SPONTANEOUS TONE IN DEOXYCORTICOSTERONE ACETATE-SALT HYPERTENSIVE RATS

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ABSTRACT

This body of work tests the hypothesis that reactive oxygen species (ROS) contribute to the modulation of spontaneous tone observed in aortic rings from deoxycorticosterone acetate (DOCA)-salt hypertensive rats. In isolated organ baths, tension developed in rings from hypertensive but not SHAM-normotensive rats in response to increases in preload. Endothelial-denudation and nitric oxide (NO) synthase inhibition with N^G-nitro-L-arginine methyl ester (L-NAME) increased spontaneous tone. These results indicate that spontaneous tone is related to the preload and that NO plays a protective role opposing tone.

Basal superoxide anion ($O_2^{\cdot-}$) production was increased in aortic rings from DOCA-salt hypertensive rats. Stretch increased $O_2^{\cdot-}$ production even further. Tempol, an $O_2^{\cdot-}$ scavenger, or apocynin, an inhibitor of NADPH-oxidase, attenuated hypertension. Spontaneous tone was abolished by superoxide dismutase (SOD), tempol, or apocynin in endothelium-intact rings but not in endothelium-denuded rings nor in L-NAME treated rings. Catalase, the enzyme that breaks down hydrogen peroxide (H_2O_2), increased spontaneous tone. Taken together, these findings suggest that $O_2^{\cdot-}$ derived from NAD(P)H-oxidase modulates spontaneous tone primarily by scavenging NO, while H_2O_2 serves as a protective mechanism.

The cyclooxygenase (COX) inhibitor, valeroyl salicylate, and the thromboxane-prostaglandin (TP) receptor antagonist, SQ 29548, inhibited spontaneous tone. 20-hydroxyeicosatetraenoic acid (20-HETE) production was increased in rings from DOCA-salt hypertensive rats. Inhibition of the CYP4A isozyme with aminobenzotriazole decreased spontaneous tone. Exogenous 20-HETE increased spontaneous tone in an endothelium-dependent manner. These effects were blocked by the COX inhibitor or the TP-receptor antagonist. Thus 20-HETE increases tone, an effect likely mediated by its COX metabolites.

Cromakalim, a K_{ATP} channel opener, abolished spontaneous tone in a glibenclamide-sensitive fashion. In aortic cells from DOCA-salt hypertensive rats, ATP-dependent $K^+(K_{ATP})$ channel currents were either absent or weak in response to challenges by elevated extracellular K^+ and by cromokalim. These findings suggest that the function of K_{ATP} channels is impaired in smooth muscle cells from aorta of DOCA-salt hypertensive rats.

The data support the hypothesis that a complex array of ROS and metabolites of arachidonic acid (20-HETE and its metabolites) interact in concert with depressed K_{ATP} channel activity to modulate spontaneous tone in the DOCA-salt model.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	Cytosolic free calcium
17-ODYA	17-octadecynoic acid
20-HETE	20-hydroxyeicosatetraenoic acid
20-HEP	20-hydroxy-endoperoxide
AA	Arachidonic acid
ABT	Aminobenzotriazole
Ang II	Angiotensin II
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
Ba^{2+}	Barium
BH_2	Dihydrobioterin
BH_4	Tetrahydrobioterin
BP	Blood pressure
Ca^{2+}	Calcium
cAMP	Cyclic 3', 5'-adenosine monophosphate
cGMP	Cyclic 3', 5'-guanosine monophosphate
COX	Cyclooxygenase
CYP	Cytochrome p-450 monooxygenase
DMSO	Dimethylsulfoxide
DOCA	Deoxycorticosterone acetate
eNOS	Endothelial nitric oxide synthase

EC_{50}	Concentration of half maximal response
EDCF	Endothelial derived constricting factor
EDHF	Endothelial derived hyperpolarizing factor
EDRF	Endothelial derived relaxing factor
EET	Epoxyeicosatrienoic acid
Em	Membrane potential
E_{max}	Maximal response
ET	Endothelin
ET_A	Endothelin type A receptor
ET_B	Endothelin type B receptor
H_2O_2	Hydrogen peroxide
HPLC	High Pressure Liquid Chromatography
i.v.	Intravenous
IP_3	inositoltriphosphate
K^+	Potassium
K_{ATP}	ATP-dependent potassium channel
K_{Ca}	Calcium activated potassium channel
K_{IR}	Inward rectifier potassium channel
K_V	Voltage dependent potassium channel
L-NAME	N^G-nitro-L-arginine methyl ester
MAP	Mean arterial pressure
mRNA	Messenger ribonucleic acid
MVB	Mesenteric vascular bed

Na^+	Sodium
NADH	Nicotinamide adenine dinucleotide dehydrogenase
NADPH	Nicotinamide adenine dinucleotide phosphate dehydrogenase
NO	Nitric oxide
$O_2^{\cdot-}$	Superoxide anion
NOS	Nitric oxide synthase
$^{\cdot}OH$	Hydroxyl radical
$OONO^-$	Peroxynitrite
PE	Phenylephrine
PG	Prostaglandin
PGI_2	Prostacyclin
PLC	Phopholipase C
PSS	Physiological saline solution
R_{max}	Maximum relaxation response
ROS	Reactive oxygen species
SD	Sprague-Dawley
SEM	Standard error of mean
SHR	Spontaneously hypertensive rat
SHR-SP	Spontaneously hypertensive rats-stroke prone
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
TALH	Thick ascending loop of Henle

TP	Thromboxane-prostaglandin
TX	Thromboxane
VSMC	Vascular smooth muscle cells
WKY-rat	Wistar-Kyoto rat

1. Introduction

When subjected to an increased transmural pressure, blood vessels develop a spontaneous, intrinsically initiated contraction. The magnitude of spontaneous tone is elevated in hypertension. The increased spontaneous tone may contribute to the increase in resistance observed in small resistance vessels and the decrease in compliance of large conducting vessels. In this introduction, the contributions of the endothelium, oxidative stress, arachidonic acid metabolites and potassium channels in modulating vascular smooth muscle tone are reviewed. In addition, the roles of these endogenous metabolites in various rats models of hypertension are reviewed.

1.1. Spontaneous tone

1.1.1. Definition

Blood vessels can contract spontaneously to increase the basal tone in response to stretch and in the absence of any exogenous vasoconstrictor agonist or circulatory factor. This phenomenon, also known as the "Bayliss effect", was demonstrated by WM Bayliss in 1902. He suggested that this response is the mechanism that maintains tissue blood flow constant in response to changes in blood pressure. The contraction of blood vessels on applying a stretching force *in vitro* is designated "myogenic tone". This tone is considered similar to the vascular tone that develops in response to the change in the level of transmural pressure *in vivo* (Hwa & Bevan,1986a). The myogenic behavior of blood vessels provides the background tone upon which other vasomotor influences act. In physiological conditions, myogenic tone is restricted to systemic resistance arteries (Uchida & Bohr,1969). In hypertension, along with an augmentation of myogenic tone in small arteries, larger conducting vessels including aorta can contract in

response to increases in intraluminal pressure (Sunano et al., 1996; Rinaldi & Bohr,1989). This pressure induced spontaneous contraction is thought to be acquisition of myogenic behavior by large arteries in hypertension. The terms "myogenic tone" and "spontaneous tone" are used interchangeably in the literature for smaller vessels. But while describing the acquired myogenic behavior in aorta of hypertensive animals, there seems to be a preference in using the term spontaneous tone (Di Wang et al., 1999; Rinaldi & Bohr,1989; Northcott et al., 2002). Accordingly, the term spontaneous tone was adopted in this thesis.

Two properties appear to characterize the myogenic phenomenon. First, the intensity of the tone increases as the stretch increases (Grande et al., 1979; Hwa & Bevan,1986a; Bevan,1985). Second, the magnitude of the tone depends on extracellular Ca^{2+} (Grande,1989; Grande & Mellander,1978). How stretch induces contraction of blood vessels remains obscure. It is thought that stretch activates L-type voltage-gated Ca^{2+} channels, which facilitates Ca^{2+} influx (Rapacon-Baker et al., 2001). There are some suggestions that stretch activated cation channels also contribute to the development of spontaneous tone (Zhou & Kung,1992). The Ca^{2+} influx in aortic cells of hypertensive animals in response to stretch is not linked to release of Ca^{2+} from intracellular stores, since ryanodine did not attenuate spontaneous tone (Shima & Blaustein,1992). Generally, it is thought that an increase in transmural pressure serves as a positive feedback factor increasing spontaneous tone. Stretch also induces the release of vasodilator factors, which serves as a negative feed back mechanism. The relative balance between the positive and negative feedback factors determines its myogenic tone (Folkow,1989). This thesis identifies several factors that are likely to play a role in modulating spontaneous tone in one rat model of hypertension.

1.1.2. Spontaneous tone in rat model hypertension

Rat models of hypertension can be divided into primary and secondary groups according to their etiology (Pinto et al., 1998). Primary hypertension can be subdivided into two subgroups: genetic models, characterized by Mendelian type of inheritance (e.g. spontaneously hypertensive rats (SHR); Dahl -salt sensitive rats), and environmental models (e.g. stress induced hypertension). Secondary models of hypertension can be induced experimentally either by manipulation of renovascular structure (e.g. classic Goldblatt hypertension) or by manipulation of endocrine function (e.g. deoxycorticosterone acetate (DOCA)-salt hypertension).

Because the work done for this thesis was performed in the DOCA-salt model of hypertension, the literature reviews on this model will be emphasized. Nevertheless, when appropriate, comparisons will be made to high renin model of hypertension.

The DOCA-salt rat represents an experimental model of mineralocorticoid hypertension. As reviewed by Paul M Stewart, prevalence rates for mineralocorticoid hypertension are 0.5-2% in unselected patients with essential hypertension. By contrast, specialist centers have reported a much higher prevalence rates of 5-12% in the hypertensive population (Stewart,1999). Hypertension with hypokalaemia and suppression of plasma renin activity is the hallmark for mineralocorticoid hypertension. Mineralocorticoid-based hypertension is characterized by increased sodium and water retention. Increased volume overload induces expansion of the extracellular fluid compartment, which suppresses endogenous plasma renin activity. The administration of DOCA, in combination with unilateral nephrectomy and a high salt diet induces this form of low renin hypertension (Gomez-Sanchez et al., 1996). This DOCA-salt model of hypertension is different from other experimental forms of hypertension including

SHR, 2K1C, angiotensin II (Ang II)-induced hypertension, which are high renin hypertension and so various endocrine system play a different role in these rats.

Spontaneous tone is enhanced in hypertension (Fitzpatrick & Szentivanyi,1980; Izzard et al., 1996). Enhanced spontaneous tone has been reported in several experimental models of hypertension including the SHR (Sekiguchi et al., 1998), the Ang II-infused model (Di Wang et al., 1999), and the DOCA-salt model (Rinaldi & Bohr,1989). In hypertension, augmentation of myogenic behavior in small arteries and arterioles are expected to contribute to the elevation in peripheral resistance and blood pressure. The acquisition of spontaneous tone by conducting vessels such as the aorta in hypertension may favor elevation of systolic blood pressure by decreasing compliance.

1.1.3. Role of calcium

During myogenic contraction, there is an increase in intracellular free calcium concentration $[Ca^{2+}]_i$ (Meininger et al., 1991). Increases in transmural pressure during stretch increase $[Ca^{2+}]_i$ and myosin light chain phosphotase (Zou et al., 1995). The increases of $[Ca^{2+}]_i$ from the extracellular space occurs via activation of L-type calcium channels, and it has an obligatory role in the development of spontaneous tone (Miriel et al., 1999). In isolated vascular smooth muscle cells (VSMC) stretch can also increase $[Ca^{2+}]_i$ via a stretch-activated cation channel (Davis et al., 1992).

In aorta from DOCA-salt hypertensive rats, spontaneous tone was increased remarkably (Rinaldi & Bohr,1989). Incubation of the aorta with a Ca^{2+} entry blocker, D-600, or an L-type Ca^{2+} channel blocker attenuated the spontaneous tone in these rats suggesting that Ca^{2+} influx through an L-type Ca^{2+} channel was essential for the generation of spontaneous tone in this model of hypertension.

1.2. Role of endothelium in vascular tone

Endothelium contributes in the regulation of mean arterial pressure (MAP) through endothelium derived factors. These factors include vasodilators nitric oxide (NO), prostacyclin (PGI_2) and a novel hyperpolarizing factor and the vasoconstrictors endothelin (ET), thromboxane (TX), leukotrienes and superoxide anion ($O_2^{\cdot-}$). In the peripheral circulation, these local factors either alone or via their interaction with other factors exert their effects to modulate vascular tone. In kidney these factors play an important role in the long-term regulation of blood pressure.

1.2.1. Endothelium derived vasodilator factors

1.2.1.1. Nitric oxide

Before the discovery of the NO, it was well known that the endothelium produces a factor that causes vasodilatation. It was named endothelium derived relaxing factor (EDRF). The characterization of EDRF as NO, by the Nobel Prize laureates F. Murad, R. F. Furchgott and L. J. Ignarro was a breakthrough in endothelial research.

1.2.1.1.1. Nitric oxide synthase

NO is synthesized by the enzyme nitric oxide synthase (NOS). There are three isoforms of NOS (Michel & Feron,1997). nNOS (neuronal) was originally purified and cloned from neuronal tissue. nNOS is now known to be much more widely distributed beyond neuronal tissue. iNOS (inducible), originally purified and cloned from an immunoactivated macrophage cell line, is now known to be widely distributed including in VSMC. eNOS (endothelial) is the third mammalian NOS isoforms. It was initially isolated from vascular endothelium, but has since been discovered in cardiac myocytes, brain, platelets and elsewhere. All three isoforms of

NOS share a similar overall scheme. It involves the five-electron oxidation of the terminal guanido nitrogen of the amino acid, L-arginine, to form NO plus L-citrulline, a complex reaction involving molecular oxygen and NADPH as a co-substrate. NO synthesis by NOS depends upon its binding to the ubiquitous calcium regulatory protein calmodulin. In response to an agonist binding to its receptor in living VSMC, there is increased production of IP_3 in endothelium. Increased IP_3 induces increased $[Ca^{2+}]_i$ and activation of the Ca^{2+}/calmodulin pathway. This evokes increased NO production by eNOS (Illiano et al., 1992). Both eNOS and nNOS are calcium-dependent, but iNOS is able to bind with calmodulin with extremely high affinity in a calcium-independent manner.

1.2.1.1.2. Mechanism of action of NO

NO induces vasorelaxation through cyclic guanosine monophosphate (cGMP)-dependent and cGMP-independent mechanisms. This gas activates guanylyl cyclase and increases the production of its second messenger, soluble cGMP (Murad,1986). Accumulation of cGMP causes decreased phosphoinositide metabolism and formation of inositol phosphates, including inositoltriphosphate (IP_3), by decreasing phospholipase C activity. cGMP also causes decreased $[Ca^{2+}]_i$ and decreased activity of myosin light chain kinase. Decreased $[Ca^{2+}]_i$ subsequently causes hyperpolarization of VSMC acting directly on the Ca^{2+} dependent K^+ channel (Murad,1999). cGMP also activates the ATP dependent K^+ channel to facilitate hyperpolarization of vascular smooth muscle. Aside from these cGMP-dependent mechanisms, NO can result in nitration reactions with thiols in proteins to form nitrothiols, and it can react with $O_2^{\cdot-}$. In some blood vessels an important cGMP independent pathway of NO action seems to be inhibition of the production of 20-HETE (Sun et al., 2000). Therefore, NO can also exert its effect through both cGMP-dependent and independent pathways.

1.2.1.1.3. NO in hypertension

NO plays an important role in regulating vascular tone under normal physiological conditions. Administration of inhibitors of NOS *in vivo* results in vasoconstriction and elevation of blood pressure (BP) indicating basal release of NO (Rees et al., 1989). Most forms of hypertension are associated with endothelial dysfunction. In SHR, eNOS mRNA expression is similar to that in the normotensive control rats (Seki et al., 1997), but the cGMP pathway is impaired (Kojda et al., 1998). Also, $O_2^{\cdot-}$ production is increased in SHR. The increased inactivation of NO by increased oxidative stress or a defect in the signal transduction system, rather than a defect in NO formation seems to be the major factor for decreased NO bioavailability reported in most forms of hypertension. In N^G-nitro-L-arginine methyl ester (L-NAME) induced hypertension, there is an overexpression of ET, suggesting that NO seems to play an important role in the regulation of ET-1 gene expression (Sventek et al., 1996). In summary, it is clear that NO exerts a vasorelaxant effect that serves to counteract vasoconstrictor influences. A dysfunctional NO system could contribute to hypertension by leaving vasoconstrictor mechanisms unopposed.

1.2.1.1.4. Role of NO in spontaneous tone

The role of NO in modulating spontaneous tone has received some attention. In SHR, it has been reported that endothelium denudation and application of an inhibitor of NOS, N^G-monomethyl-L-arginine caused an increase in active tone in aortic rings (Sunano et al., 1996). In Ang II -dependent hypertension, endothelium denudation and NOS-inhibition did not change the magnitude of spontaneous tone in aortic rings, suggesting an impaired bioavailability of NO (Di Wang et al., 1999).

The role of NO in modulating spontaneous tone in the DOCA-salt model of hypertension has received little attention. In view of the importance of the NO system in the regulation of vascular

tone, it seems reasonable to postulate that this system might play a major role in modulating spontaneous tone in the DOCA-salt model. A major objective of the work described in this thesis was to ascertain this role.

1.2.1.2. Role of vasodilator prostaglandin

In the vascular endothelium, PGI_2 is the major vasodilator cyclooxygenase (COX) metabolite of arachidonic acid (AA). It also prevents platelet aggregation (Bunting et al., 1976). PGI_2 mediates vasodilatation by increasing cyclic 3', 5'- adenosine monophosphate (cAMP), which leads to a decrease in the amount of $[Ca^{2+}]_i$ (Claesson et al., 1977). Intravenous injection of PGI_2 showed a dose-dependent antihypertensive effect in conscious rats with spontaneous or chronic renal hypertension. In normotensive rats, PGI_2 also decreased blood pressure but the dose-response curve was shifted to the right in these rats. In the SHR, there is an enhanced formation of PGI_2, which is thought to be an adaptive response to the elevated levels of catecholamines in the circulation (Pace-Asciak et al., 1978). PGI_2 secretion was also enhanced in the endothelial cells from SHR compared with Wistar-Kyoto rats (WKY) (Chao et al., 1995). In the SHR there is also an increased production of vasoconstrictor prostaglandin suggesting overall elevated COX activity in the SHR (Ishimitsu et al., 1991). In DOCA-salt hypertension, increased vascular PGI_2 generation due to increased PGI_2 synthase has been reported with no change in the level of vasoconstrictor prostaglandin (Uehara et al., 1991; Ishimitsu et al., 1991). Recently, it has been shown that increased peroxynitrite ($OONO^-$) associated with oxidative stress can inactivate PGI_2 (Zou et al., 1999). Thus hypertension would appear to have effects on PGI_2 levels. In animal studies, it has been shown that inhibition of COX with indomethacin does not have any effect in normotensive rats but it does elevate blood pressure in the SHR (Levy,1977). These effects are

not specific to the generation of vascular PGI_2, as inhibition of COX metabolites in other regions, notably the kidney could play a role.

In the aorta from SHR, application of indomethacin depressed spontaneous tone (Sekiguchi et al., 1998) suggesting that, though both vasoconstricting and vasodilating prostaglandin are increased in SHR, it is the vasoconstricting prostanoid that dominates. *However, the role of PGI_2 in modulating spontaneous tone in the DOCA-salt model has not been studied. In view of the increased generation of PGI_2 in these rats, with no change in vasoconstrictor prostaglandin generation, it seems reasonable to suggest that PGI_2 might serve to suppress spontaneous tone in DOCA-salt hypertensive rats. If this is the case, then an inhibitor of COX should exaggerate spontaneous tone. Accordingly the effects of indomethacin and valeroyl salicylate on spontaneous tone were tested.*

1.2.1.3. Endothelial derived hyperpolarizing factor (EDHF)

NO and PGI_2 are the most important endothelium derived vasodilators. However, agonist mediated vasorelaxation is not completely blocked by inhibition of NOS and COX *in vitro*. The vasodilatation is associated with hyperpolarization of the cell suggesting the existence of EDHF. EDHF–mediated relaxation is unmasked experimentally only after blockade of endothelial NO synthesis, suggesting that under normal physiological conditions EDHF formation is suppressed (Bauersachs et al., 1996). The physiological role of EDHF is difficult to demonstrate since NO and PGI_2 production must be inhibited to reveal the role of EDHF. In situations where NO-mediated vasorelaxation is impaired, such as hypertension and coronary heart disease, EDHF release may become a crucial reserve mechanism for maintenance of vascular tone. EDHF shares some commonality with NO as it is also synthesized in vascular endothelium in a Ca^{2+}/calmodulin dependent manner (Illiano et al., 1992). But, unlike NO and PGI_2, EDHF

induces hyperpolarization of VSMC without intracellular increases of cyclic nucleotides (cAMP and cGMP) (Garcia-Pascual et al., 1995). EDHF directly activates small and intermediate conductance calcium-dependent potassium channels and causes hyperpolarization of VSMC (Eichler et al., 2003). Different substances have been suggested as candidates for EDHF depending on the species and vascular bed. Candidates for EDHF are K^+ (Edwards et al., 1998), gap junctions (Fukuta et al., 1999), epoxyeicosatrienoic acids (EETs) (Campbell et al., 1996), hydrogen peroxide (H_2O_2) (Matoba et al., 2000), anandamide (Randall & Kendall,1998) and NO itself (Simonsen et al., 1999). Most studies show that in big vessels such as the aorta endothelium-dependent vasorelaxation is dominated by NO and PGI_2 with little contribution by EDHF (Sekiguchi et al., 1998). But some studies show a significant contribution of EDHF in maintaining vascular tone in aorta in various pathophysiological conditions including pregnancy (Bobadilla et al., 1997), streptozocin induced diabetes mellitus (Shen et al., 2003) and SHRs (Iglarz et al., 1999).

It appears unlikely that EDHF plays a significant role in modulating spontaneous tone in the aorta, a large conducting vessel, but its contribution cannot be excluded. If it does play a role, one could expect it to suppress tone. Accordingly, one might predict a deficit of EDHF in the DOCA-salt model. Admittedly, exaggerated effects of other factors in the aorta of DOCA-salt hypertensive rat may make it difficult to explore the role of EDHF in modulating spontaneous tone.

1.2.2. Endothelial derived vasoconstrictors

ET-1 is the most important endothelium-derived vasoconstrictor. Other endothelium- derived vasoconstrictors are vasoconstrictor prostanoids and reactive oxygen species (ROS). Details on

vasoconstrictor prostanoids like prostaglandin H_2 (PGH_2) and TXA_2 and endothelial derived reactive oxygen species are discussed in subsequent sections.

1.2.2.1. Endothelin

1.2.2.1.1. Mechanism of action of ET-1

ET-1 was discovered in porcine aortic endothelial cells as a novel vasoconstrictor (Yanagisawa et al., 1988) that was more potent than Ang II and arginine vasopressin (AVP). The discovery of ET ended the search for an EDCF reported by other investigators earlier (Gillespie et al., 1986). The vascular actions of ET are mediated by one of two receptor subtypes, ET_A and ET_B, which are coupled to G-protein (Takigawa et al., 1995). Both ET_A and ET_B receptors activate phopholipase C (PLC). ET-1 binding to the ET_A receptor results in a sustained vasoconstriction due to an extremely slow dissociation of the peptide from its receptor. Activation of PLC causes phosphatidylinositol hydrolysis, formation of IP_3 and diacylglycerol (Enoki et al., 1995). Formation of IP_3 by ET-1 mobilizes Ca^{2+} from intracellular stores. This initiates a transient increase in $[Ca^{2+}]_i$ (Simonson et al., 1990) is followed by sustained increase of $[Ca^{2+}]_i$ due to the influx of extracellular Ca^{2+} through either dihydropyridine-sensitive voltage-dependent Ca^{2+} channels (Iwamuro et al., 1998) and/or receptor-operated nonselective cation channels (Zhang et al., 1998). Binding of ET-1 to the ET_B receptor present on endothelial cells causes vasodilatation via production of NO (Kobari et al., 1994) and PGI_2 (Zellers et al., 1994). Therefore, the overall vascular effects of ET-1 depend on the balance of ET_A-mediated and ET_B-mediated effects, though ET_A- mediated actions seem to be the predominant ones in vasculature.

1.2.2.1.2. Role of ET-1 in DOCA-salt hypertension

ET-1 plays an important role in the development of hypertension in the DOCA-salt model. ET-1 content is increased in aorta and mesenteric arteries from DOCA-salt hypertensive rats (Lariviere et al., 1993). ET-1 levels are higher in DOCA-salt hypertensive rats compared with SHRs (Schiffrin et al., 1995b). DOCA and salt treatment can also increase the expression of ET-1 in SHRs (Schiffrin et al., 1995a).

Both non-selective ET_A/ET_B and selective ET_A receptor antagonists have been reported to attenuate the development of hypertension and decrease BP in DOCA-salt hypertensive rats (Yu et al., 1998; Yu et al., 2001b). In cardiomyocytes and fibroblasts from DOCA-salt hypertensive rats, ET-1 increased $[Ca^{2+}]_i$ but ET-1 signaling pathways were blunted (Touyz et al., 1998). Therefore ET-1 may not play a critical role in the pathophysiology of the severe concentric cardiac hypertrophy present in DOCA-salt hypertensive rats. Recently it has been reported that ET-1 increased vascular $O_2^{\cdot-}$ production in DOCA-salt hypertensive rats via ET_A/NADPH oxidase mediated pathways (Li et al., 2003a). ET_A receptor antagonism has been shown to decrease aldosterone induced oxidative stress (Pu et al., 2003) suggesting that the capacity of ET-1 to increase oxidative stress is not limited to DOCA-salt hypertension. Despite these regional differences, there is convincing evidence supporting a role of ET-1 in the pathogenesis of hypertension particularly the DOCA-salt hypertensive model.

1.2.2.1.3. ET-1 in spontaneous tone

In view of the major role played by ET-1 in the pathogenesis of DOCA-salt hypertension, it seems reasonable to postulate that ET-1 might contribute to spontaneous tone in DOCA-salt hypertensive rats. Indeed, the observations by Rinaldi and Bohr that endothelial-denudation reduced spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats would appear to suggest that EDCF maintains spontaneous tone in these rats (Rinaldi & Bohr,1989). In

addition, it has been suggested that in the SHR a component of the enhanced myogenic response is mediated via ET-1(Falcone & Meininger,1999). *Thus, an objective of the work presented in this thesis was to determine the effect of ET_A and ET_B antagonists on spontaneous tone in DOCA-salt model.*

1.2.3. Endothelial dysfunction and spontaneous tone

The term endothelial dysfunction generally refers to an impairment of endothelium-dependent vasorelaxation caused by a loss of NO bioactivity in the vessel wall. Experimentally, a compromised acetylcholine-induced dilatation response is defined as endothelial dysfunction. The loss in NO bioavailability may be caused by several mechanisms. Decreased expression of the enzyme eNOS is one such mechanism. In hypertension, a decrease in the expression of eNOS has been associated with renal tissue injury. Conversely, short term experimental overexpression of eNOS is associated with improved endothelial function in stroke-prone SHR (Alexander et al., 2000). A lack of a cofactor or substrate for eNOS can also lead to endothelial dysfunction. L-arginine improves endothelial function in Dahl-salt hypertension (Zhou et al., 2001). L-arginine also improves endothelial dysfunction in human essential hypertension (Lekakis et al., 2002). Supplementation of tetrahydrobiopterin (BH_4), the essential cofactor for eNOS for NO synthesis, prevents the earlier rise of blood pressure in rats with subtotal nephrectomy (Podjarny et al., 2003). In a human study it has also been shown that administration of BH_4 improved acetylcholine-induced vasodilatation in hypertensive individuals (Higashi et al., 2002). These findings suggest that an adequate supply of cofactors for NOS are necessary for adequate NO production. Alteration of the cellular signaling mechanism for NO production, such that eNOS is not properly activated (Shimokawa et al., 1991), may also cause

decreased NO bioavailability. Finally, accelerated NO degradation by ROS may reduce bioavailability of NO.

Although endothelial dysfunction is frequently assumed to be synonymous with a decrease in NO function, in the pure sense of the term, it refers to a situation where any component of the endothelium is either abnormally elevated or reduced in activity. Thus overexpression of components of the ET system or underexpression of components of the EDRF/EDHF system could be related to endothelial dysfunction and could conceivably contribute to spontaneous tone.

1.3. Role of reactive oxygen species in modulating vascular tone

ROS are critical for normal functioning of cardiac and vascular cells. Excessive production of ROS may cause tissue injury by oxidizing macromolecules, a process termed oxidative stress. ROS species also play an important role in vascular dysfunction and alteration of endothelial function in hypertension. Increased oxidative stress related to overproduction of oxygen derived free radicals, most importantly $O_2^{\cdot-}$, has been documented in many hypertensive rat models including the SHR (Yamamoto et al., 1992), Ang II induced hypertension (Oskarsson & Heistad,1997), Dahl hypertensive rats (Swei et al., 1997) and DOCA-salt hypertensive rats (Somers et al., 2000). Increased $O_2^{\cdot-}$ subsequently produces increased H_2O_2 and hydroxyl radical ($^{\cdot}OH$), which can also contribute to vascular responses.

1.3.1. Superoxide anion

$O_2^{\cdot-}$ is generated by 1-electron reduction of molecular oxygen. The presence of unpaired electrons causes $O_2^{\cdot-}$ to be chemically unstable and highly reactive. The enzymes that are

thought to be the potential vascular sources of $O_2^{\cdot-}$ are endothelial nitric oxide synthase (eNOS), NAD(P)H oxidase and xanthine oxidase.

1.3.1.1. Nitric oxide synthase

NOS catalyses the electron transport from the electron donor, NADPH, to a prosthetic heme group to produce NO and L-citrulline. Fully reduced 5,6,7,8-BH_4 is an essential cofactor required for NOS to transfer electrons to a nitrogen of its substrate, L-arginine, to form NO (Vasquez-Vivar et al., 1998; Gorren & Mayer,2002). It has been reported that under certain conditions, NOS produces $O_2^{\cdot-}$ and H_2O_2 in addition to NO (Pou et al., 1992). Reduced bioavailability of BH_4 or in the presence of the partially oxidized BH_4 analogue, 7,8-dihydrobiopterin (BH_2), NOS generates $O_2^{\cdot-}$ and not NO (Vasquez-Vivar et al., 2002). This phenomenon is referred as NOS "uncoupling". The ratio between oxidized and reduced BH_4 metabolites regulates NO and $O_2^{\cdot-}$ formation from eNOS.

eNOS can become uncoupled in a variety of pathophysiological conditions. Uncoupling of eNOS is suggested to be an important source of $O_2^{\cdot-}$ production in stroke-prone SHR (Hamilton et al., 2001) and in the NADPH-oxidase knock- out mouse model of hypertension (Landmesser et al., 2003). Recently, it has been demonstrated that, $OONO^-$, the product of the reaction between NO and $O_2^{\cdot-}$, can oxidize BH_4 and that this may lead to uncoupling of NOS (Kuzkaya et al., 2003; Laursen et al., 2001).

In a review article, Hua Cai and DG Harrison (Cai & Harrison,2000) suggested that uncoupling of eNOS may contribute to vascular dysfunction in at least three different ways. Firstly, the diminished NO production itself contributes to impaired vasorelaxation. Secondly, $O_2^{\cdot-}$ formation from the uncoupled eNOS contributes to the oxidative stress. Finally eNOS can

be uncoupled partially to produce both NO and $O_2^{\cdot-}$ simultaneously and thereby increase $OONO^-$, which may contribute to oxidative stress and further uncoupling of eNOS.

1.3.1.2. NAD(P)H oxidase

The NAD(P)H oxidases are the membrane-associated enzymes that catalyze the 1-electron reduction of oxygen using NADH or NADPH as the electron donor. NADPH is the major substrate of the vascular system (Griendling et al., 2000). Depending on the functional structure, NAD(P)H oxidase is divided into two groups, neutrophil NAD(P)H oxidase and VSMC NAD(P)H-oxidase. The neutrophil oxidase consists of 4 major subunits: gp91phox, p22phox, p47phox, and p67phox. mRNA for these subunits have been also demonstrated in endothelial cells (Bayraktutan et al., 1998) and adventitial cells (Pagano et al., 1997). These subunits are configured in such a way that the oxidase utilizes intracellular NADP or NADPH and transfers the electrons across the membrane to extracellular oxygen (Meier et al., 1991). However, in VSMC, NAD(P)H oxidase does not express gp91phox, which is substituted by its homologue nox1 (Lassegue et al., 2001). The functional arrangement of these subunits results in intracellular production of $O_2^{\cdot-}$ and H_2O_2 in VSMC. The activity of the vascular NAD(P)H oxidase is increased by various vasoactive agonists including Ang II (Griendling et al., 1994), ET-1 (Li et al., 2003a), thrombin (Patterson et al., 1999), platelet-derived growth factor (Sundaresan et al., 1995) and tumor necrosis factor-α (De Keulenaer et al., 1998).

