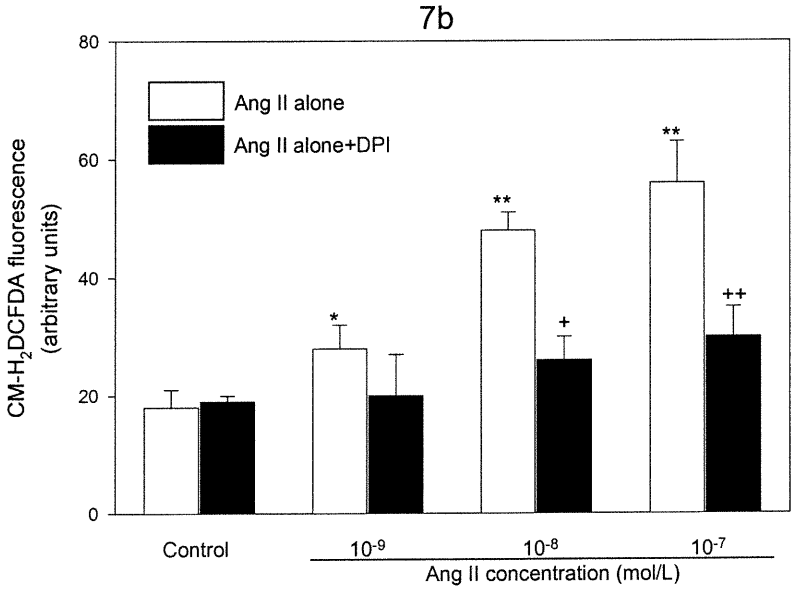

7b
Ang II alone
Ang II alone+DPI
CM-H_2DCFDA fluorescence (arbitrary units)
0
20
40
60
80
*
**
**
+
++
Control
10^{-9}
10^{-8}
10^{-7}
Ang II concentration (mol/L)

Figure 3.1.8

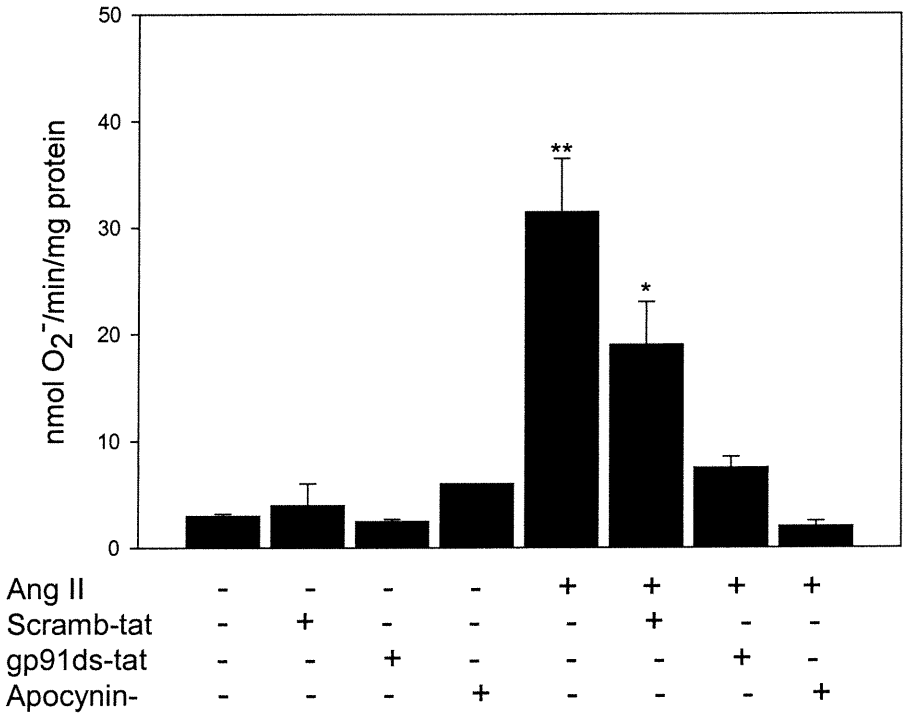

50
40
30
20
10
0
nmol O_2^-/min/mg protein
**
*
Ang II - - - - + + + +
Scramb-tat - + - - - + - -
gp91ds-tat - - + - - - + -
Apocynin- - - - + - - - +

FIGURE LEGENDS

Figure 3.1.1.

Characterization of cells as VSMCs and subcellular localization of gp91phox by confocal immunofluorescence microscopy. Fig 1A, labeling with anti-α-smooth muscle actin antibody 1B, labeling with anti-smooth muscle myosin monoclonal antibody, 1C, labeling with anti-calponin monoclonal antibody, 1D, labeling with monoclonal anti-human fibroblast surface protein, clone 1B10 antibody, 1E, labeling with anti-Von Willebrand factor antibody, 1F, dual labeling with phalloidin (red fluorescence) and a monoclonal anti-p91phox antibody (clone 54.1) (fluorescein conjugate, green fluorescence). gp91phox appears to have a reticular staining extending toward the cell membrane. Image were taken at the midplane level, using x63 objective (figures 1A-1E) and x100 objective (image 1F).

Figure 3.1.2.

Representative RT-PCR products of RNA, extracted from VSMCs from human small arteries (HVSMC), human aortic smooth muscle cells (ASMC), and rat VSMCs (RVSMC). The human colon carcinoma cell line CaCo2 was used as a positive control for human nox1 and human and rat neutrophils (PMN) were used as positive controls for gp91phox. Experiments were performed in duplicate using 4 different preparations.

Figure 3.1.3.

Expression of NAD(P)H oxidase subunits in control and Ang II (10^{-7} mol/L)-stimulated VSMC from human small arteries (HVSMC). Abundance of gp91phox and p22phox was assessed in membrane fractions, whereas p40phox, p47phox and p67phox were assessed in cytosolic fractions. Human neutrophils (PMN) served as a positive control. Results are presented as % expression relative to control conditions, taken as 100%. Results are means±SEM of 4-6 experiments. *$p<0.05$, **$p<0.01$ vs control counterpart.

Figure 3.1.4.

Translocation of cytosolic subunits by Ang II. Western blot analysis of p47phox and p67phox in cytosolic and membrane fractions following Ang II (10^{-7} mol/L) stimulation (10-15 mins). Upper graphs demonstrate decreased abundance of p47phox and p67phox in cytoplasmic fractions and lower panels demonstrate increased abundance of the subunits following Ang II stimulation. Data are expressed as % expression relative to control, taken as 100%. Results are means±SEM of 4-6 experiments. *$p<0.05$, **$p<0.01$ vs control counterpart.

Figure 3.1.5.

Representative immunoblots demonstrating effects of actinomycin D (act) and cycloheximide (cyclo) on Ang II-induced expression of NAD(P)H oxidase subunits. Cells were pre-incubated (30 mins) with either actinomycin D (0.5 μg/mL) or cycloheximide (0.5 μg/mL) and then treated with vehicle (control) or

with Ang II (10^{-7} mol/L) for 2 hours. Expression of gp91phox and p22phox was assessed in membrane fractions whereas the other subunits were assessed in cytosolic fractions. *p<0.05, **p<0.01 vs control, +p<0.05, ++p<0.01 vs cycloheximide group.

Figure 3.1.6.

Effects of Ang II on serine-phosphorylation of p47phox. A polyclonal p47phox antibody was used to immunoprecipitate (IP) p47phox from cytoplasmic fractions of vehicle-treated and Ang II-stimulated (15 mins) HVSMCs. Upper panel is a representative immunoblot (IB), which was performed using anti-phosphoserine antibody. Lower panel demonstrates corresponding bar graphs. Results are means±SEM of 3 cell preparations. *p<0.05 vs control.

Figure 3.1.7.

Fig 7a. Bar graphs demonstrate Ang II effects on NADPH oxidase activity in VSMCs from human small arteries. VSMCs were exposed to Ang II (10^{-8}mol/L) or vehicle for 15 mins. Assays were prepared in whole cell homogenates and in membrane and cytosolic fractions. Each bar represents the mean±SEM of 3 experiments. *p<0.05 vs control counterpart. *Fig 7b.* Graphs demonstrate Ang II effects on CM-H_2DCFDA fluorescence in the absence and presence of DPI (10^{-5} mol/L). Fluorescence was measured 30 minutes after Ang II addition. Results are means±SEM of 6-8 experiments with each experimental field comprising 14-20 cells. *p<0.05, **p<0.01 vs control, +p<0.05, ++p<0.01 vs Ang II counterpart.

Figure 3.1.8.

Bar graphs demonstrate Ang II effects on NADPH oxidase activity in HVSMCs in the absence and presence of scrambled-tat (scramb-tat) ($5x10^{-6}$ mol/L), gp91ds-tat ($5x10^{-6}$ mol/L), or apocynin ($30x10^{-6}$ mol/L). Cells were pre-exposed to the different agents for 30 minutes prior to Ang II (10^{-7} mol/L) stimulation (15 mins). Results are means±SEM of 5 experiments. *$p<0.05$, **$p<0.01$ vs other groups.

Project II: Role of ROS in the Development of Hypetension in SHR-SP

3.2
Antioxidant effects of vitamins C and E are associated with altered activation of vascular NAD(P)H oxidase and superoxide dismutase in stroke-prone SHR.

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Running title: Antioxidants, oxidative stress and hypertension in SHRSP

Hypertension 2001; 38:606-611

My contribution to this study:

Preparation of rat diets,

Detection of vascular $\bullet O_2^-$ by lucigenin chemiluminescence,

Measurement of NADPH oxidase activity,

Measurement of total antioxidant status (TAS),

Measurement of superoxide dismutase activity,

Analysis of data.

ABSTRACT

Ascorbic acid (vitamin C) and alpha tocopherol (vitamin E) have antioxidant properties that could improve redox-sensitive vascular changes associated with hypertension. We determined whether vitamins C and E influence vascular function and structure in hypertension by modulating activity of NADPH oxidase and superoxide dismutase (SOD). Adult stroke-prone-SHR (SHRSP) were divided into 3 groups: control (C) (n=6), vitamin C (vit C) (1000 mg/day) (n=7), and vitamin E (vit E) (1000 IU/day) (n=8) groups. All rats were fed 4% NaCl. Blood pressure was measured weekly. After 6 weeks treatment rats were killed and mesenteric arteries mounted as pressurized preparations. Vascular $\bullet O_2^-$ generation and NADPH oxidase activity were measured by chemiluminescence. Vascular SOD activity and plasma total antioxidant status (TAS) were determined spectrophotometrically. Blood pressure increased from 212±7 mmHg to 265±6 in controls. Treatment prevented progression of hypertension (vit C 222±6 to 234±14, vit E 220±9 to 227±10 mmHg). Acetylcholine-induced vasodilation was improved ($p<0.05$) and media to lumen ratio was reduced ($p<0.05$) in the treated rats. $\bullet O_2^-$ was lower in vitamin groups compared with controls (vit C 10±4, vit E 9.6±3.5, C 21±9 nmol/min/mg; $p<0.05$). Both vitamin-treated groups showed significant improvement ($p<0.01$) in TAS. These effects were associated with decreased activation of vascular NADPH oxidase (vit C 46±10, vit E 50±9, C 70±16 nmol $\bullet O_2^-$/min/g dry weight, $p<0.05$) and increased activation of SOD (vit C 12±2, vit E 8±1, C 4.6±1 U/mg, $p<0.05$).

