ENDOTHELIUM AND SMOOTH MUSCLE FUNCTION IN RAT MESENTERIC VASCULATURE

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M.D., Bethune Medical University, 1983
M.Sc., The University of British Columbia, 1994

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pharmacology & Therapeutics, Faculty of Medicine)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2001

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Vancouver, Canada

Date April 27 2001

DE-6 (2/88)

ABSTRACT

The mesenteric arterial bed (MAB) comprises medium and small arteries as well as arterioles. They are important in generating and controlling peripheral resistance, thereby regulating blood flow and maintaining blood pressure. This hemodynamic function is mainly determined by the smooth muscle tone and contractility of mesenteric arteries and arterioles. Endothelial cells lining blood vessels help smooth muscle in this function by releasing various vasoactive substances. Abnormal vascular reactivity and impaired endothelium function has been found in several forms of hypertension. The purpose of the research in this dissertation was to study some cellular mechanisms involved in regulating smooth muscle reactivity and endothelium vasodilator function in rat mesenteric vasculature, and their abnormalities in hypertensive states.

Cl^- currents represent a depolarizing mechanism in vascular smooth muscle cells, thus in the first part of the study the contribution of Cl^- channels to α_1-adrenoceptor-mediated vasoconstriction was studied in mesenteric arteries in vitro and in vivo from sham normotensive and two-kidney, one-clip (2K1C) hypertensive rats. Blockade of Cl^- channels with niflumic acid (NFA) significantly inhibited cirazoline-induced vasoconstriction in isolated MAB from both groups of rats. Cirazoline-evoked vasoconstriction was also significantly inhibited following removal of Cl^- from the perfusion buffer. Removal of Cl^- resulted in a significantly greater inhibition of cirazoline-mediated vasoconstriction in MAB from sham rats as compared with 2K1C rats. In vivo, intravenous infusion of cirazoline caused a dose-dependent decrease in superior mesenteric vascular conductance. Pretreatment with NFA significantly attenuated the cirazoline-mediated decrease in vascular conductance.

To further investigate how the Cl^- channel blockade impaired α_1-adrenoceptor-mediated vasoconstriction, the inhibitory effect of NFA on cirazoline-induced vasoconstriction in isolated MAB was compared with that produced by the voltage-operated calcium channel (VOC) blocker nifedipine (NFDP). The extent to which the contractions to cirazoline were reduced by nifedipine compared to NFA plus NFDP was similar. Thus, effects of NFA and NFDP were not additive. In addition, in the absence of extracellular Ca^{2+}, the transient phasic contraction to cirazoline was not affected by NFA, or by NFDP. NFA also had no effect on contraction induced by the depolarizing agent KCl. These observations suggest that Cl^- channels play an important role in α_1-adrenoceptor-induced vasoconstriction in mesenteric blood vessels. They may act by producing membrane depolarization, thereby indirectly inhibiting activation of VOCs. The contribution of Cl^- channels in α_1-adrenoceptor-mediated vasoconstriction in mesenteric blood vessels from 2K1C hypertensive rats appears to be reduced. This effect may reflect an adaptive change due to increased vascular resistance in hypertension.

In the second part of the dissertation, the role of Cl^- channels in endothelium-dependent relaxation to acetylcholine (ACh) in superior mesenteric artery and the factors that mediate the endothelium-dependent relaxation were investigated. The aorta was also studied as a comparison. ACh concentration-dependently relaxed phenylepinephrine (PE)-induced tone in rat endothelium-intact mesenteric arteries and aorta. Inhibition of Cl^- channels with NFA had no effect on the dilator responses to ACh in either mesenteric arteries or aorta. The BK_{Ca} antagonist, TEA, decreased the potency (pD_2) to ACh without affecting the maximum response (R_{max}) in mesenteric arteries, whereas it had no effect in aorta. In the presence of NFA plus TEA, there was no further inhibition seen in mesenteric arteries as compared to

TEA alone. In contrast, in the aorta, the pD_2 to ACh was significantly inhibited by NFA plus TEA although without changing the R_{max}. In addition, neither NFA nor TEA alone, nor TEA plus NFA had any effect on relaxation to the Ca^{2+} ionophore A23187 in aorta. These data suggest that besides BK_{Ca}, Cl^- channels play a functional role in ACh-induced endothelium-dependent relaxation in aorta, possibly by preventing the depolarization-mediated inactivation of receptor-operated Ca^{2+} channels (ROC), thereby resulting in a sustained Ca^{2+} influx and NO synthesis. By contrast, in mesenteric arteries, K^+ channels, but not Cl^- channels, mediated the relaxation. In addition, we found that indomethacin has no effect on, while L-NMMA only slightly impaired, the relaxation to ACh, suggesting that the effect of PGI_2 is negligible, while the contribution of NO is small in mesenteric arteries. Furthermore, the L-NMMA/ indomethacin-insensitive component of the relaxation response of mesenteric artery to ACh was greatly inhibited in the presence of SK_{Ca} and BK_{Ca} antagonists. High K^+ (30 mM) further decreased the maximum relaxation to ACh, but did not abolish it. Thus, the observations suggest that EDHF contributes to a large part of the ACh-induced vasorelaxation in rat superior mesenteric arteries. Another relaxing factor or (possibly more than one) that is distinct from EDHF, such as NO and PGI_2, may also play a role.

In the third part of the dissertation, the contribution of endogenous EDRF (NO) and endothelium-derived contraction factors (prostaglandins) to reactivity to NE in MAB from hypertensive Zucker obese rats with hyperinsulinemia and insulin resistance was studied. The influence of insulin on the NE response was examined. There was no major difference in pressor responses to NE in MAB between hypertensive Zucker obese and normotensive Zucker lean rats, except for a small decrease in responsiveness to the highest concentration of NE (90 nmol) tested. Inhibition of NO synthesis with L-NMMA enhanced the

vasoconstriction to NE, while blockade of prostanoid production by indomethacin decreased the NE response. A pathophysiological concentration of insulin (200 mU/l) potentiated responses to the two lowest concentrations of NE (0.3 and 0.9 nmol) used in MAB from Zucker obese rats, but not lean rats. The potentiating effect of insulin was further enhanced after blockade of NO synthesis, while it was prevented by inhibition of prostanoid production. These data suggested that NE-induced vasoconstrictor responses are normally modulated by concurrent release of NO and vasoconstrictor cyclooxygenase product(s) in MAB from both obese and lean Zucker rats. Insulin increases the release of contracting cyclooxygenase product(s) and enhances reactivity to NE in MAB from obese rats. This altered action of insulin may play a role in hypertension in this hyperinsulinemic/insulin resistant model.

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^{125}I	^{125}iodine
2K1C	Two-kidney, one-clip
ACh	Acetylcholine
Ang II	Angiotensin II
ANOVA	Analysis of variance
BK_{Ca}	large conductance calcium-activated potassium channels
BP	Blood pressure
Ca^{2+}	Calcium ion
Cl^-	Chloride ion
COX	Cyclooxygenase
CTX	Charybdotoxin
E_{Cl}	Chloride equilibrium potential
ECs	Endothelial cells
ED_{50}	The molar concentration of agonist which produces 50% of the maximum effect
EDHF	Endothelium-derived hyperpolarizing factor
ER	Endoplasmic reticulum
Er	Reverse membrane potential
ET-1	Endothelin-1
ETOH	Ethanol
IbTX	Iberiotoxin
$I_{Cl(Ca)}$	Calcium-activated chloride current/channel

$I_{K(Ca)}$	Calcium-activated potassium current
Indo	Indomethacin
IP_3	Inositol 1,4,5-triphosphate
k	Ion efflux rate (elimination constant per min)
$K_{(Ca)}$	Calcium-activated potassium channels
K^+	Potassium ion
KCl	Potassium chloride
L-NMMA	N^{ω}-monomethyl-L-arginine
MAB	Mesenteric arterial bed
MAP	Mean blood pressure
Na^+	Sodium ion
NE	Norepinephrine
NFA	Niflumic acid
NFDP	Nifedipine
NO	Nitric Oxide
NOS	Nitric oxide synthase
O_2^-	Superoxide anion
pD_2	-log ED_{50}
PGH_2	Prostaglandin endoperoxides H_2
PGI_2	Prostacyclin
P_{open}	Open probability
R_{max}	The maximum response of an agonist
ROCs	Receptor-operated calcium channels

SK_{Ca}	Small conductance calcium-activated potassium channels
SMAC	Superior mesenteric arterial conductance
SMAF	Superior mesenteric arterial blood flow
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
TEA	Tetraethylammonium
TxA_2	Thromboxane A_2
V_m	Membrane potential
VOCs	voltage -operated calcium channels
VSMC	Vascular smooth muscle cell

ACKNOWLEDGEMENTS

I would like to give my profound gratitude to my supervisor Dr. Kathleen M. MacLeod for her accepting me as her student to complete my doctoral degree program, for her advice, understanding, consideration and constant support throughout the last course of my research.

I am also greatly indebted to Dr. Casey van Breemen, my co-supervisor, for giving me the opportunity to continue my study under his supervision as a Ph.D. student in the Department of Pharmacology. His brief but crucial advice always greatly inspired me. Without his consideration and support it is hard to imagine that I could have completed my program successfully.

I would like to thank the members of my research committee: Dr. John H. McNeill and Dr. Ismail Laher for their support and advice.

My special thanks to my former supervisor Dr. Reza Tabrizchi. With his instruction I started my doctoral program and completed most of my initial research.

I would like to thank Dr. David V. Godin, the head of the Department of Pharmacology, for his financial support and for his constant concern with my progress. I am also thankful to all the professors in the department who gave me courses in the past years for their valuable knowledge and helpful advice.

I would also like to thank the Faculty of Pharmaceutical Sciences, especially my home division: the Division of Pharmacology and Toxicology. Everyone there has been very friendly so that I feel it as though I was at my home department. Thanks to Billy Chow and Violet Yuen for their help with glucose and insulin assay; to Dr. Linfu Yao for his technical advice, and to Billy Chow, Swamy Subramanian, Lili Zhang and Andrea Bardell for making our laboratory a happy and warm place to work.

DEDICATION

In the memory of my Mom

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To my Dad and my brothers Ningyi, Xian and Yong

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To Helai

for the love in the long journey of my study towards both Master's and Ph. D. degree in Canada

INTRODUCTION

I. OVERVIEW

In normal circumstances, the cardiovascular system delivers blood to the tissues in amounts corresponding to the metabolic demand, and at a pressure that allows appropriate diffusion across the capillaries. An important part of this process is mediated by the resistance vessels, which measure 20 to 500 μm in lumen diameter and consist of small arteries (with a lumen larger than 100 μm) and arterioles (with a lumen smaller than 100 μm) (Davis et al. 1986; Mulvany and Aalkjaer 1990; Schiffrin 1992). Adjustment of the resistance of these vessels through changes in their lumen diameter permits regulation of tissue blood flow and aids in control of blood pressure, thus allowing appropriate distribution of cardiac output. This hemodynamic characteristic of the resistance vessels is mainly determined by the smooth muscle tone of the resistance arteries, which is governed by local, neuronal and humoral factors. Endothelial cells lining blood vessels, in particular, help smooth muscle in this function by producing and releasing various vasoactive substances. Knowledge of mechanisms involved in the regulation of smooth muscle tone in resistance arteries is thus of major importance for our understanding of the regulation of peripheral resistance under normal conditions and the pathogenesis of diseases such as hypertension where the peripheral resistance is altered.

The splanchnic circulation receives about 25% of total cardiac output in resting man (Folkow and Neil 1971). It may possibly receive up to 30% of cardiac output in rats under resting conditions (Folkow and Neil 1971; Nilsson 1985). This makes it an important region

for maintaining cardiovascular homeostasis (Lundgren 1983). The mesenteric vascular bed, one of the major vasculature beds within the splanchnic circulation, possesses great potential for demand related up- or down-regulation of the blood flow to the intestines (Mitchell and Blomqvist 1971). Resistance to blood flow in the mesenteric vascular bed is therefore of great hemodynamic importance. As representatives of resistance vasculature and highly reactive muscular vessels, rat mesenteric arteries have been extensively used in research in past decades.

Besides their use in *in vivo* studies of relationships between blood pressure, blood flow and vascular resistance, various preparations of isolated mesenteric arteries have been used in *in vitro* work. For instance, the isolated perfused mesenteric arterial bed (McGregor 1965) is frequently used to study the regulation of the integrated contractile activity of the mesenteric arterial vasculature as a whole. The isolated small arteries (usually the 2^{nd} and 3^{rd} order branches of the superior mesentery) are widely used to investigate the structure and function of the individual resistance arteries (Bevan and Osher 1972; Duling et al. 1981; Halpern et al. 1984; Mulvany and Halpern 1976). The superior mesenteric artery, while in all likelihood a conduit artery, is also frenquently used to compare the properties of muscular arteries with aorta and other elastic arteries, as well as with those muscular arteries in different tissues.

The overall purpose of the studies presented in this dissertation is to investigate some of the cellular mechanisms that regulate smooth muscle contraction and relaxation in mesenteric arteries, and to study how neurotransmitters, circulating hormones such as insulin, and vasoactive substances released from endothelium interact to modulate mesenteric vascular tone in normal and hypertensive states.

Specifically, the study consisted of 3 parts. The purpose of the first part was to investigate the contribution of Cl^- channels to α_1-adrenoceptor-mediated vasoconstriction in mesenteric arteries from normal and hypertensive rats. The second part of the study was designed to examine the role of Cl^- as well as K^+ channels in endothelium-dependent relaxation in superior mesenteric artery, and the factors that mediate the endothelium-dependent relaxation. And finally, the purpose of the third part of the study was to evaluate the regulation of NE-induced vasoconstriction in the perfused mesenteric arterial bed from obese rats with hypertension. The effect of insulin was examined in the latter investigation.

The following review focuses mainly on α_1-adrenoceptor-mediated excitation-contraction coupling mechanisms and on mechanisms by which the endothelium modulates vascular tone, and how these are altered by hypertension. The general characteristics of mesenteric vasculature that may be distinct from other systemic vascular beds and that may help to understand the mechanisms that regulate vascular tone in the mesenteric vasculature are also addressed.