The vascular NAD(P)H oxidases are essential in various vascular responses including growth and migration. These enzymes have also been linked to pathological states, notably hypertension. Chronic infusion of Ang II in rats resulted in hypertension in association with increased NAD(P)H oxidase derived $O_2^{\cdot-}$ generation (Rajagopalan et al., 1996). Enhanced

NAD(P)H oxidase driven $O_2^{\cdot-}$ production is associated with an upregulation of p22phox mRNA expression in the aorta of adult SHR (Zalba et al., 2000). The increased production of $O_2^{\cdot-}$ in aorta of DOCA-salt hypertensive rats is associated with increased NAD(P)H oxidase activity (Wu et al., 2001).

In human internal mammary arteries and saphenous veins, NAD(P)H oxidase has been demonstrated to be the source of basal $O_2^{\cdot-}$ production (Berry et al., 2000). In VSMC from healthy subjects, Ang II stimulated $O_2^{\cdot-}$ production has been shown to be mediated via phopholipase D-dependent NADH/NADPH oxidase sensitive pathways (Touyz & Schiffrin,1999). This Ang II induced oxidative stress is exaggerated in VSMC from essential hypertensive patients (Touyz & Schiffrin,2001). Recently it has been reported that human VSMC express a functionally active gp91phox-subunits similar to neutrophilic oxidase (Touyz et al., 2002). Thus both animal and human studies strongly suggest the importance of NAD(P)H oxidase in the generation of $O_2^{\cdot-}$ in hypertension.

1.3.1.3. Xanthine oxidase

The enzyme xanthine oxidase catalyses the oxidation of hypoxanthine and xanthine in the process of purine metabolism. During this reaction, the enzyme reduces molecular oxygen leading to production of both $O_2^{\cdot-}$ and H_2O_2. Xanthine oxidase inhibitors lower the blood pressure in SHR (Nakazono et al., 1991) and prevent the increased free radical production in SHR (Chen & Suzuki,1989). Oxypurinol, an inhibitor of xanthine oxidase, normalized increased $O_2^{\cdot-}$ associated with early stages of experimental atherosclerosis caused by diet-induced hypercholesterolemia (Ohara et al., 1993). In human subjects, oxypurinol improves endothelial vasodilator function in hypercholesterolemic but not in hypertensive patients (Cardillo et al., 1997). Thus the role of this enzyme seems to be mainly in hypercholesterolemia.

1.3.2. Oxidative stress and endothelial dysfunction

Among all the ROS, $O_2^{\cdot-}$ is critically involved in the breakdown of NO. Even before EDRF was identified as NO, it had been reported that superoxide dismutase (SOD) prevented the inactivation of EDRF (Rubanyi & Vanhoutte,1986). It is now clear that the reaction of $O_2^{\cdot-}$ with NO leads to the production of $OONO^-$ (Beckman et al., 1990). Under normal physiological conditions endogenous antioxidant defenses scavenge $O_2^{\cdot-}$ and minimize its reaction with NO. In a number of disease conditions, increases in $O_2^{\cdot-}$ reduces NO bioavailability leading to endothelial dysfunction (Laight et al., 1998). $OONO^-$ itself is believed to be a potent oxidant that induces the oxidation of proteins, DNA and lipids and causes nitration of tyrosine residues in vascular cells (Ballinger et al., 2000). Recently it has been reported that BH_4 is an important target for oxidation by $OONO^-$, which leads to uncoupling of eNOS. $OONO^-$ also inhibits PGI_2 synthesis (Zou & Ullrich,1996), which may subsequently compromise vascular function.

The interaction between NO and $O_2^{\cdot-}$ occurs almost 3 times faster than the reaction rate for $O_2^{\cdot-}$ with SOD (Thomson et al., 1995). SOD catalyses the reaction of $O_2^{\cdot-}$ with water to produce H_2O_2. The vascular action of H_2O_2 remains obscure because H_2O_2 has been shown to cause both vasoconstriction (Yang et al., 1998; Shen et al., 2000) and vasorelaxation (Yang et al., 1999; Bharadwaj & Prasad,1995) depending upon the tissue. Some authors have suggested H_2O_2 to be the EDHF (Matoba et al., 2000). H_2O_2 is not a free radical *per se* but has oxidizing effects that contribute to oxidant stress. H_2O_2 can cross the cell membrane and in the presence of Fe^{2+}, H_2O_2 breaks down to produce highly reactive hydroxyl ($^{\cdot}OH$) radicals. Another source of $^{\cdot}OH$ radicals is $OONO^-$. It can be protonated to form peroxynitrous acid, which in turn can yield $^{\cdot}OH$ radicals. However, contractile responses elicited by H_2O_2 in aorta from hypertensive rats has been suggested to be via the COX pathway and not through $^{\cdot}OH$ radicals (Rodriguez-Martinez et al.,

1998). ·OH radicals can oxidize lipids, damage cell membranes, and oxidize thiol groups. ·OH radicals can also play an important role in diabetes and atherosclerosis and contribute to H_2O_2 induced vasodilatation (Prasad & Bharadwaj,1996). In human studies it has been shown that deferoxamine, an iron chelator that inhibits generation of ·OH radicals improved the responses of the coronary microvasculature to sympathetic stimulation in type-1 diabetes mellitus (Hattori et al., 2002).

Though increased $O_2^{\cdot-}$ is associated with altered vascular relaxation in various rat models of hypertension, it is not the only ROS that reacts with NO. Lipid radicals (LO. and LOO.) can react with NO to form respectively LONO and LOONO (O'Donnell et al., 1997). ·OH radicals also may react with NO and contribute to endothelial dysfunction (Pieper et al., 1997). It has been also demonstrated that only oxidized LDL inhibits the endothelium-dependent vascular relaxation (Tanner et al., 1991), indicating another mechanism for ROS in endothelial dysfunction.

1.3.3. DOCA-salt hypertension and oxidative stress

Vascular $O_2^{\cdot-}$ formation is enhanced in DOCA-salt hypertensive rats (Somers et al., 2000). Administration of membrane targeted SOD has been reported to improve acetylcholine-induced vasorelaxation in aortic rings of DOCA-salt hypertensive rats, suggesting that $O_2^{\cdot-}$ contributes to endothelial dysfunction in this model. In DOCA-salt hypertensive rats, increased activity of NAD(P)H oxidase in the aortic wall and increased $O_2^{\cdot-}$ production have been reported (Beswick et al., 2001a). Long term oral administration of antioxidants, including the $O_2^{\cdot-}$ scavenger, tempol, or of the NADPH oxidase inhibitor, apocynin, decreased systolic blood pressure and reduced $O_2^{\cdot-}$ generation in DOCA-salt hypertensive rats (Beswick et al., 2001b). Thus,

considerable evidence suggests that NAD(P)H-oxidase derived $O_2^{\cdot-}$ contributes to the elevation in blood pressure in the DOCA-salt model.

As reviewed earlier, ET-1 plays a role in the maintenance of total peripheral resistance and BP in DOCA-salt hypertension (Yu et al., 2001a). ET also contributes to the hemodynamic effects of AVP since the ET antagonist, bosentan, reduced the increase in total peripheral resistance evoked by AVP in this model (Yu et al., 2001b). Brattleboro rats, that are AVP deficient, failed to develop hypertension and increased ET gene expression in response to DOCA-treatment, confirming that AVP exerts its vascular effects in part though ET-1 in the DOCA-salt model (Intengan et al., 1999). Recently it has been reported that AVP induces vascular $O_2^{\cdot-}$ production through activation of the V_1 receptor and that ET-1 is an intermediary signaling molecule in AVP stimulated $O_2^{\cdot-}$ formation in DOCA-salt hypertensive rats (Li et al., 2003b). ET-1 stimulated $O_2^{\cdot-}$ production in DOCA-salt rats. This effect was prevented is prevented by an ET_A receptor antagonist or the NADPH oxidase inhibitor, apocynin, suggesting that in DOCA-salt hypertension ET-1 increases vascular $O_2^{\cdot-}$ via the ET_A-NADPH-oxidase pathway (Li et al., 2003a).

In summary, considerable evidence suggests that $O_2^{\cdot-}$ contributes to the elevation of BP in the DOCA-salt model of hypertension. Part of this contribution of $O_2^{\cdot-}$ appears to be as a mediator of the vascular actions of AVP and ET-1, two peptides contributing to the elevations in BP. In Ang II dependent hypertension, $O_2^{\cdot-}$ plays an important role in mediating spontaneous tone. *However, the possibility that $O_2^{\cdot-}$ may contribute to the increased spontaneous tone observed in vessels from DOCA-salt hypertensive rats has not been explored. Thus, the major objective of the work described in this thesis was to determine the contribution of oxidative stress, particularly, that of $O_2^{\cdot-}$ to the generation of spontaneous tone in the DOCA-salt model.*

1.4. Arachidonic acid metabolites in modulating vascular tone

AA is a long chain (20-carbon) polyunsaturated fatty acid, found in virtually all tissues across the body. Several tissue or organ specific stimuli can cause a transient increase in cytosolic arachidonate by activating phopholipase A_2 or other phospholipases. Endogenous agonists such as angiotensin II and norepinephrine and even mechanical stimuli may cause release of arachidonic acid from the cell membrane. AA can be oxygenated by three enzyme systems, cyclooxygenase (COX), lipoxygenase and cytochrome P-450 monooxygenase (CYP).

1.4.1. Alteration of cyclooxygenase metabolites

COX metabolizes AA to an endoperoxide, prostaglandins (PG)G_2 and PGH_2, which in turn are metabolized by tissue specific enzymes to PGI_2, PGE_2, PGD_2, and $PGF_{2\alpha}$. PGI_2, a potent vasodilator, has been reviewed earlier in this introduction. PGH_2 is converted by thromboxane synthase to TXA_2, a potent vasoconstrictor and facilitates platelet aggregation. TXA_2 causes vasoconstriction by activating the prostaglandin-thromboxane (TP) receptor. An important function of COX metabolites of AA is their contribution to the inflammatory response. Non streroidal anti-inflammatory drugs (NSAIDs) block COX. TXA_2 plays a critical role in platelet aggregation. COX metabolites also appear to play a significant role in modulating vascular tone in hypertension. Accelerated synthesis of TXA_2 has been reported in SHR in response to stimuli that induce a release of AA (Shibouta et al., 1981). In DOCA-salt hypertension, it has been reported that vascular PGI_2 generation is increased, which may serve to limit the elevations in BP in this model (Uehara et al., 1991). On the other hand, the impaired adenylate cyclase pathway in DOCA-salt hypertension may mitigate the beneficial effects of PGI_2 (Millette et al., 2000). Overproduction of vasoconstrictor prostanoids such as PGH_2 and its subsequent conversion to TXA_2 can lead to increases in vascular tone. Inhibition of NO by L-NAME increases BP. The

L-NAME induced pressor response is suggested to be partly mediated by TXA_2 (Nafrialdi et al., 1994). It seems likely that the relative levels of vasoconstrictor and vasodilator prostanoids determine their effects in hypertension.

In addition to metabolizing AA, COX contributes to oxidative stress by generating ROS (Kontos,1987). COX- induced ROS has been suggested to impair vasodilatation in coronary microvessels (Oltman et al., 2003). COX may metabolize CYP derivatives of AA to active or inactive metabolites. Recently it has been shown that COX activates an endothelial derived vasoconstricting factor (Yang et al., 2003). Finally, $OONO^-$ levels are increased in hypertension and $OONO^-$ can increase COX activity.

In summary, it is evident that COX is a versatile enzyme and its activity can be altered in a complex manner in hypertension. In the DOCA-salt model, increased production of PGI_2 by COX may inhibit spontaneous tone. On the contrary, COX may contribute to oxidative stress in this model to aggravate spontaneous tone. *Accordingly, the contribution of COX on spontaneous tone and oxidative stress in DOCA-salt rats was tested.*

1.4.2. Alteration of lipoxygenase metabolites of AA

Lipoxygenase metabolites of arachidonic acid play a role in agonist evoked vasoconstriction. The 5-lipoxygenase metabolites, cysteinyl leukotrienes, evoked dose-dependent contractions in aorta of hypertensive SHR but not in aorta of normotensive WKY (Stanke-Labesque et al., 2001). In SHR, cysteinyl leukotrienes contributed to the enhanced vasoconstriction evoked by Ang II but not that evoked by ET-1 (Shastri et al., 2001). In hypertension resulting from coarctation of the aorta, lipoxygenase metabolites play an important role in the increase in BP in these rats (DelliPizzi et al., 2000). In DOCA-salt hypertensive rats, endoperoxides seem to be the main derivative contributing to altered vasodilatation. Lipoxygenase metabolites do not

seem to play a role in this model of hypertension (Cordellini,1999). This may be because DOCA-salt hypertension is a low renin model of hypertension and ET-1 and not Ang II play the key role in modulating vascular tone in these rats.

1.4.3. Alteration of Cytochrome P_{450} metabolites of AA

CYP genes encode for membrane-bound, heme-containing enzymes that catalyse NADPH-dependent oxidation of various drugs, chemicals and fatty acids (linolenic, linoleic, palmitic acid and arachidonic acid). CYP enzymes metabolize AA in the brain, lung, kidney and peripheral vasculature to generate epoxyeicosatrienoic acids (EETs) and 20-hydroxyeicosatetraenoic acid (20-HETE) and these compounds play critical roles in the regulation of renal and cardiovascular function.

1.4.3.1. Epoxyeicosatrienoic acids

EETs are produced by endothelial cells (Fisslthaler et al., 2000). Cultured endothelial cells express mRNA encoding for epoxygenase enzymes and produce EETs on incubation with AA (Johnson et al., 1985; Revtyak et al., 1988). One study also shows that cultured VSMC can produce EETs (Hasunuma et al., 1991). EETs hyperpolarize VSMC and reduce vascular tone (Campbell et al., 1996). In small renal (Zou et al., 1996) and mesenteric arteries (Proctor et al., 1987) of the rat, EETs are potent vasodilator compounds. It appears that most of the isomers of EETs are direct vasodilators in most vascular beds, but 5,6-, and 8,9-EETs can be metabolized by COX to either vasodilator or vasoconstrictor metabolites (Roman,2002).

The mechanism by which EETs hyperpolarize VSMC is by increasing open-state probability of K_{Ca} channels (Li et al., 1997). In the renal circulation of the rat, the vasodilator response to EETs is dependent on activation of adenylyl cyclase and protein kinase A (Imig et al., 1999). Overall, the available evidence suggests that depending on the species and vascular bed, EETs

can cause vasodilatation both via protein kinase-dependent and independent pathways. EETs can increase $[Ca^{2+}]_i$ in endothelial cells. The increase in $[Ca^{2+}]_i$ can stimulate the formation and release of NO, PGI_2 and TXA_2. (Graber et al., 1997). EETs also potentiate AVP -induced elevation in $[Ca^{2+}]_i$ in aortic VSMC (Thibonnier et al., 1993). The release of these factors can modulate the direct vasodilatory effect of EETs either by augmenting or by opposing it.

In some vessels e.g. coronary arteries EETs are thought to be the EDHF (Campbell et al., 1996; Pratt et al., 2001). The fact that EETs are produced by the endothelium and evoke hyperpolarization of VSMC supports this notion. In contrast, EETs do not appear to serve as EDHF in other vascular beds (e.g. hepatic arterial bed) (Zygmunt et al., 1996).

1.4.3.2. 20-hydroxyeicosatetraenoic acid

Enzymes of the CYP4A, 4B, and 4F families catalyze the ω-hydroxylation of fatty acids. Several isoforms in these families produce 20-HETE when incubated with AA (Hardwick et al., 1987; Kimura et al., 1989). In contrast to EETs, which are found in endothelium, 20-HETE is produced in VSMC (Roman et al., 2000). Small arteries throughout the body express CYP4A and are thought to produce 20-HETE. Both 20-HETE and EETs act predominately as autocrine factors. These compounds are very lipophilic and are found in higher concentrations in plasma, urine, or interstitial fluid (Roman,2002). They are rapidly incorporated into membrane phospholipids and are released from these stores in response to stimuli that activate phospholipase. After release from the cell membrane, EETs and HETEs are rapidly metabolized by β-oxidation to 18- and 16-carbon products. In many tissues, CYP and COX enzymes can further metabolize EETs and HETEs. 20-HETE metabolites of COX have been shown to modify its biological action. The metabolism of CYP metabolites of AA by COX is both tissue and

species specific. The effect of COX on 20-HETE is thought to be through the formation of 20-hydroxy-PGE_1 or PGI_2 in the endothelium.

1.4.3.2.1. Renal action of 20-HETE

20-HETE plays an important role in the regulation of tubular reabsorption of sodium. In the proximal tubules, 20-HETE inhibits renal Na^+-K^+-ATPase activity (Schwartzman et al., 1985). In the thick ascending loop of Henle (TALH), 20-HETE plays a major role in the regulation of chloride transport by inhibiting Na^+-K^+-2Cl- cotransport (Carroll et al., 1991). 20-HETE blocks the K^+ channel in the apical membrane of TALH cells and so limits K^+ availability for transport via the Na^+-K^+-2Cl- cotransport (Wang & Lu,1995). By blocking K^+ recycling, 20-HETE inhibits sodium reabsorption and facilitates natriuresis. Thus, the effect of 20-HETE in TALH is mainly antihypertensive.

1.4.3.2.2. Vascular action of 20-HETE

20- HETE constricts mesenteric (Chu et al., 2000), renal (Roman & Harder,1993) and skeletal muscle arterioles (Frisbee et al., 2001). After inhibition of the production of endogenous 20-HETE by 17-octadecynoic acid (17-ODYA), exogenous 20-HETE reduces the diameter of vessels. The vasoconstrictor response to 20-HETE in peripheral arteries of rats and rabbits is blocked by indomethacin or an endoperoxide receptor antagonist, suggesting a role for COX metabolites of 20-HETE in its biological function (Escalante et al., 1993; Escalante et al., 1989).

20-HETE evokes vasoconstriction via inhibition of the K_{Ca} channel. In VSMC isolated from cat cerebral arteries, inhibition of 20-HETE with 17-ODYA activated the large-conductance K_{Ca} channel and this effect was completely abolished by addition of 20-HETE (Harder et al., 1994). In larger vessels such as the aorta, exogenous 20-HETE induced vasoconstriction (Escalante et al., 1993). The mechanism by which 20-HETE blocks the K_{Ca} channel is still obscure.

Structural analogs of 20-HETE block the vasoconstrictor response to 20-HETE suggesting that there is some type of receptor for 20-HETE in VSMC (Alonso-Galicia et al., 1999). Very recently, there has been some suggestion about the structure of the 20-HETE receptor (Yu et al., 2003). 20-HETE activates protein kinase C in VSMC from rat renal (Sun et al., 1999) and cat cerebral circulation (Lange et al., 1997). In the rat aortic VSMC, 20-HETE directly activates the mitogen-activated protein (MAP) kinase-signaling pathway (Uddin et al., 1998). 20-HETE is also involved in the norepinephrine and Ang II induced activation of MAP kinase in these cells (Muthalif et al., 1998). In contrast to its predominant vasoconstrictor action, 20-HETE dilated bovine coronary arteries by stimulating the release of PGI_2 from the endothelium (Pratt et al., 1998). 20-HETE is also a dilator of pulmonary artery (Yuan et al., 1996), likely through activation of the NO system. In summary, although 20-HETE may dilate the coronary and pulmonary beds, its predominant action is vasoconstriction.

1.4.3.2.3. 20-HETE and other vasoconstrictors

Vasoconstrictors such as Ang II, AVP and norepinephrine activate phopholipase in VSMC and increase the release of AA and 20-HETE. Inhibiting the formation of 20-HETE attenuates vasoconstrictor responses to AVP (Carroll et al., 1996), Ang II (Alonso-Galicia et al., 2002), ET-1 (Oyekan et al., 1998), and norepinephrine (Uddin et al., 1998) in renal and mesenteric arteries of rat. Recently ET-1 has been linked to renal production of CYP metabolites of AA in the isolated rat kidney (Oyekan et al., 1997). ET-1 and 20-HETE have similar renal effects. Both decrease glomerular blood flow and both induce diuresis and natriuresis. ET-1 has been associated with renal release of 20-HETE. Therefore, 20-HETE has been considered the second messenger for ET-1 in kidney (Oyekan & McGiff,1998a).

1.4.3.2.4. Interaction of NO and 20-HETE

The interaction between NO and 20-HETE varies among the different vascular beds. In microsomes prepared from renal arterioles, a NO donor, sodium nitroprusside, dose dependently inhibited the formation of 20-HETE. NO is also known to inhibit some heme-containing proteins including NOS and CYP. Therefore, NO induced inhibition of 20-HETE is considered to serve as a cGMP independent pathway of NO (Sun et al., 1998). Inhibition of the formation of 20-HETE blunts the effects of L-NAME on blood pressure suggesting that NO inhibits the CYP4A metabolite of AA (Oyekan & McGiff,1998b). In contrast to its effect elsewhere, in the pulmonary artery, 20-HETE induced vasodilatation is endothelium-dependent and COX-dependent (Jacobs et al., 1999). The mechanism by which 20-HETE causes vasodilatation is thought to be by increasing $[Ca^{2+}]_i$, which increases NO (Yu et al., 2002).

1.4.3.3. Effects of 20-HETE on myogenic tone

20-HETE plays an important role in the modulation of myogenic responses in arterioles that regulate blood flow in various vascular beds including renal (Imig et al., 1999), cerebral (Gebremedhin et al., 2000), mesenteric (Wang et al., 2001) and skeletal muscle (Frisbee et al., 2001). *In vitro,* elevations in transmural pressure stimulates release of 20-HETE. Blockade of the formation of 20-HETE prevents pressure-induced contraction of these vessels. It is suggested that activation of stretch-activated channels facilitate Ca^{2+} influx followed by an increase in the $[Ca^{2+}]_i$, which in turn stimulates the release of AA from the cell membrane and promotes the formation of 20-HETE. 20-HETE blocks the K_{Ca} channel and prevents hyperpolarization of VSMC associated with the elevation of $[Ca^{2+}]_i$. By maintaining the membrane potential in a more depolarized state, 20-HETE potentiates Ca^{2+} influx and myogenic tone.

1.4.3.3.1. Role of 20-HETE in experimental hypertension

In Dahl rats, which are deficient in CYP4A activity in the renal medulla (Roman et al., 1997), the decrease in outer medullary 20-HETE production and the associated lack of inhibition of Na^+-K^+-$2Cl^-$ cotransport in the TALH appears to contribute to hypertension (Ma et al., 1994). 20-HETE production is increased in kidney of the SHR. The level of 20-HETE is almost 27 fold higher and other CYP metabolites of AA are 3 to 6 fold higher in SHR (Omata et al., 1992). The elevated levels of 20-HETE were related to the age of the animal and to the increase of blood pressure. Inhibition of CYP activity decreased 20-HETE level and blood pressure in SHR but not in normotensive control groups (Escalante et al., 1991). Aminobenzotriazole (ABT), an inhibitor of CYP4A and ω-hydroxylation, also reduced the BP in the SHR (Su et al., 1998). Ang II stimulates the formation of 20-HETE in the renal circulation of rats (Croft et al., 2000). Blockade of the synthesis of 20-HETE attenuated the vasoconstrictor actions of Ang II in renal arteries *in vitro* and *in vivo* (Alonso-Galicia et al., 2002). Recently, R. J. Roman reported that chronic administration of ABT attenuated the development of Ang II induced hypertension (Roman,2002).

The renal excretion of ET-1 and 20-HETE are four- fold higher in DOCA-salt hypertensive rats (Oyekan et al., 1999). Chronic administration of ABT reduced the synthesis of 20-HETE and reduced BP in the DOCA-salt model (Muthalif et al., 2000). These results suggest that 20-HETE contributes to the development of DOCA-salt hypertension presumably by potentiating the vasoconstrictor actions of ET-1.

In view of the significant role played by 20-HETE in modulating myogenic tone in systemic arterioles and its role in the pathogenesis of DOCA-salt hypertension, it seems reasonable to postulate that 20-HETE might contribute to the generation of spontaneous tone in the DOCA-

salt model. If this is the case, then the production of 20-HETE should be elevated in aorta from DOCA-salt hypertensive rats and inhibition of 20-HETE production should attenuate spontaneous tone. Thus an objective of the work presented in this thesis was to determine the role of 20-HETE on spontaneous tone.

1.5. Role of potassium channel in modulating vascular tone

Potassium (K^+) channels in the cell membrane regulate the membrane potential of VSMC. Four distinct types of K^+ channels have been identified in arterial smooth muscle: voltage-dependent K^+ (K_V) channels, Ca^{2+} activated K^+ (K_{Ca}) channels, inward rectifier K^+ (K_{IR}) channels and ATP sensitive K^+ (K_{ATP}) channels (Nelson & Quayle,1995). There is considerable variability in the expression of the 4 channel subtypes among the various vascular beds (Michelakis et al., 1997). This diversity may contribute to the variation in the responses to various vasodilator and vasoconstrictor agonists and to the variations in the myogenic responses among the different regional beds.

The opening of a K^+ channel in VSMC causes K^+ efflux, which promotes membrane potential (Em) hyperpolarization. K^+ efflux closes voltage-dependent Ca^{2+} channels, decreasing Ca^{2+} entry to the cell and lowering intracellular Ca^{2+}. As a major regulator of VSMC Em, K^+ channel activity is an important determinant of vascular tone and blood vessel diameter.

1.5.1. Types of K^+ channels

K_V channels are also known as delayed rectifier K^+ channels. K_V channels activate and inactivate in response to membrane depolarization and so serve an important buffering function against depolarization and vasoconstriction (Nelson & Quayle,1995). As the cell is depolarized in response to pressurization or vasoconstrictors, K_V channels open, and this limits further membrane depolarization (Knot & Nelson,1998). All VSMC have at least one K^+ current that is

activated by membrane depolarization (Nelson & Quayle,1995). In some blood vessels 4-aminopyridine, a pharmacological blocker of K_V channels enhances arterial depolarization suggesting that K_V channel, activity exists in those vessels under basal conditions (Knot & Nelson,1995). cAMP (Aiello et al., 1995) and NO/cGMP (Yuan et al., 1996) may activate K_V channels in some blood vessels and these channels may be inhibited by protein kinase C (Aiello et al., 1996).

K_{Ca} channels, found virtually in all smooth muscle, are activated by intracellular Ca^{2+} and membrane depolarization (Nelson & Brayden,1993). The activation of a few K_{Ca} channels permit K^+ to pass readily through them exerting a relatively large influence on Em, hence the name "big conductance K^+ channels" (BK_{Ca}). Vasodilators that increase intracellular cAMP and cGMP (Nelson & Quayle,1995) and epoxides of AA (Campbell et al., 1996) can activate K_{Ca} channels. "Ca^{2+} sparks", focal increases in Ca^{2+} levels due to release of Ca^{2+} through ryanodine receptors in the sarcoplasmic reticulum (SR), appear to play the most important role in regulating K_{Ca} channel activity and vascular tone (Nelson et al., 1995). Most of the vasoconstrictors including adrenaline, Ang II, ET-1, 20-HETE, and serotonin depolarize VSMC by inhibition of K_{Ca} channel activity (Worley et al., 1991). Pharmacological blockers of K_{Ca} channels are external tetraethylammonium (TEA), charybdotoxin and iberiotoxin.

In contrast to K_V and K_{Ca} channels, which are activated by membrane depolarization, K_{IR} channels are activated by membrane hyperpolarization. The K_{IR} channel in arterial smooth muscle is very sensitive to inhibition by extracellular Ba^{2+}. Extracellular Ba^{2+} also blocks other K^+ channels but only at much higher concentrations. K_{IR} channels play an important role in the K^+ induced dilatation (Nelson & Quayle,1995; Edwards et al., 1988).

K_{ATP} channels are inhibited by intracellular ATP and activated by ADP. K_{ATP} channels respond to changes in the cellular metabolic state as well as to a number of endogenous vasodilators. K_{ATP} channels have been identified in various smooth muscle cells including VSMC (Nelson & Brayden,1993). K_{ATP} channels in different tissues exhibit considerable variation in their single channel properties. Sulfonylurea drugs, such as glibenclamide, block whole cell K_{ATP} current. Cromakalim, pinacidil and diazoxide are the selective K_{ATP} channels openers.

Glibenclamide, a K_{ATP} channel blocker, evokes depolarization and vasoconstriction in the coronary circulation (Imamura et al., 1992) and in mesenteric arteries (Garland & McPherson,1992). In contrast to the coronary and mesenteric circulation, glibenclamide did not affect basal tone in the renal (Loutzenhiser & Parker,1994), cerebral (Brayden,1991), and pulmonary arteries (Wiener et al., 1991). The responsiveness to K_{ATP} channel openers varies among different sizes of arteries. In smaller peripheral arteries, e.g. renal, femoral and mesenteric arteries, relaxation to levcromakalim, a K_{ATP} channel opener, was higher than in large conduit arteries such as aorta (Nagao et al., 1992). Although these results suggest a heterogeneous distribution of K_{ATP} channels in the cardiovascular system, the glibenclamide sensitive K^+ channel seems to be important in regulating membrane potential and current in rat aorta (Mishra & Aaronson,1999).

Most of the vasodilators that cause glibenclamide sensitive relaxation increase cAMP levels. Calcitonin gene related peptide, an endogenous opener of K_{ATP} channels (Abdelrahman et al., 1992), acts through the adenylate cyclase-cAMP protein kinase-A pathway (Quayle et al., 1994). NO and atrial natriuretic factor also act in part through activation of K_{ATP} channels (Armstead,1997), suggesting the possibility that elevation of cGMP-dependent protein kinase could activate K_{ATP} channels in smooth muscle. In contrast, Ang II (Miyoshi & Nakaya,1991),

AVP (Wakatsuki et al., 1992) and ET-1 (Miyoshi et al., 1992) have been shown to inhibit K_{ATP} channels in cultured smooth muscle cells.

1.5.2. K^+ Channel in experimental hypertension

The resting Em is more depolarized in VSMC from arteries of hypertensive animals compared with normotensive animals (Cheung,1984; Shoemaker & Overbeck,1986). The increased depolarization of the membrane potential in hypertensive animals is associated with increased vascular resistance and myogenic tone (Falcone et al., 1993). K^+ channel activity is altered in hypertension. Evidence suggests that the function of the K_V channel is decreased in hypertension. Whole cell current recorded in isolated thoracic aorta myocytes suggests that the contribution of K_V channels to resting K^+ conductance is significantly lower in SHR, compared with normotensive WKY rats (Cox,1996). The current through K_V channels was found to be substantially lower in interlobar artery myocytes from DOCA-salt hypertensive rats (Martens & Gelband,1996). The functional role of K_{Ca} in VSMC is enhanced in chronic hypertension. In hypertensive animals, pharmacological inhibition of these channels depolarize and constrict arteries from various vascular beds including the mesenteric vascular bed (Asano et al., 1993) and aorta (Rusch et al., 1992). Increased Ca^{2+} influx and K_{Ca} channel function have been detected in prehypertensive SHR (Asano et al., 1995). On the other hand, pharmacological or surgical interventions that cause hypertension may also increase the activity of K_{Ca} channels, suggesting it may be the consequence of elevated blood pressure (Sobey,2001). There is some evidence that vascular K_{IR} channel function may be altered during chronic hypertension (McCarron & Halpern,1990). Ba^{2+}-sensitive vasodilator responses to >7mM K^+ are impaired in cerebral arteries isolated from stroke-prone SHR. In DOCA-salt hypertensive pigs, K^+ induced dilatation was enhanced suggesting increased K_{IR} channel activity (Webb,1982). Synthetic K_{ATP}

channel activators are less potent dilators *in vivo* in chronic hypertension. The dilatation responses to pharmacological openers of K_{ATP} channels are decreased in VSMC from the mesenteric artery of the SHR (Ohya et al., 1996) and in L-NAME induced hypertension (Kalliovalkama et al., 1999). These findings suggest that K_{ATP} channel dysfunction may also attenuate with responses to endogenous vasodilator stimuli. Several studies clearly demonstrated that impairment of K_{ATP} channel-mediated vascular responses can be restored to near normal levels by long-term treatment of high blood pressure (Ohya et al., 1996; Kalliovalkama et al., 1999), indicating the role of K_{ATP} channels in the pathogenesis of chronic hypertension. In addition, though under normal condition K_{ATP} channels have a very low open-state probability in VSMC and have little influence on the resting membrane potential, ROS such as $O_2^{\cdot-}$, H_2O_2, and $OONO^-$ regulate the current through K_{ATP} channels. NO acts in part through activation of K_{ATP} channels (Armstead,1997). $O_2^{\cdot-}$ increases the open-state of K_{ATP} channels in ventricular cells from guinea pigs (Tokube et al., 1996). H_2O_2 and $OONO^-$ can induce relaxation via K_{ATP} channels (Wei et al., 1996). Therefore, increased oxidative stress associated with the hypertensive state may exaggerate the dysfunction of impaired K_{ATP} channels in some forms of hypertension.