Our results demonstrate that vitamins C and E reduce oxidative stress, improve vascular function and structure and prevent progression of hypertension in SHRSP. These effects may be mediated via modulation of enzyme systems that generate free radicals.

INTRODUCTION

Oxidative damage induced by reactive oxygen species is due to increased production of superoxide anion ($\bullet O_2^-$) and its metabolites and/or to reduced bioavailability of antioxidant defenses. This imbalance between prooxidants and antioxidants gives rise to cellular oxidative stress, which plays an important role in the pathogenesis of hypertension (1-3). Reactive oxygen species may act through several mechanisms to mediate vascular change in hypertension: 1) direct actions on endothelial cells and vascular smooth muscle cells (VSMC) resulting in structural and functional damage, 2) scavanging of the important vasodilator nitric oxide (NO), 3) production of peroxynitrite, a potent constrictor and lipid-oxidizing radical, 4) effects on endothelial cell eicosanoid metabolism and 5) oxidative modification of low-density lipoproteins (4,5).

Many studies support a role for altered redox status in hypertension. At the cellular level, concentrations of $\bullet O_2^-$ and H_2O_2 are increased and activity of NAD(P)H oxidase, the major $\bullet O_2^-$ -generating enzyme in vascular cells, is increased (6-8). Endothelium-dependent vasodilation is impaired in hypertension, probably due to increased quenching of NO by $\bullet O_2^-$ (9). Treatment with antioxidants such as allopurinol (10) and hydrosoluble coenzyme Q10 (11)

improve endothelial function and lower blood pressure in hypertensives, who have been shown to have decreased concentrations of plasma antioxidants (12). Emerging evidence demonstrates that vitamins with antioxidant properties, such as ascorbic acid (vitamin C) and alpha tocopherol (vitamin E), also have blood pressure-lowering effects.

A large epidemiological study recently reported that dietary intake of ascorbic acid correlates inversely with hypertension and its clinical sequelae (13). In mild-moderate hypertensive patients, treatment with ascorbic acid (500 mg/day) significantly improved systolic and diastolic blood pressure (14) and increased plasma HDL cholesterol in female hypertensives (15). Vitamin C normalized vascular hyperresponsiveness to norepinephrine, as measured by forearm blood flow, in hypertensive patients (16). Furthermore, impaired endothelium-dependent vasodilation in peripheral and epicardial arteries in hypertensive subjects was improved by intra-arterial infusions of ascorbic acid (17,18). In experimental models of hypertension, vitamin C, alone or in combination with vitamin E, accelerates degradation of s-nitroglutathione, increases synthesis of NO and reduces blood pressure (19,20). Data relating to antihypertensive effects of vitamin E are conflicting. Most clinical trials failed to demonstrate beneficial effects of vitamin E supplementation in hypertensive patients (21,22). However in experimental models of hypertension vitamin E reduces blood pressure. In SHR, dietary supplementation of alpha-tocopherol for 3 months prevented development of increased blood pressure, reduced lipid peroxides in plasma and vessels and enhanced the total antioxidant status

(23,24). These effects were attributed to increased activation of vascular nitric oxide synthase (NOS) by alpha tocopherol (24-26). In Dahl salt-sensitive rats vitamin E administration ameliorated renal and vascular injury, but did not significantly reduce blood pressure (27).

Mechanisms underlying putative blood pressure-lowering effects of antioxidant vitamins have not been fully elucidated. Both vitamins C and E, which are potent scavangers of free radicals, stimulate activation of NOS activity and increase NO synthesis in endothelial cells (19,24-26). These effects could contribute to improved endothelial-dependent vasodilation in hypertension. Vitamin E inhibits expression of adhesion molecules (28), which could influence cell-cell interactions and consequently vascular structural changes associated with hypertension. Furthermore, gamma-tocotrienol has been shown to improve superoxide dismutase activity in vessels from SHR (29), suggesting that antioxidant vitamins alter activity of enzyme systems that generate reactive species.

The aim of the present study was to investigate whether vitamins C and E influence progression of blood pressure elevation in stroke prone SHR (SHRSP) by modulating the vascular redox state through changes in activation of NADPH oxidase and superoxide dismutase. Furthermore we tested the hypothesis that blood pressure effects of antioxidant vitamins ameliorate vascular functional and structural changes associated with hypertension.

METHODS

Animal experiments. At 16 weeks of age, SHR-SP were divided into three groups: control group (n=6), vitamin C-treated group (n=7) (ascorbic acid 1000 mg/day) and vitamin E-treated group (n=8) (α tocopherol 1000 IU/day). Ascorbic acid was added to drinking water and α tocopherol was mixed in sesame oil and added to the chow. All rats were placed on a high salt diet by adding 4% NaCl to the food to accelerate the progression and severity of hypertension. Rats were studied for 6 weeks. Systolic blood pressure (SBP) was measured weekly by the tail-cuff method and recorded on a model 7 polygraph fitted with a 7-P8 preamplifier and PCPB photoelectric pulse sensor (Grass Instruments Co, Cambridge, MA, USA). The average of 3 pressure readings was obtained. Rats were killed by decapitation at 22 weeks of age.

Study of small arteries. Superior mesenteric arteries were taken from the part of the mesenteric vascular bed that feeds the jejunum 8 to 10 cm distal to the pylorus and placed in cold physiological salt solution. A third-order branch of the mesenteric arterial tree (2 mm long) was dissected and mounted in a pressure myograph chamber as we described (30). Intraluminal pressure was set to 45 mm Hg with a servocontrolled pump. Endothelium-dependent relaxation was assessed by measuring the dilatory response of small arteries pre-contracted with norepinephrine ($5x10^{-5}$ M) to cumulative doses of acetylcholine (Ach) (10^{-7} to 10^{-5} M). Previous studies in salt-loaded SHRSP demonstrated that inhibition of

NO generation with NO synthase blockers abolishes Ach-stimulated responses, indicating that Ach-mediated vasodilation depends on endothelium-derived NO in this model (31). Endothelium-independent relaxation was assessed in norepinephrine pre-contracted vessels exposed to sodium nitroprusside (10^{-7} to 10^{-4} M). Lumen and media dimensions were measured with the intraluminal pressure maintained at 45 mmHg. Media Cross-Sectional Area was calculated as $(\pi/4) \cdot (D_e^2-D_i^2)$, where D_e and D_i were external and lumen diameters, respectively.

Detection of vascular $\bullet O_2^-$ by lucigenin chemiluminescence. The thoracic aorta was cleaned of adherent adipose tissue and 5 mm long rings were cut and incubated in Hepes buffer. Rings were maintained at 37°C for 30 minutes, rinsed, then gently transferred to test tubes containing warmed Hepes buffer and lucigenin (5 μmol/L). Lucigenin chemiluminescence was then recorded every 1.8 seconds for 3 minutes with a luminometer (AutoLumat LB953, EG&G Berthold, Munich, Germany). Chemiluminescence was expressed as counts/sec. Luminescence was also measured in tubes containing buffer and lucigenin without vascular rings and these blank values were subtracted from the chemiluminescence signals obtained from the aortic rings. $\bullet O_2^-$ generation was quantified against a standard curve of $\bullet O_2^-$ generation by xanthine/xanthine oxidase as previously described (6,8). Tissue $\bullet O_2^-$ formation was expressed as nmol/min/g dry tissue weight.

Measurement of total antioxidant status (TAS). Blood was collected from tail arteries in EDTA-containing tubes. Plasma was obtained by centrifuging blood at 1000 g for 10 mins. Plasma TAS was measured using the Calbiochem® total antioxidant status assay kit (Calbiochem-Novobiochem Corp. San Diego, CA) according to the manufacturer's instructions. The assay relies on the ability of antioxidants in the plasma to inhibit oxidation of 2,2' Azino-di-[3-ethylbenz-thiazoline sulphonate] (ABTS) to $ABTS^{\bullet+}$ by metmyoglobin. The amount of $ABTS^{\bullet+}$ produced is monitored by reading the absorbance at 600 nm. Under these reaction conditions, the antioxidants in the plasma cause suppression of the absorbance at 600 nm to a degree which is proportional to their concentration. The final plasma antioxidant concentration was obtained using the following formula: antioxidant concentration (mmol/L) = factor x (absorbance of blank-absorbance of sample); factor = concentration of standard/(absorbance of blank-absorbance of standard).

Measurement of NADPH oxidase activity.

Aortic segments were prepared as described above for measurement of $\bullet O_2$. NADPH oxidase was measured as described previously (32). Activity of NADPH oxidase was measured in a luminescence assay with 5 μmol/L lucigenin as the electron acceptor and 100 μmol/L NADPH as the substrate. The reaction was started by the addition of 100 μL of sample. Luminescence was measured every 1.8 seconds for 3 minutes in a luminometer (AutoLumat LB 953, Berthold). A buffer blank was subtracted from each reading. The amount of $\bullet O_2^-$ generated

was calculated by comparison with a standard curve using xanthine/xanthine oxidase (8). Protein content of the samples was measured by the Biorad method (Biorad Lab, Hercules, CA) and values of activity expressed as nmol $\bullet O_2^-$/min/g dry weight.

Measurement of superoxide dismutase activity.

Aortic segments were washed with 0.9% NaCl containing 0.16 mg/mL heparin to remove erythrocytes, which interferes with the assay. The tissue was homogenized, centrifuged and exposed to 400 μL extraction reagent (ethanol/chloroform 62.5/37.5 (V/V)). The sample was then vortexed and centrifuged at 3000 g for 5 minutes. Activity of superoxide dismutase was measured using a kit from Calbiochem (Calbiochem-Novobiochem Co. La Jolla CA). The assay kit utilizes 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo(c) fluorine reagent. This reagent undergoes alkaline autoxidation, which is accelerated by superoxide dismutase. Autoxidation of the reagent yields a chromophore, which absorbs maximally at 525 nm. The assay was performed according to manufacturer's instructions. Activity of superoxide dismutase was expressed as U/mg protein.

Data analysis

Data are presented as means ± SEM. Statistical analysis was performed using Analysis of Variance for Repeated Measures or Student's T-test. $p<0.05$ was considered significant.