II. CHARACTERISTICS OF RAT MESENTERIC VASCULATURE

1. Structure and Constituents

Generally, the arterial network in the rat mesenteric vasculature comprises the superior mesenteric artery, a medium-sized artery with a diameter range greater than 460μm, and 16-20 freely dividing small arteries, each of which further subdivides into a few branches before joining the mesenteric arterial arcade (with a lumen diameter about 200μm) that runs parallel to the intestinal wall. From the arcade arise many smaller arteries, which further

branch out, as arterioles, over the intestine (Hebel and Stromberg 1976; Lee et al. 1983b; Mulvany et al. 1978).

Similar to other arteries, the vascular wall of mesenteric arteries consists of an outer tunica adventitia, a central tunica media, and an inner tunica intima. The main cellular constituents of the vessels are the smooth muscle in the media and endothelium in the intima. The superior mesenteric artery is more muscular in structure than aorta. The smooth muscle in the superior mesenteric artery consists of 60% of the total volume of tunica media. There are approximately 6-8 layers of smooth muscle cells (SMC) arranged between 4-6 layers of elastic laminae around the vessel wall. (Lee et al. 1983a; Lee et al. 1983b). The walls of small mesenteric arteries become thinner as the arteries narrow, and there are only fragmented or no elastic laminae within the media (Lee et al. 1983a; Lee et al. 1983b). Smooth muscle cells in the small arteries and arterioles are arranged circumferentially. The number of SMC layers decreases with the decrease in diameter. The small arterioles have only a single layer of smooth muscle cells (Lee et al. 1983a; Miller et al. 1987); however the volume fraction of smooth muscle cells within the media increases with the decrease in the diameter of the vessels, being 70% in small arteries and 85% in larger arterioles (Lee et al. 1983a). The endothelial cells are separated from the media by an internal elastic lamina and form a continuous cover. Parts of endothelial cells project into the vascular smooth muscle layer forming myoendothelial junctions at various points along the arteries and arterioles (Lee et al. 1983a). Perivascular nerves are localized near the media within the adventitia. (Furness 1973; Lee et al. 1983a).

2. Localization of Peripheral Resistance

Localization of peripheral resistance is important in understanding how the mesenteric arterial bed regulates peripheral resistance. Therefore, measurements of pressure in individual vessels have been made. The percentage drop in pressure is indicative of the portion of resistance formed in the specific vessels.

Based on the results from direct measurements of the microcirculatory pressure in intestinal vasculature of anaesthetized rats, early work indicated that small arteries and the largest arterioles in the mesentery contribute approximately 23% to 57% and the small arterioles in the intestinal wall 18% to 47%, of the total vascular resistance (Bohlen 1983; Gore and Bohlen 1977; Meininger et al. 1986). Recently, in conscious freely moving rats, Fenger-Grone *et al* (Fenger-Gron et al. 1995) measured the pressure along the mesenteric vascular bed and showed that about 31% of the systemic blood pressure drop occurs in the arcade small arteries, about a 51% drop occurs in the intestinal microcirculation including arterioles, capillaries, venules and small veins, while 5% of systemic pressure dissipates in superior mesentery, 6% in arcade veins and 7% in remaining veins plus the hepatic circulation. The results indicate that both small mesenteric arteries and microcirculatory vessels contribute significantly to peripheral resistance. In addition, when pressure was measured simultaneously with superior mesenteric blood flow and then vascular resistance was calculated (Fenger-Gron et al. 1997), both small mesenteric arteries and microvessels were shear to contribute to increased resistance during norepinephrine (NE)-induced smooth muscle contraction in conscious freely moving rats, indicating that the mesenteric arterial bed not only contributes to generating peripheral vascular resistance but also contributes to its control.

3. The Control of Mesenteric Circulation

Smooth muscle tone and the resistance of the mesenteric arterial bed, as in other resistance vessels, are controlled by various intrinsic and extrinsic factors including physical forces, such as blood pressure and blood flow (Bevan and Laher 1991; Busse and Fleming 1998; Schubert and Mulvany 1999; Sun et al. 1992), neural stimuli (Bevan et al. 1980; Kawasaki et al. 1988; Nilsson et al. 1986), circulating hormones, and locally synthesized vasoactive substances, especially endothelium-derived substances (Sowers 1996 and references therein). The influence of these factors varies in different vascular beds. For instance, the strength of intrinsic myogenic responses that are evoked by transmural pressure is much less in rat mesenteric vessels as compared with the same size skeletal muscle and cerebral vessels, where myogenic responses are predominant (Coombes et al. 1999; Lagaud et al. 1999; Osol et al. 1991; Watanabe et al. 1993; Wesselman et al. 1996). On the other hand, the fact that the neural regulation of blood flow is predominant in the splanchnic region has long been recognized. (Bohlen 1984; Furness and Marshall 1974)

4. Sympathetic Vasoconstriction in the Mesenteric Arterial Bed

Rat mesenteric blood vessels are densely innervated with sympathetic nerves that mediate vasoconstriction (Furness and Marshall 1974; McGregor 1965; Nilsson et al. 1986). Normally occurring sympathetic vasoconstriction tonically regulates mesenteric vascular tone (Altura 1967). It has also been suggested from *in vitro* studies that the sympathetic neurotransmitter NE augments the myogenic response regulating mesenteric arterial tone (Chlopicki et al. 1996; Wesselman et al. 1996).

In anaesthetized rats, direct observation under a microscope showed that sympathetic nerve stimulation constricts all the mesenteric arteries except precapillary arterioles that have

no adrenergic nerves in close association with them; the magnitude of the contraction is greater in arteries than in arterioles (Furness and Marshall 1974). *In vitro* studies on isolated arteries have shown similar results, in which the maximal neurogenic response paralleled the sympathetic innervation density, being greater in the small mesenteric arteries, less in superior mesenteric artery and least in aorta (Nilsson et al. 1986). The mechanisms by which sympathetic nerve stimulation causes vasoconstriction involve in both α- and non-α-adrenergic mechanisms (Hirst and Edwards 1989 and references therein). The latter produce excitatory junction potentials (e.j.ps.); while the former release NE that activates post-junctional α-adrenoceptors (Bevan et al. 1980; Bowman and Rand 1980). The importance of these two mechanisms varies both from artery to artery and from species to species and depends on the parameters of stimulation used. It has been demonstrated that in rat mesenteric arteries, sympathetic vasoconstrictor responses are predominantly mediated by NE released from adrenergic nerve terminals (Kawasaki et al. 1988; Nilsson 1984; Nilsson et al. 1986). The α-adrenergic receptors activated by NE in rat mesenteric artery appear to be predominantly α_1 (Chen et al. 1996; Colucci et al. 1980; Colucci et al. 1981). Thus, the activation of α_1-adrenoceptors by NE is vital to control mesenteric vascular resistance, thereby regulating blood flow and blood pressure.

III. ALPHA$_1$-ADRENOCEPTORS AND VASOCONSTRICTION

1. The α_1-Adrenoceptors

In blood vessels, α_1-adrenoceptors are present throughout the vasculature but are more prominent on the arterial side. Besides the neurotransmitter NE, α_1-adrenergic receptors are also activated by circulating catecholamines, NE and epinephrine. Many

observations have demonstrated that NE released from sympathetic neurons activates the receptor in much the same way it would if applied exogenously (Raat et al. 1998). In addition, a wide variety of antagonists such as phentolamine and prazosin, and agonists such as phenylepinephrine (PE), methoxamine and cirazoline can selectively block or stimulate α_1-adrenergic receptors and distinguish them from other adrenergic receptors (Bylund et al. 1998; Ruffolo et al. 1991). In vascular smooth muscle, α_1-adrenoceptors serve a primary role in the control of smooth muscle constriction (Vargas and Gorman 1995).

2. Alpha$_1$-Adrenoceptor Subtypes

Three α_1-adrenoceptor subtypes have been identified by cloning. These were originally named α_{1c}, α_{1b}, $\alpha_{1a/d}$, and were subsequently renamed α_{1a}, α_{1b}, α_{1d}, respectively. They now have been classified on the basis of pharmacological evidence as α_{1A} (α_{1c}), α_{1B} (α_{1b}) and α_{1D} ($\alpha_{1a/d}$) adrenoceptors (Bylund et al. 1998 and references therein). A fourth α_1-adrenoceptor subtype has been postulated and is designated as α_{1L} based on its low affinity for prazosin (Oshita et al. 1991). Recently it has been suggested that the α_{1L} subtype may represent a particular conformational state of the α_{1A}-adrenoceptor (Ford et al. 1997). The three adrenoceptor subtypes α_{1A}, α_{1B} and α_{1D} differ in their amino acid sequence and their affinity for a variety of synthetic agonists and antagonists (Bylund et al. 1994; Graham et al. 1996; Hwa et al. 1995). They were reported to be heterogeneously distributed along the rat arterial tree (Piascik et al. 1994). According to pharmacological studies, contractions induced by exogenous NE and/or peripheral nerve stimulation are believed to be predominantly mediated by the activation of α_{1A}-adrenoceptors in the perfused mesenteric arterial bed of rats (Chen et al. 1996; Kong et al. 1994; Williams and Clarke 1995; Zhu et al. 1999). A small

number of α_{1B} -adrenoceptors may also be activated by NE release in this vascular bed (Kong et al. 1994). The α_{1A}-adrenoceptor has been found to be responsible for vasoconstriction evoked by application of NE or peripheral nerve stimulation in other resistance vascular beds (Blue et al. 1992; Eltze et al. 1991; Zhu et al. 1997). In superior mesenteric artery, as well as in other conduit vessels, the α_{1D}-adrenoceptor subtype has been suggested to mediate NE- and PE-induced contraction, at least in part (Buckner et al. 1996; Hussain and Marshall 1997; Yousif et al. 1998). Recently, using subtype-selective antibodies as tools, α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors were all detected in aorta, caudal, femoral, iliac, renal, superior mesenteric, and mesenteric resistance arteries. However, despite the expression of all adrenoceptors, only a single adrenoceptor seems to mediate the contractile response in the renal (α_{1A}) and femoral (α_{1D}) arteries (Hrometz et al. 1999). Based on our current knowledge, the role of the other α_1- adrenoceptor subtypes, if they exist in the mesenteric resistance arteries, is not clear.

3. Alpha$_1$-Adrenoceptor Signaling and Ca^{2+} Mobilization

α_1-adrenoceptors are intrinsic membrane glycoproteins and members of the G protein-coupled receptor (GPCR) super family. Stimulation of α_1-adrenoceptors results in activation of various effector enzymes including PLC, PLA_2 and PLD via different G proteins, (Insel et al. 1998; Llahi and Fain 1992; Nishio et al. 1996; Perez et al. 1993; Ruan et al. 1998; Schwinn et al. 1991; Wu et al. 1992). It has been generally accepted that the major effector that transduces α_1-adrenoceptor signals is the enzyme PLC (Cotecchia et al. 1990), which is likely to be PLC- β in vascular smooth muscle (Lee and Severson 1994). α_1-adrenoceptors couple to PLC predominantly via pertussis toxin-insensitive G proteins of the

$G_{q/11}$ family (Boyer et al. 1992; Smrcka et al. 1991; Taylor et al. 1991; Wu et al. 1992). Activated PLC catalyzes the hydrolysis of the membrane lipid, phosphatidylinositol-4,5-bisphosphate (PIP_2), to yield the second messengers, diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) (Abdel-Latif 1986). DAG activates protein kinase C (PKC), which may play a central role in phosphorylation of variety of cellular proteins that involved in the transduction of α_1-adrenoceptor activation into the final biological response (Berridge 1981; Horowitz et al. 1996; Mironneau et al. 1991; Nishizuka 1995; Walsh et al. 1994). At the same time, IP_3 binds to specific receptors (IP_3 receptor) on sarcoplasmic reticulum (SR) and causes Ca^{2+} release from the intracellular stores (Berridge 1993; Iino 1990; Lepretre et al. 1994). The IP_3- released Ca^{2+} can in turn activate a calcium-induced calcium release channel (CICR), which causes calcium release from a second SR pool (Baro and Eisner 1995; Karaki et al. 1997), and can activate several other classes of Ca^{2+}-sensitive ion channels on the cell membrane, such as calcium-activated K^+ ($K_{(Ca)}$) and Cl^- ($I_{Cl(Ca)}$) channels which modulate the cell membrane properties (Amedee et al. 1990a; Amedee et al. 1990b; Byrne and Large 1988b; Pacaud et al. 1989a). Activation of α_1-adrenoceptors also leads to influx of Ca^{2+} from the extracellular space (Ruffolo et al. 1991 and references therein). Depending on the species and tissue, α_1- adrenoceptors are directly or indirectly coupled to several different Ca^{2+} channels including voltage-operated Ca^{2+} channels (VOCs) (Bolton 1979; Bulbring and Tomita 1987; Nelson et al. 1988; Van Breemen et al. 1978), receptor-operated Ca^{2+} channels (ROCs) (Bolton 1979; Bulbring and Tomita 1987; Ruegg et al. 1989; Van Breemen et al. 1978), and non-selective cation channels (Amedee et al. 1990a; Byrne and Large 1988b; Loirand et al. 1991). However, the mechanism of signaling from the α_1-adrenoceptor activation to Ca^{2+} influx is still not very clear. Another Ca^{2+} influx pathway is the Ca^{2+}

release-activated Ca^{2+} channels (CRAC), which are activated by SR depletion after receptor stimulation, and have a variable sensitivity to dihydropyridine Ca^{2+} channel blockers (Low et al. 1991; Putney 1990). The channels that are insensitive to dihydropyridines have been suggested to be non-specific cation channels (Wayman et al. 1996). This pathway is believed to be important for the refilling of the depleted SR and makes a variable contribution to contractile response depending on the smooth muscle type (Gibson et al. 1998; Karaki et al. 1997; Putney 1987 and references therein). Ca^{2+} influx from the extracellular space can also activate CICR in intact guinea pig aorta, rat portal vein and rat mesenteric artery (Gregoire et al. 1993; Ito et al. 1991). An increase in free cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) by α_1-adrenoceptor activation plays a predominant role in α_1- adrenoceptor-mediated biological events, especially in regulation of smooth muscle contraction.(Fig 0.1)