1.5.3. K^+ channels in myogenic tone

As reviewed earlier in this introduction, spontaneous tone depends on Ca^{2+} influx via voltage-dependent Ca^{2+} channels. Under normal physiological conditions, a feedback mechanism counteracts the stretch induced increase in $[Ca^{2+}]_i$ and membrane depolarization to maintain the resting tone. It has been suggested that activation of K_{Ca} channels would increase K^+ efflux, which would counteract the depolarization to maintain the resting tone. Indeed, K_{Ca} channels can be activated by both $[Ca^{2+}]_i$ and membrane depolarization (Brayden & Nelson,1992). In the

SHR, increased activity of K_{Ca} channels has been reported to regulate myogenic tone (Asano et al., 1993). It has been suggested that, in hypertension, K_{Ca} channel activity may be increased to protect blood vessels against increased myogenic tone. Reduced K_V activity reported in hypertension could be an important factor underlying the depolarized Em and enhanced myogenic tone in hypertensive animals. Since NO activates K_V, impaired bioavailability of NO in hypertension could exaggerate depressed K_V channel activity, thereby contributing further to spontaneous tone. In diabetic rats, diminished responsiveness to K_{ATP} channel openers contributes to myogenic tone (Zimmermann et al., 1997). A similar impaired response to K_{ATP} channel openers reported in the SHR and in L-NAME induced hypertension may contribute to spontaneous tone in these models. These findings suggest that the activity of K^+ channels is an important modulator of spontaneous myogenic tone.

In view of the function of K^+ channels, it seems reasonable to postulate that part of the functional changes that contribute to spontaneous tone in vessels of the DOCA-salt model is related to alterations in K^+ channel function in VSMC. If this is the case, an inhibitor of K_{Ca} channels, iberiotoxin, would be expected to increase the magnitude of spontaneous tone in aorta of DOCA-salt hypertensive rats due to overactivity of these channels and it should induce tone in SHAM-normotensive rats. In addition, diminished responsiveness to a K_{ATP} channel opener may contribute to spontaneous tone in the DOCA-salt model. Accordingly, the effects of glibenclamide/cromakalim and iberiotoxin on spontaneous tone were tested.

1.6. Summary of the rationale for the work

The DOCA-salt model represents a low renin, ET-1/AVP dependent, volume overload form of hypertension. Large vessels such as the aorta in DOCA-salt hypertensive rats acquire a spontaneous increase in tone in response to stretch, which is thought to be myogenic in nature.

The aorta is a large conducting vessel. Spontaneous tone in large vessels could conceivably decrease compliance contributing to systolic hypertension. Reports from only a handful of studies on this phenomenon in DOCA-salt hypertensive rats demonstrate that spontaneous tone is endothelium-dependent and dependent on Ca^{2+} entry into aortic VSMC through L-type Ca^{2+} channels. Spontaneous tone has also been reported in experimental forms of high renin hypertension such as in the SHR and Ang II -dependent hypertension. In these rats oxidative stress and COX metabolites have been suggested to contribute to the generation of spontaneous tone, but the contribution of these factors to spontaneous tone in the DOCA-salt model has not been explored. In addition, K^+ channel activity and 20-HETE have been reported to modulate myogenic tone under some conditions. However the roles of K^+ channels and 20-HETE in the development of spontaneous tone in the DOCA-salt model have not been investigated. Several studies have demonstrated that the increased vascular production of ROS particularly $O_2^{\cdot-}$ in DOCA-salt hypertensive rats compromises NO bioavailability in these rats. ET-1 and AVP contribute to the increased $O_2^{\cdot-}$ production in these rats via NAD(P)H-oxidase. In addition, the inhibition of $O_2^{\cdot-}$ and 20-HETE attenuated the increase in BP in these rats. In view of the involvement of the ET-1/ AVP system, oxidative stress, and CYP metabolites in the development and maintenance of hypertension in the DOCA-salt hypertensive model, it is reasonable to postulate that one or more of these factors could contribute to the generation of spontaneous tone in the aorta of these rats.

Thus, the major goal of this project was to explore the contribution of the endothelium, endothelium-derived factors, ROS, 20-HETE and K_{ATP}-channels to the generation of spontaneous tone in DOCA-salt hypertensive rats.

1.6.1. Hypothesis and predictions of the working hypotheses

1.6.1.1. Hypothesis

Stretch of the vasculature induces a complex array of ROS (NO, $O_2^{\cdot-}$, H_2O_2) and metabolites of arachidonic acid (20-HETE and its metabolites) that interact in concert with depressed K_{ATP} channel activity to modulate membrane depolarization, opening of L-type Ca^{2+} channels and ultimately spontaneous tone in the DOCA-salt model of hypertension.

1.6.1.2. Working hypotheses and predictions

I. *Stretch induces parallel mechanisms in aortic rings from DOCA-salt hypertensive rats which, depolarizes the rings to generate spontaneous tone.* A prediction of this hypothesis is that arotic rings form DOCA-salt hypertensive rats but not from SHAM-normotensive rats should generate spontaneous tone in response to stretch.

II. *Endothelium contributes to the generation of spontaneous tone in aorta from DOCA-salt hypertensive rats by the production of ET-1.* A prediction of this hypothesis is that endothelium-denudation and ET_A or ET_B antagonists should attenuate spontaneous tone.

III. *Endothelium modulates spontaneous tone by producing NO in aortic rings from DOCA-salt hypertensive rats.* A prediction of this hypothesis is that endothelium-denudation and inhibition of NOS should exaggerate spontaneous tone.

IV. *DOCA-salt treatment induces hypertension and evokes stretch induced spontaneous tone in aorta by increasing $O_2^{\cdot-}$ generation, which increases spontaneous tone by inactivating NO in these rats.* A prediction of this hypothesis is that $O_2^{\cdot-}$ generation should be higher in DOCA-salt treated rats while scavengers of $O_2^{\cdot-}$ should decrease BP and decrease the magnitude of spontaneous tone in these rats. Another prediction

is that interventions that mute $O_2^{\cdot-}$ should reduce spontaneous tone when the NO system is functional but not when it is absent (endothelial-denudation) or inhibited (L-NAME treatment). In addition, the contributions of other ROS such as H_2O_2, $OONO^-$, and $^{\cdot}OH$ radical to the modulation of spontaneous tone were tested.

V. *DOCA-salt treatment evokes spontaneous tone in aorta in part by increased 20-HETE production.* A prediction of this hypothesis is that the production of 20-HETE should be increased in the aorta of DOCA-salt hypertensive rats and the inhibition of endogenous 20-HETE should attenuate spontaneous tone.

VI. *DOCA-salt treatment evokes spontaneous tone in aorta by impairing K_{ATP} channel function in aortic VSMC.* A prediction of this hypothesis is that responsiveness to K_{ATP} channel openers should be diminished in aortic rings and in VSMC isolated from aorta of DOCA-salt hypertensive rats.

2. Materials and Methods

2.1. Animals and animal preparation

All procedures and protocols performed were approved by the animal care committee at the University of Saskatchewan, Saskatoon, in accordance with the regulations established by the Canadian Council of Animal Care. Male Sprague-Dawley rats were purchased from Charles River (Montreal, PQ) at 6 wk of age and were housed in our animal quarters under standardized conditions. All rats were maintained on a 12-hours light/day cycle and received standard laboratory rat chow and water *ad libitum*.

All surgical procedures were performed in anesthetized rats. Anesthesia was induced by inhalation of 4% isoflurane and maintained with 2% isoflurane. An analgesic (0.03 mg kg^{-1} buprenorphine) was administered i.m. post-operatively. At the age of 8-10 weeks, the right kidney was removed through dorsal flank incision. One week later these rats were assigned randomly into one of two groups: a DOCA-salt treated or a SHAM treated group. In the DOCA-salt treated group, a silastic strip impregnated with 100 mg. kg^{-1} body weight of DOCA (Aldrich Chemical, Milwaukee, WI) was implanted subcutaneously in the midscapular region. A catheter connected to a radiotelemetry capsule (TA11PA-C40, Data Sciences, St. Paul, MN) was inserted into the left femoral artery and pushed so that its tip reached the abdominal aorta above the iliac bifurcation for monitoring BP. The capsule containing the transducer and radiotransmitter was positioned in the left flank region subcutaneously. From this point on, these rats were given a 0.9% NaCl and 0.2% KCl solution for drinking *ad libitum* over a 3-week period. Under this regimen, this group of rats developed hypertension. In the SHAM group, a DOCA-free silastic strip was implanted subcutaneously and tap water was supplied for drinking over the next 3

weeks. The silastic strip was made in a plastic mold from a mixture of medical grade elastomer and silastic curing agent (10:1 w/w) with or without DOCA (10mg/cm).

2.2. Chemicals

The chemicals used in the experiments and their sources are listed below:

General: Deoxycorticosterone Acetate (DOCA) was purchased from Sigma Aldrich Canada Ltd. (Oakville, ON). Isoflurane was purchased from Abbott Laboratories Limited (Saint-Laurent, PQ). Buprenorphine hydrochloride was purchased from Reckitt & Colman Pharmaceuticals Inc (Richmond, VA).

Chemicals used in functional studies: Kreb's solution salts were of analytical grade and obtained from BDH, Toronto, ON. Lucigenin, N^G-nitro-L- arginine methyl ester (L-NAME), sodium nitroprusside (SNP), catalase, acetylcholine, phenylephrine, indomethacin, tiron, tempol, superoxide dismutase (SOD), apocynin, aminobenzotriazole (ABT), glibenclamide, deferoxamine, and iberiotoxin were purchased from Sigma-Aldrich (Oakville, ON). Endothelin (ET-1), BQ 123 and BQ-788 were purchased from American Peptide Company (Sunnyvale, CA). 20-HETE, valeroyl salicylate and SQ 29,548 were obtained from Cayman Chemical Company (Ann Arbor, MI). Cromakalim was purchased from Tocris Cookson Inc., Ballwin (MO, USA).

All drug solutions and buffer were prepared fresh every day just before the experiment. Stock solutions for cromakalim and glibenclamide were made in DMSO. Stock solutions for SQ 29548 were prepared in aqueous solution and stored at -80°C. Stock solutions for indomethacin were made with warm absolute alcohol. Stock solutions for ET-1 were made with 0.2 M acetic acid and stored at -80°C. Stock solutions of BQ-788 were prepared in 5% $NaHCO_3$. All other chemicals were dissolved on the day of the experiment in aqueous solution and working

solutions were prepared by diluting with Kreb's buffer. Drugs added to the organ baths were in aliquots of <1% of the bath solution volume (10 ml). All experiments were done with an appropriate vehicle control.

Chemicals used for calcium studies: Fura-FF was purchased from Molecular Probe Inc. (Eugene, OR).

Chemicals used in molecular study (Western blot analysis and immunohistochemistry): OCT-compound (VWR, Edmonton, AB), nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA), monoclonal mouse anti-β-actin antibody (Sigma Aldrich, Oakville, ON), monoclonal mouse anti-eNOS antibody (Transduction Laboratories, Mississauga, ON), fluorescent anti-mouse (Jackson Immuno Research, Western Grove, PA), fluorescent anti-rabbit secondary antibody (Sigma, Saint Louis, MO), chemiluminescence (ECL, Amersham Pharmacia Biotech, Baie d'Urfe, PQ).

Chemicals used in patch clamp analysis: Albumin, papain, dithioerythritol, collagenase, and hyaluronidase were purchased from Sigma-Aldrich (Oakville, ON).

Chemicals used in HPLC assay: N,N– diisopropylethylamine (catalyst) was purchased from Sigma-Aldrich (Oakville, ON) and 2-(2,3 – naphthalimino) ethyl trifluoromethanesulfonate (dye) from Molecular Probe (Eugene, OR).

2.3. Equipment

2.3.1. Radiotelemetry System

The components of the radiotelemetry system are as follows: 1) an implantable capsule (TA11PA-C40, Data Sciences, St. Paul, MN) that contains a highly stable semiconductor strain gauge sensor and a battery powered electronic module to process the pressure signal and transmit

it from the animals to the receiver. 2) A 10-cm long catheter attached to the capsule. The catheter is filled with a viscous gel and the tip of the catheter is coated with an anti-thrombogenic film. 3) A receiver (RLA 1020) that detects the signal from the implanted transmitter and transfers to the acquisition system. 4) A barometric pressure reference (C11PR) that measures the atmospheric pressure to allow for the telemetered absolute pressure to be converted to a gauge pressure (relative to atmospheric pressure). 5) A consolidation matrix (BCM100) that multiplexes the signals from a number of receivers and provides power to the receivers. 6) A data acquisition system (DATAQUEST IV) that receives data from the receiver, filters corrupt samples from the incoming data stream, and stores the data after subtracting the atmospheric pressure.

2.3.2. Apparatus for mesenteric vascular bed perfusion

The components of the mesenteric vascular bed (MVB) perfusion apparatus are as follows: 1) a Harvard Apparatus Variable Speed Peristaltic Pump that pumps the Kreb's buffer from the jacketed storage chamber to the mesenteric vascular bed preparation. The flow was set at 5ml/min. 2) A thermocirculator (Harvard Apparatus, South Natick, MA) that maintains the buffer at 37°C in the jacketed storage chambers. 3) Tubes and valves: various sizes of tubing are connected together in order to conduct buffer from the storage chamber to the organ baths. Three-way valves inserted into the circuit facilitated switching of buffer. A second set of three-way valves, with a port for 1 cc syringes, was inserted midway in the tubing to allow for the injection of drugs. A third set of three-way valves was placed at the head of the apparatus to allow for the attachment of the cannulated MVB. These valves were also connected to a set of custom made glass T-tubes (Department of Chemistry, University of Saskatchewan). The T-tubes had rounded angles to facilitate the extraction of air bubbles trapped in the tubing. The

suction required to remove the air bubbles was achieved with a Gilson 2 minipump plus (Villiers le Bell, France). 4) A strain gauge transducer (Beckmen, Palo Alto, CA) was placed into the circuit just prior to the T-tube to record perfusion pressure of the preparation. Under conditions of constant flow, changes in perfusion pressure reflect changes in vascular resistance. The changes in perfusion pressure evoked by various interventions were recorded on a polygraph (Grass Inst. Co., Quincy, MA).

2.3.3. Apparatus for aortic ring tension study

The equipment for the aortic ring tension studies is as follows: 1) REDI-CUT fine wire with diameter 0.01inch (Alltech Associates, Inc. Deerfield, IL) is used to make triangular stirrups. Two of these stirrups are passed though the lumen of the aortic rings. A piece of silk thread connects the stirrups with the transducers and organ chambers. 2) A thermocirculator (Harvard Apparatus, South Natick, MA) that maintains the buffer at 37°C. 3) Custom made double layered vertical glass organ chambers (Department of Chemistry, University of Saskatchewan) that house the rings during experiments. Warm water is circulated through two layers of the glass chamber to maintain the buffer in the organ chamber at 37°C. The base of the chamber is attached to a tube with valve to facilitate emptying of the chamber. 4) Force displacement transducers (Model FT03, Grass Instruments, West Warwick, RI) that sense the tension caused by isometric contraction of the aortic rings. 5) Model 7E Polygraph, Model 7 DAF DC driver amplifier from Grass Instrument Co records the signals.

2.3.4. Apparatus for superoxide anion measurement: Luminometer

A TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) measures the light emitted from an interaction of lucigenin with $O_2^{\cdot-}$. Lucigenin added in the buffer with tissue gains an electron

from $O_2^{\cdot-}$ and emits light. The luminometer was set to read and integrate the arbitrary units of light emitted over a 30 sec period. The readings over this period are averaged.

2.3.5. Apparatus for 20-HETE assay

A High Pressure Liquid Chromatography (HPLC) system has the following components: 1) A Hitachi L-7200 Autosampler that automatically withdraws a fixed volume of sample from the sample vial and injects it into the mobile phase of a liquid chromatograph. 2) A Hitachi L-7100 pump that pumps the sample through the mobile phase at a constant flow rate. 3) A C^{18} reverse-phase HPLC column (4.6X250 mm, Waters Symmetry, Milford, MA) that separates CYP metabolites including 20-HETE using a mobile phase containing 81% methanol, 0.1% acetic acid. 4) A Hitachi L-7485 fluorescence detector is used to read the sample set at 259 nm and an emission cutoff below 394 nm. 5) A Hitachi interface D-7000 that converts the reading from the fluorescence detector to a digital signal. Data was processed in Windows NT (version 4) using a Hitachi model D-7000 Chromatography data sampler software (version 4).

2.3.6. Miscellaneous

Fluorimeter designed to monitor Ca^{2+} fluorescence (JASCO, CAF-100 Ca^{2+} analyzer, Japan Spectroscopic, Tokyo, Japan).

Apparatus for western blot analysis: Bausch and Lomb Spectronic Reader to read protein concentrations at 595 nm. Film was developed with AFA X-ray film processor (AFP Imaging Corp., Elmsford, NY). Density analysis was done with UN-SCAN-IT software from Silk Scientific Corporation.

Apparatus for patch clamp analysis: The following apparatus was used for patch clamp analysis: 1) An inverted phase contrast microscope (Olympus IX70) was used to see the cells for patching. 2) Axopach-200B patch clamp amplifier (Axon Instruments Inc.) was used to record

the currents. 3) Digidata 1200 interface and a pClamp software (version 7, Axon Instruments Inc.) was used to analyze the current data.

2.4. Methods

2.4.1. Implantation of the radiotelemetric devices

Mean blood pressure (MBP) was measured via radiotelemetry. The zero offset of each transducer was verified to be within ± 4 mm Hg before implantation of the radiotelemetric capsule. During the insertion of DOCA and SHAM strips, an incision was made in the left groin and the catheter of a telemetry device was inserted into the left femoral artery so that the tip of the catheter was in the abdominal aorta above the iliac bifurcation. The position of the tip of the catheter was verified when the rats were sacrificed for isolation of aorta. The capsule containing the transducer and radiotransmitter was positioned in the left flank subcutaneously.

2.4.2. Functional Studies

The composition of the modified Kreb's buffer solution: (in mM) NaCl 118, KCl 4.7, $MgCl_2$. $6H_2O$ 1.2, $CaCl_2$. $2H_2O$ 2.6, KH_2PO_4 1.2, $NaHCO_3$ 25, Glucose 11.1, pH 7.4. Buffer was maintained at 37°C and oxygenated with 95% O_2/ 5% CO_2.

2.4.2.1. Isolation and preparation of the mesenteric vascular bed

Under pentobarbital anesthesia (60 mg kg^{-1} body weight, i.p.) an incision was made in the abdominal wall to open the cavity. The superior mesenteric artery was identified and gently separated from the connective tissue with cotton swabs. Using a pair of curved forceps, two pieces of silk suture were placed under the superior mesenteric artery near its junction with the aorta. A small incision was made in the abdominal aorta and a catheter (PE 90) attached to a 21 G needle inserted through the incision. The sutures under the superior mesenteric artery were

tied around the catheter to secure the catheter within the superior mesenteric artery. The iliocaecal junction was identified and a suture was tied at the distal end of superior mesenteric artery. The MVB was removed from the intestinal wall. The superior mesenteric artery with the attached catheter was harvested by cutting the abdominal aorta above and below its attachment. The MVB was placed in Kreb's buffer and then connected to the perfusion apparatus.

2.4.2.2. Isolation and preparation of aortic rings

After three weeks of DOCA or SHAM treatment, rats were sacrificed under pentobarbital anesthesia (60 mg. kg^{-1}.body weight, i.p.). The chest cavity was opened and an incision was made in the heart to drain off the blood. The lungs, mediastinum and inferior vena cava were removed to expose the aorta. The thoracic aorta was separated from the connective tissue without stretching the vessel and placed in Kreb,s buffer. The thoracic aorta was cleaned of adherent fat. Seven mm long rings were cut. In some rings, a piece of soft tissue paper was wrapped around a PE50 tube and gently passed through the lumen of the aortic ring to remove the endothelium. Rings were mounted on triangular stirrups for isometric tension recording in organ chambers containing 10 ml of Kreb's buffer. One stirrup was anchored to the base of the bath while the other was attached to an FT0.3 isometric force transducer connected to a Grass 7E polygraph. The Kreb's buffer was changed every 15 minutes during the experiment.

2.4.3. Assays

2.4.3.1. Fura-FF assay

The relationship between the concentration of tiron and Ca^{2+} fluorescence was measured in buffered solutions in the absence of any biological tissue. A series of buffered solutions containing a fixed concentration of Ca^{2+} (1 mM) and increasing concentrations of tiron were

prepared. Fura-FF, a fura-2 derivative, was added to each sample tube to yield the final concentration of 1 μM. Experiments were performed with a fluorimeter designed to monitor Ca^{2+} fluorescence. Each sample was excited at 340 nm and 380 nm, and emitted light was collected at the photomultiplier through a 500 nm filter. The ratio of the fluorescence due to excitation at 340 nm to that at 380 nm ($R_{340/380}$) is directly proportional to the concentration of free Ca^{2+} in the buffer. Concentration-response curves to tiron were constructed from the data. The binding constant for tiron with calcium was calculated in this competition assay from the Cheng – Prusoff equation (Cheng & Prusoff,1973) shown below:

$$K_i = \frac{IC_{50(tiron)}}{1 + [fura\text{-}FF]/K_{d(fura\text{-}FF)}}$$

where K_i is the inhibitory constant for tiron, $IC_{50(tiron)}$ is the inhibitory concentration of tiron that reduces binding of Ca^{2+} to fura-FF by 50%, and K_d is the dissociation constant for fura-FF. In preliminary work, it was determined that tiron did not directly interfere with fura-FF fluorescence.

2.4.3.2. Measurement of superoxide anion

The composition of HEPES buffer solution was as follows: (in mM) HEPES 20, NaCl 119, KCl 4.6, $MgSO_4$ 2, Na_2HPO_4 0.15, KH_2PO_4 0.39, $NaHCO_3$ 5, Glucose 5.6, pH 7.4 . $CaCl_2$. $2H_2O$ 1.6. pH 7.4.

Aortic rings were rinsed with Kreb's buffer and transferred to test tubes containing 2 ml of HEPES buffer. Rings were incubated for several minutes and then transferred to test tubes containing 500 μl of HEPES buffer (pH 7.4) at 37° containing lucigenin (5 μM). Higher concentrations of lucigenin (250 μM) have been associated with interfering with redox cycling but lower concentrations (5 μM) have been shown not to interfere (Tarpey et al., 1999). Rings

were incubated with lucigenin for 15 min. The tubes were transferred to the luminometer. The arbitrary units of light emitted were read over a preset period of 30 seconds and integrated by the luminometer. Repeated measurements were collected over 5 minutes and averaged (reading 1). A cell permeable nonenzymatic scavenger of $O_2^{\cdot-}$, tiron (10 mM), was then added to quench the $O_2^{\cdot-}$ dependent chemiluminescence. After one minute, repeated measurements were taken again and eight readings over 5 minutes were averaged (reading 2). At the end of the experiment, aortic rings were blot dried and weighed. The difference between the initial set of readings and the readings after tiron was taken to calculate $O_2^{\cdot-}$ production.

Formula for calculation of $O_2^{\cdot-}$:

Superoxide anion = [{2X(Reading 1-2)}X1000]/ weight of the tissue in mg

2.4.3.3. Protein preparation and Western blot analysis

After isolation, a section from the thoracic aorta was frozen in liquid nitrogen immediately and stored at -80°C. The frozen tissue was then homogenized in lysis buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 0.1% SDS (sodium dodecyl sulfate), 1% Triton X-100, 1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 1,000 *g* at 4°C for 15 min. The supernatant was transferred to a fresh tube and centrifuged at 30,000 *g* at 4°C for 30 min. Protein concentration in the supernatant was determined using the Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). Fifty μg of total protein was electrophoretically size-separated on a 7% SDS-polyacrylmide gel and then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) at 4°C overnight. The membrane was blocked with 5% nonfat dry milk in TBS-T (pH 7.6, 20 mM Tris base, 137 mM NaCl, and 0.1% Tween 20) at room temperature for 1 hour and then incubated with 1:2000 diluted monoclonal mouse anti-eNOS antibody (Transduction Laboratories, Mississauga, ON) at 4°C overnight. The eNOS

protein was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Baie d'Urfe, PQ). The membrane was then stripped in stripping buffer containing 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris-HCl pH7.6 at 50°C for 30 min and reprobed with 1: 10,000 monoclonal mouse anti-β-actin antibody (Sigma Aldrich, Oakville, Ontario). The densitometric value of the samples was evaluated by densitometry (UN-SCAN-IT, Silk Scientific Corporation).

2.4.3.4. HPLC Assay for 20-HETE

1) Lipid Extraction: 1 g of thoracic aortic tissue was homogenized in 2 volumes of chloroform and 1 volume of methanol. The homogenate was centrifuged at 1000 g for 10 min at 4°C. The top phase (methanol) was discarded and the bottom phase (chloroform layer) was transferred to a fresh tube and dried under nitrogen.

2) Addition of internal standard and purification of lipid: 10 – 15 µl (100 – 150 ng) internal standard (WIT) was added to the dried phase followed by addition of methanol. The contents were dried under nitrogen and re-suspended with 200 µl of acetonitrile and 800 µl of H_2O. The re-suspended sample was vortexed briefly and transferred to a pre washed Sep-Pak Vac column. The column was washed twice with 1 ml of 30% acetonitrile in H_2O and eluted with 500 µl of ethyl acetate. The sample was dried under nitrogen

3) Fluorescent Labeling: 20 µl of 36.4 mM 2-(2,3 – naphthalimino) ethyl trifluoromethanesulfonate (dye) and 10 µl of N,N– diisopropylethylamine (catalyst) were added to the sample and vortexed thoroughly (20 sec) then incubated in the dark at room temperature for 30 min. It was then dried under nitrogen and re-suspended with 200 µl of acetonitrile and 800 µl of H_2O. The re-suspended sample was transferred to a pre-washed Sep-Pak Vac column and washed with 50% acetonitrile in H_2O six times to remove the extra dye. Then it was eluted

with 500 µl of ethyl acetate and dried under nitrogen. Finally, the sample was resuspended in 100 ul of methanol and transferred to a HPLC assay tube.

4) A standard curve was constructed by plotting the ratio of the fluorescence from varying concentrations of 20-HETE to a fixed amount of internal standard (WIT). The amount of 20-HETE in an unknown sample was determined from the standard curve.

2.4.4. Immunohistochemistry

Sections of the thoracic aorta were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 overnight at 4°C. After washing with physiological buffer solution, aortic sections were immersed serially in 10, 20 and 30% sucrose in physiological buffer solution at 4°C until the tissues floated. The aorta was then embedded in OCT-compound (VWR, Edmonton, AB) and frozen in liquid nitrogen, and stored at -80°C. Cross sections of the aorta were cut into 6 µm and placed onto poly-L-lysine coated slides. Slides were washed with 0.1% BSA in physiological buffer solution and then were incubated with 0.1% triton X-100 in PBS for 5 minutes at room temperature. The sections were blocked in 1:30 goat serum in physiological buffer solution for 30 minutes at room temperature. This was followed by incubating with 1:50 diluted monoclonal mouse anti-eNOS antibody (Transduction Laboratories, Mississauga, ON) and 1:100 diluted polyclonal rabbit anti-nitrotyrosine antibody (Molecular Probes, Eugene, OR) at 4°C overnight. After washing with PBS, aortic sections were incubated in the dark with either 1:200 diluted fluorescent anti-mouse (Jackson ImmunoResearch, Western Grove, PA) or 1:200 diluted fluorescent anti-rabbit secondary antibody (Sigma, Saint Louis, MO) at room temperature for 1 hour.

2.4.5. Electrophysiology: for K_{ATP} channel activity

Sections of freshly isolated rat thoracic aorta were kept in ice cold physiological saline solution (PSS). The aorta was cut into small pieces and incubated at 37°C in PSS containing 1 mg/ml albumin, 0.75 mg/ml papain, and 1 mg/ml dithioerythritol for 40 min. Then it was transferred to PSS in which 1 mg/ml collagenase and 1 mg/ml hyaluronidase were added. Single cells were released by gentle triturating through a Pasteur pipette, stored in the PSS solution at 4°C, and used within the same day of preparation.

Isolated VSMC were placed in a petri dish mounted on the stage of an inverted phase contrast microscope (Olympus IX70). The whole–cell patch–clamp technique was used to record K_{ATP} channel currents. Currents were recorded with an Axopach-200B patch clamp amplifier (Axon Instruments Inc.), controlled by a Digidata 1200 interface and a pClamp software (version 7, Axon Instruments Inc.). Membrane currents were filtered at 1 kHz with an eight-pole Bessel filter and stored on the hard disk of a computer for off-line analysis.

At the beginning of each experiment, the junctional potential between the pipette solution and the bath solution was electronically adjusted to zero. No leakage subtraction was performed to the original recordings, and all cells with visible changes in leakage currents during the course of study were excluded from further analysis. The pipette (intracellular) solution had the following composition: (in mM) KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10, EGTA 10; pH adjusted to 7.3 with KOH. Seals were made in 5.4 mM K^+ extracellular solution containing: (in mM) NaCl 135, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 1.2, HEPES 10, Glucose 5. Solution pH was adjusted to 7.3 with NaOH, but most recordings were made under conditions of symmetrical 140 mM K^+ using an extracellular solution of the same composition as described above, except that NaCl was omitted

from the 140 mM KCl solution. The experimental bath (volume 2 ml) was perfused continuously with extracellular solution at a rate of 2 ml. min^{-1}.

2.5. Experimental protocol

2.5.1. Study1: Characterization of spontaneous tone

2.5.1.1. Relationship of spontaneous tone to hypertensive state and preload

BP was recorded in conscious unrestrained rats after 3 weeks of DOCA and SHAM treatment. Individual rat cages were placed on receivers (RLA 1020, Data Science) for recording of BP. Two days prior to the recording, these rats were conditioned in the recording room. Subsequently the BP was recorded for five days. The first two hours were devoted to the establishment of a stable baseline.

Thoracic aortic rings were mounted in organ chambers. During the first hour after mounting the aortic rings in the organ chamber, the tension (preload) was increased progressively to the optimal tension of 5g. Five grams was chosen as the baseline tension since at this tension contraction induced by 120 mM of KCl was maximum. Since spontaneous tone was evident in rings from DOCA-salt hypertensive rats, SNP (10^{-7} M) was introduced for the last 20 minutes to allow final adjustment of the preload under passive conditions. In this way, the method for setting the preload was standardized and spontaneous tone could not contribute to the preload. On the removal of SNP by washing, aortic rings from DOCA-salt hypertensive rats, but not those from SHAM-control normotensive rats, showed an increase in tension. In a few experiments, a preload of 1, 3, or 5 g was set in the presence of SNP and the peak increase in tension following washout of SNP was plotted as a function of the preload.

Various treatments were administered either acutely after rings developed spontaneous tone or by preincubation in the bath before stretching the tissue to its optimal preload. Following SNP washout aortic rings were allowed to stabilize for 90 minutes. For acute treatment, readings were taken after 30-45 minutes to allow the maximum response to reach a plateau stage. In order to normalize the data from different rings, the contractile response to 120 mM of KCl was determined in each ring at the end of each experiment. The mean peak increase in tension (g) that developed spontaneously following washout of SNP was expressed as a percentage of the contractile response (g) to 120 mM KCl.

2.5.1.2. Role of extracellular calcium

In these experiments, the preload was applied to the aortic rings in a Ca^{2+} free buffer. Subsequently Ca^{2+} was replaced in the buffer to determine the effect of Ca^{2+} on spontaneous tone. In some experiments, aortic rings were treated with nifedipine (10^{-7} M) to explore the role of L-type calcium channel on spontaneous tone.

2.5.1.3. Role of endothelium in spontaneous tone

Spontaneous tone was recorded in endothelium-intact and endothelium-denuded aortic rings from DOCA-salt hypertensive rats and SHAM-normotensive rats. The loss of vasodilatory responses to acetylcholine (1 μM) was used to confirm endothelium-denudation. The role of ET-1 was examined by incubating rings with the ET_A antagonist, BQ123 (10 μM), and the ET_B antagonist, BQ788 (1.2 nM, 1.3 and 10 μM), either alone or in combination. Loss of ET-1 induced vasoconstriction was used to confirm the effectiveness of the compounds. In a few rings spontaneous tone was recorded in the presence of the NOS inhibitor, L-NAME (300 μM).