RESULTS

Blood pressure. Systolic blood pressure (SBP) increased progressively for the first two weeks following salt-loading and then plateaued at ≈ 263 mmHg in the control group (figure 3.2.1). Vitamin C and vitamin E prevented progression of blood pressure elevation. Six weeks after salt loading, SBP was significantly lower in the vitamin C (234±14 mmHg) and vitamin E (227±10 mmHg) groups compared to untreated controls (265±6). Body weight did not differ significantly between groups throughout the duration of the experiment (data not shown).

Effects of vitamins C and E on vascular structure and function. The media to lumen ratio was significantly reduced in the vitamin C (9.1±0.9%, p<,0.05) and vitamin E groups (6.3±0.8%, p<0.01) compared with controls (13.0±1.0%). Media thickness was also significantly reduced (p<0.05) in the vitamin C (19.9±1.2 μm) and vitamin E groups (17.8±0.0.8 μm) versus controls (23.6±1.0 μm). Media cross sectional area was not influenced by either vitamin treatment (data not shown).

As shown in figure 3.2.2, vitamin C and vitamin E significantly increased (p<0.05) maximal Ach-induced relaxation compared with controls. These results indicate improved endothelium-dependent function. Endothelium-independent vasorelaxation in response to maximal sodium nitroprusside concentration was similar in all groups (control 70±14%, vitamin C 75±11%, vitamin E 68±16%).

Effect of vitamins on vascular $\bullet O_2^-$ generation and plasma antioxidant status. Superoxide anion concentration, as measured by lucigenin chemiluminescence, was significantly lower in the vitamin-treated groups compared with the control group (figure 3.2.3). These findings were associated with significantly increased plasma total antioxidant concentrations in the vitamin C (1.2±0.1 nmol/L, $p<0.05$) and vitamin E (1.4±0.1 nmol/L, $p<0.01$) -treated groups compared with the control group (0.85±0.06 nmol/L).

Effect of vitamins on activation of NADPH oxidase and superoxide dismutase. Activity of NADPH oxidase, a major source of $\bullet O_2^-$ in the vasculature, was significantly lower in salt-loaded SHRSP following 6 weeks of treatment with vitamin C or vitamin E compared with untreated rats (figure 3.2.4a). These findings were associated with a significant rise in superoxide dismutase activity in the vitamin-supplemented rats compared to the control group (figure 3.2.4b).

DISCUSSION

The major findings of the present study are that 1) blood pressure-lowering effects of vitamin C and vitamin E are associated with improved endothelium-dependent vasodilation and amelioration of vascular structural changes, 2) vitamin supplementation decreases vascular oxidative stress and improves total antioxidant status, and 3) antioxidant properties of vitamins C and E are associated with decreased activation of NADPH oxidase and increased activity of superoxide dismutase. These data suggest that in addition to the

known free radical scavanging properties of antioxidant vitamins, they may influence the vascular redox state in severely hypertensive rats by modulating $\bullet O_2^-$-generating enzyme systems.

Vitamins C and E prevented the progression of hypertension in salt-loaded SHRSP. Previous studies investigating effects of these vitamins in experimental models of hypertension, have shown, for the most part, significant antihypertensive effects (19,23-25). Clinical studies reported blood pressure-reducing actions of vitamin C, particularly in elderly patients (13-18). However, clinical data relating to vitamin E have been disappointing. Results from the Hope trial (21) and the Collaborative Group of the Primary Prevention Project (22), where hypertensive patients were treated with vitamin E, 400 IU/day or 300 mg/day respectively, did not demonstrate any clinically relevant effects on blood pressure. Reasons for these conflicting data may relate, in part, to the fact that in experimental studies vitamin E is supplemented at higher doses (800-1000 IU/day) than those used in clinical trials (300-500 IU/day).

In the model studied here the blood pressure-lowering actions of vitamins C and E were accompanied by improved endothelium-dependent vasodilation and decreased vascular hypertrophy suggesting that these processes are redox-sensitive. Our findings are in keeping with others that salt-induced hypertension, as well as SHRSP, are hypertensive models of oxidative stress (3,9). Mechanisms whereby antioxidant vitamins influence vascular function could be through free radical scavanging, which decreases NO quenching by $\bullet O_2^-$

thereby increasing bioavailability of the potent vasodilator NO. Vitamins C and E have also been shown to directly stimulate activity of NOS by increasing intracellular tetrahydrobiopterin, which would further increase NO synthesis (17,24,29). Although we did not assess the NO system in our model, previous studies demonstrated that antioxidant vitamins increase NOS activity and NO generation in arteries from SHR (23,24). Free radicals cause extensive cellular damage, facilitate lipid peroxidation, increase intracellular free Ca^{2+} concentrations, and stimulate inflammatory- and growth-signaling events in vascular smooth muscle cells (33-35). These processes could contribute to vascular structural changes associated with hypertension, especially since $\bullet O_2^-$ and H_2O_2 stimulate hypertrophy and hyperplasia (35). In both vitamin-treated groups, media thickness was less than that in control untreated rats. These results suggest that antioxidants improve integrity of vascular structure, possibly by preventing the cellular damage induced by oxygen free radicals.

Supplementation with vitamins C and E decreased vascular $\bullet O_2^-$ and increased total plasma antioxidant status. These findings confirm the antioxidant properties of vitamins C and E and indicate that treatment influences generation of oxygen free radicals and improves antioxidant defenses in our model. Processes contributing to these effects are probably related to the direct scavanging actions of vitamins C and E. However, it is possible that these vitamins may also influence the vascular redox state by modulating activity of enzyme systems that generate reactive oxygen species. We demonstrate the

novel findings that in the vitamin-treated rats, activation of NADPH oxidase is decreased and activity of superoxide dismutase is increased. Since NADPH oxidase is the major source of superoxide anion in vascular cells, decreased activation of the enzyme would result in reduced generation of the oxygen free radical. On the other hand, enhanced activation of superoxide dismutase would lead to increased dismutation of $\bullet O_2^-$, which further decreases $\bullet O_2^-$ concentration. From our study we can not elucidate which of these systems is more important but together they could contribute to overall reduction in generation of reactive oxygen species and improved oxidative status, as we observed in the treated rats. Mechanisms whereby antioxidant vitamins influence NAD(P)H oxidase and superoxide dismutase are ill-defined, but they may play an important role in the regulation of protein expression of the enzymes at the transcriptional or post-translational levels (36). It is also possible that vitamins C and E directly influence biological activity of the enzymes. Vitamin E, which is hydrophobic and located within the cell membrane (36-38) could alter cell membrane-associated NAD(P)H oxidase by inhibiting or interrupting complex formation of the NAD(P)H oxidase subunits. On the other hand, vitamin C is located in the cytoplasmic and mitochondrial compartments, which are superoxide dismutase-rich regions (36,38). In addition to the direct actions of vitamin C, some of its effects could be mediated via vitamin E, which can act as a pro-oxidant or an anti-oxidant (36,38). Vitamin C prevents the pro-oxidant activity of vitamin E by decreasing the activity of α-tocopheroxyl-radical to α-

tocopherol, thereby acting as a co-antioxidant and further contributing to increased total antioxidant status and reduced oxidative stress.

Findings from our study confirm the role of redox-sensitive processes in the pathogenesis of hypertension in salt-loaded SHRSP. However it is still unclear whether elevated amounts of free radicals initiate the development of hypertension or whether they are a consequence of the disease process itself. Furthermore, it may be possible that salt itself could influence vascular redox status. We can also not exclude the possibility that some of the vascular effects of vitamins C and E observed in our model may be due to blood pressure-lowering effects and not necessarily to direct actions of treatment. Elucidation of these aspects await clarification

In summary we have demonstrated that chronic treatment with vitamins C and E prevents progression of blood pressure elevation in severely hypertensive rats. These effects are associated with improved endothelium-dependent vasodilation, decreased vascular hypertrophy and increased plasma antioxidant concentration. In addition, we have identified that vitamins C and E modulate activity of NADPH oxidase and superoxide dismutase, which could contribute, at least in part, to decreased vascular $\bullet O_2^-$ and improved antioxidant status. Our data support an important role for oxidative stress in the pathogenesis of hypertension in salt-loaded SHRSP.

Acknowledgements

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Figure 3.2.1

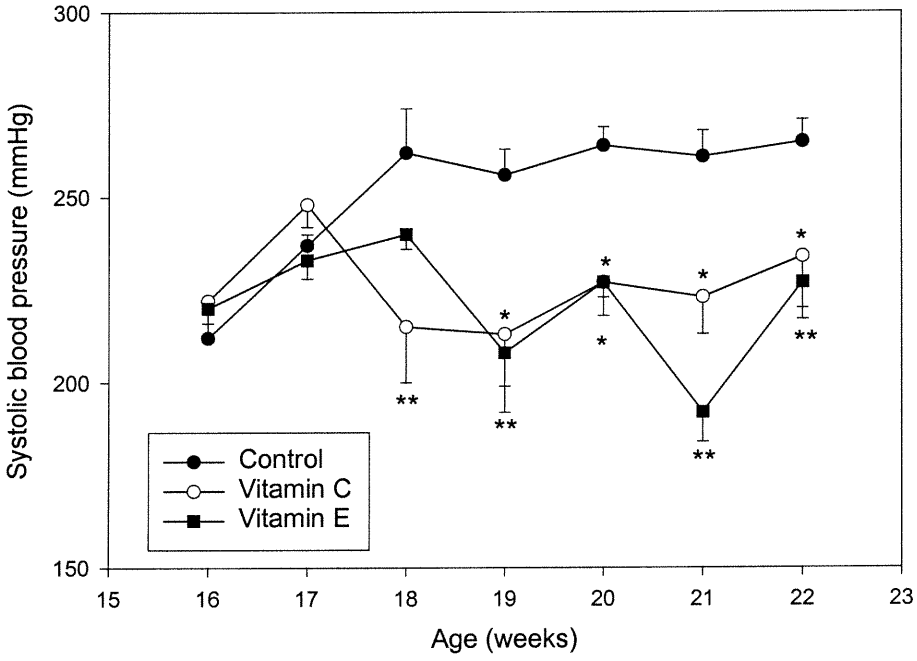

Systolic blood pressure (mmHg)
300
250
200
150
15
16
17
18
19
20
21
22
23
Age (weeks)
Control
Vitamin C
Vitamin E
*
**