4. Calcium and α_1-Adrenoceptor -Induced Contraction

The contraction of vascular smooth muscle by activation of α_1-adrenoceptors mainly depends upon the increased $[Ca^{2+}]_i$ that results from both Ca^{2+} release from intracellular organelles (i.e. SR) and influx from extracellular space (Ruffolo et al. 1991; Somlyo and Somlyo 1994). This is thought to occur by the Ca^{2+}-calmodulin dependent activation of myosin light chain kinase, which then phosphorylates myosin light chain (MLC). Phosphorylated myosin can interact with actin and so induce contraction. In addition, α_1-adrenoceptor agonists can increase the Ca^{2+} sensitivity of MLC phosphorylation by inhibition of MLC phosphotase activity, and therefore increasing contraction at a constant level of $[Ca^{2+}]_i$ (Somlyo and Somlyo 1994). The enhancement of contractile filament Ca^{2+} sensitivity, and the fact that this enhancement may be mediated through PKC during α_1- adrenoceptor

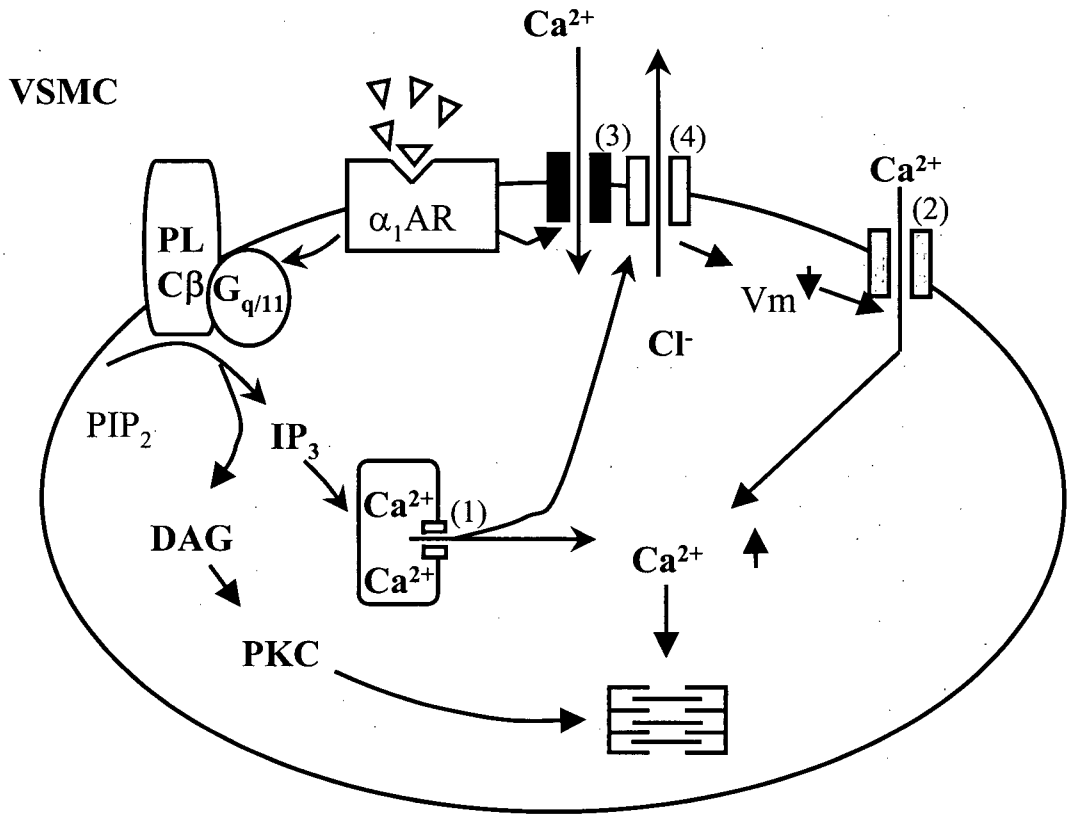


Figure 0.1 Major α_1-adrenoceptor signaling pathways that mediate vascular smooth muscle contraction. (1) IP_3 receptor, (2) voltage-gated Ca^{2+} channels, (3) receptor-operated Ca^{2+} channels (4) $I_{Cl(Ca)}$, (Please see text for details).

activation, have been demonstrated in rat mesenteric arteries (Buus et al. 1998; Drenth et al. 1989; Jensen et al. 1992; Raat et al. 1998).

Ca^{2+} release from the SR by IP_3 produced on receptor activation is mainly responsible for the initial peak of the agonist-induced contraction. This initial response is referred to as the "phasic" response. The sustained contraction, which is referred to as the "tonic" response, is caused mainly by Ca^{2+} influx (Karaki et al. 1997; Minneman 1988). This conclusion is based on the observations that in vascular smooth muscle NE and other agonists induce only a transient contraction in the absence of external Ca^{2+}, and that agonist-induced IP_3 production is also transient (Abdel-Latif 1986 and references therein). In addition, depletion of SR Ca^{2+} stores by ryanodine, a drug with selectivity for the SR (Julou-Schaeffer and Freslon 1988; Sutko et al. 1985), inhibited only the initial portion, but not the sustained portion of agonist -induced contraction (Iino et al. 1988; Julou-Schaeffer and Freslon 1988; Kanmura et al. 1988). On the other hand, it has been found that the tonic responses are largely inhibited in Ca^{2+}-free solution or in the presence of Ca^{2+} channel blockers, without affecting the initial phasic response (Somlyo 1985; van Breemen and Saida 1989). Ca^{2+} influx may also contribute to the initial portion of contraction, whereas intracellular release could also contribute to the tonic response depending on the concentration of the agonist and the arteries (Weber et al. 1995).

Several studies indicate that the contribution of Ca^{2+} from different sources to contraction in resistance vessels may differ from that in large conduit arteries. (Ashida et al. 1988; Cauvin et al. 1984; Jensen et al. 1992; Low et al. 1996; Sato et al. 1988). It has been reported that ryanodine inhibited the NE-induced contraction by 52% in rat aorta and 14% in bovine tail artery without changing high K^+-induced contractions. A Ca^{2+} channel blocker

almost completely abolished high K^+-induced contractions and reduced NE-induced contractions by 45% in the aorta and 82% in the tail artery (Ashida et al. 1988). In rabbit mesenteric arteries (Cauvin et al. 1984), NE-induced contractions and NE-stimulated ^{45}Ca efflux decreased in Ca^{2+}-free 2mM EGTA solution, while the sensitivity of NE-induced contractions to inhibition by the Ca^{2+} channel blocker diltiazem increased, in a graded fashion from proximal to distal arteries. It was also shown that ^{45}Ca influx induced by NE in the resistance vessels was approximately 10,000 fold more sensitive to the action of diltiazem than that in rabbit aorta (Cauvin et al. 1984). These data indicate a decreasing release of intracellular Ca^{2+} and an increasing dependence on extracellular Ca^{2+} for NE-induced contractions as one proceeds from proximal to distal arteries. In addition, a functional study on the relative contribution of extracellular Ca^{2+} and Ca^{2+} stores to NE- induced smooth muscle contraction in arteries and arterioles from different species also suggested that although an agonist-releasable Ca^{2+} pool is present at all levels of the vasculature, the role of the SR diminishes as the arteries become smaller, while Ca^{2+} fluxes across the plasma membrane predominate during α_1- adrenoceptor activation (Low et al. 1996). In the perfused rat hindlimb, Zhu et al (Zhu et al. 1998) demonstrated that the NE-induced maximum response was decreased by 92% following perfusion with Ca^{2+}-free medium. Nifedipine concentration-dependently inhibited NE-induced contractions with a maximum inhibition of 65% and the residual nifedipine-insensitive response was further inhibited by Cd^{2+}, suggesting the NE response in this preparation is mediated largely via an influx of extracellular Ca^{2+}, mainly via nifedipine-sensitive Ca^{2+} channels. Furthermore, in rat mesenteric small arteries, the main effect of NE on $[Ca^{2+}]_i$ was suggested to be mediated through voltage-dependent, dihydropyridine-sensitive Ca^{2+} channels, because when force,

membrane potential and $[Ca^{2+}]_i$ were measured simultaneously during stimulation with NE or potassium, a similar relationship between $[Ca^{2+}]_i$ and membrane potential was seen for both forms of activation (Nilsson et al. 1994). The inhibition of NE-induced contraction by nifedipine was also reported in these blood vessels (Chen et al. 1996). These results support the view that the contractile responses of resistance vessels are heavily dependent on the presence of extracellular Ca^{2+} and that Ca^{2+} entry occurs mainly though the VOCs during α_1-adrenoceptor activation.

5. Calcium Influx Channels, Voltage Dependence and Activation by α_1-Adrenoceptors.

With patch-clamp and molecular biology techniques, six subtypes of VOCs including L-, N-, P-, Q-, R-, and T-type have been demonstrated in vascular smooth muscle (Ganitkevich and Isenberg 1990; Hofmann and Klugbauer 1996; Nelson et al. 1990b), whereas no direct evidence for existence of ROCs has been observed (Droogmans et al. 1987; Karaki et al. 1997; Nelson et al. 1990b; Nilsson et al. 1994). The predominant VOCs in arterial smooth muscle have been found to be L-type Ca^{2+} channels, which are selectively sensitive to inhibition by the dihydropyridines (Tsien et al. 1988). At present, the major Ca^{2+} influx pathway is considered to be voltage-dependent L-type Ca^{2+} channels (hereafter simply called Ca^{2+} channels), since both the maintained arterial tone and the increase in $[Ca^{2+}]_i$ upon α_1-adrenoceptor activation can be strongly inhibited by dihydropyridines (Kuriyama et al. 1995; Minneman 1988; Nilsson et al. 1994) (and references mentioned above).

It is well known that membrane depolarization opens Ca^{2+} channels (Godfraind 1986; Kuriyama et al. 1995). The steady-state fractions of time that a Ca^{2+} channel is open (P_{open}) increase exponentially with membrane depolarization from relatively hyperpolarized

potentials. That the voltage dependence of P_{open} is over a wide voltage range has been demonstrated (Nelson et al. 1990b; Nelson et al. 1988). This increase in P_{open} with membrane potential is limited by the promotion of a long-lived closed state called inactivation. The Ca^{2+} channel is rapidly desensitized during prolonged depolarization, but is not completely inactivated (Fleischmann et al. 1994; Imaizumi et al. 1991; Nakayama et al. 1996). It has been shown that the relationship between Ca^{2+} influx through Ca^{2+} channels and membrane potential (V_m) can be very steep, with 3 mV depolarization or hyperpolarization increasing or decreasing Ca^{2+} influx as much as two-fold (Nelson et al. 1990b; Nelson et al. 1988). In addition, the relationship between smooth muscle V_m and arterial tone as the relationship between the V_m and Ca^{2+} (see above) is also very steep, so that even V_m changes of a few millivolts cause significant changes in blood vessel diameters (Nelson et al. 1990b and references therein). Mesenteric artery tone is very sensitive to V_m in the range between -46 and -20 mV (Cheung et al. 1999). The threshold for contraction induced by KCl is approximately -46mV. Maximum contraction was attained when the arteries were depolarized to -20 mV. Thus, 1mV depolarization resulted in an approximately 4% increase in tone. (This relationship was not altered in spontaneously hypertensive rats). Smooth muscle cells in arteries and arterioles, *in vitro*, have stable membrane potentials between -60 and -75 mV (Hirst and Edwards 1989). V_m values measured *in vivo* are in the range of -40 to -55 mV (Nelson and Quayle 1995 and references therein). The membrane potential of arterial smooth muscle cells *in vivo* falls in the same range in which the current through Ca^{2+} channels is strongly voltage dependent (Nelson et al. 1990b).

It has long been known that in vascular smooth muscle, α_1-adrenoceptor-mediated contraction is usually accompanied by a depolarization (Bolton et al. 1984; Byrne and Large

1988a; Mulvany et al. 1982; Nanjo 1984; Suzuki and Kou 1983; Takata 1980). For example, NE depolarized mesenteric arteries that were not subjected to transmural pressures, with the degree of depolarization depending on the concentration [2-4 mV with 0.5 μM NE (Nelson et al. 1990a); <5 and 25 mV with 10 μM NE in guinea pig (Bolton et al. 1984) and rat (Mulvany et al. 1982) mesenteric arteries, respectively]. A steep relation between membrane depolarization and tension development in rat mesenteric arteries was also found on exposure to NE (Cheung et al. 1999; Mulvany et al. 1982). Thus it is not surprising that α_1-adrenoceptor stimulation activates Ca^{2+} channels by causing depolarization (Pacaud et al. 1991). It has been suggested that agonists open L-type channels by depolarizing the cell membrane through activation of nonselective cation channels (Amedee et al. 1990a), inhibition of K^+ channels and/or activation of Cl^- channels (Pacaud et al. 1992; Suzuki 1981). However, it has been shown that NE, which was applied to the solution bathing the extrapatch membrane, also increased P_{open} of single Ca^{2+} channels in patches on single cells isolated from rabbit mesenteric artery without any change in membrane potential (Nelson et al. 1988). It has been suggested that agonists may open Ca^{2+} channels directly or indirectly through an intracellular second messenger and GTP-binding protein in the absence of membrane depolarization (Karaki et al. 1997 and references therein; Nelson et al. 1988). The reason for the discrepancy is not clear. It may be due to functional differences between species or tissues, or differences in the methods used, or the existence of the two mechanisms, parallel and synergistic. Nevertheless, in rat mesenteric small arteries, challenge with NE caused membrane depolarization, elevated $[Ca^{2+}]_i$, and induced contraction. There was a strong correlation between membrane potential and $[Ca^{2+}]_i$ when membrane potential, $[Ca^{2+}]_i$ and force were simultaneously measured, suggesting that α_1-adrenoceptor activation

elevated $[Ca^{2+}]_i$ by depolarization-induced calcium influx through voltage-sensitive channels in these vessels (Nilsson et al. 1994).