2.5.1.4. Endothelial nitric oxide synthase (eNOS) protein expression

Western blot analysis was done to detect eNOS protein expression in aorta from DOCA-salt hypertensive rats and SHAM normotensive rats, and immunohistochemistry was done to localize eNOS protein in these vessels.

2.5.2. Study 2: Contribution of oxidative stress to spontaneous tone

2.5.2.1. Blood pressure recording

During the course of DOCA and SHAM treatment, each group of rats were randomly divided into four subgroups: untreated rats, rats treated with tempol (10^{-3} M) or tiron (10^{-2} M) or apocynin (1.5×10^{-3} M). Tempol, tiron, or apocynin was added to the drinking water for three weeks. Individual rat cages were placed on receivers (RLA 1020, Data Science) for recording of BP. Two days prior to the recording, these rats were conditioned in the recording room. Subsequently, the BP was recorded for five days. The first two hours were devoted to the establishment of a stable baseline.

2.5.2.2. Specificity of antioxidants

Specificity of the various antioxidants, superoxide dismutase (SOD, 150 units ml^{-1}), apocynin (100 μM), catalase (1000 U/ml), tempol (10^{-4} M) and tiron (10^{-2} M) were tested in parallel experiments. The effect of these interventions on spontaneous tone was compared to the responses evoked by phenylephrine (1 μM) and KCl (30 mM).

2.5.2.3. Effects of tiron unrelated to that of quenching superoxide anion

2.5.2.3.1. MVB perfusion

The MVB preparation was allowed to stabilize for 1 hour to attain a steady baseline. Then several "wake-up" doses of phenylephrine (100 μM) were injected to evoke repeatable vasoconstrictor responses before proceeding to the specific experimental studies.

2.5.2.3.1.1. Effect of tiron on KCl induced contraction

Buffered solutions containing 90 mM KCl and adjusted for isotonicity were perfused through the MVB to induce a sustained vasoconstrictor response. In preliminary experiments, 90 mM KCl evoked a maximum vasoconstriction that persisted for at least two hours. In these KCl preconstricted preparations, 0.01, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 10 and 100 mM of tiron in KCl buffer was perfused through the MVB in the presence and absence of tempol. Each concentration was perfused for 10 minutes in a cumulative fashion and the decreases in perfusion pressure evoked by tiron were recorded. Constructed concentration-response curves to tiron in KCl depolarized tissues were constructed from the data.

2.5.2.3.1.2. Effect of tiron on Ca^{2+}-evoked vascular responses

The MVB was depolarized by 90 mM KCl in the presence of Ca^{2+} free buffer. When the perfusion pressure returned to baseline, buffered solutions containing 0.2, 0.4, 0.8, 1.6, 2.5 and 5 mM Ca^{2+} were perfused through the MVB in the presence and absence of tiron (10mM). Each concentration of Ca^{2+} was perfused for 10 minutes in a cumulative fashion and the increases in perfusion pressure evoked by each concentration of Ca^{2+} were recorded.

2.5.2.3.2. Fura-FF competitive binding assay

A series of buffered solutions containing 1 mM Ca^{2+} and 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 10 mM of tiron were prepared. Fura-FF, a fura-2 derivative, was added to each tube to yield the

final concentration of 1 μM. Tubes were placed in a fluorimeter to read the fluorescence after excitation at 340 and at 380 nm.

2.5.2.4. Superoxide anion dependent component of spontaneous tone

The role of $O_2^{\cdot-}$ was examined by preincubating aortic rings in the presence and absence of the $O_2^{\cdot-}$ scavenger, SOD (150 units ml^{-1}), or apocynin (100 μM), a NADPH-oxidase inhibitor. Parallel experiments were conducted in endothelium-intact and endothelium-denuded preparations. Experiments were done using allopurinol (1 and 10 μM), an inhibitor of xanthine oxidase, or L-NAME (300 μM), an inhibitor of NOS, or indomethacin (50 μM) or valeroyl salicylate (3 mM), inhibitors of COX, to explore the contributions of these enzymes to $O_2^{\cdot-}$ generation. The role of hydrogen peroxide (H_2O_2) was determined by recording spontaneous tone in the presence and absence of catalase (1000 units ml^{-1}), an enzyme that reduces H_2O_2 to H_2O and O_2, and deferoxamine (60 μM), a scavenger of the hydroxyl radical.

2.5.2.5. COX dependent component of spontaneous tone

The role of COX metabolites in modulating spontaneous tone was determined by incubating aortic rings with one of two COX inhibitors, indomethacin (50 μM) or valeroyl salicylate (3 mM), or with the thromboxane/prostaglandin (TP) receptor antagonist, SQ 29548 (3 μM) or the TXA_2 synthase inhibitor, furegrelate (50 μM). Parallel experiments were performed in endothelium-intact and endothelium-denuded aortic rings.

2.5.2.6. Measurement of superoxide anion

2.5.2.6.1. Lucigenin chemiluminescence

$O_2^{\cdot-}$ generation was determined in untreated aortic rings from DOCA-salt hypertensive rats and SHAM-normotensive rats to establish a baseline. After tension experiments, aortic rings were removed from organ baths and $O_2^{\cdot-}$ generation was determined. In some experiments, $O_2^{\cdot-}$ production was measured after isolated aortic rings had been incubated in the organ bath for one hour without a preload (unstretched) and in rings subjected to a preload of 5g for one hour (stretched).

2.5.2.6.2. Immunohistochemistry

Immunohistochemistry was done in aortic sections from DOCA-salt hypertensive rats and SHAM-normotensive rats to localize and quantify 3-nitrotyrosine protein in aorta.

2.5.3. Study 3: Contribution of 20-HETE to the generation of spontaneous tone

2.5.3.1. Detection of 20-HETE in aortic tissue

20-HETE was determined in thoracic aortic tissue from DOCA-salt hypertensive rats and SHAM normotensive rats using a fluorescent HPLC assay.

2.5.3.2. Effects of 20-HETE and ABT in aortic ring tension studies

In some rings, endogenous production of 20-HETE was blocked by incubating the rings with ABT (10 μM), an inhibitor of CYP4A that blocks ω-hydroxylation. In other rings, responses to exogenous 20-HETE (1 μM) were recorded in the presence and absence of ABT. To determine the contribution of COX metabolites of 20-HETE, responses to 20-HETE were also recorded in the presence of the COX inhibitor, valeroyl salicylate (3 mM), or a TP- receptor antagonist, SQ 29548 (3 μM) or the TXA_2 synthase inhibitor, furegrelate (50 μM).

2.5.4. Study 4: Contribution of K_{ATP} channel to the generation of spontaneous tone

2.5.4.1. Effects of K_{ATP} channel opener in preconstricted aortic rings

Aortic rings from DOCA-salt hypertensive rats and SHAM-normotensive rats were stretched gradually to 1.5 g passive load. After an one hour stabilization period, aortic rings were preconstricted with 10^{-6} M phenylephrine and responses to cromakalim (10^{-10} to10^{-5} M) were recorded in the presence and absence of endothelium. Inhibition of the cromakalim-induced response by glibenclamide (10^{-5} M) was taken to confirm the involvement of the K_{ATP} channel.

2.5.4.2. Effects of K_{ATP} channel modulators on spontaneous tone

The effects of cromakalim and glibenclamide were evaluated in rings after the rings developed spontaneous tone in a cross-over design in parallel experiments.

2.5.4.3. Whole cell patch clamp analysis for K_{ATP} currents

Membrane capacitance of VSMC isolated from the aorta of DOCA-salt hypertensive and SHAM-normotensive rats were determined. The initial experiments were designed to establish whether cromakalim could activate K_{ATP} currents in freshly isolated aortic smooth muscle cells. The recording conditions (cell dialysis with 10 mM EGTA and a holding potential of – 60 mV) were therefore chosen to minimize activity of Ca^{2+}-activated and delayed rectifier potassium channels. Cells were dialyzed with 0.3 mM ATP to enhance K_{ATP} currents. In aortic VSMC from SHAM-control rats, the K^+ gradient was reversed by increasing the extracellular K^+ concentrations from 5.4 to 140 mM. Under these conditions, inward currents were recorded. K_{ATP} channel currents were recorded in the presence of the K_{ATP} channel opener, cromakalim (1μM), and the K_{ATP} channel inhibitor glibenclamide (10 μM).

2.6. Data analysis

All values are expressed as means ± SEM. The EC_{50} values were derived from log concentration-response curves. Comparisons between groups were based on analysis of variance (ANOVA). If ANOVA indicated significance, simultaneous multiple comparisons were based on Scheffe's multiple comparison procedure. When there were only two groups, statistical comparisons were done by Student's t-test. Significance was accepted when p was <0.05.

3. Results

N.B. Negative data, particularly that related to the SHAM-groups, are not always shown.

3.1. Study 1: Characterization of spontaneous tone

3.1.1. Control BP values

After 3 weeks of DOCA treatment, the baseline MAP for DOCA salt hypertensive rats was significantly higher than that in SHAM treated rats (158 ± 3 vs 100 ± 3 mmHg, $p<0.05$; $n = 9$ in each group).

3.1.2. Relationship of spontaneous tone to hypertensive state and preload

In response to stretch aortic rings from DOCA-salt hypertensive rats, but not rings from SHAM normotensive rats, developed spontaneous tone (Figure 1). The increase in tone was often accompanied by oscillations in tension. The relationship between preload and spontaneous tone is shown in Figure 2. In these experiments, a preload of 1, 3 or 5 g was set in the presence of SNP and the peak increase in tension following washout of SNP was plotted as a function of the preload. Tone increased as a function of the preload suggesting a direct relationship between stretch and contraction. In aortic rings from DOCA-salt hypertensive rats at 5g resting tension, the developed peak tension was 2.2 ± 0.16 g, which represented 39.2 ± 3.1 % of the maximum contraction to 120 mM KCl (5.36 ± 0.38 g). Maximum contractile responses to 120 mM of KCl were significantly lower in aortic rings from SHAM-normotensive rats (4.1 ± 0.4 g). Aortic rings from normotensive SHAM-control rats failed to develop tone after washing out SNP (1.1 ± 0.57 % of KCl contraction).

3.1.3. Role of extracellular calcium

Spontaneous tone generated in rings from DOCA-salt hypertensive rats was completely abolished in the presence of a Ca^{2+} free buffer. Replacing the buffer with Ca^{2+} (2.6 mM) restored spontaneous tone after 90 minutes (from 0.025 ± 0.012 % to 25.33 ± 3.4 % of the maximum KCl contraction). The L-type calcium channel blocker, nifedipine (10^{-7} M), also completely blocked spontaneous tone (n = 5, $p<0.001$) compared to control values recorded in the absence of nifedipine (0.00% vs 36.77 ± 2.3 % of maximal contractile response).

3.1.4. Role of the endothelium in spontaneous tone

3.1.4.1. Effect of endothelium-denudation

In aortic rings from DOCA-salt hypertensive rats, endothelium-denudation increased the magnitude of spontaneous tone compared to the rings with intact endothelium. A typical tracing from a single experiment is shown in Figure 3. The exaggerated peak increase in tension was associated with a loss of the oscillations in tension. Endothelial-denudation failed to evoke spontaneous tone in rings isolated from SHAM control rats (Figure 4).

3.1.4.2. Effect of ET_A and ET_B receptor antagonist on spontaneous tone

Pretreatment or the acute administration of the ET_A receptor antagonist, BQ123 (10 μM), and the ET_B receptor antagonist, BQ788 (1.2 nM, 1.3 and 10 μM), had no significant effect on the development of spontaneous tone in the aortic rings from DOCA-salt hypertensive rats. These antagonists together did attenuate contractions evoked by ET-1.

3.1.4.3. Effect of L-NAME on spontaneous tone

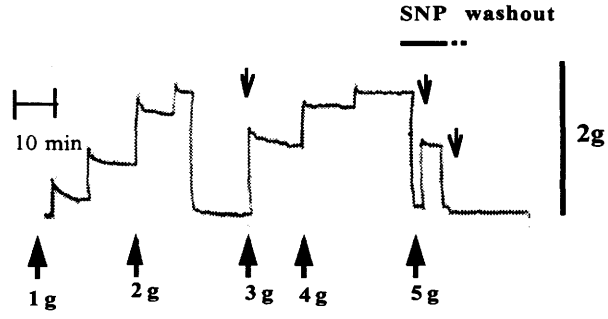
Pretreatment of aortic rings from DOCA-salt hypertensive rats with L-NAME (300 μM) increased spontaneous tone (Figure 5). In addition to pretreatment, L-NAME enhanced

spontaneous tone when it was added to the bath subsequent to the development of spontaneous tone. L-NAME did not increase the magnitude of spontaneous tone any further in endothelium denuded rings from DOCA-salt hypertensive rats. In aortic rings from SHAM control rats, L-NAME failed to evoke spontaneous tone either in the presence or absence of endothelium (Figure 6).

3.1.4.4. Endothelial-nitric oxide synthase (eNOS) protein expression

eNOS protein was present in the aorta of both DOCA-salt and SHAM rats. There was no significant difference in eNOS protein expression in the aorta of DOCA-salt and SHAM rats as determined with Western blot analysis (Figure 7) or with immunohistochemistry (Figure 8).

a. SHAM normotensive rats



b. DOCA-salt hypertensive rats

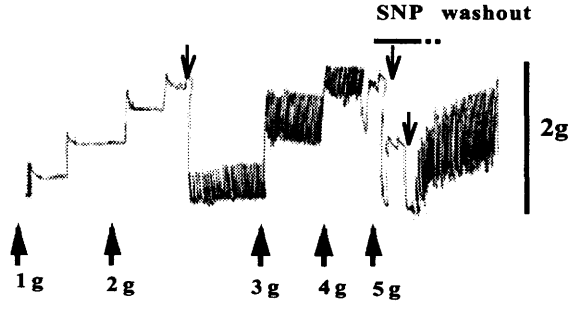


Figure 1. Typical tracing illustrating the development of spontaneous tone

Comparison of the development of spontaneous tone in aortic rings isolated from SHAM-control rats (Panel a) and DOCA-salt hypertensive rats (Panel b). The rings were stretched over a period of one hour in 1g increments (solid arrows) to an optimum tension of 5 grams for development of smooth muscle tone. Sodium nitroprusside (SNP; 10^{-7} M) was added to the bath for the last 20 minutes to allow adjustment of preload under passive conditions. Arrows pointing down indicate readjustment of the baseline.

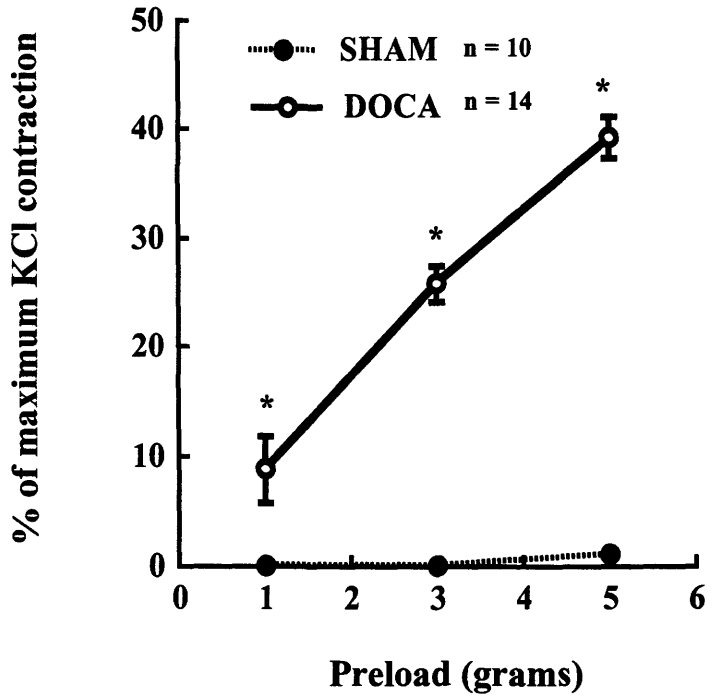


Figure 2. Relationship of spontaneous tone to preload

Values are mean ± SEM and expressed as a percentage of the maximal contractile response evoked by 120 mM of KCl. If not shown, error bars are within the range of the symbol. * $p < 0.05$ compared with SHAM-control group.

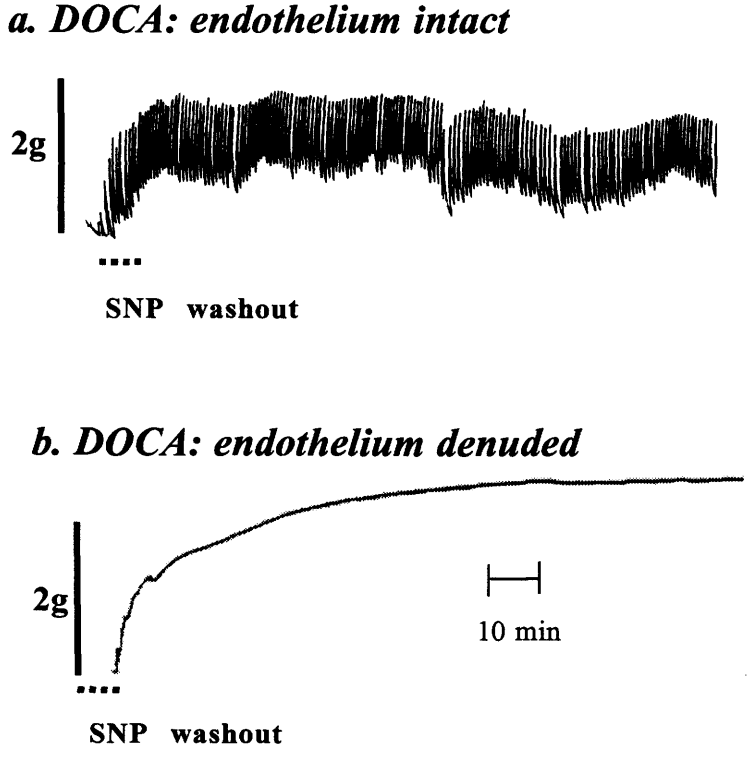


Figure 3. Typical tracing illustrating the effects of endothelium-denudation

Spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats in the presence (panel a) and in the absence of endothelium (panel b). SNP— sodium nitroprusside.

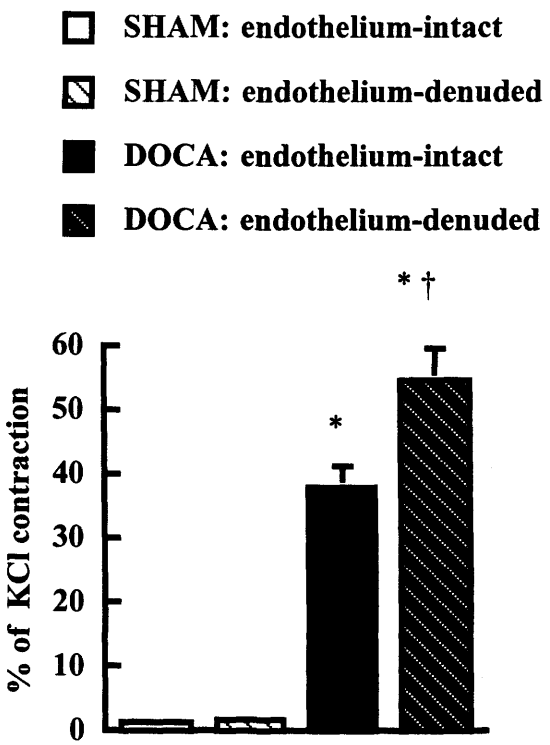


Figure 4. Pooled data on the effects of endothelium-denudation

Magnitude of spontaneous tone in aortic rings from SHAM-normotensive and DOCA-salt hypertensive rats in the presence and absence of endothelium. Pooled values expressed as a percentage of the maximum contractile response evoked by 120 mM KCl. Values are mean ± SEM, n = 8 in each group. * $p<0.05$ compared with rings from SHAM-normotensive rats and † $p<0.05$ compared with endothelium-intact rings.

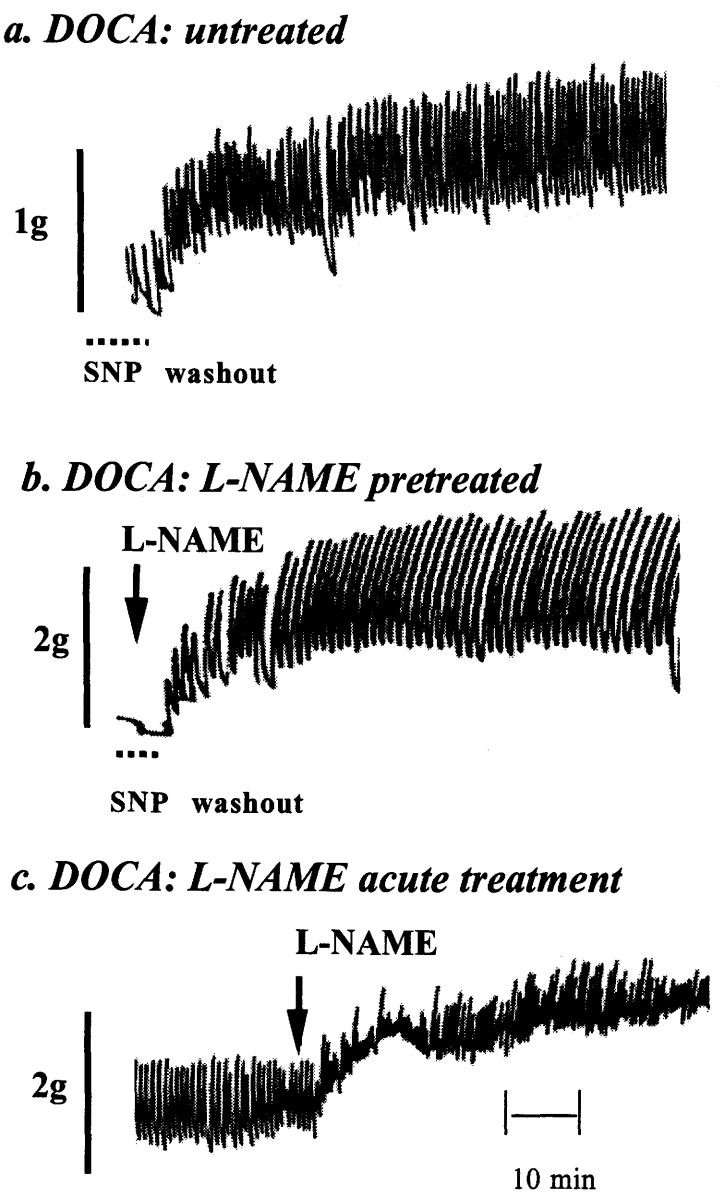


Figure 5. Typical tracing illustrating the effects of L-NAME

Spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats when a ring was untreated (panel a), when a ring was pretreated with 300 μM of N^G-nitro-L- arginine methyl ester (L-NAME) (panel b), and when a ring was treated with 300 μM of L-NAME after spontaneous tone had developed (panel c). SNP— sodium nitroprusside.

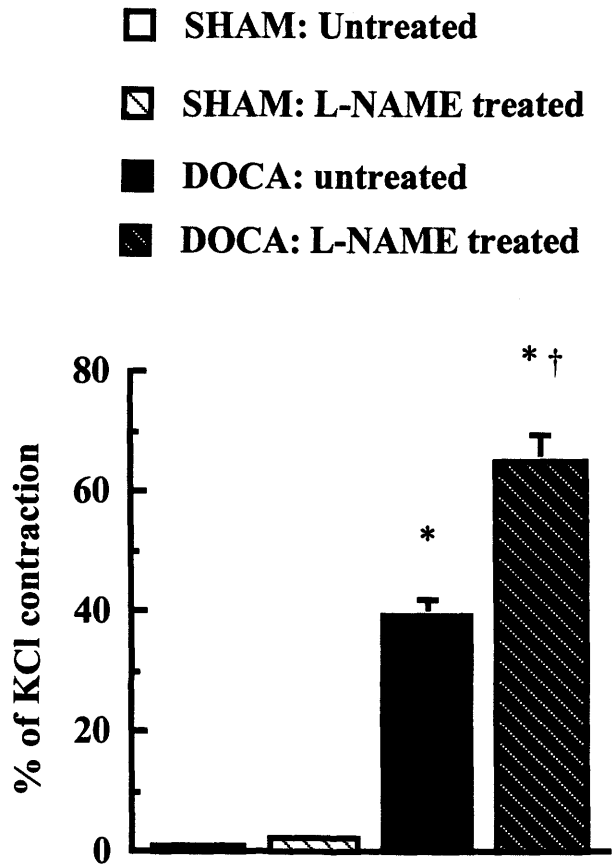


Figure 6. Pooled data on the effects of L-NAME on spontaneous tone

Magnitude of spontaneous tone in the aortic rings from SHAM-normotensive and DOCA-salt hypertensive rats in the presence and absence of pretreatment with N^G-nitro-L- arginine methyl ester (L-NAME, 300 μM). Pooled values are expressed as a percentage of maximum contractile response to 120 mM KCL. Values are mean ± SEM, n = 10 in each group. * $p<0.05$ compared with rings from SHAM-normotensive rats and † $p<0.05$ compared with untreated rings from DOCA-salt hypertensive rats.

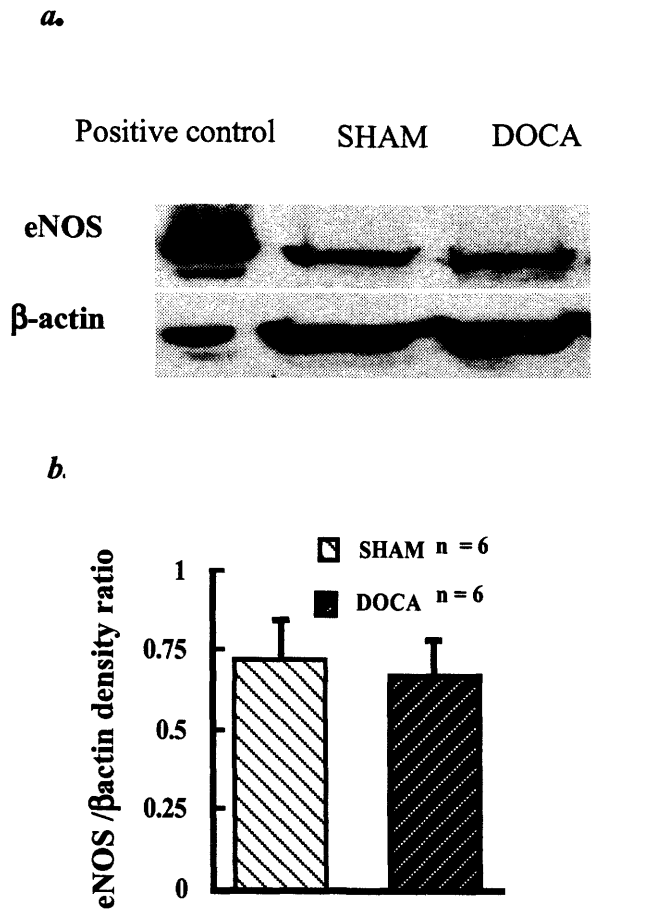


Figure 7. Western blot analysis of eNOS protein expression

Endothelial nitric oxide synthase (eNOS) protein expression (Western blot analysis) from a single experiment in aorta from SHAM-control and DOCA-salt hypertensive rats (panel a). The pooled data are expressed as the ratio between eNOS and β-actin (panel b).

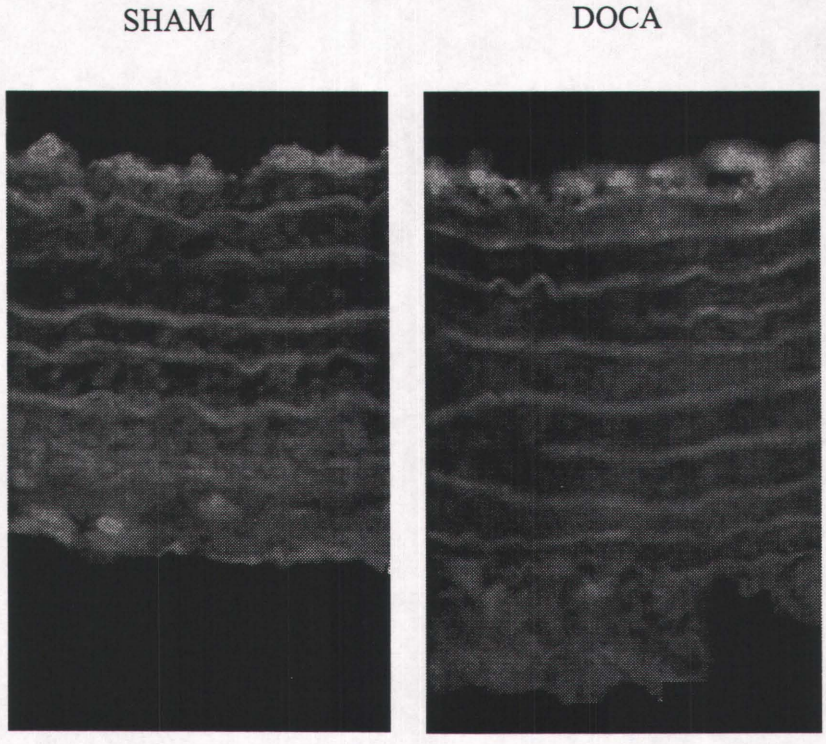


Figure 8. Immunohistochemical analysis of eNOS protein expression

Endothelial nitric oxide synthase (eNOS) protein expression (immunohistochemistry) from a single experiment in aorta from SHAM and DOCA-salt hypertensive rats. The images are original magnification X 40. The images shown are representative of similar results observed in preparations from 8 rats.

3.2. Study 2: Contribution of oxidative stress

3.2.1. Responses to oral antioxidant administration on blood pressure

Administration of tempol (10^{-3} M) or tiron (10^{-2} M) or apocynin (1.5 X10^{-3} M) in the drinking water for three weeks during the course of DOCA-treatment prevented the increase in MAP in DOCA-salt hypertensive rats (Table 1). In SHAM normotensive rats tempol or tiron had no significant effect on the MAP, $O_2^{\cdot-}$ generation, or spontaneous tone. Apocynin was not tested in SHAM-control rats. These treatments inhibited the increase in $O_2^{\cdot-}$ generation and the development of spontaneous tone in aorta of DOCA-salt hypertensive rats (Table 1).

Acute treatment with the anti-oxidants for one week after hypertension had developed yielded largely unremarkable results. Tempol (10^{-3} M) or apocynin (1.5 X10^{-3} M) for one week failed to decrease the elevated MAP, $O_2^{\cdot-}$ generation, or spontaneous tone in both DOCA-salt hypertensive and SHAM-normotensive rats. Tiron (10^{-2} M) did reduce MAP in the DOCA-salt group (125 ± 7 mmHg, n = 6) and inhibited $O_2^{\cdot-}$ generation.

Table 1. Effect of oral administration of antioxidant on mean arterial pressure, superoxide production and spontaneous tone generation

Groups	Mean Arterial Pressure (mmHg)	Superoxide anion production (milliunits. mg^{-1}. min^{-1})	Spontaneous tone (% of maximum contractile response to 120 mM KCl)
SHAM- control	107 ± 4 (16)	875 ± 135	1.25 ± 0.6
+ Tempol	103 ± 6 (6)	758 ± 199	1.00 ± 0.5
+ Tiron	96 ± 4 (6)	715 ± 73	0.50 ± 0.25
DOCA-salt	161 ± 2 (21)*	3166 ± 232*	39.2 ± 2.3*
+ Tempol	108 ± 5 (6)†	824 ± 265†	4.80 ± 2†
+ Tiron	99 ± 3 (6)†	702 ± 97†	0.00†
+ Apocynin	122 ± 0.9 (4)†	1041 ± 314†	9.30 ± 6.1†

Effect of 3- weeks of tempol (10^{-3} M) and tiron (10^{-2} M) treatment on the mean arterial pressure, $O_2^{\cdot-}$ generation and spontaneous tone in SHAM-normotensive rats and DOCA-salt hypertensive rats and apocynin (1.5×10^{-3} M) in DOCA-salt hypertensive rats. The numbers in parentheses indicate number of animals for each group. * $p < 0.05$ compared with the SHAM-normotensive control animals; † $p<0.05$ compared with DOCA control animals.