Figure 3.2.2

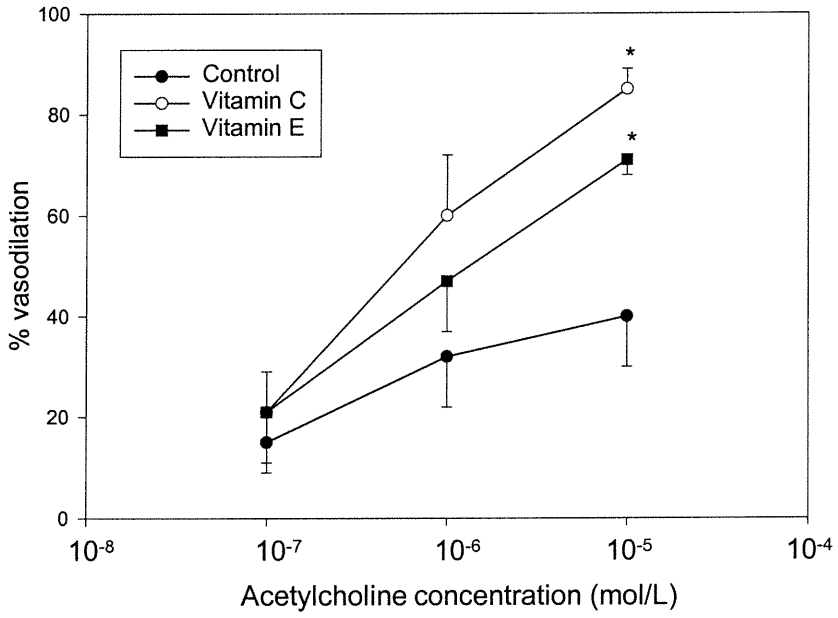

100
80
60
40
20
0
% vasodilation
Control
Vitamin C
Vitamin E
*
*
10-8
10-7
10-6
10-5
10-4
Acetylcholine concentration (mol/L)

Figure 3.2.3

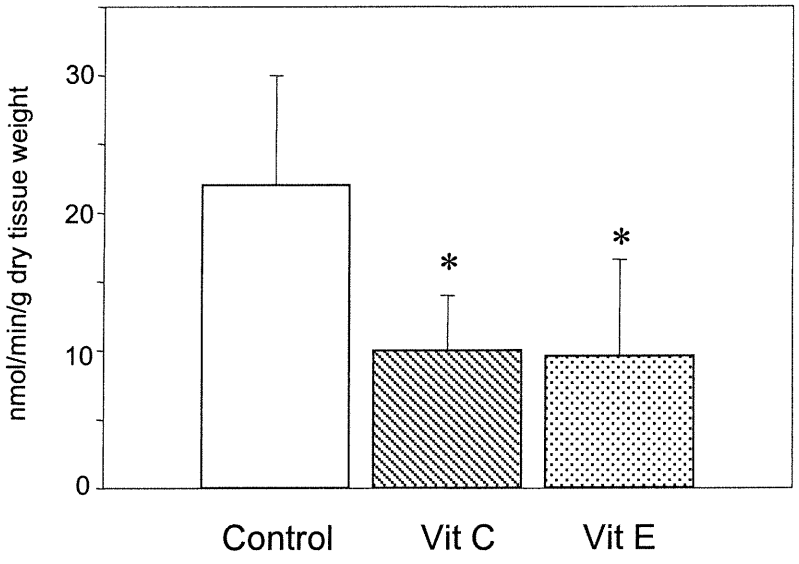
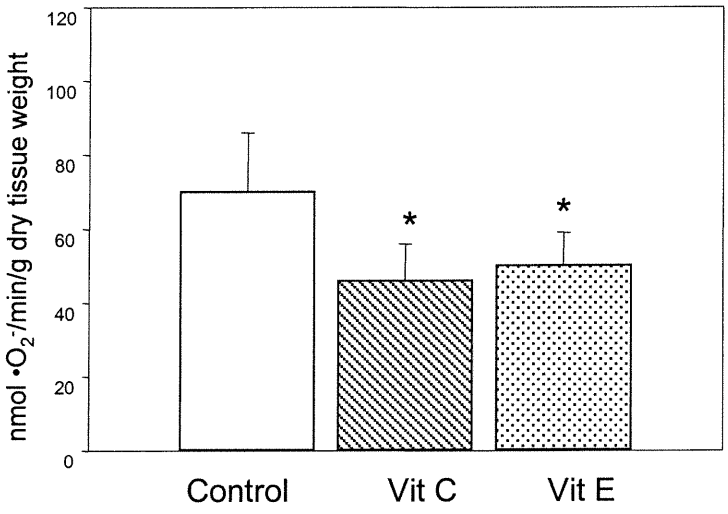


Figure 3.2.4

4a



4b

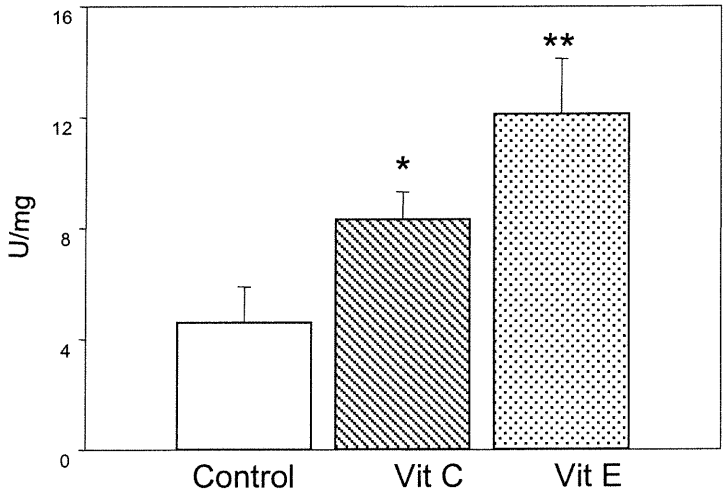


Figure Legends

Figure 3.2.1

Line graphs demonstrate systolic blood pressure in control and vitamin-treated groups. From 16 weeks of age, SHRSP were fed a high salt diet (4% NaCl) *p,0.05, **p<0.01 vs control counterpart.

Figure 3.2.2

Line graphs demonstrate vascular relaxations to acetylcholine in control and vitamin-treated salt-loaded SHRSP. Segments of small mesenteric arteries were mounted as pressurized systems as described in the Methods section. Relaxations were studied after the arteries had been pre-constricted with norepinephrine ($5x10^{-5}$ M). Each data point is expressed as the mean ±SEM. *p<0.05 vs control group.

Figure 3.2.3

Vascular $\bullet O_2^-$ production assessed by lucigenin chemiluminescence (5 μmol/L). Aortic segments from untreated and vitamin-treated rats were studied. Results are means ±SEM. *p<0.05, vs control.

Figure 3.2.4

Bar graphs demonstrate activity of NADPH oxidase (figure 3.2.4a) and superoxide dismutase (figure 3.2.4b) in aortic segments from untreated and vitamin-treated salt-loaded SHRSP. Results are expressed as means ± SEM. *p<0.05, **p<0.01 vs control group.

3.3
Chronic Treatment with a Superoxide Dismutase Mimetic Prevents Vascular Remodeling and Progression of Hypertension in Salt-Loaded Stroke-Prone SHR

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Running title: Tempol effects on BP and vascular structure and function in SHRSP

Am J Hypertens 2001 (in press)

My contribution to this study:

Preparation of rat diets,

Detection of vascular $\bullet O_2^-$ by lucigenin chemiluminescence,

Measurment of total antioxidant status (TAS),

Analysis of data.

ABSTRACT

Oxidative stress has been implicated in the pathogenesis of hypertension. The aim of the present study was to determine whether increased generation of vascular superoxide anion ($\bullet O_2^-$) contributes to blood pressure elevation by influencing vascular function and structure in severely hypertensive rats. Sixteen-week-old stroke-prone SHR (SHRSP) (n = 12) were randomly divided into two groups to receive the superoxide dismutase mimetic, tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl) (1 mmol/L in the drinking water) or ordinary tap water. Both groups were fed a high salt diet (4% NaCl). Systolic blood pressure (SBP) was measured weekly for 6 weeks by the tail cuff method. Rats were then killed and vascular structure (media to lumen ratio) and endothelial function (acetylcholine (Ach)–induced vasodilation) were assessed in small mesenteric arteries mounted as pressurized preparations. Vascular $\bullet O_2^-$ concentration was measured by lucigenin (5 μmol/L) chemiluminescence. Plasma total antioxidant status was assessed spectrophotometrically. SBP increased from 212±7 to 265±6 mmHg in the control group, whereas progression of hypertension was prevented in the tempol-treated group (209±5 to 208±8 mmHg). Tempol significantly reduced ($p<0.01$) the media:lumen ratio (7.2±0.01%) compared with controls (12.0±0.01%). Maximal Ach-induced dilatation was altered in control rats (40±9%), and was not significantly influenced by tempol (57±17%). Vascular $\bullet O_2^-$ concentration was significantly lower ($p<0.01$) and plasma total antioxidant concentration was significantly

higher ($p<0.05$) in the treated group compared with controls. In conclusion, the superoxide dismutase mimetic tempol prevents progression of hypertension. These processes are associated with attenuated vascular remodeling, decreased vascular $\bullet O_2^-$ concentration and increased antioxidant status. Our data suggest that oxidative stress plays an important role in vascular damage associated with severe hypertension in salt-loaded SHRSP.

INTRODUCTION

Oxidative stress, which is the imbalance between prooxidants and antioxidants, has been implicated in the pathogenesis of experimental and essential hypertension (1,2). The major vascular oxidant is superoxide anion ($\bullet O_2^-$), which is rapidly dismutated to hydrogen peroxide (H_2O_2) by the antioxidant enzyme superoxide dismutase (SOD) (3). $\bullet O_2^-$ and H_2O_2 have recently been identified as important intracellular and intercellular second messengers that modulate signaling pathways associated with vascular contraction and growth (4,5). Mechanisms whereby these reactive oxygen species regulate contraction may be through modulation of intracellular Ca^{2+} and/or via quenching of the vasodilator NO to form peroxynitrite, thereby depleting NO in endothelial cells (6,7). In addition $\bullet O_2^-$ and H_2O_2 are mitogenic and stimulate vascular smooth muscle cell hyperplasia and hypertrophy by activating mitogen-activated protein kinases and induction of proto-oncogene expression (8-10). Consequently free-radical-mediated oxidative processes play a key role in regulating vascular function and structure.

In hypertension, small and large arteries are characterized by vascular remodeling and decreased endothelium-dependent vasodilation (11,12), which may be due, at least in part, to increased generation of reactive oxygen species. Increased oxidative stress has been demonstrated in many models of experimental hypertension. In SHR (13-15), Ang II-induced hypertension (16,17),

DOCA-salt hypertension (18), Dahl salt-sensitive hypertension (19) and 2 kidney 1 clip (2K1C) renovascular hypertension (20), vascular production of $\bullet O_2^-$ is increased. In swine chronically infused with Ang II, blood pressure elevation was closely associated with increased plasma isoprostane F2α, a marker of oxidative stress (21). Treatment with the $\bullet O_2^-$ scavanger, SOD, significantly reduced blood pressure in Ang II-infused hypertensive rats (16). Tempol, a metal-independent, membrane-permeable SOD mimetic, normalized blood pressure and renal vascular resistance and reduced renal excretion of 8-iso-prostane F2α in SHR (14, 22). Moreover, tempol has recently been shown to decrease vascular resistance in the brain, heart, liver, kidney and intestine in Ang II-induced hypertensive rats (23). Hypertension has also been associated with low levels of endogenous antioxidants such as vitamin C, vitamin E and glutathione peroxidase (24-26).