6. Possible Role of Cl^- Channels in Ca^{2+} Influx and Smooth Muscle Contraction

6.1. Increase in Cl^- Conductance Resulting in Membrane Depolarization in VSMC

A possible role of Cl^- ions in agonist-induced Ca^{2+} entry is suggested by the fact that the manner in which VSM cells handle Cl^- sets up an ideal system for producing and maintaining membrane depolarization (see below). It is known that the intracellular Cl^- level ($[Cl^-]_i$) is many times higher than that predicted by passive distribution in smooth muscle (Aickin and Brading 1982; Casteels 1981; Koncz and Daugirdas 1994). Vascular smooth muscle cells accumulate Cl^- intracellularly through several processes (Chipperfield et al. 1993; Davis 1992; Davis et al. 1993), including Na^+-K^+-$2Cl^-$ cotransport, $Cl^-/HCO3^-$ exchange, and a third component, possibly an ATP-dependent transporter (Davis 1996). Estimates of the Cl^- equilibrium potential {E_{Cl} = -60 log (extracellular Cl^- concentration /intracellular Cl^- concentration)}, measured using either radiolabeled Cl^- flux (Kreye et al. 1977; Wahlstrom 1973a) or ion selective microelectrodes (Davis 1996), range between -11 and -50 mV. In any given vascular tissue, E_{Cl} has always been measured to be roughly 15-30 mV more positive than resting V_m (-45 to -65 mV approximately). This is consistent with values measured directly with ion selective microelectrodes in other types of smooth muscle cells (E_{Cl} between -30 to -20 mV) (Aickin and Brading 1990). Therefore, any neurotransmitter or local mediator which increases Cl^- conductance will produce efflux of Cl^-, drive the membrane potential toward E_{Cl} and hence evoke depolarization in VSM. If the Cl^- conductance-mediated depolarization is sufficient to increase significantly the open

probability of VOCs, it will result in an increased Ca^{2+} entry and subsequent smooth muscle contraction.

6.2. *Activation of α_1-Adrenoceptors Increases Cl^- Conductance and Induces Membrane Depolarization in VSMC.*

Adrenergic stimulation has frequently been shown to increase total membrane conductance while inducing depolarization and vasoconstriction (Bolton et al. 1984; Byrne and Large 1987; Casteels et al. 1977; Mekata and Niu 1972; Takata 1980). Radiolabeled ion flux studies have shown that stimulation of the α-adrenoceptor increases the membrane permeability to Cl^- ions in veins and arteries (Casteels et al. 1977; Smith and Jones 1985; Videbaek et al. 1990; Wahlstrom 1973b; Wahlstrom and Svennerholm 1974). Besides Cl^- conductance, an increase in a nonspecific cation conductance may also contribute to NE-induced depolarization in rabbit portal vein (Amedee and Large 1989). In rat small mesenteric arteries NE increased Cl^- efflux when producing depolarization, without altering the rate of K^+ efflux or Na^+ influx, indicating that NE increased the membrane Cl^- permeability (Videbaek et al. 1990). A similar result has been shown in rat portal vein (Wahlstrom 1973b). That the α_1-adrenoceptor-induced depolarization resulted from an increase in conductance to Cl^- ions has also been confirmed by microelectrode recording and patch pipette studies in guinea pig intact mesenteric veins, and isolated cells from rat and rabbit portal vein (Amedee and Large 1989; Byrne, Large 1988b; Pacaud et al. 1989b; Van Helden 1988). In short segments of guinea pig mesenteric vein, it was found that NE evoked a depolarization, and stimulated an inward current with a reversal potential (Er) about -22 mV (close to the expected E_{Cl}), which was shifted to more positive values when Cl^- in the external solution was replaced with an impermeant anion (Van Helden 1988). The alteration

of Er is in the same direction as the change in E_{Cl} on substitution with a low-Cl^- solution (Aickin and Brading 1982), indicating NE activates a Cl^- conductance to produce depolarization. In addition, microelectrode recording in whole tissues has also shown that the depolarization produced by NE was greatly attenuated on prolonged exposure to low Cl^- solution. (Van Helden 1988). This observation is consistent with that in other types of smooth muscles (Large 1984). In low external Cl^-, it is expected that the $[Cl^-]_i$ will also fall (Aickin and Brading 1982; Davis et al. 1991; McMahon and Jones 1988), and therefore the overall membrane Cl^- conductance is low when external Cl^- is reduced. Consequently, the reduction in the depolarization to NE in low-Cl^- conditions implies that a Cl^- conductance increase is responsible for NE-induced depolarization. Moreover, alteration of the Cl^- equilibrium potential produced similar changes in the reversal potential of the NE-induced response recorded with microelectrode or patch pipette techniques in VSM cells (Amedee and Large 1989; Byrne and Large 1988b; Pacaud et al. 1989b). The increased Cl^- conductance leading to membrane depolarization thus may be an important mechanism that indirectly opens VOCs and induces Ca^{2+}-dependent vasoconstriction in response to α_1-adrenoceptor activation (Mironneau and Macrez-Lepretre 1995).

6.3. Agonist-Activated Cl^- Channel in VSMC.

Agonist-induced Cl^- currents have now been identified in isolated vascular myocytes from several types of blood vessels, and can be activated by a number of agonists that depolarize and contract arteries. NE-activated Cl^- currents, as mentioned above, were also found in cells of rabbit ear artery (Amedee et al. 1990b) and pulmonary artery (Wang and Large 1993). The $I_{Cl(Ca)}$ was blocked by the selective α_1-adrenoceptor antagonist prazosin

(Amedee et al. 1990b; Pacaud et al. 1989b), suggesting NE evokes $I_{Cl(Ca)}$ via α_1-adrenoceptors. Endothelin elicited a similar Cl^- current in pig coronary, human mesenteric artery and rat renal resistance artery and aortic smooth muscle cells (Gordienko et al. 1994; Klockner and Isenberg 1991; Van Renterghem and Lazdunski 1993), as did vasopressin and ATP in cultured aortic cells (Droogmans et al. 1991; Van Renterghem and Lazdunski 1993), as well as histamine in freshly isolated cells from rabbit pulmonary artery (Wang and Large 1993). These agonist-activated Cl^- currents are Ca^{2+} dependent ($I_{Cl(Ca)}$) (Droogmans et al. 1991; Lamb et al. 1994; Pacaud et al. 1992; Pacaud et al. 1989a; Hirakawa et al. 1999). With simultaneous patch-clamp recording and intracellular Ca^{2+} measurements, it was demonstrated in rat portal vein cells that NE did not open Ca^{2+} channels, but increased $[Ca^{2+}]_i$ and evoked a Ca^{2+}-activated Cl^- current at a holding potential of -50 mV, which is about the resting potential in physiological conditions. These effects were blocked when heparin, an IP_3 receptor inhibitor, was included in the pipette solution (Pacaud et al. 1991). In addition, when intracellular Ca^{2+} stores were depleted by caffeine, subsequent application of the agonists in the presence of caffeine failed to evoke a significant rise in $[Ca^{2+}]_i$ and did not induce $I_{Cl(Ca)}$ (Pacaud et al. 1992). Furthermore, in Ca^{2+}-free external solution, NE induced a transient rise in $[Ca^{2+}]_i$ and was still able to activate a Cl^- current (Pacaud et al. 1992; Pacaud et al. 1989b). NE-induced $I_{Cl(Ca)}$ was blocked by caffeine, but could be recorded in a Ca^{2+}-free (+EGTA) bath solution in rabbit ear artery and pulmonary artery cells (Amedee et al. 1990b; Wang and Large 1993). Taken together, these data suggest that the Cl^- current evoked by α_1-adrenoceptor activation results from an increase in the intracellular concentration of calcium released from internal stores. $I_{Cl(Ca)}$ activated by the release of calcium from intracellular stores stimulated by ATP and histamine was also reported in VSMC from pig aorta and

rabbit pulmonary artery (Droogmans et al. 1991; Wang and Large 1993). Although it has been demonstrated that prolonged exposure to Ca^{2+}-free solution gradually reduced and eventually abolished $I_{Cl(Ca)}$ (Amedee et al. 1990b; Droogmans et al. 1991; Wang and Large 1993; Hirakawa et al. 1999) and that depolarizing pulses which produced Ca^{2+} entry through VOCs also activated $I_{Cl(Ca)}$ (Lamb et al. 1994; Pacaud et al. 1989a), the extent to which Ca^{2+} entry from extracellular sources can sustain activation of these channels is unknown. It has been suggested that in smooth muscle, pharmacological receptors are linked to $I_{Cl(Ca)}$ by a G protein-IP_3-intracellular Ca^{2+} store pathway (Loirand et al. 1990; Pacaud et al. 1993). Thus, agonists which stimulate IP_3-dependent mobilization of Ca^{2+} from intracellular stores could activate $I_{Cl(Ca)}$. On the other hand, Pacaud (Pacaud et al. 1991) suggested that the activation of Cl^- channels in rat portal vein is a prerequisite for enhanced opening of voltage-dependent Ca^{2+} channels in response to NE.

6.4. Physiological Role of $I_{Cl(Ca)}$

6.4.1. $I_{Cl(Ca)}$ blockers

The evaluation of the physiological role of $I_{Cl(Ca)}$ has been slow, since available $I_{Cl(Ca)}$ blockers are relatively non-selective. The most commonly used Cl^- channel blockers, including anthracene-9-carboxylic acid (A-9-C), the stilbene derivatives, 4-acetamido 4-isothocyanostilbene-2,2'-disulfonic acid (SITS) and 4,4'-diisothiocyanostilbene-2, 2'disulfonic acid (DIDS) and the fenamate, niflumic acid (NFA), have been characterized as $I_{Cl(Ca)}$ inhibitors in VSM (Large and Wang 1996 and references therein); (Kirkup et al. 1996a). In addition, 5-nitro-2-(3-phenylpropylamino)-bezoic acid (NPPB) (Kirkup et al. 1996b), ethacrynic acid, indanyloxyacetic acid (IAA) (Greenwood et al. 1995), diphenylamine-2, 2'-dicarboxylic acid (DPC) (Baron et al. 1991) and another two fenamates,

flufenamic acid and mefenamic acid (Greenwood and Large 1995) were also found to inhibit $I_{Cl(Ca)}$ in VSM. None of these compounds are specific for $I_{Cl(Ca)}$. For example, fenamates are also found to activate large conductance, Ca^{2+}-sensitive K^+ channels ($BK_{(Ca)}$) in porcine coronary artery membranes (Ottolia and Toro 1994) and in whole-cell recordings from canine coronary and rabbit portal vein (Greenwood and Large 1995; Xu et al. 1994). A-9-C, IAA and ethacrynic acid were reported to do the same in rabbit portal vein (Toma et al. 1996). However, there are marked differences in the concentrations required to inhibit $I_{Cl(Ca)}$ and evoke $I_{K(Ca)}$ for NFA. In rabbit portal vein, NFA inhibited spontaneous transient inward currents (STICs, calcium-activated Cl^- currents) with an IC_{50} of approximately 2×10^{-6} M but evoked potassium current only at concentrations greater than 1×10^{-4} M (Greenwood and Large 1995), indicating a high selectivity for $I_{Cl(Ca)}$. With A-9-C, IAA and ethacrynic acid, there is either no concentration difference or a slight concentration difference (around 2 fold, for ethacrynic acid) between inhibition of $I_{Cl(Ca)}$ and activation of $BK_{(Ca)}$, indicating these antagonists exhibit a lower degree of selectivity for $I_{Cl(ca)}$. (Greenwood et al. 1995; Hogg et al. 1994b; Toma et al. 1996). In addition, ethacrynic acid and IAA also evoked a glibenclamide-sensitive current (Toma et al. 1996), and mefenamic acids have been reported to potentiate the α_1-adrenoceptor-activated nonselective cation channels in rabbit portal vein (Yamada et al. 1996), and in the concentration range that flufenamic acid decreases STIC ($I_{Cl(Ca)}$) amplitude, these compounds also reduced both the amplitude and frequency of spontaneous transient outward currents (STOCs, Ca^{2+}-activated K^+ currents, $I_{K(Ca)}$) (Greenwood et al. 1995). The potency of other channel blockers, such as DIDS and SITS, against $I_{Cl(Ca)}$ (IC_{50} greater than 10^{-4} M) is less than that of their well-established effects on Cl^--HCO_3^- exchange in smooth muscle e.g. see (Aickin and Brading 1983). In addition, ATP-

induced cation currents in rabbit ear artery are potently inhibited by stilbene derivatives, demonstrating that these agents are unsatisfactory as selective $I_{Cl(Ca)}$ blockers, at least in some VSM (Amedee et al. 1990b). NPPB, another potent compound against $I_{Cl(Ca)}$, at 10 μM was selective for the $I_{Cl(Ca)}$, but at 30 μM also inhibited the calcium current by around 70% in rat portal vein (Kirkup et al. 1996b). In contrast, in rat cerebral arteries, a significant block of calcium channels was observed even at 10 μM NPPB (Doughty et al. 1998). These results suggest there may be different effects or varied degree of activity for some of these compounds in different tissues. As mentioned above, it seems that NFA is the most potent and selective inhibitor of $I_{Cl(Ca)}$. It has also been demonstrated that in rabbit and rat portal vein NFA potently inhibited NE-evoked $I_{Cl(Ca)}$ with IC_{50} values of 6×10^{-6} M and 1-100 μM respectively. At concentrations up to 5×10^{-5} M it did not inhibit the influx of divalent cations (measured using Ba^{2+} as a carrier) induced by membrane depolarization, and at concentrations between 10 to 100 μM, it did not inhibit VOCs at all, suggesting NFA did not inhibit voltage-gated calcium channels (Hogg et al. 1994a; Kirkup et al. 1996a). These observations offer the possibility that NFA may be a useful tool to evaluate the role of $I_{Cl(Ca)}$ in agonist-induced vasoconstriction. However, NFA ($2x\ 10^{-6}$ and $5\ x10^{-5}$ M) has been shown to enhance NE-stimulated $I_{K(Ca)}$, and it has been suggested that NFA may increase the amount of Ca^{2+} released from the intracellular store in response to stimulation with NE in rabbit portal veins (Hogg et al. 1994a). However, this characteristic of NFA may be tissue-specific, since in rat portal vein at concentrations less than $3\ x10^{-5}$ M, NFA had no effect on the magnitude of the caffeine- or NE-stimulated $BK_{(Ca)}$ (Kirkup et al. 1996a).