3.2.2. Specificity of antioxidants

SOD, apocynin, and tempol inhibited the spontaneous tone but had no significant effect on the phenylephrine and KCl induced contractile responses both in the presence and absence of endothelium in aortic rings from DOCA-salt hypertensive rats (Table 2). Catalase increased the magnitude of spontaneous tone but did not affect the phenylephrine and KCl induced contractile response. However, tiron not only abolished spontaneous tone, it also inhibited phenylephrine and KCl induced contractions in the presence and absence of endothelium. Importantly, tiron inhibited the maximum contractile response induced by 120 mM of KCl. These results together with the observation that tiron increased the clotting time led to the notion that tiron may exert effects other than those related to quenching of $O_2^{\cdot-}$. In view of the observations described above it was postulated that tiron might bind Ca^{2+}.

3.2.2.1. Effect of tiron on Ca^{2+}-binding in buffered solutions

In the absence of tissue and in buffered solutions containing a fixed concentration of Ca^{2+} (1 mM), tiron evoked dose-dependent decreases in the ratio of fura-FF fluorescence (Figure 9). This inhibitory effect of tiron on Ca^{2+} binding to fura-FF was maximal at 10 mM with an IC_{50} of 0.8 ± 0.05 mM. With a K_d for fura-FF of 5.5 μM and its concentration in the assay buffer of 1.0 μM, the K_i for tiron was calculated to be 0.692 ± 0.036 mM.

3.2.2.2. Effect of tiron on KCl-preconstricted vessels

Tiron evoked concentration-dependent decreases in perfusion pressure in the MVB preconstricted with 90 mM of KCl both in the presence and absence of tempol (Figure 10). On first inspection, it appears that, if anything, there was a tendency of tempol to enhance rather than reduce the vasorelaxant effect of tiron at least at the lower doses of 0.2 and 0.4 mM.

However, ANOVA indicated that values expressing the relationship between the dose of tiron and the decrease in perfusion pressure were not significantly different in the presence and absence of tempol ($p = 0.09$). Importantly, the R_{max} and the EC_{50} values for tiron were not significantly different in the presence and absence of tempol (Table 3).

3.2.2.3. Effect of tiron on Ca^{2+} evoked responses

With 90 mM KCl in the perfusate, Ca^{2+} evoked concentration-dependent increases in perfusion pressure of the MVB (Figure 11). Perfusion with tiron did not alter the basal perfusion pressure significantly. However, the curve expressing the relationship between the concentration of Ca^{2+} and the decreases in perfusion pressure was shifted dramatically to the right when 10 mM tiron had been added to the perfusate.

Table 2. Effect of antioxidants on spontaneous tone and agonist evoked contractions

Groups	Spontaneous tone	Phenylephrine (1μM)	KCl (30 mM)
	% of maximum contractile response to 120 mM KCl		
Endothelium intact:			
Untreated	38.2 ± 3.1	72.5 ± 2.5	72.0 ± 3.4
SOD	1.80 ± 0.9*	73.4 ± 3.8	77.1 ± 5.2
Apocynin	0.30 ± 0.3*	71.5 ± 1.2	72.6 ± 2.7
Catalase	58.7 ± 6.3*	69.6 ± 0.47	70.6 ± 0.5
Tempol	6.20 ± 2.5*	65.9 ± 0.37	72.3 ± 0.42
Tiron	0.02 ± 0.02*	56.23 ± 5.2*	63.1 ± 4.5*
Endothelium denuded:			
Untreated	54.7 ± 4.9†	74.5 ± 1.7	73.7 ± 4
SOD	59.0 ± 5.2†	70.4 ± 3.4	72.7 ± 3.2
Apocynin	54.5 ± 1.2†	73.5 ± 2.3	73.3 ± 1.5
Catalase	71.0 ± 3.4†*	75.3 ± 6.5	76.1 ± 2.7
Tempol	50.7 ± 2 †	70 ± 34	70.56 ± 1.8
Tiron	0.00†*	59.2 ± 5.2*	61 ± 2.3*

Effect of superoxide dismutase (SOD, 150 Units ml^{-1}), apocynin (100 μM), catalase (1000 Units ml^{-1}), tempol (10^{-4} M) and tiron (10^{-2} M) on spontaneous tone, phenylephrine (1 μM) and KCl (30 mM) induced contraction in aortic rings from DOCA-salt hypertensive rats in the presence and absence of endothelium. Data are mean ± SEM expressed as a percentage of maximum contraction response to 120 mM of KCl (g) obtained at the end of the tension experiments. n = 5 to 8 in each group. * $p<0.05$ compared with untreated rings. † $p<0.05$ compared with endothelium intact rings.

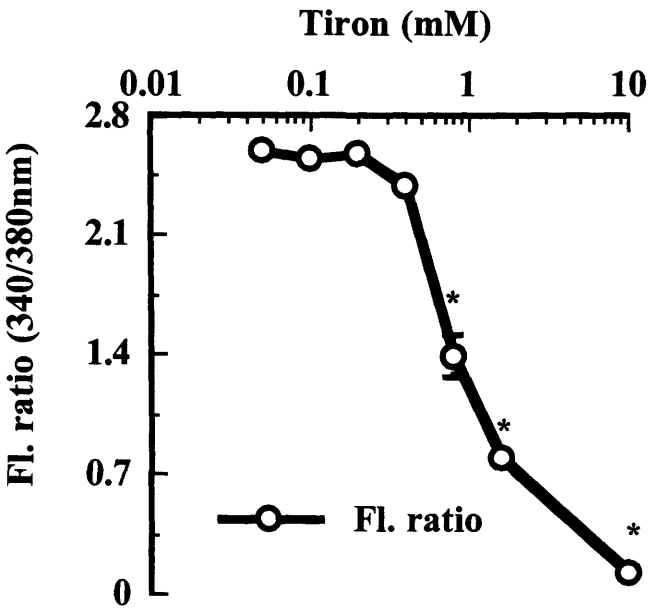


Figure 9. Relationship of the ratio of fura-FF fluorescence to the concentration of tiron

Fluorescence (340 nm/380 nm) was determined in buffered solutions in the absence of tissue. Values are means ± SEM, n = 6. If not shown, error bars are within the height of the symbol. *$p<0.001$ compared with values in the absence of tiron.

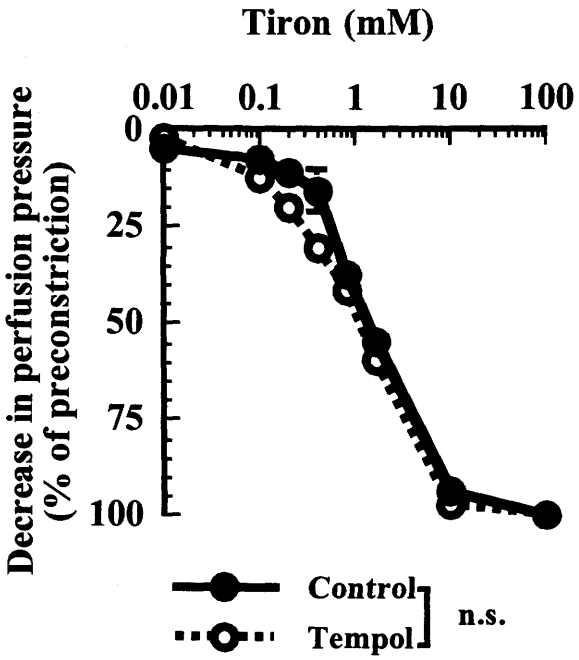


Figure 10. Effect of tiron on perfusion pressure

Relationship of the decrease in the perfusion pressure to increasing concentrations of tiron in the perfused rat superior mesenteric arterial bed preconstricted with 90 mM KCl in the presence and absence of tempol 100 μM. Values are means ± SEM expressed as a percentage of the preconstriction, n = 6. If not shown, error bars are within the height of the symbol. n.s. indicates values for the two groups were not significantly different (ANOVA).

Table 3. Potency and efficacy of tiron in the presence and absence of tempol

Groups	EC_{50} (mmol/L)	R_{max} (mmHg)
Control	1.46 ± 0.33	43.58 ± 2.6
Tempol(100μM)	1.34 ± 0.27 $^{p=0.74}$	46.12 ± 1.87 $^{p=0.52}$

Responses were recorded in the rat mesenteric vascular bed preconstricted with 90 mM KCl. Values are mean ± SEM of six separate concentration-response curves. R_{max} is the maximum decrease in perfusion pressure evoked by tiron. This value was 100% of the preconstriction evoked by 90 mM KCl. EC_{50} is the concentration that evoked 50% of the maximum response to tiron. p indicates statistical significance compared with the control group.

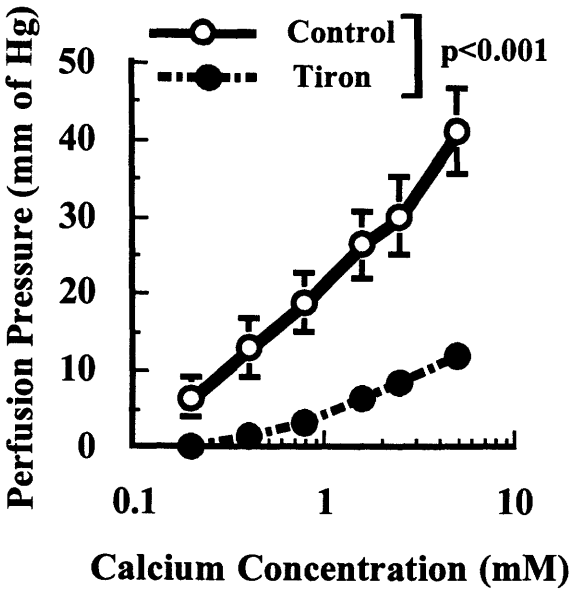


Figure 11. Effect of tiron on Ca^{2+} evoked responses

Relationship of the increase in perfusion pressure of the perfused rat superior mesenteric arterial bed in response to increasing concentrations of calcium in the presence and absence of 10 mM tiron. Values are means ± SEM, n = 6. If not shown, error bars are within the height of the symbol. p indicates statistical significance between the two curves.

3.2.3. Contribution of superoxide anion

3.2.3.1. Effects of superoxide dismutase and apocynin on spontaneous tone

Aortic rings from DOCA-salt hypertensive rats that had been pretreated with SOD (150 Units ml^{-1}) did not develop spontaneous tone after washout of SNP (Figure 12, panel c). In other rings, SOD (150 Units ml^{-1}) administered acutely after spontaneous tone had developed resulted in a dramatic decrease in tone (Figure 12, panel b). Importantly, SOD did not prevent the generation of spontaneous tone in the endothelium-denuded or L-NAME treated aortic rings from DOCA-salt hypertensive rats (Figure 13). Another cell permeable scavenger of $O_2^{\cdot-}$, tempol (100 μM), also inhibited spontaneous tone in endothelium-intact aortic rings from DOCA-salt hypertensive rats and also had no effect on the tone in endothelium-denuded and L-NAME treated aortic rings. Pretreatment of aortic rings from DOCA-salt hypertensive rats with the NADPH-oxidase inhibitor, apocynin (100 μM), inhibited the generation of spontaneous tone (Figure 14). Apocynin did not affect the tone generation in endothelium-denuded and L-NAME treated aortic rings (Figure 15). In the aortic rings from SHAM-control rats, SOD and apocynin had no effect on generation of spontaneous tone.

3.2.3.2. Effect of DOCA treatment on superoxide production

$O_2^{\cdot-}$ production in isolated aortic rings incubated in the organ bath for one hour without a preload (unstretched) and in rings subjected to a preload of 5g for one hour (stretched) is shown in Figure 16. The basal $O_2^{\cdot-}$ production (unstretched rings) was much higher in rings isolated from the DOCA-salt hypertensive rat than in those isolated from SHAM rats. Exposure of the rings to a preload of 5g for one hour increased $O_2^{\cdot-}$ production in the DOCA-salt group. In the

SHAM-group rings, the tendency of increased $O_2^{\cdot-}$ generation in response to stretch was not statistically significant (p=0.72).

3.2.3.3. Localization of 3-nitrotyrosine by immunohistochemistry

3-nitrotyrosine protein moieties were assessed as a marker of oxidative stress to confirm the chemiluminescence findings. Immunohistochemistry performed with a polyclonal antibody raised against 3-nitrotyrosine revealed greater staining in endothelium, smooth muscle layers and adventitia, in aorta from DOCA-salt hypertensive rats compared with SHAM-normotensive rats (Figure 17). Immunoreactivity was not observed when the anti 3-nitrotyrosine antibody was preincubated with 3-nitrotyrosine antibody (10 mM) or when the primary antibody was omitted, indicating that the staining was specific.

3.2.3.4. Effects of endothelium denudation and L-NAME on superoxide production

$O_2^{\cdot-}$ production was not significantly changed by either L-NAME pretreatment or endothelium-denudation in untreated aortic rings from DOCA- salt hypertensive rats compared with untreated rings (Figure 18).

3.2.3.5. Effects of SOD and tempol

In DOCA-salt hypertensive rats, $O_2^{\cdot-}$ was decreased dramatically in SOD treated aortic rings (Figure 18). There was no significant difference in $O_2^{\cdot-}$ generation among aortic rings from SHAM control rats and SOD-treated aortic rings from DOCA-salt rats. In endothelium-denuded and L-NAME treated rings from DOCA-salt hypertensive rats, SOD decreased the generation of $O_2^{\cdot-}$ rings significantly (Figure 18) even though it did not affect the spontaneous tone in these rings (Figure 13). Pretreatment with tempol also decreased the $O_2^{\cdot-}$ level in rings from DOCA-

salt hypertensive rats (data not shown). Neither SOD nor tempol had any effect on aortic rings from SHAM rats.

3.2.3.6. Effects of apocynin

$O_2^{\cdot-}$ was decreased dramatically in apocynin-treated aortic rings from DOCA-salt hypertensive rats (Figure 18). In endothelium-denuded and L-NAME treated rings from DOCA-salt hypertensive rats, apocynin decreased the generation of $O_2^{\cdot-}$ (Figure 18) even though it did not affect the spontaneous tone (Figure 15). Apocynin did not affect $O_2^{\cdot-}$ production in rings from SHAM rats.

3.2.3.7. Effects of nifedipine

Pretreatment with nifedipine (10^{-7} M) did not affect $O_2^{\cdot-}$ production in either endothelium-intact or endothelium-denuded aortic rings from DOCA-salt hypertensive rats and SHAM-normotensive rats (Figure 19).

3.2.3.8. Effects of xanthine oxidase inhibition

Acute administration or pretreatment of aortic rings from DOCA-salt hypertensive rats and SHAM-normotensive rats with allopurinol (1 and 10 μM), an inhibitor of xanthine oxidase, did not have any significant effect on spontaneous tone both in the presence and absence of endothelium (39.65 ± 2.56 vs 41.66 ± 3.28 % of maximum contractile response). Allopurinol also had no significant effect on the $O_2^{\cdot-}$ generation in both groups of rats (Figure 19).

3.2.3.9. Effects of indomethacin and valeroyl salicylate

Acute administration or pretreatment of aortic rings from DOCA-salt hypertensive rats and SHAM-normotensive rats with indomethacin (50 μM) or valeroyl salicylate, inhibitors of COX, had no significant effect on the $O_2^{\cdot-}$ generation in both groups of rats (Figure 19).

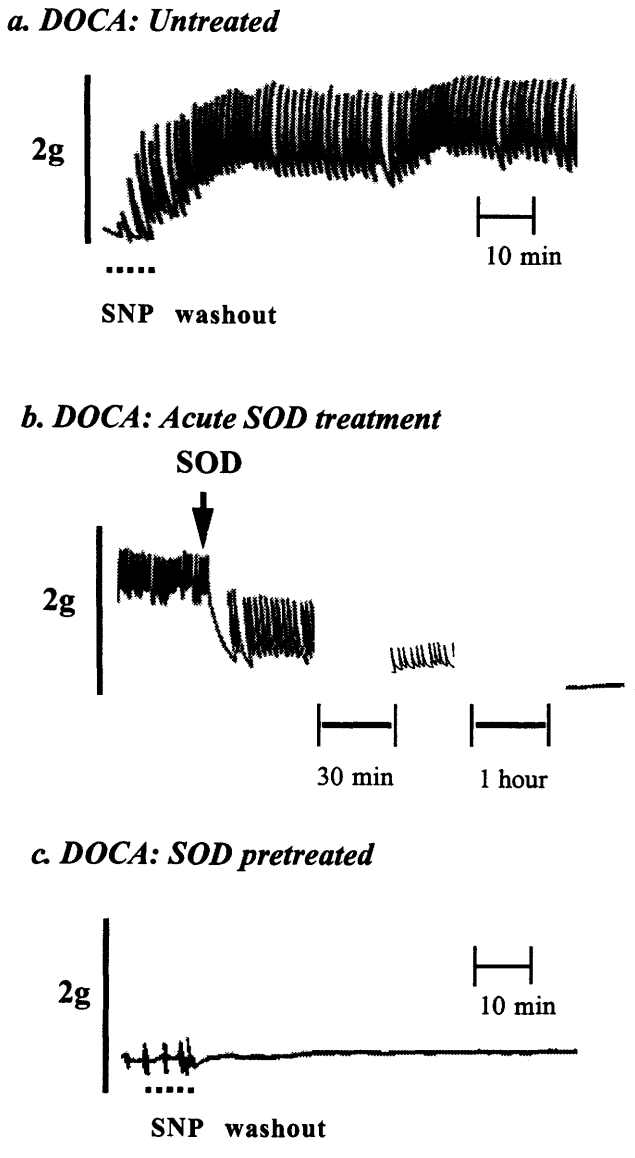


Figure 12. Typical tracing illustrating the effects of superoxide dismutase

Typical tracing of tension developed in aortic rings isolated from DOCA-salt hypertensive rats when a ring was untreated (panel a), acutely treated (panel b) and pretreated with 150 Units ml^{-1} of superoxide dismutase (SOD; panel c). SNP— sodium nitroprusside.

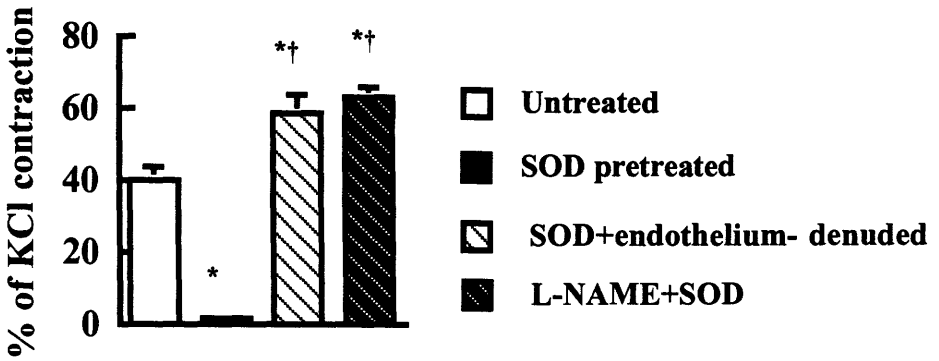


Figure 13. Pooled data on the effects of superoxide dismutase on spontaneous tone

Effect of SOD on the magnitude of spontaneous tone in endothelium intact, endothelium-denuded and N^G-nitro-L- arginine methyl ester (L-NAME) treated rings from DOCA-salt hypertensive rats. Pooled values are expressed as a percentage of maximum contractile response to 120 mM KCl. Values are mean ± SEM, n = 7 in each group. * $p<0.01$ compared with SOD-untreated rings. † $p<0.05$ compared with endothelium intact untreated rings.

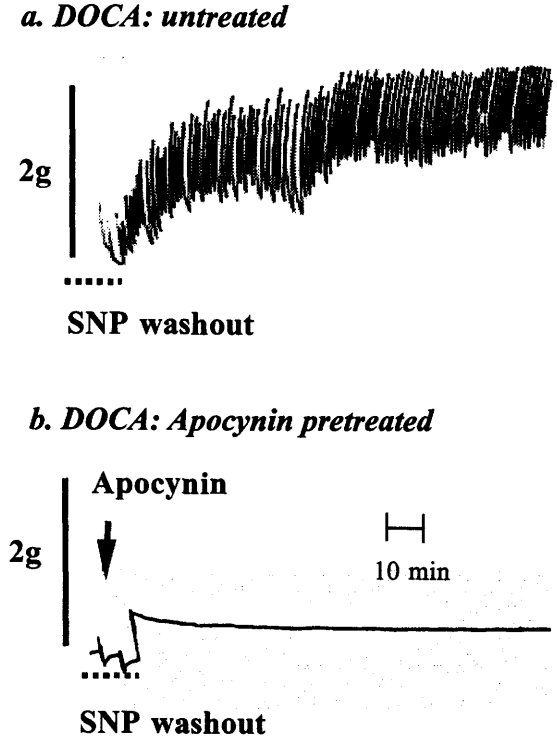


Figure 14. Typical tracing of the effects of apocynin on spontaneous tone

Typical tracing of tension developed in aortic rings isolated from DOCA-salt hypertensive rats when a ring was untreated (panel a) and when a ring was pretreated with 100 μM of apocynin (panel b). SNP— sodium nitroprusside.

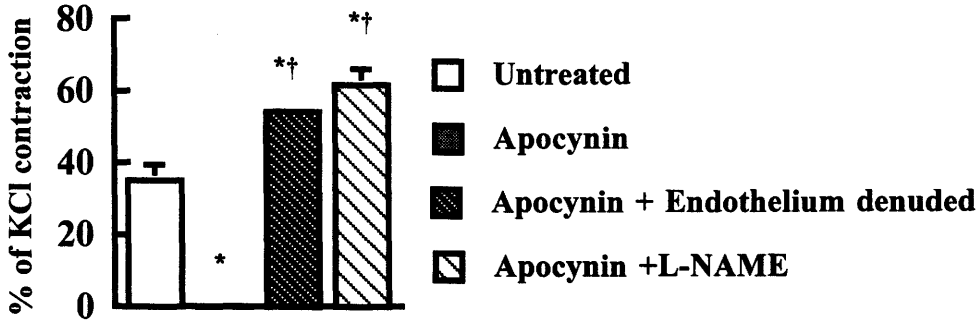


Figure 15. Pooled data on the effects of apocynin on spontaneous tone

Effect of apocynin on the magnitude of spontaneous tone in endothelium-intact, endothelium-denuded and N^G-nitro-L- arginine methyl ester (L-NAME) treated rings from DOCA-salt hypertensive rats. Pooled values are expressed as a percentage of maximum contractile response to 120 mM KCl. Values are mean ± SEM, n = 5 in each group. * $p<0.01$ compared with apocynin-untreated rings. † $p<0.05$ compared with endothelium-intact untreated rings.

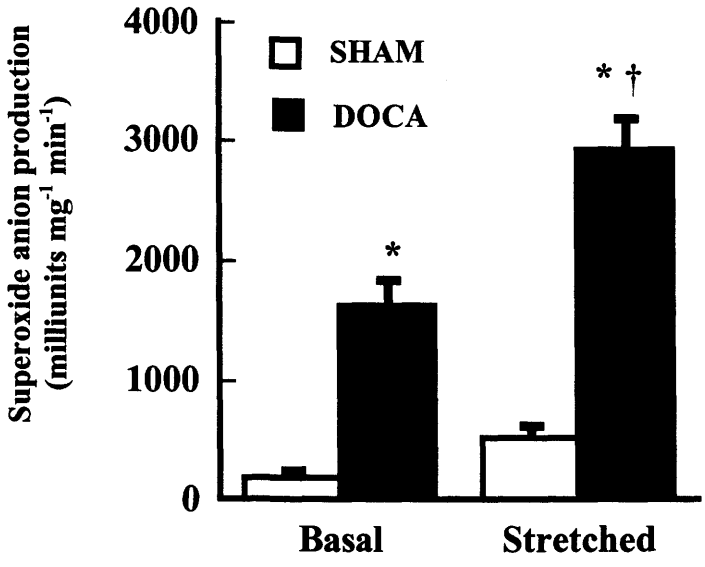


Figure 16. Superoxide production in unstretched or stretched aortic rings

Superoxide production determined by the lucigenin chemiluminescence method in either unstretched (Basal) or stretched aortic rings of DOCA-salt hypertensive rats and SHAM control rats. Values are mean ± SEM expressed in arbitrary units. mg^{-1}. min^{-1}. n = 8 for each group. * $p<0.001$ compared with superoxide level in normotensive SHAM rats. † $p<0.001$ compared with the basal superoxide level in unstretched rings from DOCA-salt hypertensive rats. P=0.72, SHAM basal vs SHAM stretched.

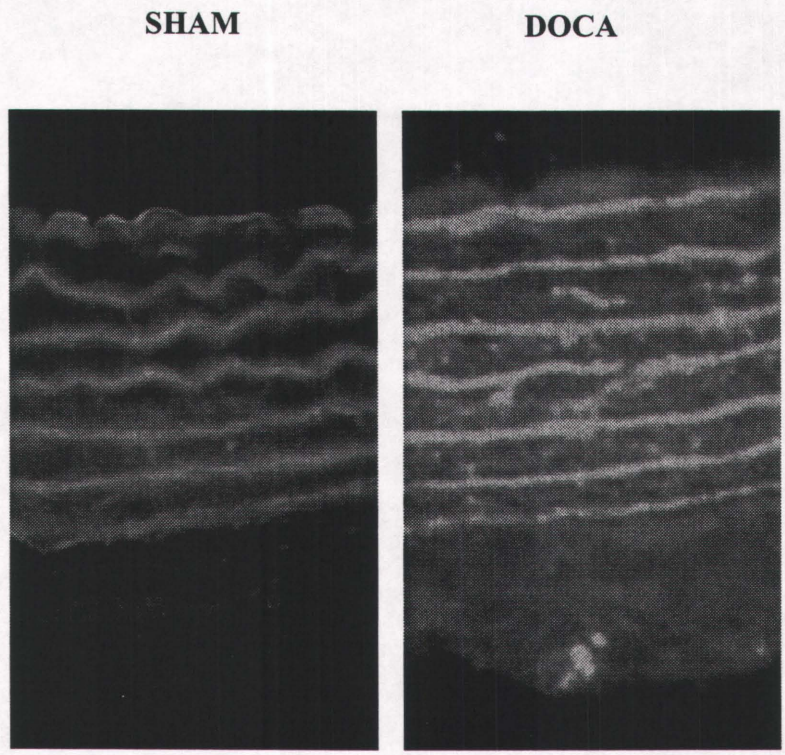


Figure 17. Localization of 3-nitrotyrosine

Photographs show immunohistochemical localization of 3-nitrotyrosine in aorta of SHAM-normotensive and DOCA-salt hypertensive rats. 3-nitrotyrosine staining was notably increased in all layers of the aortic wall in DOCA-salt hypertensive rats. The aortic medial area was also significantly increased in DOCA-salt hypertensive rats. The images are original magnification X40. The images shown are representative of similar results observed in preparations from 10 rats.

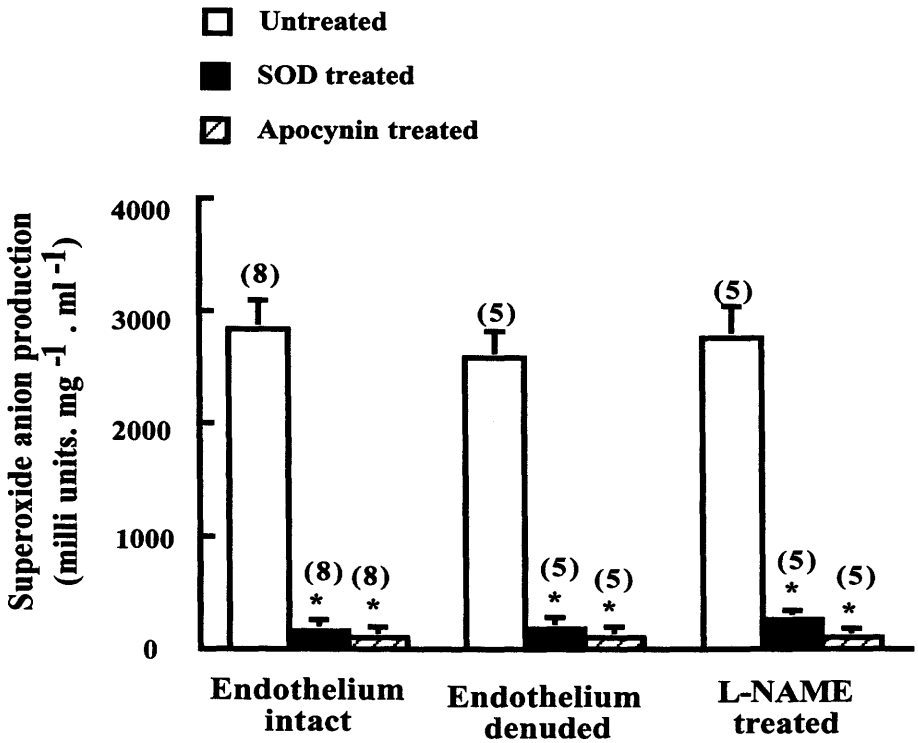


Figure 18. Effects of superoxide dismutase (SOD) and apocynin on superoxide production

Effects of 150 Units ml^{-1} of superoxide dismutase (SOD) and 100 μM of apocynin on superoxide production determined by the lucigenin chemiluminescence method in endothelium-intact, endothelium-denuded and N^G-nitro-L- arginine methyl ester (L-NAME) treated aortic rings from DOCA-salt hypertensive rats. Values are expressed as the mean ± SEM. The number on the top of each column indicates number of animal for that group. If not shown, error bars are within the range of the symbol. *$p<0.001$ compared with superoxide level in SOD/apocynin untreated rings.

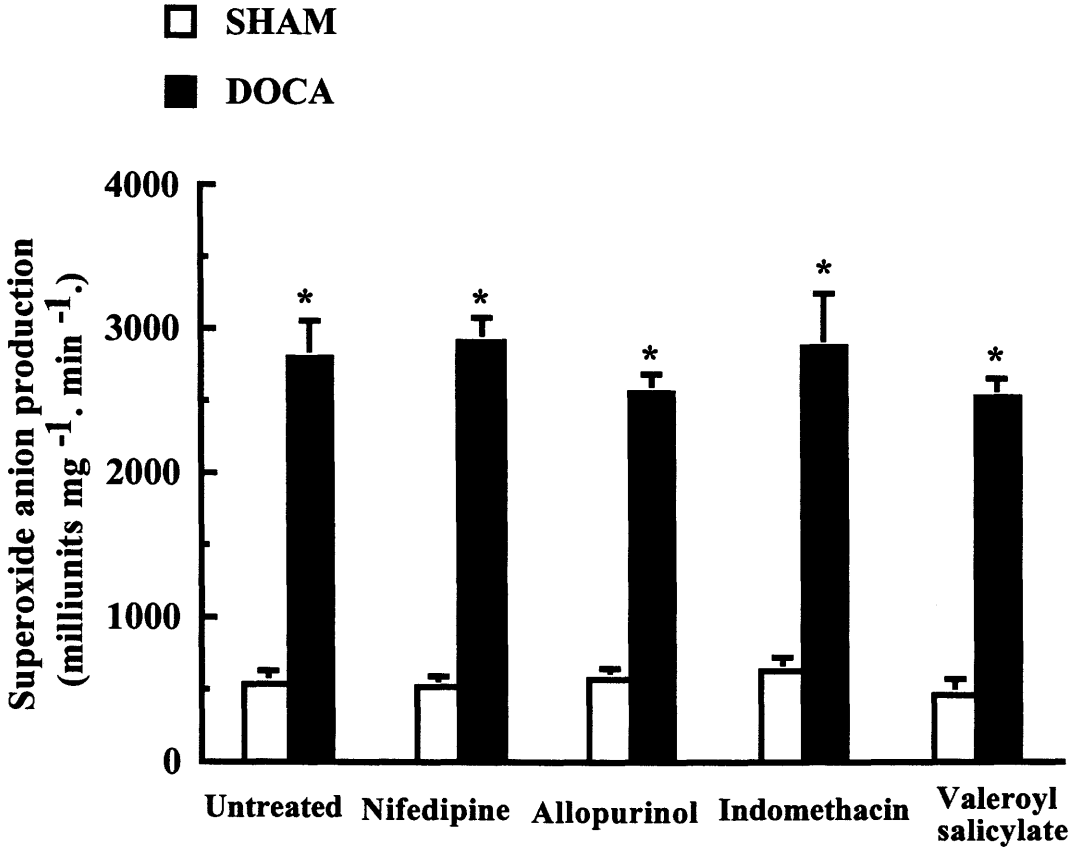


Figure 19. Effects of nifedipine, allopurinol, indomethacin and valeroyl salicylate on the superoxide production

Effects of nifedipine (10^{-7} M), allopurinol (10 μM), indomethacin (50 μM) and valeroyl salicylate (3 mM) on superoxide production determined by the lucigenin chemiluminescence method in aortic rings from SHAM-normotensive rats and DOCA-salt hypertensive rats. Values are expressed as mean ± SEM, n = 6 for each group. * $p<0.001$ compared with superoxide level in aorta of SHAM-normotensive rats.