Although these observations implicate oxidative stress in the pathogenesis of hypertension, the extent to which reactive oxygen species contribute to vascular functional and structural alterations in hypertension, particularly severe hypertension, remains unclear. Therefore, the objective of the present study was to determine the vascular oxidative stress and plasma antioxidant status in salt-loaded stroke-prone SHR, a model of malignant hypertension. In addition, we investigated the role of $\bullet O_2^-$ on progression of hypertension, vascular structure and endothelial function, by assessing effects of 6-week treatment of tempol.

METHODS

Animal experiments

The study was conducted according to recommendations from the Animal Care Committee of the Clinical Research Institute of Montreal (IRCM) and the Canadian Council of Animal Care. Male SHRSP were studied. Rats were bred at the IRCM, with the original breeding pair obtained from the National Institutes of Health (Bethesda, Maryland). They were housed at 22°C and 60% humidity under a 12-hour light/dark cycle and were maintained on standard chow. At 16 weeks of age, rats were divided into two groups: control group (n=6) and tempol-treated group (n=6) (1 mmol/L tempol mixed in the drinking water). Tempol is readily stable in water and was prepared fresh daily. All rats were placed on a high salt diet by adding 4% NaCl to the food to accelerate the progression and severity of hypertension. Rats were studied for 6 weeks. Systolic blood pressure (SBP) was measured weekly by the tail-cuff method and recorded on a model 7 polygraph fitted with a 7-P8 preamplifier and PCPB photoelectric pulse sensor (Grass Instruments Co, Cambridge, MA, USA). The average of 3 pressure readings was obtained. Rats were killed by decapitation at 22 weeks of age.

Study of small arteries

Superior mesenteric arteries were taken from the part of the mesenteric vascular bed that feeds the jejunum 8 to 10 cm distal to the pylorus and placed in cold physiological salt solution (PSS) of the following composition: NaCl 120 mmol/L, $NaHCO_3$ 25 mmol/L, KCl 4.7 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4$ 1.2

mmol/L, $CaCl_2$ 2.5 mmol/L, ethylenediaminetetraacetic acid (EDTA) 0.026 mmol/L, and glucose 5.5 mmol/L. A third-order branch of the mesenteric arterial tree (approximately 2 mm in length) was carefully dissected 1 mm from the intestine and cleaned of all adherent connective tissue under a dissecting microscope. The arterial segments were mounted in a pressure myograph chamber as previously described (11) and slipped onto two glass microcannulae. The axial length of the arterial segment was adjusted by carefully positioning the cannula until vascular walls were parallel without any stretch. Intraluminal pressure was set to 45 mm Hg with a servocontrolled pump. Vessels were then equilibrated for 1 hour with PSS which was bubbled with 95% air (21% O_2) and 5% CO_2 to give a pH of 7.4-7.45 and heated to 37°C.

Endothelium-dependent and -independent relaxation were assessed by measuring the dilatory response of small arteries pre-contracted with norepinephrine ($5x10^{-5}$ M) to cumulative doses of acetylcholine (Ach) (10^{-7} to 10^{-5} M) and sodium nitroprusside (10^{-7} to 10^{-4} M). Thereafter, mesenteric arteries were deactivated with Ca^{2+}-free PSS containing 10 mmol/L EGTA for 30 minutes to eliminate tone. Lumen and media dimensions were measured with the intraluminal pressure maintained at 45 mmHg. Media Cross-Sectional Area was calculated as $(\pi/4) \cdot (D_e^2-D_i^2)$, where D_e and D_i were external and lumen diameters, respectively.

Detection of vascular $\bullet O_2^-$ by lucigenin chemiluminescence

The method for measuring lucigenin chemiluminescence was based on that described by Harrisons group (27). The descending thoracic aorta was cleaned of adherent adipose tissue and 5 mm long rings were cut and incubated in Hepes buffer (in mmol/L, NaCl 119mM, HEPES 20Mm, KCl 4.6mM, $MgSO_4$ 1.0mM, Na_2HPO_4 0.15mM, KH_2PO_4 0.4mM, $NaHCO_3$ 5mM, $CaCl_2$ 1.2mM, glucose 5.5mM), which was bubbled continuously with 95% air (21%O_2)-5% CO_2 to maintain pH 7.4. Rings were maintained at 37°C for 30 minutes, rinsed, then gently transferred to test tubes containing warmed Hepes buffer and lucigenin (5 μmol/L), an acridylium dinitrate. Lucigenin chemiluminescence was then recorded every 1.8 seconds for 3 minutes with a luminometer (AutoLumat LB953, EG&G Berthold, Munich, Germany). Chemiluminescence was expressed as counts/sec. Luminescence was also measured in tubes containing buffer and lucigenin without vascular rings and these blank values were subtracted from the chemiluminescence signals obtained from the aortic rings. $\bullet O_2^-$ generation was quantified against a standard curve of $\bullet O_2^-$ generation by xanthine/xanthine oxidase as previously described (27). Tissue $\bullet O_2^-$ formation was expressed as nmol/min/g dry tissue weight.

Measurement of total antioxidant status (TAS)

Blood was collected from tail arteries in EDTA-containing tubes. Plasma was obtained by centrifuging blood at 1000 *g* for 10 mins. Plasma TAS was measured using the Calbiochem® total antioxidant status assay kit (Calbiochem-

Novobiochem Corp. San Diego, CA) according to the manufacturers' instructions. The assay relies on the ability of antioxidants in the plasma to inhibit oxidation of 2,2' Azino-di-[3-ethylbenz-thiazoline sulphonate] (ABTS) to $ABTS^{\bullet+}$ by metmyoglobin. The amount of $ABTS^{\bullet+}$ produced is monitored by reading the absorbance at 600 nm. Under theses reaction conditions, the antioxidants in the plasma cause suppression of the absorbance at 600 nm to a degree which is proportional to their concentration. The final plasma antioxidant concentration was obtained using the following formula: antioxidant concentration (mmol/L) = 1.5 mmol×[(absorbance of H_2O added – initial blank-H_2O absorbance) – (absorbance of sample added – initial blank-sample absorbance)] / [(absorbance of H_2O added – initial blank-H_2O absorbance) – (absorbance of standard added – initial blank-standard absorbance)].

Data analysis

Data are presented as means ± SEM. Statistical analysis was performed using Analysis of Variance for Repeated Measures or Student's T-test. Differences between means were analyzed for "simple main effects" using a *post hoc* Student Newman-Keul's test. $p<0.05$ was considered significant.

RESULTS

Blood pressure

Salt loading resulted in a progressive increase in SBP from 212 ± 7 to 265 ± 6mmHg in the control group. Tempol prevented progression of blood

pressure elevation (209 ± 5 to 208 ± 8 mmHg) (figure 3.3.1). From 2 weeks of treatment until the termination of the experiment at 6 weeks, SBP was significantly lower ($p<0.01$) in the treated group compared with control counterparts. Body weight was not significantly different between groups throughtout the duration of the experiment (data not shown).

Effects of tempol on vascular structure and function

Figure 3.3.2 demonstrates the morphometric characteristics of small mesenteric arteries from control and tempol-treated groups. Lumen diameter was not different between groups. The media width and media to lumen ratio were significantly greater ($p<0.01$) in control rats compared with tempol-treated rats. Media cross sectional area was not influenced by tempol treatment.

Figure 3.3.3 shows the vasorelaxatory response of small arteries to cumulative doses of Ach. Maximal Ach-induced dilation was altered in control rats (40 ± 9%). Tempol treatment increased maximal Ach-induced relaxation (57 ± 17%) but significance was not achieved. Endothelium-independent vasorelaxation in response to maximal sodium nitroprusside concentration was similar in both groups (≈70%, data not shown). These data indicate that neither endothelium-dependent nor endothelium-independent vasodilation are influenced by tempol treatment in salt-loaded SHRSP.

Effect of tempol on vascular $\bullet O_2^-$ generation and plasma antioxidant status

As demonstrated in figure 3.3.4a, tempol significantly reduced ($p<0.01$) superoxide levels in aortic rings from salt-loaded SHRSP. These findings were

associated with significantly increased ($p<0.05$) plasma total antioxidant levels in the tempol-treated group compared with the control group (figure 3.3.4b).

DISCUSSION

The major finding in the present study is that chronic treatment with tempol attenuates progression of hypertension in salt-loaded SHRSP, a model of malignant hypertension. Moreover, we demonstrate that this membrane permeable SOD mimetic prevents vascular remodeling and that these processes are associated with decreased generation of vascular $\bullet O_2^-$ and increased plasma antioxidant levels. The effect of tempol on vasodilatory responses did not reach significance. Our data suggest that oxidative stress plays an important role in the pathogenesis of salt-mediated hypertension in SHRSP. Mechanisms whereby $\bullet O_2^-$ increases blood pressure in this model appear to be through redox-sensitive processes that influence vascular remodeling.

Increasing evidence supports a role for reactive oxygen species in the pathogenesis of hypertension. Earlier studies suggested that only Ang II-sensitive hypertension was redox-dependent (16,17,28), but it is now clear that oxidative stress is important in many models of experimental hypertension as well as in essential hypertension (13-15,18,19,29,30). In the present study we demonstrate that $\bullet O_2^-$ quenching by tempol, prevents progression of blood pressure elevation, suggesting a role for $\bullet O_2^-$ in severely hypertensive SHRSP. Kerr et al. (31) reported that SHRSP have excess $\bullet O_2^-$ in aortic vessels, but to our knowledge, there are no data on salt-loaded SHRSP, a model of malignant

hypertension. Underlying causes for increased arterial $\bullet O_2^-$ production in hypertension have not been fully identified, but increased activiation of NAD(P)H oxidase (32), increased expression of NAD(P)H oxidase subunits, particularly p22phox (32,33), and stretch of vascular cells (34) have been implicated. Furthermore, it has recently been reported that in SHRSP, $\bullet O_2^-$ is generated by eNOS (31). In our study, the salt-dependent increase in blood pressure as well as vascular $\bullet O_2^-$ formation were attenuated by tempol, suggesting that salt itself may influence mechanisms that produce $\bullet O_2^-$. This is supported by other studies, which demonstrated that aortic superoxide generation is increased in salt-dependent hypertension (18,19).