6.4.2. $I_{Cl(Ca)}$ blockers inhibit agonist-induced vasoconstriction.

Given the general lack of selectivity of Cl^- channel blockers, it is important to study the effects of these blockers with a carefully controlled experimental design to assess the role of $I_{Cl(Ca)}$ in contractile mechanisms under physiological condition. There have been several experiments with Cl^- channel antagonists in whole tissue preparations, suggesting the involvement of a Cl^- conductance in agonist-induced contraction of smooth muscle. For instance, it was demonstrated that IAA-94 reduced endothelin-evoked contraction in rat aorta and renal arteries (Iijima et al. 1991; Takenaka et al. 1992) and the contraction induced by angiotensin II in rat renal afferent arterioles (Carmines 1995). It was also shown that NFA reduced NE-induced contraction in rat aorta and mesenteric arteries, as well as ET-1- and Ang II-induced contractions in rat pulmonary arteries. (Criddle et al. 1996; Criddle et al. 1997; Guibert et al. 1997; He and Tabrizchi 1997; Hyvelin et al. 1998; Lamb and Barna 1998). NFA and DIDS were also reporte to inhibit α_1-adrenoceptor-mediated vasoconstriction in mesenteric vascular bed in anesthetized rats (He and Tabrizchi 1997; Lamb et al. 2000). Recently, it has been reported that NPPB (3 μM) inhibited the contractile response and increase in $^{45}Ca^{2+}$ influx produced by PE, a selective α_1-adrenoceptor agonist, in rat caudal arteries (Min et al. 1999). Removal of chloride ions also impaired PE-induced contractions and $^{45}Ca^{2+}$ influx, while NPPB had no effect on PE-induced contraction in Cl^--free buffer. These results thus provide some evidence of the role of Cl^- channels in α_1-adrenoceptor-mediated Ca^{2+} influx and contraction. It is of interest to further assess the contribution of Cl^- conductance to contractile responses to α_1-adrenoceptor in mesenteric arteries since, as mentioned previously, in mesenteric arteries the membrane potential has an important modulating influence on the tension response to NE (Cheung et al. 1999; Mulvany

et al. 1982), and the main effect of NE on $[Ca^{2+}]_i$ has been found to be mediated though voltage-dependent, dihydropyridine-sensitive Ca^{2+} channels (Nelson et al. 1988).

IV. ENDOTHELIUM-MEDIATED REGULATION OF MESENTERIC ARTERIAL TONE

The single layer of endothelial cells (ECs) that lines the luminal side of mesenteric arteries and all other blood vessels plays an important role in regulating blood vessel function. The EC layer serves in part as a protective covering and permeability barrier to the movement of substances through the blood vessel wall. In addition, ECs also have an active role in regulating vascular tone by releasing various vasoactive substances: relaxing and contracting factors. Vessel tone is dependent on the balance between these factors, as well as on the ability of the smooth muscle cells to respond to them.

1. Endothelium-Derived Vasorelaxing Factors

A large body of evidence shows that ECs synthesize and release nitric oxide (NO), prostacyclin (PGI_2) and an unidentified endothelial-derived hyperpolarizing factor(s) (EDHF) that cause blood vessels to dilate (Furchgott and Vanhoutte 1989; Furchgott and Zawadzki 1980; Garland et al. 1995; Moncada and Vane 1978b; Palmer et al. 1987 and references therein).

1.1. Nitric Oxide (NO):

1.1.1. NO synthesis

NO is formed from the guanidine-nitrogen terminal of L-arginine plus molecular oxygen by a heme-containing enzyme called NO synthase (NOS) (Ignarro 1990a; Palmer et al. 1988). There are three isoforms of NOS: the constitutive endothelial NOS (eNOS, NOS

III), and neuronal NOS (nNOS, NOS I), which are mainly present in endothelial and neuronal cells, and inducible NOS (iNOS, NOS II) that is only found in cytokine-activated cells and does not seem subject to any cellular control mechanisms (Forstermann et al. 1994; Marsden et al. 1992; Nishida et al. 1992; Palmer et al. 1988). Activation of the constitutive NOS is Ca^{2+}-calmodulin-dependent and requires reduced nicotinamide adenine dinucleotide phosphate (NADPH), 5,6,7,8-terahydrobiopterin (BH4) and flavin mononucleotide for optimal activity (Busse et al. 1993; Knowles and Moncada 1994; Mayer and Werner 1995; Moncada et al. 1991). The production of NO can be inhibited by interfering with any of the above factors (Moncada et al. 1991; Xie et al. 1992). Analogues of L-arginine, such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA), N^G- nitro-L-arginine methyl ester (L-NAME), are potent competitive inhibitors of NOS activity and selectively inhibit NO formation. These have been very useful in providing insight into the role of NO in the vasculature (Knowles and Moncada 1994; Mayer et al. 1989; Moncada et al. 1991; Rees et al. 1989; Rees et al. 1990).

1.1.2. Mechanism of NO release in endothelial cells

a). Agonist-stimulated NO release

In endothelium, NO generation by eNOS is stimulated by various neurohumoral substances including ACh, bradykinin, histamine, ADP, ATP, etc., which strictly depend on an increase in $[Ca^{2+}]_i$ (Busse et al. 1989; Busse et al. 1993; Freay et al. 1989; Furchgott and Vanhoutte 1989; Griffith et al. 1986; Long and Stone 1985; Lopez-Jaramillo et al. 1990; Luckhoff and Busse 1986; Luscher 1990; Singer and Peach 1982) (Fig. 0.2). The agonist-induced increase in $[Ca^{2+}]_i$ involves both a transient IP_3-mediated release of Ca^{2+} from

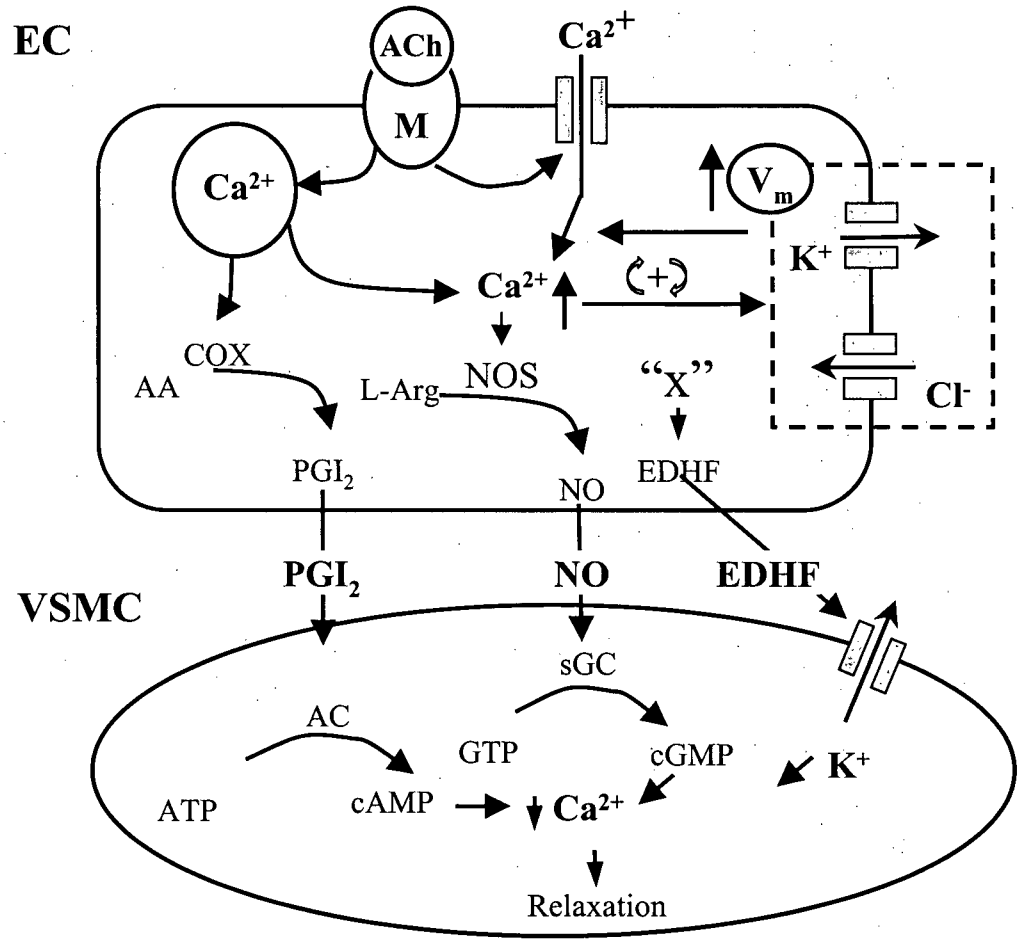


Figure 0.2 Schematic diagram illustrating pathways of Ach-induced release of EDRFs in endothelial cells and relaxation of smooth muscle by the EDRFs. Modified from Vanhoutte (1997) . ACh binding to muscarinic receptor increase in IP_3 which contrabutes to the increase in cytoplasmic Ca^{2+} by releasing it from endoplasmic reticulum (ER). Muscarinic receptor activation induces an influx of Ca^{2+} into the cytoplasma. The resulting increase in $[Ca^{2+}]_i$ activates NOS to produce NO and leads to the release of EDHF. The increased $[Ca^{2+}]i$ also accellerates the formation of PGI_2 from arrachidonic acid (AA) by COX. Stimulation with ACh also produces hyperpolarization of membrane potential (Vm) due to activation of $K_{(Ca)}$ and Cl^- channels by the rise in Ca^{2+}. The resuling hyperpolarization accentuates Ca^{2+} influx due to the increased electrochemical gradient for Ca^{2+} and thereby by a positive feed back loop potentiaes the release of EDRFs. NO causes relaxation by activating the formation of cGMP from GTP. PGI_2 causes relaxation by activating the formation of cAMP from ATP. EDHF cause hyperpolarization and relaxation by opening K+ channels.

intracellular stores (Freay et al. 1989; Jaffe et al. 1987; Pirotton et al. 1987; Wang et al. 1995b) and a small but more sustained transmembrane influx of Ca^{2+} from the extracellular space (Wang et al. 1995a). Several lines of evidence have shown that release of NO absolutely requires Ca^{2+} influx from the extracellular space (Luckhoff and Busse 1990; Luckhoff et al. 1988; White and Martin 1989). However, the regulation and pathway(s) for agonist-induced Ca^{2+} entry remain to be elucidated (Nilius et al. 1997b). It has been suggested that agonists binding to their receptors in the plasma membrane either directly gate the Ca^{2+} channels (receptor-operated channels), or indirectly couple to the Ca^{2+} channels through a G protein (Chen and Rembold 1995) or second messenger such as IP_3 (Vaca and Kunze 1995) or IP_4 (Luckhoff and Clapham 1992) to cause Ca^{2+} entry. Another suggestion is that agonist-induced Ca^{2+} entry is a consequence of depletion of an endoplasmic reticulum Ca^{2+} (via store -operated Ca^{2+} channels) (Putney 1991), which can be achieved in the absence of agonist (Hallam et al. 1989). Recently it has been further demonstrated that in freshly isolated aortic endothelial cells, ACh and store depletion activated the same Ca^{2+} entry pathway but through parallel mechanisms (Wang and van Breemen 1997). Although in vascular endothelial cells there exists a variety of Ca^{2+}-permeable channels that are responsible for receptor-mediated Ca^{2+} entry, it is generally accepted that agonist-induced Ca^{2+} influx is controlled by membrane potential (Demirel et al. 1994; Nilius et al. 1997b and references therein). A membrane hyperpolarization caused by agonist opening K^+ channels provides an electrochemical gradient for maintained Ca^{2+} entry during agonist stimulation. A similar mechanism for modulation of the driving force has also been proposed for Cl^- channels (Hosoki and Iijima 1994; Hosoki and Iijima 1995; Wang and van Breemen 1999;

Yumoto et al. 1995) (Fig. 0.2). It has been reported that depolarizing endothelial cells by increasing the extracellular K^+ concentration or preincubation of endothelial cells or intact arteries with K^+ channel blockers decreased the duration and the magnitude of agonist-induced Ca^{2+} influx. This in turn reduced the production of NO (Luckhoff and Busse 1990) and inhibited vasorelaxation (Demirel et al. 1994). However, little information is available on whether Cl^- channels modulate NO synthesis and NO-mediated vasorelaxation.

b). Basal and constitutive release of NO

The continuous basal release of NO represents a sizable portion of the total NO-releasing capacity of native endothelial cells (Busse et al. 1993). However, the rate of NO formation under basal conditions seems to be substantially smaller in cultured endothelial cells, implying that native endothelial cells may be continuously exposed to a stimulus, such as shear stress, which affects NO synthase expression. Evidence has accumulated that mechanical force generated at the endothelium by fluid shear stress and pulsatile stretch are important in ensuring the continuous release of vasoactive endothelial autacoids (Busse and Fleming 1998). It has been suggested that eNOS may be differentially activated by receptor-dependent agonists and mechanical stimuli (Fleming et al. 1997). It has been observed that a rapid NO release in response to an onset of flow or an increase in flow above preexisting levels, like in response to receptor-dependent agonists, was Ca^{2+}/camodulin-dependent (Busse et al. 1993; Kuchan and Frangos 1994). On the other hand, the constitutive sustained release of NO from the endothelium by physical stimuli such as shear stress exerted by the flowing blood, as well as mechanical stress induced by isometric contraction, may involve $[Ca^{2+}]_i$ redistribution within the cytoskeleton/caveolae and the activation of one or more

regulatory eNOS-associated proteins without any apparent rise of $[Ca^{2+}]_i$ (Busse and Fleming 1998; Fleming et al. 1999; Hutcheson and Griffith 1996; Kuchan and Frangos 1994).