3.2.4. Effects of catalase on spontaneous tone

The role of endogenous H_2O_2 in the modulation of spontaneous tone and $O_2^{\cdot-}$ levels was tested in rings treated with catalase. In the aortic rings from DOCA-salt hypertensive rats, catalase (1000 Units ml^{-1}) increased spontaneous tone significantly (Figure 20). Catalase also increased tone significantly in endothelium-denuded, SOD-treated and L-NAME treated aortic rings from DOCA-salt hypertensive rats (Figure 21). Catalase failed to increase the tone significantly in apocynin-treated aortic rings. In aortic rings from SHAM normotensive rats, catalase evoked a significant but small increase in tone (10.35 ± 2.98 % of KCl contraction). Catalase did not have any significant effect on the $O_2^{\cdot-}$ generation in both DOCA-salt hypertensive rats and SHAM-normotensive rats.

3.2.5. Effects of deferoxamine

In aortic rings from DOCA-salt hypertensive rats, the hydroxyl radical scavenger deferoxamine (60 μM), showed a tendency to increase spontaneous tone (from 36.2 ± 3.6 % to 45.1 ± 3.98 % of the maximal contractile response), but this change was not statistically significant (n=10, p= 0.1). In aortic rings from SHAM normotensive rats, deferoxamine did not evoke spontaneous tone. Deferoxamine did not have any significant effect on the $O_2^{\cdot-}$ generation in either DOCA-salt hypertensive or SHAM-normotensive rats.

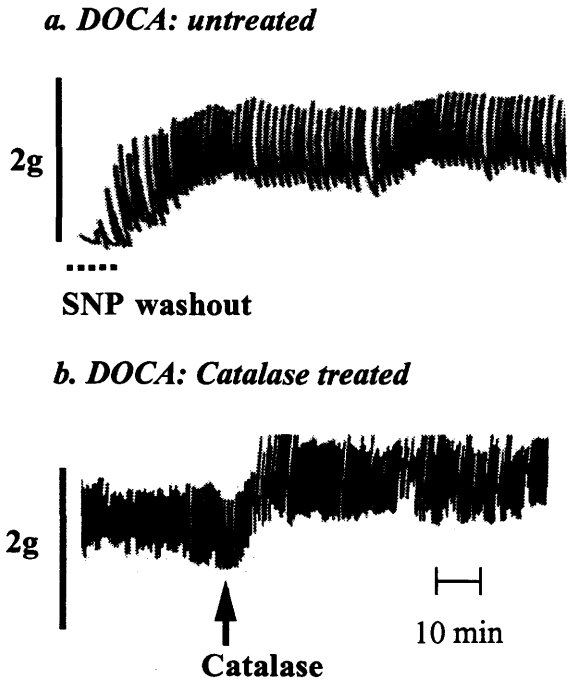


Figure 20. Typical recording illustrating the effect of catalase on spontaneous tone

Typical tracing showing the effect of catalase on spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats. Panel a, untreated ring or panel b, a ring pretreated with 1000 Units ml^{-1} of catalase. SNP— sodium nitroprusside.

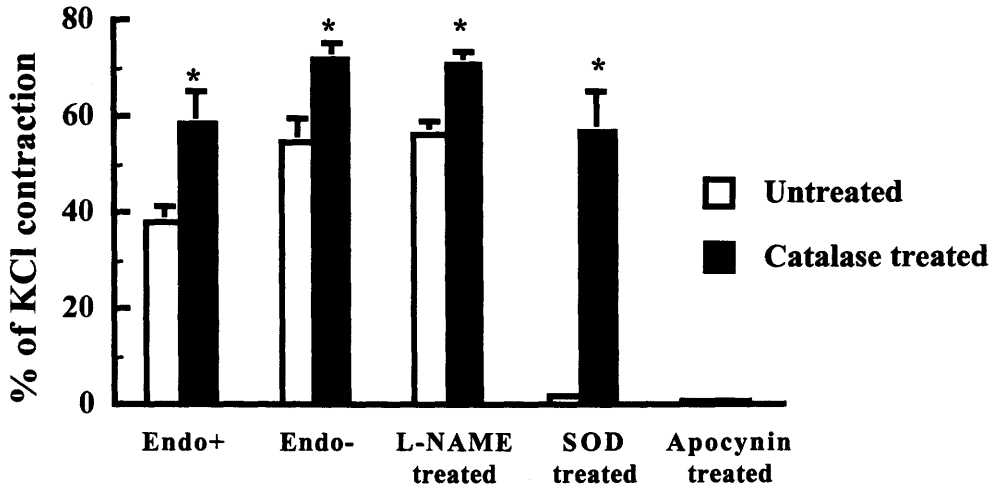


Figure 21. Pooled data on the effects of catalase on spontaneous tone

Effects of catalase on spontaneous tone in endothelium-intact (Endo+), endothelium-denuded (Endo-), or in endothelium-intact aortic rings pretreated with N^G-nitro-L- arginine methyl ester (L-NAME) or superoxide dismutase (SOD) or apocynin. Pooled data are expressed as percentages of maximum contractile responses to 120 mM KCl. Values are mean ± SEM, n = 6 in each group. *$p<0.05$ compared with untreated rings.

3.2.6. COX dependent component of spontaneous tone

Pretreatment of aortic rings with the COX inhibitors, indomethacin (50 μM) or valeroyl salicylate (3 mM), or with the TP receptor antagonist, SQ 29548 (3 μM), inhibited the generation of spontaneous tone in endothelium-intact aortic rings from DOCA-salt hypertensive rats (Figure 22 and Figure 23). In contrast, SQ 29548 failed to reduce tone and valeroyl salicylate only reduced tone modestly in endothelium-denuded rings (Figure 23). A TXA_2 synthase inhibitor, furegrelate (50 μM), had no effect on the spontaneous tone in the endothelium-intact and endothelium-denuded aortic rings from DOCA-salt hypertensive rats or SHAM-normotensive rats. In aortic rings from SHAM control rats, these agents had no significant effect. Importantly, $O_2^{\cdot-}$ generation was not significantly different in aortic rings treated with cyclooxygenase inhibitors and TP-receptor antagonists compared with the untreated rings (Figure 19).

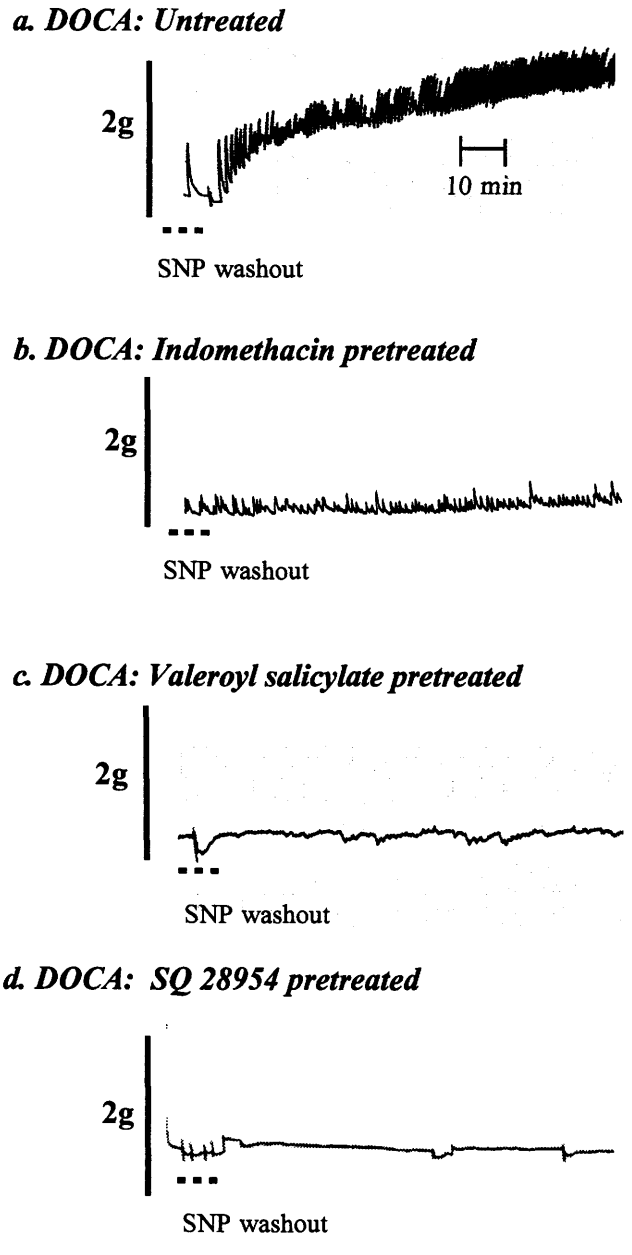


Figure 22. Typical tracing illustrating the effects of COX inhibitors and TP-receptor antagonist

Illustrative tracing showing spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats when a ring was untreated (panel a), pretreated with 50 μM indomethacin (panel b) or 3 mM of valeroyl salicylate (panel c), and with 3 μM of SQ 29548 (panel d). SNP—sodium nitroprusside.

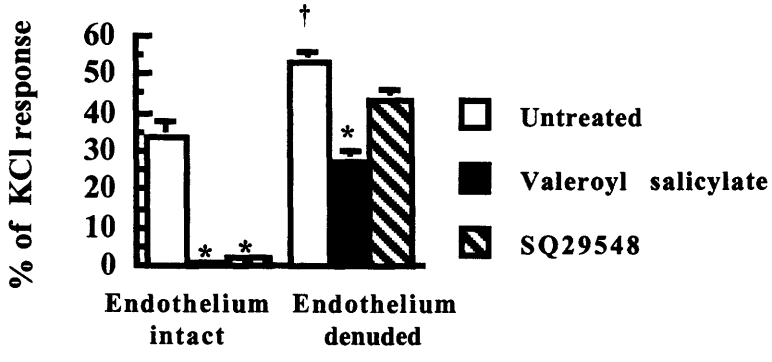


Figure 23. Pooled data on effects of cyclooxygenase inhibition and TP-receptor antagonist on spontaneous tone

Effects of pretreatment with valeroyl salicylate (3 mM) and SQ 29548 (3 μM) on spontaneous tone in the presence and absence of endothelium. Pooled values are expressed as a percentage of maximum contractile response to 120 mM KCL. Values are mean ± SEM, n = 4 in each group. *$p<0.05$ compared with untreated rings. † $p<0.05$ compared with endothelium-intact rings.

3.3. Study 3: Contribution of 20-HETE

3.3.1. HPLC assay for 20-HETE

Endogenous 20-HETE levels were higher in aortic tissue from DOCA-salt hypertensive rats compared with SHAM-normotensive tissue (26.13 ± 2.15 vs 6.59 ± 5.4 ng/g of tissue, $p < 0.05$). Figure 24 shows the HPLC peak for 20-HETE in one gram of aortic tissue from SHAM-normotensive and DOCA-salt hypertensive rats. For one gram of tissue, aortas from 7 rats were required for SHAM normotensive rats and aortas from 4 rats were required in DOCA-salt hypertensive rats.

3.3.2. Effect of 20-HETE on spontaneous tone

Exogenous 20-HETE (1 μM) increased spontaneous tone in endothelium-intact rings from DOCA-salt hypertensive rats (Figure 25 and Figure 27). Spontaneous tone was elevated in endothelium-denuded rings and 20-HETE failed to increase the tone any further. However, exogenous 20-HETE did increase the tone in L-NAME treated rings from DOCA-salt hypertensive rats. Iberiotoxin (10^{-8} M) increased the magnitude of spontaneous tone significantly both in the presence and absence of endothelium in aortic rings from DOCA-salt hypertensive rats. In the presence of the iberiotoxin-evoked increase in tone, 20-HETE failed to increase tone any further. Conversely, in the presence of 20-HETE evoked increases in tone, iberiotoxin failed to increase tone any further. In the aortic rings from SHAM-normotensive rats, neither 20-HETE nor iberiotoxin evoked spontaneous tone.

3.3.3. Effect of aminobenzotriazole

Pretreatment or acute treatment of aortic rings with aminobenzotriazole (ABT, 10^{-5}M) decreased spontaneous tone in aortic rings from DOCA-salt hypertensive rats with intact endothelium but not in endothelium-denuded rings (Figure 26 and Figure 27). However, ABT did decrease spontaneous tone in aortic rings treated with L-NAME. In the ABT treated rings, 20-HETE increased the magnitude of spontaneous tone in the presence of endothelium but failed to do that in the absence of endothelium (Figure 27). In aortic rings from SHAM-normotensive rats, ABT had no effect on spontaneous tone.

3.3.4. Effect of COX inhibition on 20-HETE evoked responses

Spontaneous tone was decreased in aortic rings pretreated with the COX inhibitor, valeroyl salicylate (3 mM). Exogenous 20-HETE (1 μM) failed to increase spontaneous tone in valeroyl salicylate treated rings both in the presence and absence of endothelium (Figure 28). Exogenous 20-HETE (1 μM) also failed to increase spontaneous tone in TP receptor antagonist, SQ 29548 (3 μM) treated rings but did increase tone in the TXA_2 synthase inhibitor, furegrelate (50 μM), treated rings.

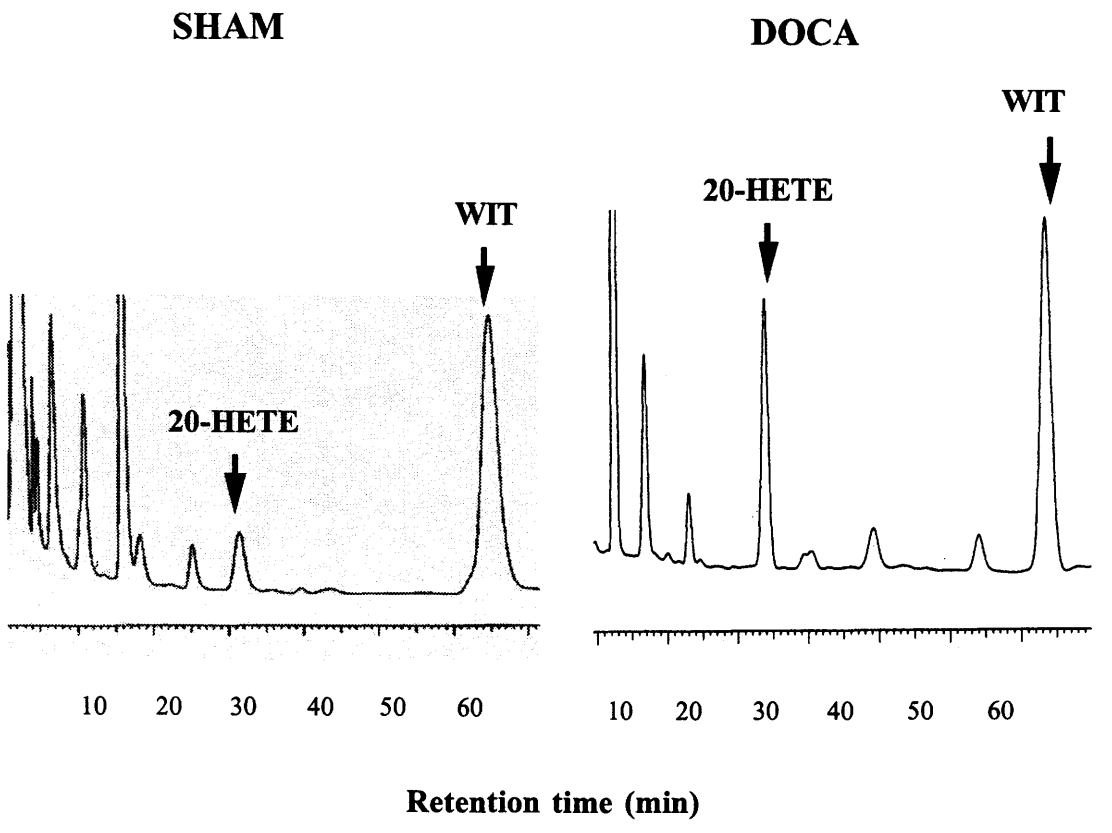


Figure 24. HPLC assay for 20-HETE

High pressure liquid chromatography (HPLC) chromatograms of 20-HETE in 1 g of aortic tissue from SHAM-normotensive rats and DOCA-salt hypertensive rats. The images shown are representative of similar results observed in 4 experiments. WIT=WIT 002, an internal standard for 20-HETE.

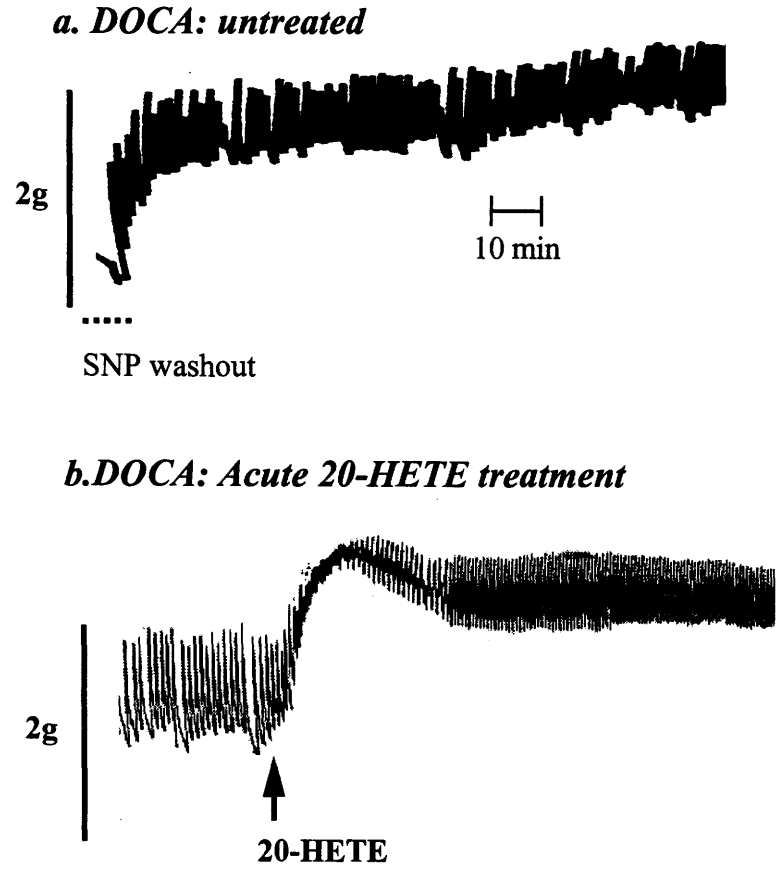


Figure 25. Typical recording illustrating the effect of 20-HETE on spontaneous tone

Spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats when a ring was untreated (panel a) and when a ring was treated acutely with 1 μM of 20-HETE (panel b). SNP—sodium nitroprusside.

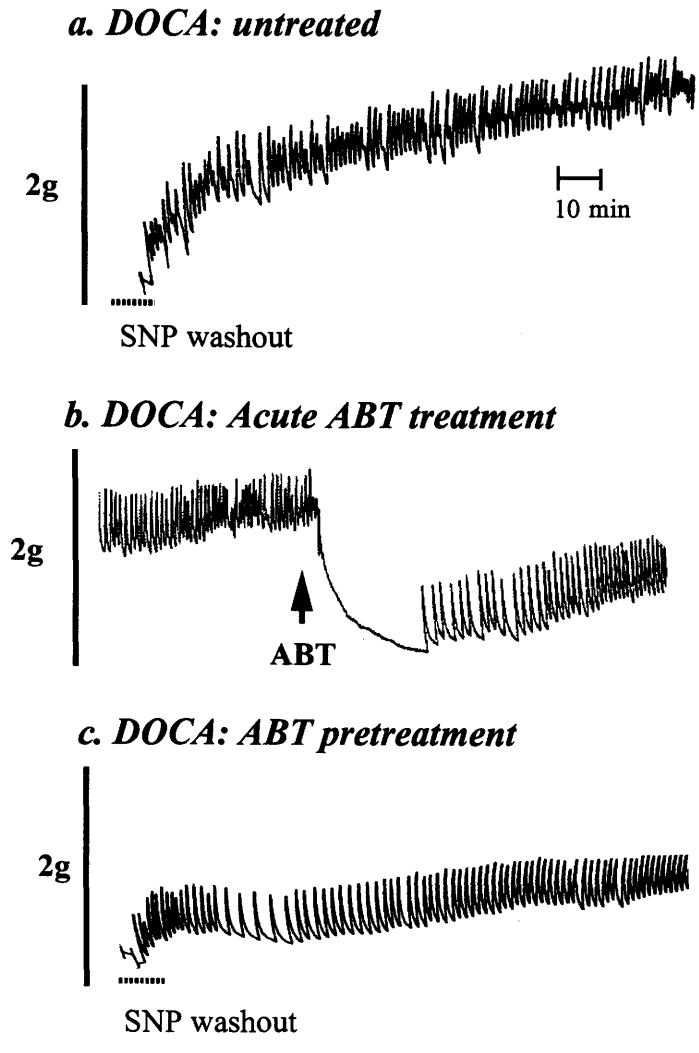


Figure 26. Typical recording illustrating the effect of ABT on spontaneous tone

Spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats when a ring was untreated (panel a), when a ring was acutely treated with 10 μM of aminobenzotriazole (ABT) after spontaneous tone had developed (panel b), and when a ring was pretreated with 10 μM of ABT (panel c). SNP—sodium nitroprusside.

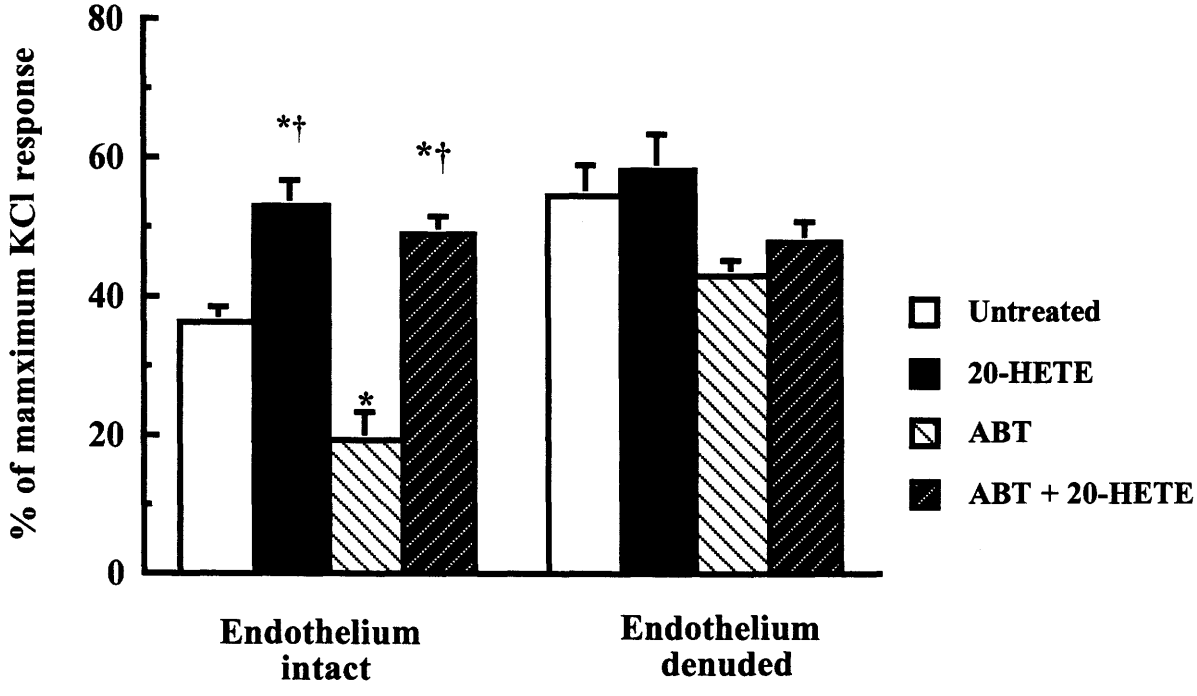


Figure 27. Pooled data on effects of 20-HETE and ABT on spontaneous tone

Effect of 20 HETE (1 μM) and aminobenzotriazole (ABT, 10^{-5}M) on spontaneous tone in aortic rings from DOCA-salt hypertensive rats in the presence and absence of endothelium. Values are mean ± SEM and are expressed as a percentage of maximal contractile response to 120 mM of KCl, n = 6 for each groups. * $P < 0.05$ compared with the untreated rings. † $P < 0.05$ compared with ABT alone treated rings.

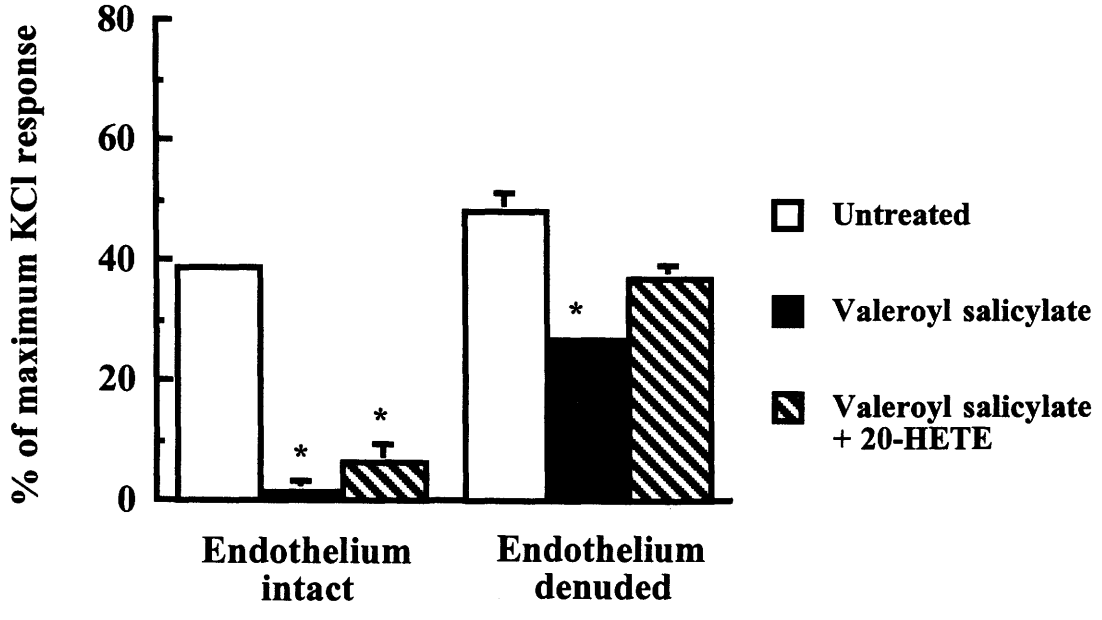


Figure 28. Cyclooxygenase modulation of effect of 20-HETE on spontaneous tone

Effect of valeroyl salicylate (3 mM) on the increase on spontaneous tone evoked by 20 HETE (1 μM) in aortic rings from DOCA-salt hypertensive rats in the presence and absence of endothelium. Values are mean ± SEM and are expressed as a percentage of maximal contractile responses to 120 mM of KCl, n = 4 for each groups. If not shown, error bars are within the height of the symbols. * $P < 0.05$ compared with the untreated rings.

3.4. Study 4: Contribution of K_{ATP} channels

3.4.1. Effect of cromakalim on phenylephrine preconstricted aortic rings

Cromakalim evoked concentration-dependent relaxation of phenylephrine preconstricted aortic rings isolated from both DOCA-salt hypertensive rats and SHAM-normotensive rats. In aortic rings with an intact endothelium (Figure 29), the dose-response curve to cromakalim was shifted to the right in DOCA-salt hypertensive rats compared with SHAM-control rats. The EC_{50} values were much higher in the DOCA-salt group (Table 4). Moreover, the maximum response (E_{max}) to cromakalim was significantly lower in aortic rings from DOCA-salt hypertensive rats. Glibenclamide, a K_{ATP} channel blocker, abolished the cromakalim-induced relaxation in rings from both DOCA-salt hypertensive rats and SHAM-control rats. In endothelium-denuded aortic rings the dose-response curve to cromakalim was also shifted the right in the DOCA salt hypertensive group compared to the SHAM group (Figure 29). The EC_{50} was higher and the E_{max} was lower in the DOCA-salt group (Table 4). There were no significant differences between EC_{50} values and between E_{max} values in endothelium-intact and endothelium-denuded preparation.

3.4.2. Effect of cromakalim on spontaneous tone of aortic rings

In aortic rings from DOCA-salt hypertensive rats, cromakalim (10^{-6} M) abolished spontaneous tone (Figure 30 and Figure 31). Cromakalim also abolished spontaneous tone in endothelium-denuded aortic rings from DOCA-salt hypertensive rats (Figure 31). Glibenclamide (10^{-5} M) had no significant effect on spontaneous tone in aortic rings from DOCA-salt hypertensive rats (Figure 31), nor on the basal tone in rings from SHAM control rats. Nevertheless, pretreatment of aortic rings with glibenclamide (10^{-5} M) prevented the inhibitory effect of cromakalim (Figure

30). Glibenclamide (10^{-5} M) also reversed cromakalim- induced inhibition of spontaneous tone when administered acutely. In aortic rings from SHAM control rats, glibenclamide and cromakalim had no significant effect.

3.4.3. Electrophysiology of K_{ATP} channel activity

Smooth muscle cells from aorta of DOCA- salt hypertensive rats and SHAM normotensive rats had a membrane capacitance of 15.98 ± 0.83 (n = 13) and 14.71 ± 0.72 pF (n = 16) respectively. In aortic smooth muscle cells from SHAM-control rats, the K^+ gradient was reversed by increasing the extracellular potassium concentration from 5.4 to 140 mM (Figure 32). Application of 1 μM cromakalim produced additional inward currents from –58.8 ± 13.4 to –111.54 ± 19.3 pA ($p<0.05$). The cromakalim-induced current was abolished by the sulphonylurea K_{ATP} channel inhibitor, glibenclamide (10 μM), which is relatively selective for K_{ATP} channels at this concentration. In contrast to the SHAM-control rats, smooth muscle cells from aorta of DOCA-salt hypertensive rats exhibited reduced or no inward currents (Figure 33). Increasing the extracellular potassium concentration from 5.4 to 140 mM increased the inward currents modestly in 5 cells out of 13 cells tested. Cromakalim at 1 μM was added to all of 13 cells tested, out of which 2 showed an increase of K_{ATP} channel activity (-3.8 vs. -61.09 and –19.7 vs. –69.3 pA respectively). In the other 11 cells there was no further significant increase of K_{ATP} channel currents. On increasing the cromakalim concentration to 10 μM, one additional cell of the 7 cells tested showed a significant increase of K_{ATP} current (-28.65 vs. –69.9 pA).

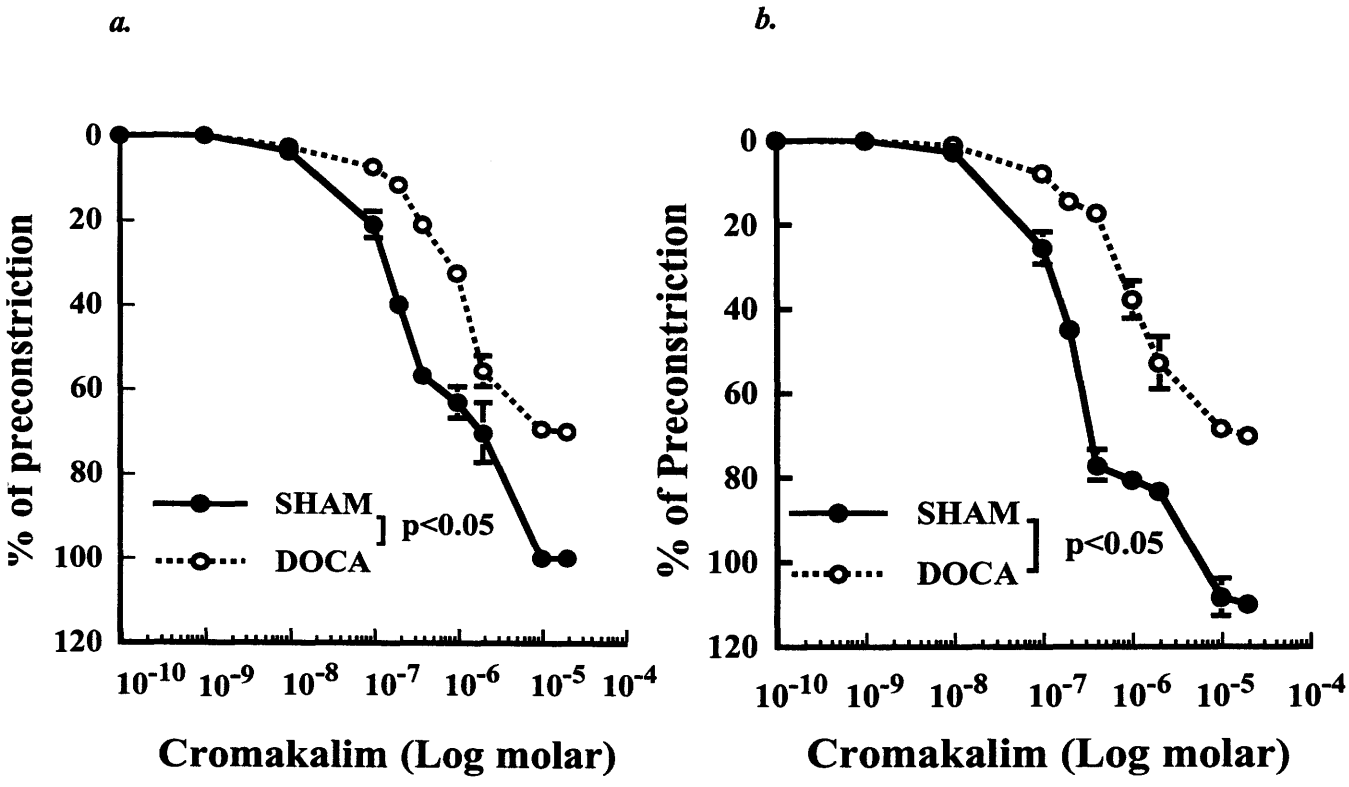


Figure 29. Effects of cromakalim on preconstricted aortic rings

Relationship of the decrease in tone to increasing concentrations of cromakalim in aortic rings from DOCA-salt hypertensive rats and SHAM-normotensive rats preconstricted with phenylephrine in the presence (panel a) and absence (panel b) of endothelium. Values are mean ± SEM and are expressed as a percentage of the preconstriction, n = 4 for each group. If not shown, error bars are within the height of the symbol. p indicates statistical significance between two groups.