Vascular pathological processes associated with hypertension include vascular remodeling, due to hyperplasia, hypertrophy and/or alterations in extracellular matrix deposition, and impaired endothelium-dependent vasodilation (11,12). These events contribute to narrowing of the arterial lumen, and consequent increased peripheral resistance and blood pressure. To evaluate whether superoxide contributes to these processes in salt-loaded SHRSP, we tested chronic effects of tempol on structural and functional changes in small mesenteric arteries (corresponding to resistance arteries). Tempol is a metal-independent, membrane permeable, stable SOD mimetic that is specific to the superoxide radical (35). In tempol-treated rats, media thickness and media:lumen ratio were significantly less than in untreated rats, suggesting that $\bullet O_2^-$. quenching by the SOD mimetic prevents vascular remodeling associated

with salt-dependent hypertension in SHRSP. However, we can not exclude the possibilty that blood pressure lowering itself, may influence, at least in part, arterial remodeling. Nevertheless, our findings suggest that $\bullet O_2^-$ does influence vascular wall structure, either directly or indirectly. Cellular mechanisms underlying these actions may be due to attenuation of superoxide–induced vascular smooth muscle cell growth (5,8,36). This is supported by *in vitro* studies, which demonstrated that mitogenic effects of oxygen free radicals are reduced when $\bullet O_2^-$ generation is inhibited (10,37).

In addition to influencing remodeling, excess $\bullet O_2^-$ may contribute to blood pressure elevation by reducing NO bioavailibilty, thereby altering endothelium-dependent vasodilation. Many studies have reported impaired vascular relaxation in hypertensive models associated with oxidative stress (38,39). Furthermore, treatment with various antioxidants, has been shown to improve endothelial dysfunction (39,40). In the present study, endothelium-dependent vasodilation was markedly altered. Although tempol improved maximal Ach-elicited vasodilation, significance was not achieved. These data indicate that increased vascular $\bullet O_2^-$ does not contribute significantly to altered vascular tone in salt-loaded hypertensive SHRSP, and that other mechanisms are probably involved. Taken together our findings suggest that in this model of severe hypertension, excess $\bullet O_2^-$ plays a more important role in modulating vascular structure than endothelial function.

Vascular $\bullet O_2^-$, generated mainly by NAD(P)H oxidase, acts extracellularly and intracellularly, where it elicits harmful effects such as lipid peroxidation, extravasation of plasma proteins, DNA damage, and inflammatory responses (41,42). To verify that tempol scavanged oxygen free radicals in our model, we measured vascular $\bullet O_2^-$ by the lucigenin chemiluminescence technique. Recently 5 μmol/L lucigenin has been shown to correlate well with electron spin resonance as a quantitative measurement of $\bullet O_2^-$ generation (43,44). Compared to the untreated group, tempol significantly reduced $\bullet O_2^-$ production, indicating effectiveness of treatment. Furthermore, tempol treatment was associated with increased plasma total antioxidant concentration, suggesting overall improvement in the oxidative state. Mechanisms whereby tempol influences antioxidant status are unclear, but it may be possible that increased scavanging of $\bullet O_2^-$ by the SOD mimetic, would increase availability of endogenous antioxidants.

In summary, we have demonstrated that the membrane permeable SOD mimetic tempol attenuates the progression of blood pressure elevation in a model of severe hypertension. These effects were associated with reduced vascular hypertrophy, decreased generation of $\bullet O_2^-$ and increased plasma antioxidant concentrations. Our findings suggest that oxidative stress plays an important role in the pathogenesis of hypertension in salt-loaded SHRSP, and that these processes may be due, in part, to redox-sensitive vascular remodeling.

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Figure 3.3.1

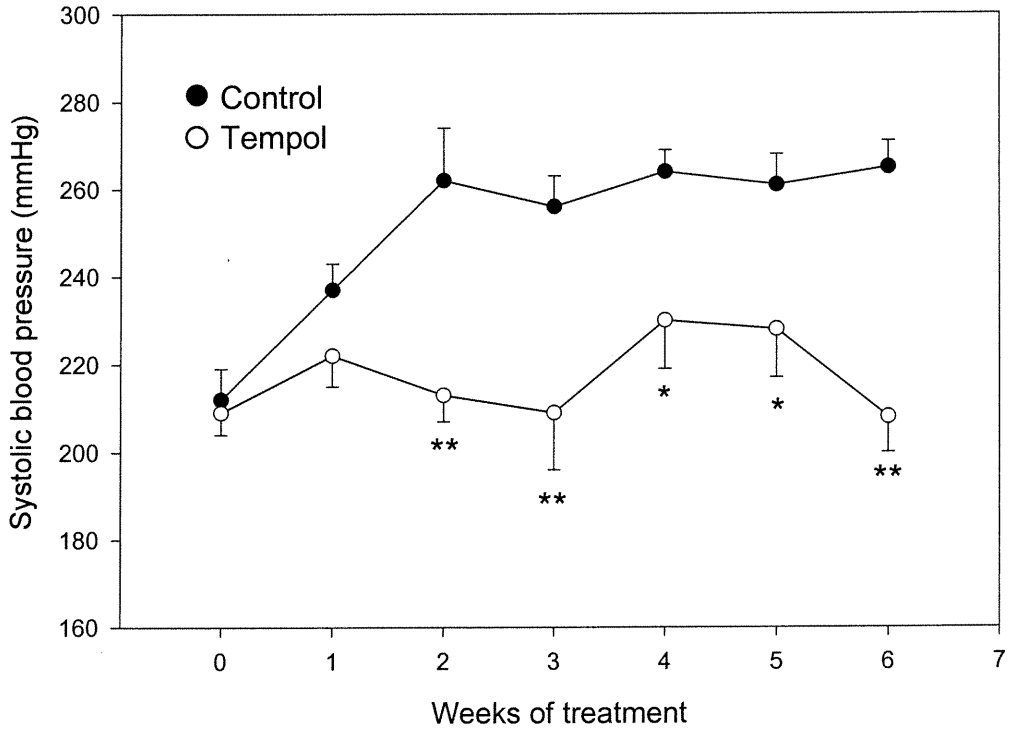
300
280
260
240
220
200
180
160
Systolic blood pressure (mmHg)
Control
Tempol
**
**
*
*
**
0
1
2
3
4
5
6
7
Weeks of treatment

Figure 3.3.2

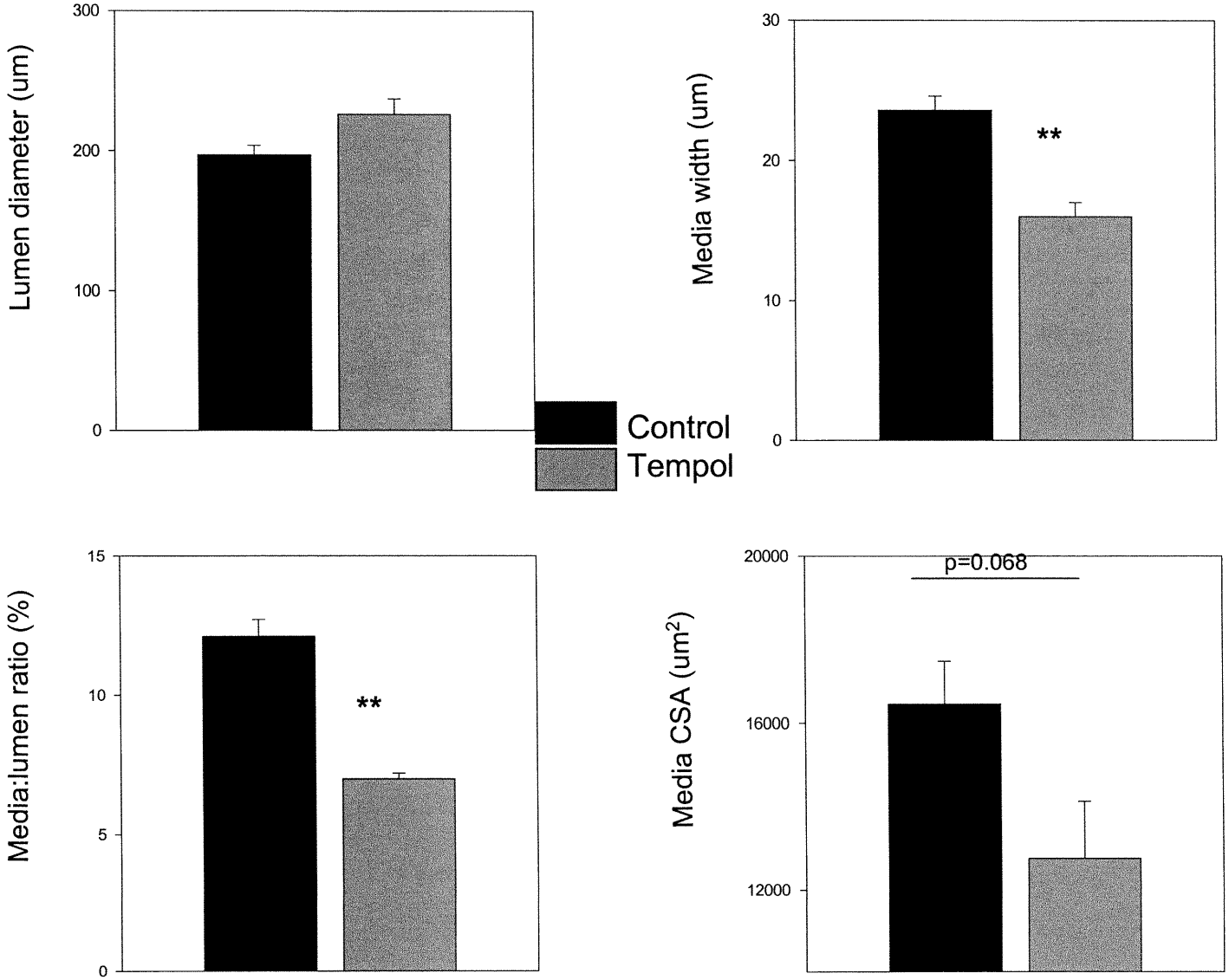
Lumen diameter (um)
300
200
100
0
Control
Tempol
Media width (um)
30
20
10
0
**
Media:lumen ratio (%)
15
10
5
0
**
Media CSA (um2)
20000
16000
12000
p=0.068