1.1.3. Mechanism of NO-mediated relaxation

NO released from vascular endothelial cells diffuses rapidly to and acts in a paracrine fashion on adjacent vascular smooth muscle cells. The smooth muscle relaxation caused by NO was first described to be mediated mainly by the activation of soluble guanylate cyclase in the smooth muscle cells of vascular wall, leading to increase in guanosine 3',5'-cyclic monophosphate (cGMP) and the subsequent activation of cGMP-dependent protein kinases, such as protein kinase G (PKG), which may modulate Ca^{2+} metabolism resulting in smooth muscle relaxation (Cornwell et al. 1991; Ignarro 1990b; Lincoln and Cornwell 1991; Salomone et al. 1996; Tewari and Simard 1997). The reliance of endothelium-dependent vasodilation on this mechanism is based on the parallel drawn between the increase in cGMP content of arterial tissue caused by endothelium-dependent vasodilators and those of nitrovasodilators, whose action is based on releasing NO (Gruetter et al. 1981; Ignarro 1989; Martin et al. 1985; Rapoport et al. 1985). In addition, endothelium-dependent relaxation to NO may be reduced by hemoglobin or methylene blue, which antagonize the rise in cGMP either by inhibiting guanylate cyclase, or by scavenging NO and preventing its stimulation of the enzyme (Edwards et al. 1986; Gruetter et al. 1981; Ignarro et al. 1987; Ignarro et al. 1986; Kruszyna et al. 1987; Martin et al. 1986; Wolin et al. 1990)

Recently, NO has been shown to produce hyperpolarization in resting tissue or to repolarize smooth muscle cells previously depolarized by an agonist (Cohen et al. 1997; Garland and McPherson 1992; Krippeit-Drews et al. 1992; Murphy and Brayden 1995a;

Parsons et al. 1994; Plane et al. 1995; Plane et al. 1998; Tare et al. 1990). K^+ channel activation by NO (either directly or via cGMP) has been observed in a number of isolated arteries (Archer et al. 1994; Bolotina et al. 1994; Bychkov et al. 1998; George and Shibata 1995; Mistry and Garland 1998; Peng et al. 1996; Plane et al. 1998; Quignard et al. 1999; Robertson et al. 1993). In rat mesenteric arteries, NO and/or NO donor hyperpolarization of the resting membrane potential have been observed, and the hyperpolarization was sensitive to glibenclamide, implicating K_{ATP} channels (Garland and Plane 1996; Garland and McPherson 1992). Electrophysiological experiments have revealed that NO and NO donors produced a cGMP-independent activation of large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) in isolated smooth muscle cells from rat small mesenteric arteries (Mistry and Garland 1998). In microvessels of rat mesentery, NO donors activated $BK_{(Ca)}$, but this effect was mimicked by cGMP and inhibited by blocking the activity of PKG (Carrier et al. 1997). In addition, ACh hyperpolarized smooth muscle in intact rat small mesenteric arteries tonically, by activating both ATP- and Ca^{2+}-dependent K^+ current (Weidelt et al. 1997). The hyperpolarization was completely blocked by an inhibitor of NOS but not by methylene blue, a guanylate cyclase inhibitor, suggesting the non-involvement of the soluble guanylate cyclase. Functional studies showed that in rat endothelium-intact isolated mesenteric resistance arteries, full relaxation to NO donors can be accounted for by a charybdotoxin (CTX)-sensitive, cyclic GMP-independent mechanism (Plane et al. 1996). CTX is an inhibitor of large and intermediate Ca^{2+}-activated K+ channels.

Evidence from the literature suggests that NO operates at multiple sites in vascular smooth muscle cells. The extent to which different mechanisms contribute to relaxation may depend on the contractile agonist, the specific endothelium-dependent relaxant used, the

tissue and the species (Ghisdal et al. 2000; Plane et al. 1998; Wolin et al. 1998) (and references above).

1.1.4. NO regulates mesenteric vascular tone

a). Effect of basal NO release:

Several lines of *in vivo* evidence suggest that constitutive levels of expression of NOS in endothelium are sufficient to influence tone in mesenteric blood vessels under basal conditions. Inhibition of NOS has been shown to produce constriction of mesenteric blood vessels and decrease mesenteric blood flow under basal conditions (Gardiner et al. 1990) and following ganglion blockade (Fozard and Part 1991). In isolated superior mesenteric arteries, the basal tone was enhanced in the presence of the NOS inhibitor, N^G-nitro-L-arginine (L-NNA) or the guanylate cyclase inhibitor, methylene blue (Wu et al. 1997). In addition, when the NO concentration was measured with an NO-specific microelectrode, the NO scavenger oxyhaemoglobin reduced the NO signal below baseline in the absence of vasoconstrictor (Simonsen et al. 1999). These results suggest the presence of continuous basal release of NO in these preparations. However, in isolated perfused mesenteric arterial beds and in pressurized and perfused mesenteric resistance arteries, NOS inhibitors did not have any effect on basal tone, but enhanced responses to vasoconstrictors, suggesting there is no basal NO release, whereas the liberation of NO requires active tone (Adeagbo et al. 1994; Amerini et al. 1995; Baisch et al. 1994; Dohi et al. 1990; Ebeigbe et al. 1990; Le Marquer-Domagala and Finet 1997; Tatchum-Talom and Atkinson 1997). In isolated mesenteric arteries, shear stress induces relaxation and this effect is totally endothelium-dependent in both large (400-500 μm) and small (150-250 μm) arteries (Takamura et al. 1999). The contribution of NO,

which was evaluated by the use of NOS inhibitors, was found to be more prominent in large arteries than in small arteries, whereas the NO-independent component was equally distributed in both sizes of arteries and was inhibited by K^+ channel blockers (Takamura et al. 1999).

b). Effects of agonist-induced NO release

In the isolated perfused mesenteric arterial bed, as in other arteries, ACh, histamine, AVP and the Ca^{2+} ionophore A23187 all induced endothelium-dependent relaxation (Adeagbo and Malik 1990; Bhardwaj and Moore 1988; Furchgott et al. 1987; Randall et al. 1988). However, it has been found since the pioneering work by Furchgott and colleagues that hemoglobin and methylene blue inhibit ACh-induced relaxation to lesser extent in the mesenteric arterial bed as compared to large blood vessels such as aorta (Furchgott et al. 1987; Khan et al. 1992). Later, the possibility that both NO and EDHF are involved in the responses of the rat mesenteric arterial bed to ACh and histamine was suggested by Adeagbo & Triggle (Adeagbo and Triggle 1993). They showed that in physiologic salt solution (PSS), ACh- and histamine-induced vasodilation of cirazoline-preconstricted mesenteric arterial beds were only partially attenuated by the NOS inhibitor N^G- nitro-L-arginine methyl ester (L-NAME). Changing the membrane potential by varying extracellular K^+ concentration $[K^+]_o$ decreased L-NAME-resistant vasodilation, indicating a role of the putative EDHF. These observations were supported by other investigators (Kamata et al. 1996a; McCulloch et al. 1997; Parsons et al. 1994; Randall et al. 1997). Since ACh and carbachol were less potent as vasorelaxants in the presence of K^+ or K^+ channel blockers than in the presence of L-NAME, a greater contribution of EDHF than NO to relaxation induced by muscarinic

receptor stimulation in rat mesenteric arterial bed has been suggested (Adeagbo, Triggle 1993; Kamata et al. 1996a; Kamata et al. 1996b; McCulloch et al. 1997; Parsons et al. 1994; Randall et al. 1997). The relative contribution of NO to endothelium-dependent relaxation on stimulation by muscarinic agonists was small, with only a minor decrease in potency of the relaxant, or with a small reduction in maximum relaxation in the presence of NOS inhibitors among these studies. In addition, Kamata *et al* showed that the effects of the endothelium-dependent relaxation induced by platelet-activating factor (PAF) (Kamata et al. 1996b), or by the Ca^{2+}-ATPase inhibitor cyclopiazonic acid (Kamata et al. 1996a) were different as compared to ACh, and suggested that a novel-relaxing factor (Kamata et al. 1996a) may exist. Furthermore, Parsons *et al* (Parsons et al. 1994) also compared ACh-induced relaxation in perfused mesenteric arterial beds with that in isolated second, third and fourth order sequential branches and concluded that the relative contribution of NO and NO-independent components was similar in sequential branches. However, different results have been presented by other researchers (see below).

In isolated small mesenteric arteries, Shimokawa *et al* (Shimokawa et al. 1996) demonstrated that the contribution of NO decreases as the vessel size becomes smaller under both basal conditions and on stimulation with ACh. They also showed that the immunoreactivity of eNOS was strongest in aorta and decreased as the vessel size became smaller in the mesenteric vasculature. Using L-NAME to inhibit NOS, Garland and McPherson (Garland and McPherson 1992) concluded that release of NO was not involved in ACh-induced vasorelaxation in isolated small mesenteric arteries from Wistar Kyoto rats. Gustafsson *et al* (Gustafsson et al. 1993) also failed to find any effect with another NOS inhibitor L-NNA in number of their experiments with small mesenteric arteries from Wistar

rats, as did Zygmunt *et al* (Zygmunt et al. 1995), using female Sprague-Dawley (SD) rats. On the other hand, it has been reported that L-NNA inhibited ACh-induced relaxation in small mesenteric arteries from Sprague-Dawley and Wistar rats, although NO contributed only a small part of the ACh- induced relaxation (Hwa et al. 1994; Plane and Garland 1996; Waldron and Garland 1994; Wu et al. 1993).

In isolated superior mesenteric artery, the relative contribution of NO also varied among different studies. It has been reported that NO solely contributed to ACh-induce relaxation in SD rats (Hwa et al. 1994), whereas other studies showed that both NO-dependent and independent relaxation contribute to ACh-induced responses in Wistar rats (Chen and Cheung 1997; Fukao et al. 1995; Nagao et al. 1992).

The results for the Ca^{2+} ionophore A23187-induced endothelium-dependent relaxation were also conflicting in mesenteric vasculature. No A23187-stimulated relaxation effect in small mesenteric arteries (Zygmunt et al. 1995), or only a NO-independent relaxation (Parsons et al. 1994), or both NO-dependent and independent relaxation evoked by A23187 (Nagao et al. 1992; White and Hiley 1997) were reported in rat mesenteric arteries. In addition, Kamata (Kamata et al. 1996b) reported that A23187-induced relaxation was not affected by either depolarization with high K^+ or by exposure to eNOS-cGMP pathway inhibitors, whereas the vasodilation was slightly but significantly inhibited by treatment with a combination of methylene blue and L-NNA in isotonic high K^+ solution, suggesting that A23187 may also produce a novel EDRF or more than one.

The reasons for the diversity of the contribution of NO to receptor-dependent agonist-induced relaxation in mesenteric vasculature among different studies is not entirely clear, but the contributing factors as mentioned above may be the differences in the strain and gender

of rats, in the way of handling the tissues, such as the initial stretch of vessels (Parkington et al. 1993; Zygmunt et al. 1994a), the nature of the relaxing agonists, and that of the contractile agonist that was used to precontract the blood vessels. Indeed, it has been observed that NE-induced contractions were reversed by ACh via both NO and NO synthase-independent smooth muscle repolarization, whereas the reversal of contraction to the thromboxane-mimetic U46619 by ACh was entirely mediated by the action of NO, independently of a change in membrane potential (Plane and Garland 1996).

c). Vasoconstrictor–induced NO release

Recently it has been demonstrated that in rat perfused mesenteric arterial bed, stimulation of perivascular sympathetic nerves releases NE and induces vasoconstriction which triggers a secondary release of endothelial NO coupled to cGMP production. In addition, exogenous NE-induced vasoconstriction is also coupled to increases in NO and cGMP release. The electrically evoked vasoconstriction and NO release were abolished by blocking either sympathetic exocytosis with guanethidine or α_1-adrenoceptors with prazosin, suggesting the NO release is stimulated by NE binding to α_1-adrenoceptors (Boric et al. 1999).

1.2. Prostacyclin (PGI_2)

1.2.1. Synthesis and release

PGI_2 was discovered in 1976 (Moncada et al. 1976a). PGI_2 production is initiated by the enzyme PLA_2, which liberates arachidonic acid from membrane phospholipids. The enzyme prostaglandin G/H synthase, which possesses cyclooxygenase (COX) activity,

converts arachidonic acid into prostaglandin endoperoxides. Subsequently, PGI_2 synthase forms PGI_2 from the endoperoxide prostaglandin H_2, which is the precursor of all prostanoids. PGI_2 is the major vasodilator prostaglandin (PG) produced by EC in most blood vessels including mesenteric arteries (Carter and Pearson 1992; Moncada and Vane 1978a; Peredo et al. 1997; Pipili et al. 1988). Inhibition of COX activity with COX inhibitors, such as indomethacin, will effectively block synthesis of PGI_2 and other prostanoids, thereby preventing their actions.

Like NO, PGI_2 synthesis/release is also stimulated by variety of endogenous mediators and drugs, as well as physiological stimuli (Bhagyalakshmi and Frangos 1989; Piper and Vane 1971). The release of PGI_2 is also believed to be triggered by an increase in $[Ca^{2+}]_i$ (Hallam et al. 1988; Long and Stone 1985). However, in bovine cultured aortic endothelial cells, NO release correlates most closely with transmembrane Ca^{2+} influx rather than Ca^{2+} release from intracellular stores, while PGI_2 release is entirely dependent on Ca^{2+} release from the stores (Luckhoff et al. 1988). Parsaee (Parsaee et al. 1992) have shown that higher levels of $[Ca^{2+}]_i$ are required for PGI_2 release than for NO release. Furthermore, inhibition of intracellular Ca^{2+}mobilization by TMB-8 attenuated bradykinin-induced PGI_2 release (Whorton et al. 1984), while exerting a minimal effect on NO release (Peach et al. 1987). Therefore, it seems that there is a difference in the Ca^{2+} source required for the release of NO and PGI_2 (Fig. 0.2).