Table 4. Potency and efficacy for cromakalim-evoked responses

	Endothelium-Intact		Endothelium-Denuded	
	EC_{50} (μM)	E_{max} (%of KCl)	EC_{50} (μM)	E_{max} (%of KCl)
DOCA	0.850 ± 0.100*	68 ± 2*	0.939 ±.069*	70 ± 2*
SHAM	0.108 ± .005	108 ± 5	0.225 ±.010	100 ± 2

EC_{50} and E_{max} for cromakalim in phenylephrine-preconstricted aortic rings from DOCA-salt hypertensive rats and SHAM-normotensive rats in the presence and absence of endothelium. n = 4 in each group. Values are mean ± SEM * $p < 0.05$ compared to SHAM control rings.

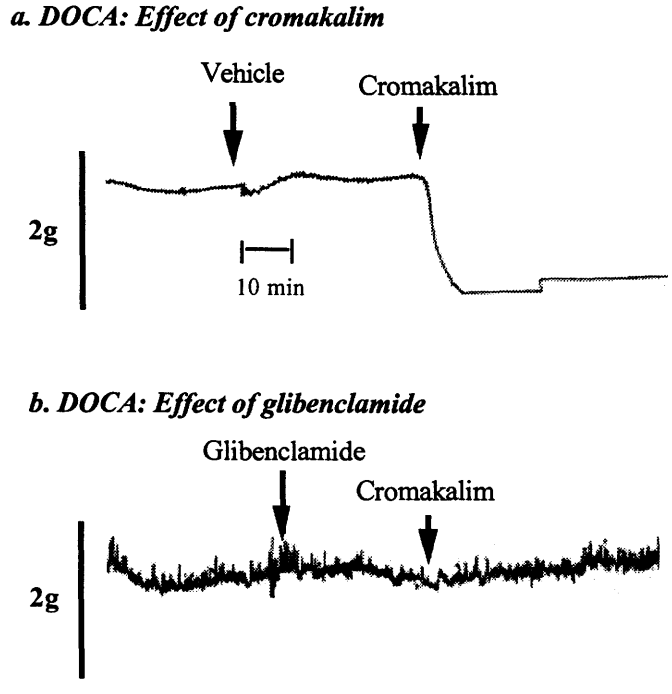


Figure 30. Effects of cromakalim and glibenclamide on spontaneous tone

Effect of 10^{-6} M cromakalim (panel a) and of 10^{-5} M glibenclamide (panel b) on spontaneous tone in aortic rings of DOCA-salt hypertensive rats.

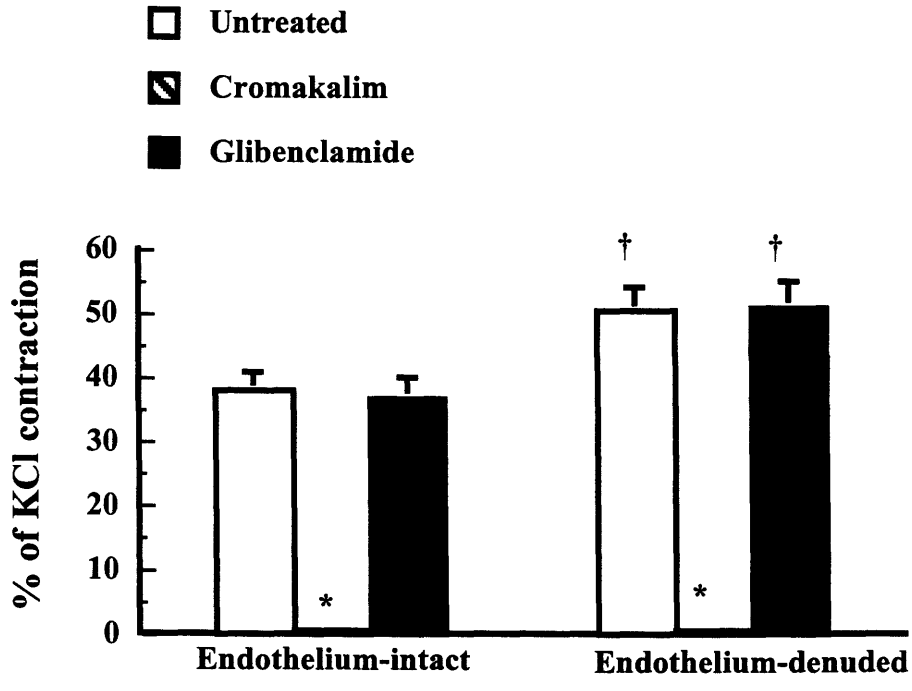
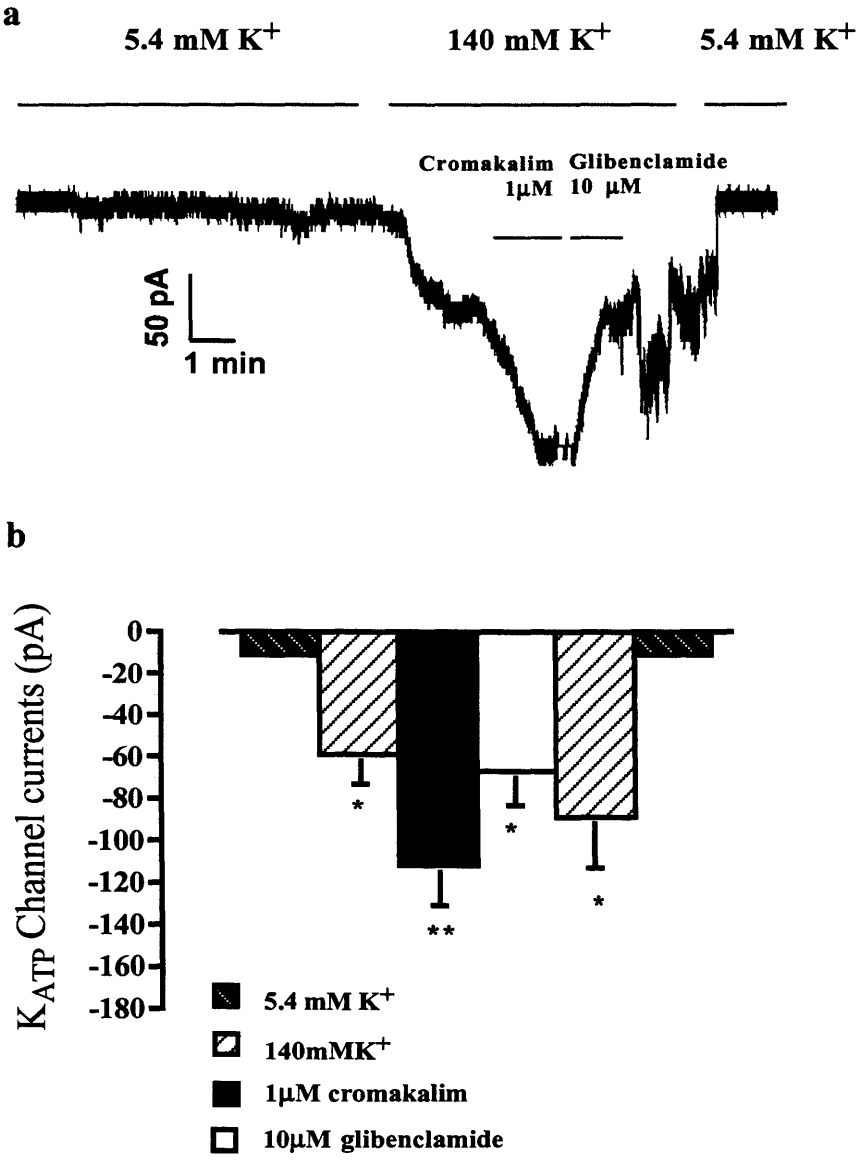


Figure 31. Pooled data on effects of cromakalim and glibenclamide on spontaneous tone

Effects of cromakalim (1 μM) and glibenclamide (10 μM) on the increase on spontaneous tone in aortic rings from DOCA-salt hypertensive rats in the presence and absence of endothelium. Values are mean ± SEM and are expressed as a percentage of maximal contractile responses to 120 mM of KCl, n = 4 for each groups. If not shown, error bars are within the height of the symbols. * $p < 0.001$ compared with the untreated rings. † $p < 0.05$ compared with the endothelium-intact rings.



Note: Figure 32. legend is on the next page.

Figure 32. K_{ATP} currents in aortic smooth muscle cells from SHAM-normotensive rat

Panel a represents a typical recording of whole-cell current in a cell held at –60 mV. The K^+ concentration was increased from 5.4 to 140 mM prior to addition of cromakalim (1 μM) and glibenclamide (10 μM). Panel b shows the pooled data. Values are the means ± SEM from 10 cells. ** $p<0.01$, * $p<0.05$ compared with control.

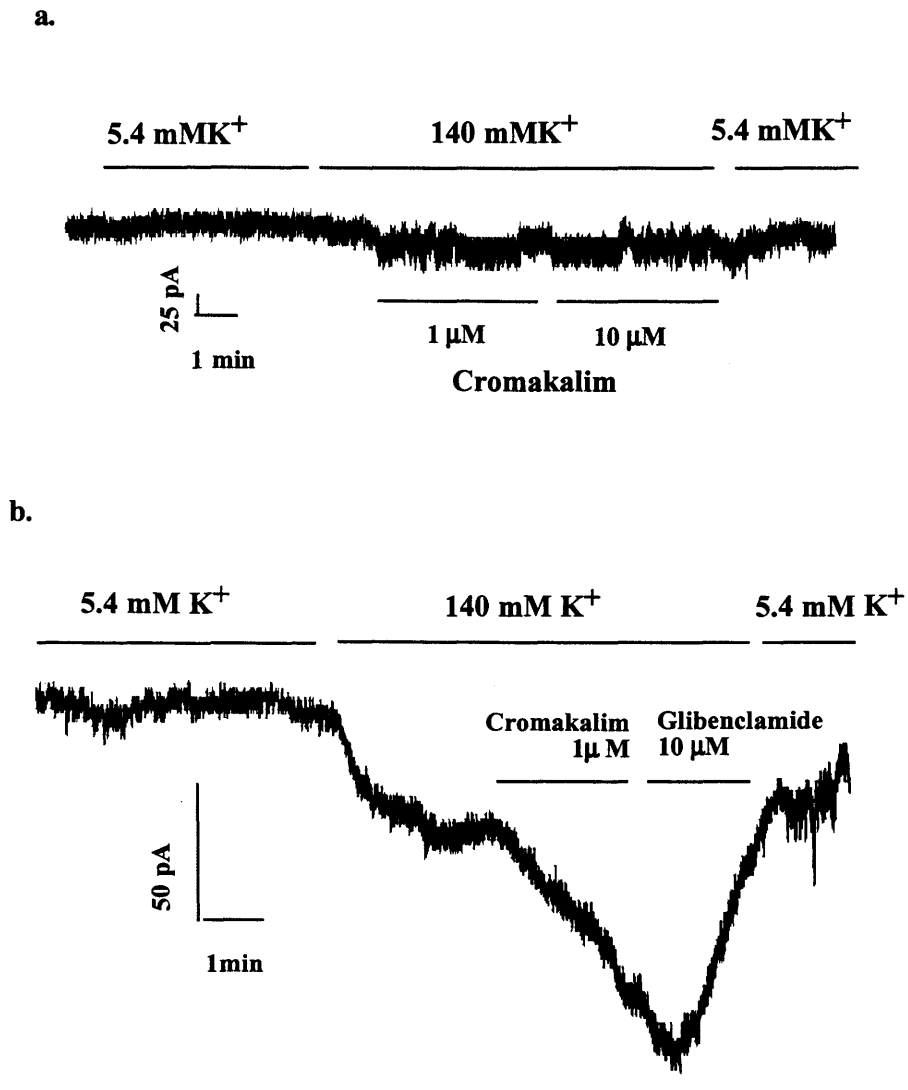


Figure 33. K_{ATP} currents in aortic smooth muscle cell from DOCA-salt hypertensive rats

Typical recording of whole-cell current in two cells held at -60 mV. K^+ concentration was increased from 5.4 to 140 mM prior to addition of cromakalim (1 μM followed by 10 μM) and glibenclamide (10 μM). Panel a is an example of a cell with virtually no response. Panel b is an example of a cell with functional K_{ATP} channels.

4. Discussion:

N.B. To facilitate the reader, a schematic illustrating the complex array of mechanisms contributing to the modulation of spontaneous tone is provided in Figure 34 on page 135.

4.1. Characterization of spontaneous tone

4.1.1. Relationship to preload and hypertensive state

The tension that developed spontaneously in aortic rings isolated from DOCA-salt hypertensive rats was directly proportional to the preload. Tension increased dramatically as the preload was increased from 1g to 5g. The final adjustment of the preload was determined in the presence of SNP to ensure that the preload was set in the relaxed passive state, a method described previously by others (Di Wang et al., 1999). Washout of the SNP resulted in reproducible increases in active tension. The development of active tension in response to stretch appears to be *the vitro* correlate of myogenic tone. In small resistance arteries, the resistance of the vessel changes in response to changes in transmural pressure (Scotland et al., 2001). When the transmural pressure increases, these vessels respond to the elevated pressure by constricting, a phenomenon termed "myogenic tone" (Bayliss WM,1902). Myogenic tone is an intrinsic property of vascular smooth muscle and has been observed in small arteries and arterioles from normotensive animals. On the other hand the aorta has been described as a quiescent muscle, with no myogenic contractions or active tone. However, the aorta of hypertensive animals does manifest active tone (Sunano et al., 1992; Sunano et al., 1996). An increase of intraluminal pressure or passive stretch triggers a spontaneous oscillatory contraction in aorta from hypertensive rats but not from normotensive rats. This spontaneous active tone has

been described as acquisition of myogenic properties by large blood vessels in the hypertensive state (Rapacon-Baker et al., 2001). This tone in large conducting vessels could decrease compliance, thereby contributing to systolic hypertension.

The increase in tension in response to stretch appears to be specific to hypertension since spontaneous tone failed to develop in aortic rings isolated from SHAM control rats even at the highest preload of 5g. Others have described spontaneous tone in other rat models of hypertension including the spontaneously hypertensive rat (SHR) (Sekiguchi et al., 1998; Sunano et al., 1996) and the angiotensin II-infused model of hypertension (Di Wang et al., 1999). Oral treatment of DOCA-salt hypertensive rats in this study with tempol and tiron prevented the increase in mean arterial pressure, $O_2^{\cdot-}$ production, and the development of spontaneous tone, findings consistent with the notion that spontaneous tone is related to the hypertensive state.

4.1.2. Role of extracellular calcium

It has been suggested that there is a calcium transient in smooth muscle of pressurized resistance arteries during myogenic tone (Miriel et al., 1999) and spontaneous active tone in aorta of hypertensive rats (Rapacon-Baker et al., 2001). In this study removal of extracellular calcium from the buffer inhibited the tone completely and addition of calcium restored the tone. This agrees with findings of other investigators that described spontaneous tone as a calcium-dependent phenomenon (Rinaldi & Bohr,1989; Di Wang et al., 1999; Hwa & Bevan,1986b). Since nifedipine prevented the tone, influx of extracellular calcium through L-type calcium channels appears to be critical for generation of spontaneous tone. Importantly, nifedipine did not interfere with $O_2^{\cdot-}$ generation in either SHAM-control rats or DOCA-salt hypertensive rats. Thus a significant role of L-type calcium channels in the increased generation of $O_2^{\cdot-}$ observed in DOCA-salt hypertensive rats can be excluded.

4.1.3. Contribution of endothelium

The development of spontaneous tone appears to be independent of the endothelium because spontaneous tone was present both in the presence and absence of the endothelium. Under our conditions, endothelial-denudation actually increased spontaneous tone suggesting the endothelium plays a protective role opposing spontaneous tone. Our results differ from an earlier report where endothelial-denudation depressed spontaneous tone in aorta of DOCA-salt hypertensive rats suggesting the release of a constrictor agent from endothelium (Rinaldi & Bohr,1989). On the other hand, these findings are consistent with observations in the SHR where endothelium-denudation also augmented spontaneous tone (Sekiguchi et al., 1998).

The possibility that endothelial derived vasoconstrictor or vasodilator factors might contribute to or modulate the tone cannot be excluded. The DOCA-salt hypertensive model is an ET dependent model of hypertension and ET-1 can increase the generation of $O_2^{\cdot-}$ in DOCA-salt hypertensive rats by stimulation of NADPH oxidase (Li et al., 2003a). Recently it has been reported that chronic blockade of ET_A receptors decreases vascular $O_2^{\cdot-}$ generation in DOCA-salt hypertension (Callera et al., 2003). Under the present experimental conditions, ET-1 does not appear to contribute to spontaneous tone directly because preincubation or acute administration of the ET_A receptor antagonist, BQ123, or the ET_B receptor antagonist, BQ788, did not have any effect on spontaneous tone. Moreover, endothelial-denudation actually increased the magnitude of spontaneous tone. These findings suggest that ET-1 *per se* does not contribute to spontaneous tone in DOCA-salt hypertensive rats.

In contrast, endothelial-derived NO plays an important role in limiting the development of spontaneous tone. Several observations are consistent with this conclusion. Firstly, removal of the endothelium increased spontaneous tone. Secondly, inhibition of nitric oxide synthase by L-

NAME increased tone in the endothelium-intact preparation. Thirdly, in endothelium-denuded preparations L-NAME did not increase the tone any further. Thus NO appears to be an important modulator of spontaneous tone. Initially, it was tempting to postulate that a deficiency in the NO system in the DOCA-salt hypertensive rat accounted for the development of spontaneous tone. However, eNOS protein expression was similar in DOCA-salt and SHAM rats. Moreover, endothelium-denudation and L-NAME did not generate spontaneous tone in rings from normotensive SHAM control rats. These findings indicate that a deficiency of the NO system *per se* cannot account for the development of spontaneous tone. Nevertheless, NO does act to suppress tone in the DOCA-salt model.

4.2. Contribution of oxidative stress

4.2.1.1. Contribution to mean arterial pressure

MAP was significantly higher in DOCA-salt hypertensive rats after 3-weeks of DOCA and salt treatment compared with the SHAM-normotensive group. The present study demonstrates that oral administration of the $O_2^{\cdot-}$ scavengers tempol or tiron or the NAD(P)H oxidase inhibitor, apocynin, in drinking water for 3-weeks during the DOCA treatment prevented the elevation of MAP in DOCA-salt hypertensive rats but did not decrease the BP in SHAM- control rats. These results indicate a role of NAD(P)H -oxidase derived $O_2^{\cdot-}$ in the increased BP in DOCA-salt hypertensive rats and are consistent with a earlier study where 28-days treatment with apocynin or tempol prevented the elevation of BP in DOCA-salt hypertensive rats (Beswick et al., 2001a; Beswick et al., 2001b). In the preliminary study it was found that acute treatment with tempol or apocynin did not decrease the MAP in DOCA-salt hypertensive rats after the BP was elevated. Tiron was the only $O_2^{\cdot-}$ scavenger that decreased the BP significantly after it was elevated. Tempol or tiron did not decrease the BP in SHAM-normotensive rats, indicating a beneficial role

of these antioxidants only in hypertensive animals. The ability of tiron to decrease BP acutely can be attributed to its propensity to bind with calcium as discussed later.

4.2.1.2. Contribution to the modulation of superoxide anion generation

3 weeks of DOCA-treatment increased the aortic $O_2^{\cdot-}$ generation in DOCA salt hypertensive rats compared with SHAM-normotensive rats. The $O_2^{\cdot-}$ production was significantly reduced in aortic rings from DOCA-salt hypertensive rats treated for 3-weeks with tempol or tiron or apocynin. The levels of $O_2^{\cdot-}$ generation in antioxidant treated DOCA-salt hypertensive rats were not statistically different from those in untreated SHAM-control rats. Tempol and tiron treatment did not have a significant effect on the $O_2^{\cdot-}$ levels on SHAM-normotensive rats. Both the elevated MAP and elevated $O_2^{\cdot-}$ production in aorta from DOCA-salt treated rats was prevented by oral administration of tempol or tiron or apocynin in drinking water. These findings are consistent with the interpretation that the decrease in MAP evoked by these anti-oxidants is likely due to their ability to decrease $O_2^{\cdot-}$ generation.

4.2.1.3. Choice of antioxidant

4, 5-dihydroxy-1, 3-benzene disulfonic acid (Tiron) is a SOD mimetic, and a cell membrane permeable scavenger of $O_2^{\cdot-}$ (Krishna et al., 1992). It has been used widely *in vitro* in concentrations of 10-30 mM to determine the role of $O_2^{\cdot-}$ in modulating a variety of biological responses (Arimura et al., 2001; Fleming et al., 2001). In the present study on BP it was found that after hypertension was established in DOCA-salt hypertensive rats, tiron was the only oral antioxidant to decrease the blood pressure. In view of these considerations tiron was selected for functional experiments.

In the initial experiments with tiron it was discovered that tiron caused concentration-dependent decreases in perfusion pressure in the perfused MVB preconstricted with 90 mM KCl.

In searching for an interpretation for this observation, it prompted to us to speculate that tiron might bind with calcium present in Kreb's buffer. This data is consistent with the interpretation that tiron binds Ca^{2+}, and that this effect inhibits Ca^{2+}-dependent biological responses. The observation that tiron evoked concentration-dependent decreases in the fluorescence ratio of fura-FF, a ratio directly proportional to the concentration of free (unbound) Ca^{2+} in the buffer, provides direct evidence of an interaction between tiron and Ca^{2+}. The fact that this experiment was performed in buffered solutions in the absence of tissue illustrates the direct nature of this interaction uncomplicated by tissue related events. The binding constant for tiron with Ca^{2+} was 0.692 ± 0.036 mM, a concentration well below the 10-30 mM often employed in biological experiments. The conclusion that tiron binds Ca^{2+} is also consistent with the data on the effects of tiron on biological responses that are Ca^{2+} dependent. The depolarizing stimulus, 90 mM KCl, induces vasoconstriction by opening voltage-gated Ca^{2+} channels. Tiron caused concentration-dependent decreases in perfusion pressure in the perfused MAB preconstricted with 90 mM KCl, an effect consistent with the notion that tiron binds Ca^{2+}. This effect of tiron was similar in the presence of 100 μM tempol, a concentration that abolished the increases in $O_2^{\cdot-}$ evoked by Ang II in aortic rings (Shastri et al., 2002). Thus, the ability of tiron to reduce perfusion pressure appears to be more consistent with its ability to bind Ca^{2+} than its proported ability to scavenge $O_2^{\cdot-}$. Moreover, the vasoconstriction evoked by increasing concentrations of Ca^{2+} in mesenteric bed preparations depolarized with 90 mM KCl was examined. Tiron shifted the concentration-response curve to Ca^{2+} dramatically to the right. Thus, the data on vasoconstrictor responses dependent on Ca^{2+} also supports the notion that tiron binds Ca^{2+}. Finally, tiron inhibited blood coagulation, a Ca^{2+} dependent response. Citrate and oxalate are often used to prevent blood from clotting because of their propensity to bind Ca^{2+}. In view of the direct interaction of tiron and

Ca^{2+} demonstrated in the fura-FF studies, it is reasonable to suggest that the effect of tiron on blood coagulation was also due to its propensity to bind Ca^{2+}, an effect similar to citrate and oxalate. Surprisingly, there appear to be no previous studies on the effects of tiron on the binding of Ca^{2+} and the consequences of this effect on biological responses. It was concluded that the interpretation of the effects of tiron in the scientific literature regarding the role of $O_2^{\cdot-}$ in a variety of biological responses may need to be reevaluated.

Tempol, another nonenzymatic, cell permeable SOD mimetic scavenger of $O_2^{\cdot-}$, did not interfere with the fluorescence ratio of fura-FF at the concentration used for functional studies (1μM to 1 mM). Therefore SOD or tempol was chosen as a scavenger of $O_2^{\cdot-}$. Though tiron was not used in the functional studies, it was used in the luminometer because it did not interfere with luminometer readings and because of its excellent ability to scavenge $O_2^{\cdot-}$. Since the L-type Ca^{2+} channel blocker, nifedipine, and removal of Ca^{2+} from extracellular buffer did not interfere with superoxide production, we suggest that tiron can safely be used in the chemiluminescence assay.

4.2.1.4. Role of superoxide anion

Spontaneous tone in aortic rings of DOCA-salt hypertensive rat appears to depend on the increased $O_2^{\cdot-}$ levels. This conclusion is based on the following observations. Firstly, $O_2^{\cdot-}$ production was increased significantly in aortic rings of DOCA-salt hypertensive rats compared to SHAM control rats. The increased $O_2^{\cdot-}$ production agrees with previous work where it had been reported that vascular $O_2^{\cdot-}$ generation is high in DOCA-salt hypertensive rats (Somers et al., 2000; Wu et al., 2001). Secondly, preincubation with the $O_2^{\cdot-}$ scavenger, SOD, completely prevented spontaneous tone in aortic rings in the presence of endothelium. We also confirmed that SOD treatment resulted in decreased $O_2^{\cdot-}$ generation. Thirdly, stretch increased the

generation of $O_2^{\cdot-}$ in rings from DOCA-salt hypertensive rats even above the elevated basal levels observed in these rats. Thus, stretch was associated with parallel increases in $O_2^{\cdot-}$ generation and spontaneous tone in the DOCA-salt animals. In contrast, stretch did not increase $O_2^{\cdot-}$ production significantly and did not evoke spontaneous tone in SHAM rats.

The mechanisms by which $O_2^{\cdot-}$ contributes to spontaneous tone may be related, at least in part, to its interaction with NO. The production of NO appears unimpaired in DOCA-salt hypertensive rats because eNOS protein expression is similar in both groups of rats, and because L-NAME and endothelial-denudation increased the magnitude of tone in these rats. We also observed that SOD failed to decrease spontaneous tone in the rings that were pretreated with L-NAME or in endothelium-denuded rings, though it was able to decrease the level of $O_2^{\cdot-}$ in those rings. Therefore we conclude that SOD decreased spontaneous tone in endothelium-intact rings by scavenging $O_2^{\cdot-}$ and thereby freeing endothelial derived NO to relax the tissue. It is also possible that in DOCA-salt hypertensive rats, increased $O_2^{\cdot-}$ is responsible for increased calcium influx in VSMC because the maximum contractile response induced by 120 mM KCl is significantly higher in aortic rings of DOCA-salt hypertensive rats compared to that in SHAM-normotensive rats.

4.2.1.5. Role of NADPH-oxidase in increased superoxide generation

Potential sources contributing to the enhanced generation of $O_2^{\cdot-}$ generation include eNOS, xanthine oxidase, and NADPH oxidase. In SHR-SP, eNOS has been reported to increase $O_2^{\cdot-}$ generation (Kerr et al., 1999). The production of $O_2^{\cdot-}$ has been ascribed to uncoupled eNOS in SHR-SP (Hamilton et al., 2001). However, in the present experiments, inhibition of eNOS with L-NAME did not interfere with the generation of $O_2^{\cdot-}$, confirming an earlier report indicating no

significant difference in $O_2^{\cdot-}$ production in de-endothelialized or L-NAME treated aortic rings from DOCA-salt hypertensive rats (Beswick et al., 2001a). Moreover, L-NAME reduces NO production, and consequently, the formation of $OONO^-$. Since spontaneous tone was not reduced in the aortic rings treated with L-NAME, it is also unlikely that $OONO^-$ accounts for spontaneous tone in DOCA-salt hypertensive rats. Since COX inhibitors decreased the tone, the possibility that $OONO^-$ had an indirect effect on the generation of spontaneous tone cannot be ruled out, a possibility that is discussed later. Inhibition of COX and xanthine oxidase also had no effect on the production of $O_2^{\cdot-}$ in both stretched and unstretched rings from DOCA-salt hypertensive rats. Therefore, the possibility of any contribution from these enzymes in the increased generation of $O_2^{\cdot-}$ in aorta of DOCA-salt hypertensive rats was excluded.

On the other hand, it has been reported that NAD(P)H oxidase activity is increased in the aorta of DOCA-salt hypertensive rats and that this increase is associated with elevated $O_2^{\cdot-}$ production (Beswick et al., 2001a). In this study, the NAD(P)H oxidase inhibitor, apocynin, prevented the generation of spontaneous tone in aortic rings with intact endothelium, and it inhibited the generation of $O_2^{\cdot-}$ in both stretched and unstretched aortic rings from DOCA-salt hypertensive rats. This result is consistent with the results of Beswick et al in DOCA-salt hypertensive rats (Beswick et al., 2001a). In view of these considerations, it was concluded that NAD(P)H oxidase derived $O_2^{\cdot-}$ generation is a major contributor to the development of spontaneous tone in the DOCA-salt hypertensive rat.

The site accounting for $O_2^{\cdot-}$ generation by NAD(P)H oxidase is unclear. NAD(P)H oxidase is expressed in endothelial cells (Zafari et al., 1998), VSMC (Griendling et al., 1994; Zalba et al., 2000) and cells in the adventitial layer (Di Wang et al., 1999). An increased inflammatory response and macrophage/monocyte activity has been reported in the DOCA-salt model of

hypertension (Beswick et al., 2001b). NAD(P)H oxidase can also be expressed by inflammatory cells such as infiltrating neutrophils (Clozel et al., 1991) and macrophages/ monocytes (Dinauer et al., 1987). In our immunohistochemistry study it was found that 3-nitrotyrosine was significantly higher in aortic walls of DOCA-salt hypertensive rats compared to that of SHAM-normotensive rats and it was distributed in all layers of the wall of the aorta. It was also found that the removal of endothelium did not interfere with the increase $O_2^{\cdot-}$ production in aortic rings from DOCA-salt hypertensive rats. In endothelium-denuded rings, SOD and apocynin did not have any effect on the spontaneous tone but decreased the generation of $O_2^{\cdot-}$ significantly. These two observations suggest that the endothelial layer is not the major source for $O_2^{\cdot-}$ in this rat model of hypertension. Although the exact location of NAD(P)H -oxidase was not determined in the present study, various observations suggested that either adventitial fibroblasts or VSMC or both contribute to the increase in $O_2^{\cdot-}$ production in DOCA-salt hypertensive rats.

4.2.1.6. Contribution of hydrogen peroxide

The possibility that H_2O_2 might play a role in modulating spontaneous tone merits consideration. SOD is known to catalyze the formation of H_2O_2 from $O_2^{\cdot-}$ and H_2O. Opinion on the vascular effects of H_2O_2 is controversial. It has been reported to contract isolated rat aortic rings (Yang et al., 1998; Shen et al., 2000). On the contrary, H_2O_2 has also been suggested to be a vasodilator in rat aorta (Yang et al., 1999), rabbit aorta (Bharadwaj & Prasad,1995) and porcine coronary arteries (Hayabuchi et al., 1998). In the functional study catalase, a scavenger of H_2O_2, evoked an increase in the intensity of spontaneous tone in aortic rings from DOCA-salt hypertensive rats in the presence or in the absence of endothelium or rings pretreated with L-NAME or SOD. Importantly, catalase did not evoke any increase in tone when rings were pretreated with the NAD(P)H oxidase inhibitor, apocynin, a situation characterized by the lack of

production of $O_2^{\cdot-}$. Since $O_2^{\cdot-}$ is an important precursor in the formation of H_2O_2, the lack of effect of catalase in apocynin-treated vessels is not surprising. Accordingly, it is suggested that in the stretched aortic rings of DOCA-salt hypertensive rats, any endogenous H_2O_2 formed from the interaction of $O_2^{\cdot-}$ and H_2O_2 acts as a vasodilator to protect the tissue against generation of spontaneous tone.