Figure 3.3.3

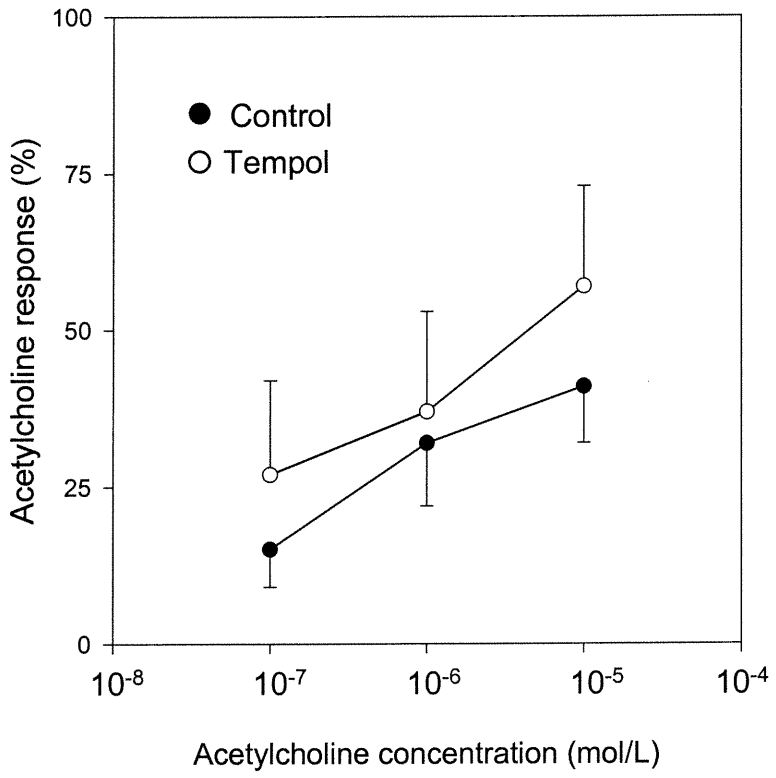
100
75
50
25
0
Control
Tempol
Acetylcholine response (%)
10-8
10-7
10-6
10-5
10-4
Acetylcholine concentration (mol/L)

Figure 3.3.4

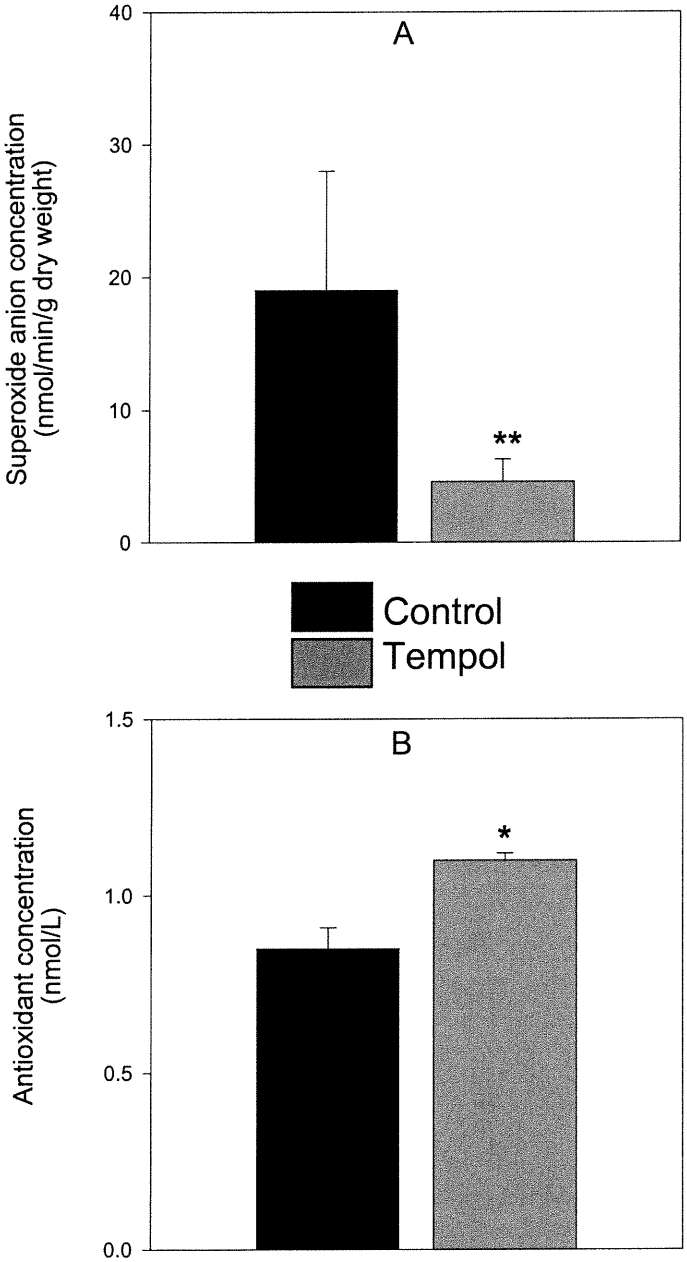
A
Superoxide anion concentration (nmol/min/g dry weight)
40
30
20
10
0
**
Control
Tempol
B
Antioxidant concentration (nmol/L)
1.5
1.0
0.5
0.0
*

FIGURE LEGENDS

Figure 3.3.1 Line graphs demonstrate systolic blood pressure in control (n = 6) and tempol-treated (n = 6) groups. From 16 weeks of age, SHRSP were fed a high salt diet (4% NaCl) and given either tap water (control group) or tap water-containing tempol (1 mmol/L) to drink. *p,0.05, **p<0.01 vs control counterpart.

Figure 3.3.2 Bar graphs demonstrate morphological characteristics of small mesenteric arteries in control and tempol groups. Arteries were obtained at the end of the experiment and examined as described in the Methods section. *p<0.05 vs control, **p<0.01 vs control.

Figure 3.3.3 Line graphs demonstrate acetylcholine (Ach) responses of small mesenteric arteries from control rats and tempol-treated rats. Vessels were precontracted with 5 x 10^{-5} norepinephrine. Arteries were obtained at the end of the experiment and mounted as pressurized systems as described in the Methods section.

Figure 3.3.4 Effects of tempol on vascular superoxide anion production and plasma antioxidant status. Figure 3.3.4a. Bar graphs demonstrate $\bullet O_2^-$ formation in aortic rings from control and tempol-treated salt-loaded SHRSP. $\bullet O_2^-$ was measured with 5 μmol/L lucigenin chemiluminescence after 6 weeks treatment. Figure 3.3.4b. Bar graphs demonstrate total antioxidant concentration in plasma from control and tempol-treated groups. Plasma was obtained at the end of the experiment. Total antioxidant status was measured spectrophotometrically using the Calbiochem® TCA kit as described in the Methods section. *$p<0.05$, **$p<0.01$ vs control.

IV. DISCUSSION

Results from these studies suggest that VSMC NAD(P)H oxidase is a major source of ROS and that inappropriate activation of NAD(P)H oxidase results in vascular oxidative stress, which may contribute to vascular changes in hypertension.

This is based on the following major findings:

1). Vascular smooth muscle cells possess a functionally active $\cdot O_2^-$ - generating enzyme system that is regulated by Ang II.

2). The major source of VSMC $\cdot O_2^-$ appears to be NAD(P)H oxidase, which resembles the neutrophil enzyme.

3). NAD(P)H oxidase – generated $\cdot O_2^-$ is increased in VSMCs from hypertensive rats.

4). Treatment with a SOD mimetic or antioxidant vitamins improves endothelial function and prevents structural changes in small arteries of hypertensive rats.

5). Antioxidant treatment of severely hypertensive rats attenuates progression and development of hypertension.

4.1 Use of lucigenin to measure vascular $\cdot O_2^-$ concentration

In our studies, vascular $\cdot O_2^-$ was measured using the acridylium dinitrite compound. However, there is some controversy as to the specificity of this probe for accurately detecting $\cdot O_2^-$. It has been shown that the concentration of

lucigenin is a critical parameter affecting the validity of this assay (145). Skatchkov et al. evaluated reduced concentrations of lucigenin (5 μmol/L) as a tool to quantify $\cdot O_2^-$ production in vascular tissue. Lucigenin-induced effects on endothelial function were assessed by isometric tension recording of isolated aortic rings suspended in organ baths. Effects of lucigenin on $\cdot O_2^-$ production were studied using spin trapping and electron spin resonance spectroscopy. Lucigenin at 250 μmol/L, but not at 5 μmol/L, caused a significant attenuation of endothelium-dependent relaxation to ACE, which was prevented by pretreatment with SOD. Spin-trapping studies revealed that lucigenin at 250 μmol/L increased vascular $\cdot O_2^-$ production several folds while 5 μmol/L lucigenin did not stimulate $\cdot O_2^-$ production. Thus, lucigenin at a concentration of 5 μmol/L seems to be a sensitive and valid probe for assessing $\cdot O_2^-$ in vascular tissue (145). Furthermore Griendling et al. point out that lucigenin has been criticized because of its ability to undergo redox cycling, in particular when NADH is used as a substrate. Low levels of lucigenin (<10 μmol/L) do not suffer from this drawback, and under these conditions, NADPH appears to be the major substrate of the vascular enzymes (146). In our experiments, NADPH oxidase activity was detected using 5 μmol/L lucigenin, a concentration that does not significantly induce auto-oxidation. Furthermore, we used NADPH as the substrate. Consequently we believe that the results presented in the present study, using lucigenin, accurately reflect intrinsic $\cdot O_2^-$ concentration.

4.2 ROS and NADPH oxidase

Vascular $\cdot O_2^-$ plays a critical role in vascular biology because it is a major source for many other ROS, particularly H_2O_2. ROS are important regulators of vascular cell functions and have been implicated in vascular pathology in hypertension (147). In our experiments, exposure of cells to Ang II increased activity of NAD(P)H oxidase almost 2-fold. In the presence of DPI, Ang II-induced activation of NAD(P)H oxidase was inhibited, indicating that NAD(P)H oxidase-derived ROS is regulated by Ang II. Other studies have shown that various vasoactive agents, in addition to Ang II (148~150), are associated with ROS production, such as endothelin-1 (151,152) and PDGF (6,150,153). There has been much controversy whether vascular NAD(P)H oxidase is the same as the leukocyte NAD(P)H oxidase. It was speculated that the oxidase might have much lower activity whose tissue distribution was much more widespread than that of the leukocyte oxidase. The activity of NAD(P)H oxidase in the VSMCs is only 1/20 that of neutrophils. Endothelial cells express only 1/100 of p22 phox and gp91 phox protein detected in leukocytes (154). This may relate to the fact that leukocyte oxidase generates ROS as part of a defense mechanism, whereas the principal function of vascular NAD(P)H oxidase provides oxidants for signaling purposes (6). It has recently been shown that all of the components of the neutrophil NADPH oxidase exist in endothelial and adventitial cells (126). mRNAs for gp91phox, p22phox, p47phox, and p67phox have been demonstrated in endothelial (51) and adventitial cells (12). In contrast, only

p22phox and p47phox have been definitively identified in VSMCs (51,155) Whereas, gp91 phox has not been found in VSMCs (29) which is pivotal for production of ROS (154). The finding that some nonphagocytic cells express p22phox in the absence of gp91phox raises the possibility that there are gp91phox isoforms that serve a similar function in these cells. Suh et al. (156) cloned a homologue of gp91phox (termed mox-1, recently re-named as nox-1, for NAD(P)H oxidase) from human colon carcinoma and rat aortic smooth muscle cells. Recently, other gp91phox homologues have been identified, as nox-2, nox-3, and nox-4 (105). To date, gp91phox has not been demonstrated in VSMCs.