1.2.2. Actions

Physiologically, PGI_2 is a local autacoid (Blair et al. 1982). In the lumen of blood vessels PGI_2 prevents platelet aggregation, and thus the release of vasoconstrictor and

growth-promoting agents. It acts in concert with nitric oxide, which also inhibits platelet aggregation (Radomski et al. 1987). PGI_2 also acts on smooth muscle cells to exert a vasorelaxant effect (Moncada and Vane 1978b). PGI_2 contributes to endothelium-dependent relaxation of several isolated blood vessels and to vasodilation of perfused organs (Forstermann et al. 1986; Holtz et al. 1984; Lamontagne et al. 1992; Vegesna and Diamond 1986). The mechanisms by which PGI_2 mediates smooth muscle relaxation involve the stimulation of specific receptors and activation of adenylate cyclase leading to an elevation of intracellular cyclic adenosine monophosphate (cAMP) (Halushka et al. 1989; Luscher and Vanhoutte 1990) (Fig. 0.2). PGI_2 was reported to activate glibenclamide-sensitive K^+ channels via the cAMP pathway, leading to smooth muscle hyperpolarization and relaxation (see review for Vanhoutte et al. 1996). However, the relative importance of PGI_2 in relation to endothelium-derived nitric oxide and other endothelium–derived vasorelaxants such as EDHF, at the level of resistance arteries, is currently unclear, both physiologically and in hypertension (Schiffrin 1996).

1.3. EDHF

1.3.1. Identity

In many blood vessels, inhibition of the synthesis of NO and PGI_2 does not result in complete loss of endothelium-dependent relaxation in response to variety of agonists, such as ACh, histamine, bradykinin, or substance P. A putative non-NO/PGI_2 mediator which hyperpolarizes vascular smooth muscle cells has been termed the endothelium-derived hyperpolarizing factor (Feletou and Vanhoutte 1999; Mombouli and Vanhoutte 1997; Quilley et al. 1997; Waldron et al. 1996). The chemical identity of EDHF is not yet established. It has been proposed that EDHF could be K^+ in small resistance arteries of rats (Edwards et al.

1998), or may be a metabolite of arachidonic acid produced by cytochrome P-450-dependent monooxygenase in coronary, mesenteric and carotid arteries of several species (Adeagbo and Henzel 1998; Chen and Cheung 1996; Hecker et al. 1994; Popp et al. 1996; Triggle et al. 1999), Alternatively, it has been suggested to be anandamide, an arachidonic acid derivative and endogenous cannabinoid, in isolated perfused mesenteric and coronary arterial beds of rats (Randall et al. 1996; Randall and Kendall 1997; Randall and Kendall 1998). However, evidence has also been presented that neither K^+ (Quignard et al. 1999; Vanheel and Van de Voorde 1999), nor a cytochrome P450 metabolite (Chataigneau et al. 1998a; Corriu et al. 1996; Fukao et al. 1997b; Van de Voorde and Vanheel 1997), nor a cannabinoid (Chataigneau et al. 1998b; Plane et al. 1997; White, Hiley 1997) meets the pharmacological criteria of an EDHF. Taken together, the collective data suggest that EDHF is not one substance, and there may be a considerable number of different mechanisms that mediate endothelium-dependent hyperpolarization in different vascular beds. Indeed, it has been suggested that endothelium-dependent hyperpolarization could involve electrical coupling through the myo-endothelial junctions, not only in small resistance arteries (Edwards et al. 1999; Yamamoto et al. 1999) but also in conduit arteries (Chaytor et al. 1998; Edwards et al. 2000). In a very recent report, Edwards et al (Edwards et al. 1999) compared responses putatively mediated by EDHF in guinea pig internal carotid and rat hepatic and mesenteric arteries, and evaluated the effect of gap junction inhibitors. They concluded that gap junctions play some role in the EDHF response in rat arteries, but the primary mechanism would appear to be mediated by K^+. In contrast, in the guinea-pig internal carotid artery, gap junctions may be the sole mechanism underlying the response attributed to EDHF, indicating that the nature of EDHF shows considerable tissue and species variability.

1.3.2. $[Ca^{2+}]_i$ dependency of EDHF release

In common with the release of NO and PGI_2, elevation of $[Ca^{2+}]_i$ in endothelial cells has also been proposed to be essential for the release of EDHF (Chen and Suzuki 1990). This hypothesis has been supported by the finding that the Ca^{2+} ionophore A23187 induces endothelium-dependent membrane hyperpolarization (Chen and Suzuki 1990; Nagao et al. 1992; Nakashima and Vanhoutte 1993). Recently, Fukao *et al* (Fukao et al. 1997c) measured ACh-induced endothelium-dependent NO/PGI_2-independent hyperpolarization in rat mesenteric artery as a marker for EDHF release. They reported that the ACh-induced release of EDHF is possibly initiated by Ca^{2+} release from an IP_3-sensitive Ca^{2+} pool as a consequence of stimulation of phospholipid hydrolysis due to phospholipase C activation, and is maintained by Ca^{2+} influx via a Ni^{2+}- and Mn^{2+}-sensitive pathway. They also indicated that the Ca^{2+} influx mechanism seems to be activated following IP_3-induced depletion of the Ca^{2+} pool. Thus, the EDHF release from mesenteric arteries seems to rely on both Ca^{2+} release from intracellular stores and Ca^{2+} entry from the extracellular space (Fig. 0.2).

1.3.3. EDHF- mediated vasodilation in mesenteric arteries.

a). Mechanisms of EDHF-mediated relaxation

The action of EDHF is believed to occur via the activation of K^+ channels, leading to hyperpolarization of the vascular smooth muscle membrane and vasorelaxation. This is based on evidence that variations in the extracellular K^+ concentration ($[K^+]_o$) control the amplitude of the endothelium-dependent hyperpolarization, and that the hyperpolarization is associated with enhanced K^+ conductance across the membrane and is blocked by some K^+ channel

blockers (Adeagbo and Triggle 1993; Chen and Suzuki 1989; Chen et al. 1991; Fukao et al. 1997a; Nagao and Vanhoutte 1992; Taylor and Weston 1988). In addition, EDHF-mediated relaxation correlates well with EDHF-mediated hyperpolarization, implying a causal relationship (Chen and Cheung 1997) and is also blocked by elevated $[K^+]_o$ or K^+ channel antagonists (Adeagbo and Triggle 1993; Fukao et al. 1995; Garland and McPherson 1992; Hansen and Olesen 1997; McCulloch et al. 1997; Randall et al. 1997; Waldron and Garland 1994; Zygmunt et al. 1994b). However, the K^+ channels mediating the response to EDHF in vascular smooth muscle have not been characterized (Fig. 0.2).

In superior mesenteric artery, when membrane potential and tension were simultaneously measured, tetraethylammonium TEA (5mM), a relatively selective inhibitor of BK_{Ca}, and apamin, a small-conductance Ca^{2+} activated K^+ channel (SK_{Ca}) antagonist, significantly inhibited ACh-induced smooth muscle hyperpolarization and relaxation that were resistant to the action of NOS inhibitors. CTX, a large- and intermediate- Ca^{2+}-activated K^+ channel antagonist, marginally inhibited both responses. However, the combination of apamin and CTX abolished both the hyperpolarization and the relaxation (Chen and Cheung 1997).

In the perfused mesenteric arterial bed, information regarding the specific K^+ channels that mediate the response to EDHF is limited. Only Adeagbo (Adeagbo and Triggle 1993) reported that apamin completely blocked the EDHF-induced relaxation to ACh.

Most of the studies that characterize the EDHF-mediated relaxation are carried out in small resistance mesenteric arteries. Vasorelaxation to ACh was reported to be attenuated by iberiotoxin (IbTX), a selective large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) blocker, TEA (5mM), 4-aminopyridine (4-AP), a blocker of delayed rectifier, voltage-dependent, K^+

channels (K_V), and $BaCl_2$ (100 μM), a selective blocker for inward rectifier K^+ channels (Nelson and Quayle 1995 and references therein). Combined pretreatment with IbTX plus L-NNA completely blocked the vasorelaxation (Hansen and Olesen 1997). In addition, apamin, TEA (1mM) and 4-AP each significantly reduced NO- and PGI_2-independent relaxations to carbachol, but had no significant effect on the response to A23187 (White and Hiley 1997). Although $BaCl_2$ and CTX alone did not show any effect, exposure of arterial segments to the combination of apamin and CTX abolished EDHF-mediated hyperpolarization (Chataigneau et al. 1998b) and NO- and PGI_2-independent relaxation (Plane et al. 1997; White and Hiley 1997) evoked by ACh or carbachol. In contrast, IbTX had no significant effect on the relaxation to carbachol either alone or in combination with apamin (White and Hiley 1997). Furthermore, in pressurized small mesenteric arteries (diameter: 70-120 μM), Lagaud et al (Lagaud et al. 1999) showed that apamin alone completely blocked, while IbTX had no effect on, ACh-induced, NO/PGI_2-independent relaxation. In contrast, the EDHF-mediated response to CPA was abolished only in the presence of apamin plus IbTX, although either apamin or IbTX alone significantly inhibited it. In addition, neither TEA, CTX, 4-PA nor $BaCl_2$, had any effect on the response to CPA. In another study, using pressurized small mesenteric arteries of the same size, Doughty (Doughty et al. 1999) reported that apamin plus CTX abolished ACh-induced dilation of either PE-stimulated or myogenic tone in the presence of L-NAME and indomethacin when the drugs were applied intraluminally. Since superfusion with both CTX and apamin was without effect on the EDHF-mediated relaxation, the authors concluded that apamin and CTX block EDHF-mediated relaxation by an action on the endothelium, and not an action in the smooth muscle.

Shear stress also induces EDHF release in mesenteric arteries. The released EDHF was noted in both large (400- 500 μm) and small vessels (150-250 μm). The EDHF-mediated component of the shear stress-induced relaxation was almost abolished by TEA and was significantly inhibited by the combination of CTX and apamin (Takamura et al. 1999).

Most studies in the mesenteric vascular bed demonstrate that the ATP-sensitive K^+ channel blocker glibenclamide has no effect on EDHF-mediated hyperpolarization and relaxation (Adeagbo and Triggle 1993; Garland and McPherson 1992; Hansen and Olesen 1997; Kamata et al. 1996a; Lagaud et al. 1999; McCulloch et al. 1997).

Collectively, the data suggest that in the mesenteric vascular bed, several K^+ channels are involved in EDHF-mediated relaxation, particularly Ca^{2+}-activated K^+ channels. The heterogeneity of K^+ channels, especially with different agonists, suggests that EDHF activity may be due to more than one chemical entity.

b). Functional contribution of EDHF to endothelium-mediated relaxation

It is evident that EDHF-induced relaxation assumes a greater functional importance than NO as artery size decreases (Hwa et al. 1994; Nagao et al. 1992; Shimokawa et al. 1996). However, the importance of EDHF is not just related to the vessel size, but may also be vascular region-dependent (Clark and Fuchs 1997; Zygmunt et al. 1995). Experiments in coronary and pulmonary arteries indicate that EDHF may represent a reserve mechanism in some large arteries under certain conditions (Drummond and Cocks 1996; Kemp et al. 1995; Kilpatrick and Cocks 1994). In the mesenteric vascular bed, EDHF seems to play an important role in mediating endothelium-dependent vasodilation (see above), in the main mesenteric artery (Chen and Cheung 1997; Fukao et al. 1995; Nagao et al. 1992) and its

small branches (Adeagbo and Triggle 1993; Hwa et al. 1994; Kamata et al. 1996a; Kamata et al. 1996b; McCulloch et al. 1997; Parsons et al. 1994; Plane and Garland 1996; Randall et al. 1997; Waldro and Garland 1994; Wu et al. 1993) although some conflicting results have been reported (Hwa et al. 1994).

2. Endothelium-Derived Contracting Factors

Vasoconstrictors derived from EC have been identified and characterized to some degree, including endothelin-1 (ET-1) and endothelium-derived contracting factor(s) (EDCF) (Luscher and Vanhoutte 1990; Vanhoutte 1989). The nature of EDCF varies with the species and anatomical site of its production. Prostaglandin endoperoxides H_2 (PGH_2), thromboxane A_2 (TxA2) and superoxide anion (O_2^-) have been suggested as possible candidates (Luscher et al. 1992). In most situations, contractions initiated by vasoconstrictors derived from endothelial cells are found in pathological conditions, particularly in hypertension, while under normal conditions relaxing factors are predominantly released from endothelial cells (Luscher et al. 1993b; Mistry and Nasjletti 1988; Purkerson et al. 1986; Vanhoutte 1996; Wilcox et al. 1996).

2.1. Endothelin-1

2.1.1. Synthesis and release

The endothelins are a family of contractile peptides made up of 21-amino acids (Yanagisawa et al. 1988). They are synthesized from larger precursors and expressed in different tissues. ET-1 is synthesized in endothelial cells, and its expression is induced by several factors including hypoxia, NE, angiotensin II, vasopressin, thrombin, insulin, cytokines and growth factors (see review for Masaki 1995). Physical stimuli, such as shear

stress, and other factors such as NO and PGI_2 decrease ET-1 production and release. ET-1 release from perfused rat mesenteric arterial bed and its enhancement by hypoxia has been observed (Rakugi et al. 1990).

The circulating levels of ET-1 are low under physiological conditions, since most peptides are secreted toward the abluminal side, i.e. toward the smooth muscle cells (Wagner et al. 1992). Therefore, ET-1 mainly acts in a paracrine and autocrine manner through two subtypes of receptors: ET_A and ET_B, which have been cloned (Arai et al. 1990; Sakurai et al. 1990).