In this study, the mechanism by which H_2O_2 attenuates spontaneous tone could not be determined. It has been shown that the $^{\cdot}OH$ radical can induce vasorelaxation in normal rats (Prasad & Bharadwaj,1996). However, treating aortic rings from DOCA-salt hypertensive rats with deferoxamine, a scavenger of $^{\cdot}OH$ radicals, failed to elevate spontaneous tone. Interestingly, catalase not only increased spontaneous tone in aortic rings from DOCA-salt hypertensive rats, it also induced the development of spontaneous tone in aortic rings from SHAM-control rats, although the magnitude of this effect was small. This finding suggests that in normotensive SHAM-rats, H_2O_2 helps to protect the vessel from developing spontaneous tone.

4.2.1.7. Contribution of COX metabolites

Both indomethacin and valeroyl salicylate reduced spontaneous tone dramatically as did the TP-receptor antagonist in aortic rings with intact endothelium. In contrast, in endothelium-denuded rings, the TP receptor antagonist failed to reduce tone and valeroyl salicylate decreased tone only slightly. Therefore, it is apparent that a COX product contributes to the development of spontaneous tone and that the major effects of these products are endothelium-dependent. The nature of the COX metabolite/s remains elusive. The first consideration was $O_2^{\cdot-}$ or TXA_2. TXA_2 is the most potent COX derived vasoconstrictor. However, the TXA_2 synthase inhibitor, furegrelate, had no effect on spontaneous tone in the endothelium-intact and endothelium-denuded aortic rings. Therefore it is clear that TXA_2 *per se* did not exaggerate spontaneous tone.

The COX inhibitors and the TP-receptor antagonist did not affect the $O_2^{\cdot-}$ production suggesting that $O_2^{\cdot-}$ was not the elusive COX metabolite.

$OONO^-$, the product of the interaction between NO and $O_2^{\cdot-}$, is known to increase COX activity (Landino et al., 1996). Recently it has been shown that $OONO^-$ can inactivate prostacyclin synthase (Zou et al., 2002). Since, COX inhibitors decreased the tone, we cannot rule out the possibility that $OONO^-$ had an indirect effect on the generation of spontaneous tone. Therefore, it is possible that increased $OONO^-$ in DOCA salt hypertensive rats serves as a link between the oxidative stress and COX system to modulate spontaneous tone.

4.2.1.8. Summary of role of reactive oxygen species

The endothelium, and in particular endothelial-derived NO, appears to play a protective role limiting the development of spontaneous tone. However, the activity of the NO system is insufficient to prevent development of tone. Strikingly, an increase in NADPH oxidase-derived $O_2^{\cdot-}$ appears to be a major factor in the generation of spontaneous tone. At least part of the effect of $O_2^{\cdot-}$ appears to be related to its interaction with NO. $OONO^-$ may play a role by interfering with COX metabolites. The generation of H_2O_2 from $O_2^{\cdot-}$ appears to be a mechanism that mitigates the development of spontaneous tone by $O_2^{\cdot-}$. Finally, a COX component also appears to contribute to the development of spontaneous tone in the DOCA-salt hypertensive rat. These events may contribute to the pathogenesis and impaired vascular reactivity in mineralocorticoid hypertension.

4.3. Contribution of 20-HETE

Endogenous 20-HETE levels in the aortic tissue from DOCA-salt hypertensive rats were higher than those in SHAM-normotensive rats. 20-HETE was measure with a method recommended for its accuracy in measuring CYP metabolites (Maier et al., 2000). As discussed

in the introduction, 20-HETE plays a significant role in the myogenic response to elevations of transmural pressure in renal, cerebral, and mesenteric arterioles. The increased 20-HETE in the aortic tissue in DOCA-salt hypertensive rats is consistent with a role for 20-HETE in modulating spontaneous tone.

Inhibition of endogenous 20-HETE production with ABT decreased the magnitude of spontaneous tone in an endothelium-dependent manner in aortic rings from DOCA-salt hypertensive rats suggesting that endogenous 20-HETE exaggerates spontaneous tone. The inhibitory effect of the CYP4A inhibitor on spontaneous tone was not as robust as the inhibitory effect of SOD or apocynin or valeroyl salicylate. There are several possible explanations for this observation. First, CYP4A inhibitors including ABT block the synthesis of 20-HETE but do not prevent the release of 20-HETE from the membrane phospholipid pool. Second, inhibition of the vasoconstrictor effect of 20-HETE following ABT treatment may be incomplete since 20-HETE activates the PKC and MAP kinase signal transduction cascade, which may have a long lasting effect on vascular tone. Third, while the concentration of ABT used in these experiments was shown to be relatively specific for the CYP4A family, we cannot exclude the possibility that, at this concentration, ABT also inhibits the formation of EETs. EETs can increase $[Ca^{2+}]_i$ concentration in endothelial cells (Graber et al., 1997) which, in turn, can stimulate the formation of NO and PGs in rings with intact endothelium. Thus, the inhibitory effect of ABT on spontaneous tone could be an attenuated one, compensated by a simultaneous decrease in NO generation. Consistent with this interpretation is the observation that ABT also had a small but significant inhibitory effect on the magnitude of spontaneous tone in L-NAME treated rings from DOCA-salt hypertensive rats. In L-NAME treated rings the absence of NO maintained spontaneous tone in the presence of ABT. Theoretically, L-NAME treatment can also increase

the generation of 20-HETE since the cGMP-independent component of NO is suggested to be by inhibition of 20-HETE production.

There are two plausible explanations for the absence of an inhibitory effect of ABT on spontaneous tone in endothelium-denuded rings from DOCA-salt hypertensive rats. First, endothelial cells produced 20-HETE and endothelial-denudation eliminated this source. Second, the endothelium mediated the vascular action of 20-HETE. While 20-HETE is thought to be produced primarily in VSMC, (Roman et al., 2000), Zhu et al reported the presence of CYP4A mRNA in endothelial cells (Zhu et al., 2002). Since ABT exhibited an inhibitory effect on L-NAME treated rings but not in endothelium-denuded rings, the possibility that the endothelium was a source of 20-HETE cannot be excluded. Alternatively, a more likely explanation is that the endothelium served to modulate the effect of 20-HETE released from the VSMC. The absence of a 20-HETE induced increase in spontaneous tone in endothelium-denuded rings supports this notion. Since 20-HETE is a substrate for COX, we explored the role of COX in modulating the effect of 20-HETE. 20-HETE failed to increase spontaneous tone in aortic rings pretreated with the COX inhibitor, valeroyl salicylate or indomethacin, suggesting that 20-HETE is metabolized by COX into a vasoactive metabolite that contributes to spontaneous tone. In endothelium-intact rings, the TXA_2 synthase inhibitor, furegrelate, did not have any effect on the 20-HETE induced increase in spontaneous tone. This suggests that COX does not generate TXA_2 from 20-HETE. It has been reported that 20-HETE is metabolized by COX to the 20-hydroxy-endoperoxides (20-HEP), 20-hydroxy PGG_2 and 20-hydroxy PGH_2 (Schwartzman et al., 1989). These findings suggest that 20-HETE may not be the primary mediator of spontaneous tone. Rather its COX metabolites, notably 20-hydroxy PGG_2 and 20-hydroxy PGH_2, likely modulate spontaneous tone in aorta of DOCA-salt hypertensive rats in an endothelium dependent manner.

4.4. Contribution of K_{ATP} channel

The data suggest that the activity of K_{ATP} channels is decreased in the aorta of DOCA-salt hypertensive rats compared with SHAM-normotensive rats. In the functional component of the study, the K_{ATP} channel activator, cromakalim, induced dose-dependent decreases in vascular tone in PE-preconstricted aortic rings, but this effect was much less in rings isolated from DOCA-salt hypertensive rats than those recorded in rings from SHAM control rats. Indeed, aortic rings from DOCA-salt hypertensive rats were less sensitive (EC_{50} in DOCA 0.85 ± 0.1 vs 0.108 ± 0.005 μM in SHAM). Cromakalim completely abolished the preconstricted tone induced by PE in aortic rings from SHAM-control rats, whereas cromakalim only attenuated the tone induced by PE in rings from DOCA-salt hypertensive rats. Thus, the efficacy (E_{max}) of cromakalim was reduced in rings from DOCA-salt hypertensive rats. Importantly, cromakalim induced dilatation responses were reversed by glibenclamide, a specific inhibitor of K_{ATP} channel activity, confirming that the cromakalim induced responses were specific to its action on the K_{ATP} channels.

The endothelium did not appear to play a role in the vasodilatation evoked by cromakalim in PE-preconstricted aortic rings. Removal of the endothelium did not affect cromakalim-induced relaxation responses. Although, there is evidence for the presence of K_{ATP} channels in vascular endothelium (Ishizaka & Kuo,1997), it is the activation of K_{ATP} channels in VSMC that seems to be dominant for modulation of vascular tone (Nelson et al., 1990a; Nelson et al., 1990b; Standen et al., 1989). Of course, recent evidence suggests abnormal endothelial function in DOCA-salt hypertensive rats (Somers et al., 2000). Since abnormal NO production by endothelium seems to contribute to the diminished responsiveness to K_{ATP} channel openers in some diseased conditions such as diabetes (Zimmermann et al., 1997), we cannot exclude the possibility that endothelial

dysfunction contributes to altered vascular reactivity in vessels isolated from DOCA-salt hyperensive rats. However, this present study identifies changes at the VSMC level that contributes to impaired relaxation responses in these rats.

The electrophysiological component of the study supports the functional data. Indeed, the whole cell patch clamp results confirmed that K_{ATP} channel activity was significantly decreased in aortic VSMC from DOCA-salt hypertensive rats. Increasing the extracellular potassium concentration from 5.4 to 140 mM or addition of cromakalim evoked inward K_{ATP} channel currents in VSMC isolated from SHAM-normotensive rats. In contrast, elevations of extracellular potassium or addition of cromakalim evoked inward K_{ATP} channel currents in only a few cells tested and the response of these cells was less than those observed in cells from SHAM normotensive rats. These results indicate that K_{ATP} channel activity is impaired in aortic VSMC isolated from DOCA-salt hypertensive rats. Interestingly, impaired responses of mesenteric arterial cells to the K_{ATP} channel opener, levcromakalim, have been reported in SHR (Ohya et al., 1996), an experimental model for genetic hypertension with high plasma renin activity. Thus, impaired K_{ATP} channel activity appears evident in both a genetic and an endocrine model of hypertension.

Strikingly, cromakalim abolished spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats. Glibenclamide blocked completely the cromakalim-induced response providing additional evidence that the effects of cromakalim were due to its effects on the K_{ATP} channel. Clearly, an increase in K_{ATP} channel activity evoked by an exogenous K_{ATP} channel opener is able to completely suppress spontaneous tone in the DOCA-salt model of hypertension. Interestingly, the patch clamp studies indicated depressed K_{ATP} channel activity in cells isolated from DOCA-salt hypertensive rats. While depressed K_{ATP} function could contribute to the

development of spontaneous tone, it begs the question about how cromakalim suppresses tone if K_{ATP} channels are impaired. Importantly, while most cells failed to generate K_{ATP} channel current in responses to cromakalim, some did. Thus, it is conceivable that the residual K_{ATP} channel activity in vessels from DOCA-salt hypertensive rats was sufficient for cromakalim to suppress tone. In the final analysis, the functional studies support this conclusion since the effect of cromakalim on spontaneous tone was dramatic.

Glibenclamide did not induce spontaneous tone in SHAM normotensive rats. Thus, a defect in K_{ATP} channel activity *alone* cannot account for spontaneous tone. Though under normal conditions, K_{ATP} channels have a very low open-state probability and have little influence on the resting membrane potential of VSMC, ROS such as $O_2^{\cdot-}$, H_2O_2 and $OONO^-$ regulate the current through K_{ATP} channels. NO acts in part through activation of K_{ATP} channels (Armstead,1997). $O_2^{\cdot-}$ increases the open state probability of K_{ATP} channels in ventricular cells from guinea pigs (Tokube et al., 1996). H_2O_2 and $OONO^-$ can induce relaxation via K_{ATP} channels (Wei et al., 1996). In DOCA-salt hypertensive rats increased oxidative stress decreases the bioavailability of NO (Beswick et al., 2001b). The possibility that decreased bioavailability of NO may exaggerate impaired vascular reactivity due to decreased function of K_{ATP} channels in vessels from DOCA-salt hypertensive rats cannot be excluded. Therefore, the contribution of $O_2^{\cdot-}$ in modulating spontaneous tone in aortic rings of DOCA-salt hypertensive rats may not be determined solely by its interaction with NO, vascular K_{ATP}-channel may have a possible regulatory effect on action of NO, $O_2^{\cdot-}$, H_2O_2 and $OONO^-$.

In the final analysis, it seems reasonable to suggest that a decrease in K_{ATP} channel activity is one contributing factor to the generation of spontaneous tone in the DOCA-salt model, but other

factors must compensate successfully to prevent the development of spontaneous tone in SHAM-normotensive rats when the K_{ATP} channel is blocked with glibenclamide.

4.5. A unifying hypothesis?

This thesis describes a complex array of seemingly disparate mechanisms that modulate spontaneous tone in the DOCA-salt hypertensive rat. This seeming disconnectedness may be more apparent than real since the many of adaptations may be related through a unified response to stretch.

In the DOCA-salt hypertensive rat, elevations in blood pressure impose a chronic increase in transmural pressure. Indeed, hypertension is characterized by increases in intramural pressure and in sheer stress. The implied stretch associated with this chronic increase in transmural pressure elicits adaptive responses. The blood vessels adapt in order to regulate blood flow and to restore the mean circumferential wall stress. It has been suggested that spontaneous tone in hypertension is one such mechanism (Meininger & Davis,1992). There are several broad hypotheses concerning the sequence of events that couple changes in intravascular pressure or stretch with alterations in vascular smooth muscle activation. These hypotheses include (1) altered membrane properties leading to activation or inactivation of ion channels, (2) modulation of membrane bound enzymes and consequently cell-signaling pathways within the VSMC, (3) length dependent contractile protein function, and (4) endothelial-dependent and independent modulation of VSMC tone.

Stretch, or more precisely the response to stretch, appears to be the common denominator. Myogenic response, basal tone, stretch activation, spontaneous tone, and intrinsic tone are all terms that have been applied to describe the response of vascular smooth muscle to stretch (Davis & Hill,1999). Ultimately, stretch must lead to elevations in $[Ca^{2+}]_i$, at least in part through depolarization of the cell membrane. The work in this thesis confirms that the function of L-type Ca^{2+} channels is critical to the generation of spontaneous tone in the DOCA-salt

hypertensive rat. The challenge then is to provide a plausible explanation for the link between stretch, the state of polarization of the membrane as determined by K^+ channel function, the opening of L-type Ca^{2+} channels, and the various regulatory or modulatory systems identified in this thesis.

Stretch-induced depolarization could result from inhibition of any of the various K^+ currents identified in smooth muscle (Kuriyama et al., 1998). A direct role for a K^+ channel in initiating spontaneous tone has not been shown, but there is evidence that K^+ currents do counteract myogenic responses. In this thesis, we demonstrate that K_{ATP} channel function is impaired in DOCA-salt hypertension. It seems plausible to suggest that the chronic stretch associated with the high blood pressure of this model may have contributed to K_{ATP} channel dysfunction, which in turn contributes to depolarization, opening of L-type Ca^{2+} channels, and spontaneous tone. This would be consistent with the hypothesis that signal transduction moieties that act, at least in part, through activation of K_{ATP} channels, such as NO and H_2O_2, would have a limited or reduced effect. Importantly, since K_{ATP} function is only impaired and not abolished, it allows exogenous administration of K_{ATP} channel activators to suppress spontaneous tone when administered in large doses.

A link between stretch, K^+ channel activity, opening of L-type Ca^{2+} channels, and ROS appears likely. Interestingly, increases in tyrosine phosphorylation, specifically related to mechanotransduction, have been demonstrated in cells subjected to stretch (Sadoshima & Izumo,1993). On the one hand, tyrosine phosphorylation may contribute to the phosphorylation of mitogen-activated protein kinase, which can regulate contractile proteins. On the other hand, tyrosine phosphorylation can also lead to NAD(P)H-oxidation and generation of $O_2^{\cdot-}$ (Touyz et al., 2003). In turn, ROS have been shown to influence the abundance and activity of L-type Ca^{2+}

channels. On the other hand, $O_2^{\cdot-}$ increases $[Ca^{2+}]_i$ through an IP_3 dependent mechanism (Wu et al., 2000). Thus, it is plausible to suggest that stretch increases $O_2^{\cdot-}$, which leads to the elevations in $[Ca^{2+}]_i$ and spontaneous tone. Alternatively, the data in this thesis is consistent with the hypothesis that $O_2^{\cdot-}$ increases spontaneous tone by inactivating NO and decreasing its bioavailability. NO, through both cGMP dependent and independent pathways, activates K_{Ca} and K_{ATP} channels and, therefore, promotes hyperpolarization of the membrane. Thus, inactivation of NO by $O_2^{\cdot-}$ would depolarize the cell and lead to the opening of L-type Ca^{2+} channels. This appears to be a plausible explanation linking stretch, K^+ channel activity, opening of L-type Ca^{2+} channels, and ROS. To add to the complexity, H_2O_2 may contribute to vascular remodeling, which may alter mechanotransduction. While stretch evoked changes in ROS may affect Ca^{2+} concentrations, changes in $[Ca^{2+}]_i$ may also regulate the generation of ROS. Indeed, the stretch associated with sheer-stress is well known to evoke activation of NOS through elevation in $[Ca^{2+}]_i$ in endothelial cells.

A link between stretch, K^+ channel activity, opening of L-type Ca^{2+} channels and products of arachadonic acid also appears likely. It has been proposed that stretch-induced production of 20-HETE, through activation of phospholipase C and release of arachidonic acid from the cell membrane (Vandenburgh et al., 1993), may sustain or even initiate myogenic responses (Harder et al., 1997). Elevations in 20-HETE would block K_{Ca} channels leading to depolarization and opening of L-type Ca^{2+} channels. Indeed, the data in this thesis demonstrated that inhibition of endogenous 20-HETE decreased the magnitude of spontaneous tone. Thus, the data is consistent the notion that stretch induced changes in 20-HETE may modulate opening of L-type Ca^{2+} channels and therefore spontaneous tone. A role for stretch induced elevations in arachidonic acid metabolites is also consistent with this hypothesis.

One important adaptive mechanism during chronic hypertension is the increase in the arterial wall thickness. This thickening of the wall occurs through an increase in smooth muscle (hypertrophy or hyperplasia) and deposition of selected components of extracellular matrix, particularly collagen. Vascular endothelium also plays an important role in the early functional adaptation in hypertension. Increases in $[Ca^{2+}]_i$ during sheer-stress can increase NO formation from endothelial cells but lately it has been reported that sheer-stress can induce NO formation by a calcium independent mechanism, namely by changing intracellular pH and tyrosine phosphorylation (Ayajiki et al., 1996). The role of mechanotransduction merits consideration. The increase in the thickness of the arterial wall may contribute to the return of the circumferential wall stress toward its normal value. The thickening results from an increase in smooth muscle and extracellular matrix, with the associated growth and remodeling processes depending on a host of regulatory signals that likely include the altered mechanical environment of the cell. Smooth muscle tone has also been shown to acutely modify the residual strain of vessels, possibly through connections to the extracellular matrix. These observations suggest that there is a two-way interaction between the extracellular matrix and VSMC force development (Zeller & Skalak,1998).

In summary, this thesis provides a plausible explanation linking stretch, membrane depolarization, voltage gated L-type channels, and spontaneous tone and the modulation of these events by ROS (NO, $O_2^{\cdot-}$, H_2O_2) and COX products (including those derived from 20-HETE).

4.6. Summary and Conclusions

Figure 34 is a schematic that depicts the major events modulating spontaneous tone.

The objective of this body of work was to characterize the spontaneous tone observed in aortic rings isolated from DOCA-salt hypertensive rats and to determine the contribution of

various ROS and their interactions in modulating this tone. In addition, the roles of 20-HETE and K_{ATP} channels in modulating spontaneous tone were examined. A plethora of complementary functional and analytical techniques were recruited to test the hypothesis. Whole animal experiments were designed to record blood pressure in DOCA-treated and SHAM-treated rats by the radiotelemetry method in the presence and absence of various treatments. Spontaneous tone was quantified in isolated organ bath experiments in the presence and absence of selected interventions. Analytical techniques included lucigenin chemiluminescence, Western blot analysis, immunohistochemistry and HPLC, were chosen to quantify the contribution of various modulators. Finally, whole cell patch clamp experiments were designed to evaluate the role of K_{ATP} channel activity.

In contrast to SHAM-normotensive rats, thoracic aortic rings from DOCA-salt hypertensive rats developed spontaneous tone in response to the stretch associated with increasing the preload from 1-5 g. Tone was higher in endothelium-denuded aortic rings than in endothelium-intact vessels. Inhibition of NOS with 300 μM L-NAME also increased spontaneous tone. These findings suggest that the endothelium and, notably, NO plays a protective role limiting the development of spontaneous tone. However, endothelium-denudation did not induce spontaneous tone in the rings from SHAM-rats indicating that the intrinsic property of spontaneous tone resides outside of the endothelium. The endothelium acts solely as a modulator of spontaneous tone.

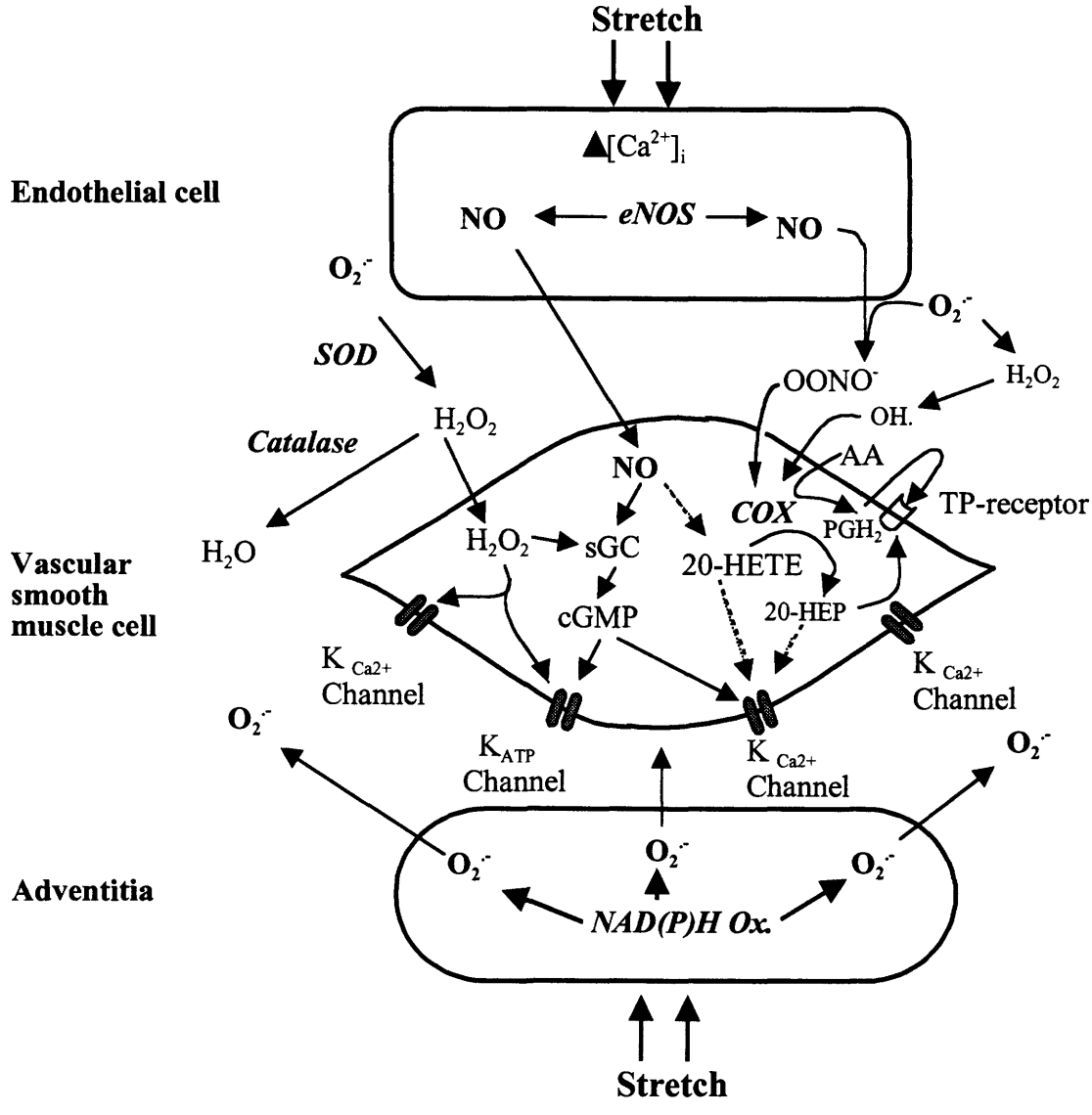


Figure 34. Proposed model for modulation of spontaneous tone

Suggested mechanism for spontaneous tone and interaction of various modulators for spontaneous tone in the aortic rings from DOCA-salt hypertensive rats

In order to explore the role of $O_2^{\cdot-}$, we first selected the $O_2^{\cdot-}$ scavenger, tiron, as it has been used widely in concentrations of 10-30 mM to determine the role of $O_2^{\cdot-}$ in modulating a variety of biological responses. However, it was discovered that tiron also binds Ca^{2+}. In competition assays in buffered solutions with no tissue present, tiron decreased the fluorescence ratio of fura-FF, a measure of $[Ca^{2+}]$: the inhibition constant (K_i) of tiron with Ca^{2+} was 0.692 ± 0.036 mM. In the mesenteric bed perfused at constant flow and preconstricted with 90 mM KCl, tiron evoked decreases in perfusion pressure of the mesenteric bed in a concentration-dependent manner (R_{max} = 43.58 ± 2.6 mmHg; EC_{50} = 1.46 ± 0.33 mM). This vasodilator effect of tiron was similar in the presence of the $O_2^{\cdot-}$ scavenger, tempol (R_{max} = 46.12 ± 1.87 mmHg; EC_{50}= 1.34 ± 0.27 mM). In the presence of 90 mM KCl, increasing concentrations of Ca^{2+} increased perfusion pressure and tiron shifted the concentration-response curve to Ca^{2+} to the right. The data indicate that tiron binds Ca^{2+} at concentrations at or below those commonly used to scavenge $O_2^{\cdot-}$, an action that may be responsible for a variety of biological responses. The interpretation in the scientific literature of effects of tiron on the role of $O_2^{\cdot-}$ may need to be reevaluated. In view of this current discovery, we abandoned the use of tiron for the functional studies and selected more specific interventions.

An important role for $O_2^{\cdot-}$ in modulating spontaneous tone appears evident. Basal $O_2^{\cdot-}$ production was increased in aortic rings from DOCA-salt hypertensive rats compared with SHAM-normotensive rats. Stretch increased the $O_2^{\cdot-}$ production even further in hypertensive but not in normotensive rats. The development of hypertension by the DOCA-salt regimen was attenuated by treatment (orally over 3 weeks) with tempol, a $O_2^{\cdot-}$ scavenger, or apocynin, an inhibitor of NAD(P)H-oxidase (Table 1, page 71). Spontaneous tone was abolished by SOD,

tempol, or apocynin in endothelium-intact rings but not in endothelium-denuded rings nor in L-NAME treated rings, even though SOD and apocynin inhibited apparent $O_2^{\cdot-}$ production in all rings. eNOS protein expression was not different in thoracic aorta from DOCA-salt hypertensive rats and SHAM-normotensive rats. 3-nitrotyrosine levels were increased in aorta from DOCA-salt hypertensive rats. 3-nitrotyrosine serves a marker for $OONO^-$, a product of the interaction betweeen $O_2^{\cdot-}$ and NO. Taken together, these findings suggest that $O_2^{\cdot-}$ derived from NAD(P)H-oxidase modulates spontaneous tone primarily by scavenging NO.

The role of H_2O_2 was also investigated. Catalase, the enzyme that breaks down H_2O_2 formed from the interaction of $O_2^{\cdot-}$ and H_2O, increased spontaneous tone in aorta from DOCA-salt hypertensive rats in a endothelium-independent manner. Catalase also increased tone in SOD-treated and L-NAME-treated aortic rings but not in apocynin-treated aortic rings. The latter intervention is characterized by decreased $O_2^{\cdot-}$ production and, consequently, decreased H_2O_2 formation. These results suggest that H_2O_2 attenuates the development of spontaneous tone and, therefore, serves as a protective mechanism.

A role for products of arachidonic metabolism is also apparent. The COX inhibitor, valeroyl salicylate, and the TP-receptor antagonist, SQ 28954, inhibited spontaneous tone. These inhibitory effects were attenuated by endothelium-denudation. 20-HETE production was increased in aortic rings from DOCA-salt hypertensive rats. Inhibition of the CYP4A isozyme with ABT decreased spontaneous tone. Exogenous 20-HETE increased the magnitude of spontaneous tone in an endothelium-dependent manner. Importantly, 20-HETE failed to increase spontaneous tone in the presence of the COX inhibitor or the TP-receptor antagonist. These results suggest that 20-HETE exaggerates spontaneous tone in an endothelium-dependent way. These effects of 20-HETE appear to be mediated by its COX metabolites.

This final study was designed to evaluate the contribution of ATP-dependent potassium channels to the changes in vascular reactivity and spontaneous tone observed in vessels isolated from DOCA-salt hypertensive rats. In PE preconstricted aortic rings, cromakalim induced dose-dependent, glibenclamide-sensitive relaxation. The dose response curve to cromakalim was shifted to the right in DOCA-salt hypertensive rats (EC_{50}: 0.850 ± 0.100) compared with SHAM-normotensive rats (0.108 ± 0.005), and the maximum relaxation (E_{max}) evoked by cromakalim was significantly lower in aortic rings from the DOCA group (68 ± 2%) compared to the SHAM group (108 ± 5%). The results were similar in endothelium-denuded rings. Spontaneous tone was observed in aortic rings (5g preload) from DOCA-salt but not SHAM rats. Cromakalim abolished spontaneous tone and the effect was blocked by glibenclamide. In whole cell patch clamp studies, increasing extracellular K^+ concentrations from 5.4 to 140 mM and the administration of cromakalim evoked dramatic increases in K_{ATP} channel currents in aortic cells isolated from SHAM rats. In contrast, in aortic cells from DOCA-salt hypertensive rats, K_{ATP} channel currents were either absent or weak in response to challenges by elevated extracellular K^+ and by cromakalim. These findings suggest that the function of K_{ATP} channels is impaired in smooth muscle cells from aorta of DOCA-salt hypertensive rats. This dysfunction may contribute to the impaired vasodilatation and spontaneous tone observed in these rats.

In conclusion, the results demonstrate that components from both the endothelium and from VSMC contribute to the generation of spontaneous tone in aorta from DOCA-salt hypertensive rats but not in aorta from SHAM-control normotensive rats. The endothelium appears particularly important as multiple endothelial mediators and pathways appear to modulate the spontaneous tone. The results provide experimental evidence of the dynamic interplay within the endothelium to control spontaneous tone. Both NO and $O_2^{\cdot-}$ and the interactions between these

two ROS appear to play major roles in modulating spontaneous tone. H_2O_2 also plays important roles in protecting against development of spontaneous tone. In addition, the results suggest that increased 20-HETE and its metabolites by endothelial COX are likely to contribute to the generation of spontaneous tone. These findings highlight the importance of addressing the role of various mediators and not only the role of NO while studying endothelial dysfunction in hypertension. Finally, impaired K_{ATP} channel function at the level of the VSMC in aorta from DOCA-salt hypertensive rats contributes to the mechanisms modulating spontaneous tone. Thus, a complex array of ROS (NO, $O_2^{\cdot -}$, H_2O_2, $OONO^-$) and metabolites of arachidonic acid (20-HETE and its COX metabolites) interact in concert with depressed K_{ATP} channel activity to modulate spontaneous tone in the DOCA-salt model. This thesis provides a plausible explanation linking stretch, membrane depolarization, voltage gated L-type calcium channels, and spontaneous tone and the modulation of these events by ROS and COX products. Together the findings suggest novel therapeutic targets in the treatment of spontaneous tone.

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