Results from our studies demonstrate that VSMCs from human small arteries posess a functionally active NADPH oxidase that resembles the neutrophil enzyme. All five leukocyte NADPH oxidase subunits, including gp91phox, were expressed in human VSMCs from human resistance arteries. Similar to findings of others, we did not detect gp91phox in human aortic smooth muscle cells nor in rat VSMCs. Reasons for the differential expression of gp91phox in VSMCs from human small and large arteries is unclear.

4.3 Stretch and ROS production

In cell culture, stretch increases endothelial and vascular smooth muscle production of ROS. In perfused isolated vessels, elevations of pressure can

increase vessel Ang II production. Zafari et al. (157) showed that increased production of H_2O_2 in response to Ang II seems to be critical in the development of vascular smooth muscle hypertrophy caused by the peptide. Intratissue formation of Ang II plays a critical role in cardiovascular remodeling. Upregulation of these alternate pathways may occur through stretch, stress, and turbulence within the blood vessel. Luscher's group (158,159) reported that stretch of vascular cells can enhance production of both $\cdot O_2^-$ and H_2O_2. These findings raise the possibility that the direct effects of hypertension, which increases stretch of VSMCs in vivo, might also increase vascular production of ROS. Also, $\cdot O_2^-$ itself induces vasoconstriction. The results of Souza et al. (160) demonstrated that exposure to NADPH elicited a 20% increase in vascular tone, which was decreased by SOD mimetics in a concentration-dependent manner, suggesting that $\cdot O_2^-$ was responsible for this phenomenon. In cultured cells, this oxidase is activated by cyclic stretch, and it is conceivable that the direct mechanical effect of hypertension on the vessel wall has a similar effect in vivo. Thus, $\cdot O_2^-$ and vasoconstriction seem to influence each other.

4.4 Oxidant stress, antioxidants and NO

In light of previous studies of renovascular hypertension, genetic hypertension, and hypertension caused by exogenous Ang II, it is suggested that hypertension of almost any cause can increase vascular oxidant stress (21). Our results showed that antioxidants ameliorate the development of hypertension in

salt-loaded SHR-SP. In the aorta, the major endothelium-derived relaxing factor is NO, which rapidly reacts with $\cdot O_2^-$ to form the $ONOO^-$. Our results showed decreased generation of $\cdot O_2^-$, possibly by decreased NADPH oxidase activity. This would lead to reduced quenching of NO, and increased bioavailability of this important vasodilator.

Endothelial production of NO is crucial in the control of vascular tone, arterial pressure, smooth muscle cell proliferation and platelet adhesion to the endothelial surface. Impaired endothelium-derived NO bioactivity is a common feature of many vascular diseases including hypertension (161,162). The action of NO is particularly sensitive to the local availability of $\cdot O_2^-$. Both endothelial elaboration of NO and arterial relaxation in response to nitrovasodilators are dependent upon intact copper-zinc SOD activity (123). Laursen et al. (122) demonstrated an excess vascular $\cdot O_2^-$ production that is linked to reduced NO bioactivity. Conversely, increasing vascular SOD activity enhances NO-mediated arterial relaxation in experimental models of hypertension. Thus, scavenging $\cdot O_2^-$ has important implications for NO bioactivity in hypertension. Ascorbic acid efficiently scavenges $\cdot O_2^-$ in hypertension (125) indicating that NO bioavailibility is improved by parenteral ascorbic acid at supraphysiologic concentrations (~10 mmol/L).

Multiple mechanisms may account for the ability of ascorbate to preserve NO. These include ascorbate-induced decreases in LDL oxidation, scavenging of intracellular $\cdot O_2^-$, release of NO from circulating or tissue S-nitrosothiols, direct reduction of nitrite to NO, and activation of either endothelial NO synthase or

smooth muscle guanylate cyclase (163). α-Tocopherol, the other important antioxidative vitamin, inhibits LDL oxidation through an LDL-specific antioxidant action, decreases cellular production and release of ROS, inhibits endothelial activation and improves the biologic activity of endothelium-derived NO through a cell- or tissue-specific antioxidant action. In addition α-tocopherol, a number of thiol antioxidants have been shown to decrease adhesion molecule expression and monocyte-endothelial interactions (162).

However, clinical data relating to vitamin E effects on blood pressure have been disappointing. Results from the HOPE trial (164) and the Collaborative Group of the Primary Prevention Project (165), where hypertensive patients were treated with vitamin E, 400 IU/day or 300 mg/day respectively, did not demonstrate any clinically significant effects on blood pressure. Reasons for these conflicting data may relate, in part, to the fact that in experimental studies vitamin E is supplemented at higher doses (800-1000 IU/day) than those used in clinical trials (300-500 IU/day). Moreover, clinical trials have a number of limitations, for example, antioxidant treatment of patients with advanced disease (secondary prevention) may not provide information relevant to disease prevention in healthy individuals (primary prevention). HOPE trial was secondary prevention trial in which more than 75% of participants were treated with aspirin or other antiplatelet agents, and many participants also received β-blockers, lipid-lowering agents, and calcium channel blockers (179).

4.5 Antioxidative Mechanisms of Vitamin C, E and Tempol

Vit C is a water-soluble vitamin that is not synthesized by humans. Ascorbate functions as a reducing agent by delivering a hydrogen atom with its single electron to a ROS with a single unpaired electron in its outer ring. Ascorbic acid can induce collagen synthesis and lipid peroxidation. Vit C may act by normalizing altered glucose metabolism, decreasing excess endogenous aldehydes and increasing free sulfhydryl groups of membrane Ca^{2+} channels leading to the normalization of cytosolic [Ca^{2+}], vascular resistance and blood pressure (166)

There are a number of potential mechanisms underlying the beneficial effects of ascorbate on endothelial function. Firstly, ascorbate may decrease the levels of $\cdot O_2^-$ and oxidized LDL (167,168). Because of the facile reaction between $\cdot O_2^-$ and NO radicals, relatively high concentrations of ascorbate (10 mmol/L) are required to effectively inhibit the reaction of NO with $\cdot O_2^-$ (168). Secondly, ascorbate may indirectly enhance endothelium-dependent vasodilation by sparing intracellular thiols, which in turn stabilize NO through the formation of biologically active S-nitrosothiols. Thirdly, reducing agents such as ascorbate have also been implicated in the rapid release of NO from S-nitrosothiols (169,170).

It was shown that physiological concentrations of ascorbate increase the synthesis and biological activity of NO in cultured endothelial cells by increasing

intracellular tetrahydrobiopterin (123,171). Thus, a very likely mechanism whereby intracellular ascorbate stimulates NOS activity is regeneration of tetrahydrobiopterin from the trihydrobiopterin radical. Such a mechanism of action of ascorbate would also prevent NOS from leaking superoxide radicals.

Vit E is an essential lipid-soluble substance that acts as a potent nonenzymatic antioxidant in mammalian lipid membranes. Vit E exerts its principal antioxidant effect as a chain-breaker to impede ROS-induced lipid peroxidation. Cell membrane polyunsaturated free fatty acids possess available hydrogen atoms and therefore are attractive targets for ROS. Vitamin E donates hydrogen atoms to ROS and prevents disruption of the polyunsaturated free fatty acid double bonds (172).

Tempol (4-hhdroxy-2,2,6,6-tetramethyl poperidinoxyl) is a stable, membrane-permeable, metal-independent superoxide dismutase mimetic. Tempol is a small molecular weight cyclic nitroxide that has been used as a spin trap for $\cdot O_2^-$ and reduces superoxide-related injury in ischemia/reperfusion, inflammation and radiation (173).

In our in vivo experiments, SBP increased from 212±7 to 265±6 mm Hg in the control group, whereas progression of hypertension was prevented in the tempol-treated group (209±5 to 208±8 mm Hg). Tempol significantly reduced the media: lumen ratio. Maximal Ach-induced dilatation was altered in control rats (40±9%), and was not significantly influenced by tempol (57±17%). The previous in vivo studies in SHR showed that mesenteric vessels generate oxygen radicals

through xanthine oxidase (174), but several observations suggest that the NADH/NADPH oxidase system accounts for the majority of $\cdot O_2^-$ generation in the vessel wall (11,175). Our results demonstrated that in the Vit C and Vit E treated groups, NADPH oxidase activity, in addition to vascular $\cdot O_2^-$ concentration, was significantly lower. Tempol did not significantly influence NADPH oxidase activity. This may relate to the property of the $\cdot O_2^-$ scavenger, since tempol functions primarily as a SOD mimetic.

In our tempol-treated rats, media thickness and media:lumen ratio were significantly less than in untreated rats, suggesting that $\cdot O_2^-$. quenching by the SOD mimetic prevents vascular remodeling associated with salt-dependent hypertension in SHRSP. However, we can not exclude the possibility that blood pressure lowering itself, may influence, at least in part, arterial remodeling. In our study, endothelium-dependent vasodilation was markedly altered. Although tempol improved maximal Ach-elicited vasodilation, significance was not achieved. These data indicate that increased vascular $\cdot O_2^-$ does not contribute significantly to altered vascular tone in salt-loaded hypertensive SHRSP, and that other mechanisms are probably involved.

The aims of our studies were to evaluate whether antioxidant therapy influences development of hypertension in salt-loaded SHR-SP, a model that develops severe hypertension rapidly. We believe that the experimental design is adequate to address this specific question. However it would be useful to investigate whether antioxidants influence blood pressure in normal conditions

as well as to evaluate whether salt is the dependent variable. These aspects could be assessed by performing additional experiments in SHR-SP which are not salt-loaded and in control WKY. Some of these issues have been addressed by others. It seems that beneficial effects of anti-oxidant therapy are not dependent on salt-sensitivity of hypertensive rats (166,176~178)

4.6 Conclusions

In conclusion, our data demonstrate the novel findings that VSMCs from human small arteries possess a functionally active gp91phox–containing leukocyte-like NADPH oxidase. This enzyme, which is a major source of vascular ROS, is regulated by Ang II. The antioxidants, Vit C & E and tempol, reduce oxidative stress, improve vascular function and structure and prevent progression of hypertension in SHR-SP. These effects may be mediated via modulation of enzyme systems that generate free radicals. Taken together, our data support a functional redox-generating system in vascular smooth muscle cells that may play a role in vascular functional and structural changes associated with development and maintenance of hypertension, at least in salt-loaded SHRSP.

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