2.1.2. ET receptors and their function in rat mesenteric vascular bed

ET_A receptors, which are present in vascular smooth muscle cells, mediate vasoconstriction and cellular proliferation (Luscher et al. 1993a), while ET_B receptors, which were thought to occur mainly on endothelial cells, mediate vasodilation by generation of NO and PGI_2 (Luscher et al. 1993a; Matsuda et al. 1993; Warner et al. 1989; Wright and Fozard 1988). It has now been demonstrated that ET_B receptors are also found on vascular smooth muscle and mediate vasoconstriction in some blood vessels (Batra et al. 1993; Moreland et al. 1992; Sumner et al. 1992). At lower concentrations (comparable to physiological plasma levels), ET-1 causes vasodilation by activation of endothelial ET_B receptors, while at higher concentrations it provokes sustained contractions by activation of ET_A (and in some blood vessels, also ET_B) receptors on the smooth muscle cells (Luscher et al. 1996; Masaki 1995; Mehta et al. 1992). In addition, ET-1 may interact with other vasoactive substance to affect smooth muscle function. It was shown that threshold doses of ET-1 potentiated responses to NE or sympathetic nerve stimulation in several blood vessels of different species including

rat mesenteric arteries (Henrion and Laher 1993; Tabuchi et al. 1989b; Wong-Dusting et al. 1991; Yang et al. 1990). Recently Kita *et al* (Kita et al. 1998) confirmed that ET-1 at sub-pressor doses enhances contractile responses to NE, and further demonstrated that this effect was mediated by ET_B receptors, in perfused rat mesenteric arterial bed. In addition, in hypertension, endothelin may also stimulate release of COX pathway-derived contracting factors to mediate endothelium-dependent vasoconstriction. It has been shown that ET stimulated TxA_2 release and evoked an endothelium-dependent contraction in aorta from spontaneously hypertensive rats (SHR) but not Wistar-Kyoto (WKY) rats (Taddei and Vanhoutte 1993). ET-1 stimulated release of PGE_2 from the perfused rat mesenteric artery was reported (Tabuchi et al. 1989a).

ET_B receptors that are responsible for endothelial-dependent relaxation have been characterized in isolated perfused mesenteric vascular bed (D'Orleans-Juste et al. 1993; Warner et al. 1993). ET_A and ET_B receptors that induce vasoconstriction have also been found in perfused mesenteric vascular bed (D'Orleans-Juste et al. 1993; Warner et al. 1993), as well as in endothelium-denuded intact small mesenteric arteries and primary cultures of smooth muscle cells isolated from the mesenteric resistance arteries (Touyz et al. 1995). In small mesenteric arteries, ET_A receptors were thought to predominate and seemed to be the critical ones involved in vasoconstriction (Deng et al. 1995). However, a clear role for ET_B receptors in mediating constrictor responses to ET-1 in small mesenteric arteries without endothelium, which was only revealed when both ET_A and ET_B receptors were blocked, was demonstrated (Mickley et al. 1997). A similar phenomenon has also been reported in rabbit pulmonary artery (Fukuroda et al. 1994) and other non-vascular tissues (Clozel and Gray 1995; Fukuroda et al. 1996). It was suggested that a receptor crosstalk occurs and therefore

blockade of both ET_A and ET_B receptors may be required for effective inhibition of ET-1-induced vasoconstriction. (Fukuroda et al. 1996; Mickley et al. 1997).

2.2. Prostanoids: PGH_2, TxA_2

2.2.1. Synthesis /release and receptor blockade

The prostaglandin endoperoxide PGH_2, as mentioned above in section III.1.2, is an intermediate in the COX pathway of arachidonic acid metabolism (Moncada and Vane 1978a). Like PGI_2, thromboxane A_2 (TxA_2) is transformed enzymatically from PGH_2, but via an alternative metabolic pathway. TxA_2 synthase, which catalyzes the transformation of PG endoperoxides into TxA_2, has been found mainly in platelets, as well as in blood vessels (Moncada and Vane 1978a). In blood vessels, prostaglandins are mainly synthesized in and released from endothelial cells (Smith 1986). TxA_2 and PGH_2 stimulate contraction of vascular smooth muscle via interaction with a common receptor (Mais et al. 1985), which can be blocked by specific receptor antagonists, such as SQ 29,548 (Auch-Schwelk et al. 1990).

2.2.2. Effect of PGH_2/TxA_2 in normal and hypertensive mesenteric vascular bed of rats

Endothelium-dependent contractions with a variety of stimuli including arachidonic acid, ACh, the Ca^{2+} ionophore A23187, 5-HT and sudden stretch, that are sensitive to inhibitors of COX, occur in veins (De Mey and Vanhoutte 1982; Miller and Vanhoutte 1985), cerebral (Katusic et al. 1988; Katusic and Vanhoutte 1989; Shirahase et al. 1987; Toda et al. 1988), and pulmonary arteries (Altiere et al. 1986), and diabetic aorta (Tesfamariam et al. 1990; Tesfamariam et al. 1989), as well as in aorta (Kato et al. 1990; Kung and Luscher 1995; Lin et al. 1994; Luscher and Vanhoutte 1986) of aging and

hypertensive rats. Both PGH_2 and TxA_2 have been implicated as the endothelium-derived contracting factor.

In mesenteric resistance arteries, PGH_2 and TxA_2 have been reported to be involved in endothelium-dependent vasoconstriction (Lang et al. 1995; Noll et al. 1997), or in impaired endothelium-dependent relaxation (Carvalho et al. 1997; Diederich et al. 1990; Jameson et al. 1993; Li and Bukoski 1993; Luscher et al. 1990; Sunano et al. 1999; Takase et al. 1994; Watt and Thurston 1989) to ACh in genetic or experimental hypertensive rats. In addition, these contracting factors may also be responsible for the change in the vascular responsiveness to some contractile agonists such as Ang II and NE in hypertensive mesenteric arteries (Carvalho et al. 1997; Noll et al. 1997). The contribution of PGH_2 and TxA_2 may be different in different models of hypertension. For example, in mesenteric resistance arteries of adult SHR and DOCA-salt hypertensive rats, PGH_2 seems to mediate the endothelium-dependent contraction elicited by ACh, which opposes relaxation by endothelium-derived nitric oxide (Diederich et al. 1990; Luscher et al. 1990). In 2K1C renovascular hypertensive rats, the blockade of TxA_2/ PGH_2 receptors with ridogrel and inhibition of TxA_2 synthase with dazoxiben normalized the impaired relaxation response to ACh in the perfused mesenteric arterial bed, while the smooth muscle response to nitric oxide, tested with sodium nitroprusside, was unaltered. This suggests that the decreased responsiveness of smooth muscle to ACh resulted from an increase in TxA_2 formation rather than a decrease in sensitivity to NO in the mesenteric resistance vessels of this model of hypertensive rats (Carvalho et al. 1997).

2.3. Superoxide Anion (O_2^-)

2.3.1 Formation

O_2^- is generated though one-electron reduction of O_2 by NAD(P)H oxidases, and other enzymes, such as xanthine oxidase (Land and Swallow 1971). It is known that O_2^- is produced via the side-chain reaction of PGH synthase in the presence of NADH or NADPH (Kukreja et al. 1986). The rate of O_2^- generation was markedly inhibited by COX inhibitors when arachidonate was used as substrate (Kukreja et al. 1986).

It has been demonstrated that cultured endothelial cells could produce O_2^- under basal conditions and during such stimulation as reperfusion or treatment with bradykinin, A23187, interferon-γ, interleukin-1 or angiotensin II (Ang II) (Katusic 1996 and references therein; Zhang et al. 1999). Recently accumulated evidence has shown that Ang II stimulates production of O_2^- in blood vessels throughout the vascular wall, especially in the endothelium and adventitia (Di Wang et al. 1999; Nakane et al. 2000). Ang II also increased O_2^- production in cultured vascular smooth muscle cells (Griendling et al. 1994; Touyz and Schiffrin 1999). COX, xanthine oxidase and NADH oxidoreductase have all been identified as sources of O_2^- in the vascular endothelium (Cosentino et al. 1994; Holland et al. 1990; Kontos 1985; Mohazzab et al. 1994; Munzel et al. 1999; Rajagopalan et al. 1996; White et al. 1996).

2.3.2. Mechanism of action

O_2^- can be inactivated by superoxide dismutase (SOD) or may react with other free radicals, such as NO (Pryor 1994). Interaction of O_2^- with NO is very rapid and leads to production of an oxidant, peroxynitrite (Beckman et al. 1990). Thus, O_2^- may decrease the concentration of NO, favoring an increase in arterial tone, and increase formation of a potentially toxic free radical that may cause oxidative injury. In addition, increased

production of O_2^- in the blood vessel wall inhibits synthesis of PGI_2, but not that of TxA_2 (Katusic and Vanhoutte 1989; Moncada et al. 1976b). This effect may also contribute to impairment of endothelium-dependent relaxation and favor an increase in arterial tone.

2.3.3. Effects of O_2^- on endothelial function in mesenteric vasculature

O_2^- has been proposed as a possible endothelium-derived contracting factor (Katusic and Vanhoutte 1989; Vanhoutte and Katusic 1988), but evidence for a direct vasoconstrictor effect of O_2^- in vascular smooth muscle cells is missing (Katusic 1996). However, it has been repeatedly reported that vasoconstriction in response to agonists, such as A23187, U46619, NE and Ang II, could be inhibited by SOD or potentiated by SOD inhibitors in canine basilar artery, rabbit renal afferent arterioles and rat aorta, respectively (Katusic et al. 1993; Katusic, Vanhoutte 1989; Kawazoe et al. 2000; Laight et al. 1998; Schnackenberg et al. 2000). The effects were endothelium-dependent, and NOS inhibitors could either restore the vasoconstriction in the presence of SOD or abolish the potentiation produced by inhibitors of SOD (Laight et al. 1998; Schnackenberg et al. 2000). The results suggested that the major mechanism responsible for participation of O_2^- in endothelium-dependent contractions is inactivation of NO. O_2^- was also reported to suppress the modulatory influence of endogenous NO on Ang II-induced afferent arteriolar constriction in diabetic rats (Schoonmaker et al. 2000) and a NE-induced pressor response in aorta from SHR rats (Wu et al. 1998).

O_2^- effects on endothelium function by increasing the breakdown of NO were also found in rat mesenteric vasculature. In rat small mesenteric arteries, it was shown that SOD caused an endothelium-dependent relaxation of NE-induced tone and potentiated

endothelium-dependent relaxation to ACh (Sunman et al. 1993). This effect of SOD has been attributed to its ability to scavenge O_2^- that inactivates basal and ACh-induced NO release. In the perfused mesenteric microcirculation of rats, application of Ang II induced an immediate production of O_2^- and vasoconstriction that was inhibited by SOD (Kawazoe et al. 1999). In addition, when NO was directly measured in isolated mesenteric small artery rings, an increased NO decomposition by O_2^- was observed in adult (15 week-old) stroke-prone spontaneously hypertensive rats (SHRSP) as compared with age-matched normotensive Wistar-Kyoto rats, although NO release remained unaffected (Tschudi et al. 1996). Furthermore, the endothelium-dependent vasoconstriction to high concentrations of ACh seen in small mesenteric arteries from prehypertensive SHR rats (4 week-old), but not in normotensive Wistar-Kyoto rats, seems to be mediated by O_2^-, which interferes with the effects of NO (Jameson et al. 1993).

V. ABNORMALITIES IN HYPERTENSION

The hemodynamic characteristic of established hypertension is an increase in total peripheral resistance. Factors thought to contribute to the increased resistance include: 1) augmented humoral responses and increased sympathetic nerve activity, 2) impaired endothelium function, and 3) structural changes, particularly in peripheral resistance vessels (Conway 1984; Folkow 1982; Mulvany 1994).

This section will mainly describe the changes in smooth muscle and endothelial function in renovascular hypertension and insulin resistance with hypertension.

1. Goldblatt 2K1C Renovascular Hypertension

1.1. 2K1C Rat Model of hypertension

The 2K1C hypertensive rat is one of the models of Goldblatt hypertension that also includes the one-kidney, one-clip (1K1C) model of experimental hypertension.

Goldblatt and his colleagues (Goldblatt et al. 1934), in 1934, produced a reliable model of renal hypertension by constricting the renal arteries of a dog with adjustable silver clamps. Very quickly, this technique was adapted for use in other small mammals. In 1939 Wilson and Byrom (Wilson and Byrom 1939) adapted the silver clip technique to rat, and successfully produced persistent hypertension by partially occluding the left renal artery and leaving the other kidney untouched (Goldblatt two-kidney one-clip hypertension). It is well established that unilateral renal ischaemia causes hypertension in humans. The 2K1C renal hypertensive rat is the experimental counterpart of human renovascular hypertension, and has been widely used for exploring the mechanisms of the genesis and sustenance of hypertension in past decades (Martinez-Maldonado 1991).

1.2. Peripheral Resistance in 2K1C Hypertensive Rats

The majority of hemodynamic studies in the 2K1C model indicate that the elevated blood pressure in both early and chronic phases depends on an increased peripheral vascular resistance (Averill et al. 1976; Hallback-Nordlander et al. 1979; Russell et al. 1983). It has been shown that mesenteric vasculature is a key area for the increased peripheral resistance in renovascular hypertension (Faber and Brody 1983; Meininger et al. 1984; Meininger et al. 1985; Teranishi and Iriuchijima 1985). The increased peripheral resistance of early phase 2K1C hypertensive rats could due to a neurohumoral mechanism causing a functional increase in vascular smooth muscle tone, but in the established phase there may be

reinforcement by structural changes within the blood vessels. This is discussed in the next section.

1.3. Structural Alterations

Structural changes develop rapidly in 2K1C hypertensive rats. Left ventricular hypertrophy could be demonstrated within 7 days of renal artery constriction and vascular changes after approximately 3 weeks (Lundgren and Weiss 1979). Later, these indirect observations using the isolated perfused hindlimb preparation were confirmed by *in vitro* morphological measurements of isolated mesenteric resistance arteries from 2K1C hypertensive rats, 4 weeks after renal artery constriction (Mulvany and Korsgaard 1983). In addition, another study (Deng and Schiffrin 1991), which investigated and quantified alterations in structure as well as in reactivity to different agents, also reported a significant reduction in external and lumen diameters, increased media width and increased media-to-lumen ratio in small mesenteric arteries from 2K1C rats. The alteration occurred within 6 weeks of development of hypertension and was accompanied by significant increases in active wall pressure produced in response to NE and arginine vasopressin (AVP). Thus, it was suggested that rapid and early structural changes, which enhance vascular reactivity to vasoconstrictors, might contribute to the maintenance of the elevated blood pressure. However, structural changes in the resistance vessels cannot be the sole determinant of the raised peripheral resistance, because removal of the constricting clip in 2K1C hypertensive rats resulted in a rapid fall in blood pressure to normal levels within 24h (Ferrario 1974; Russell et al. 1983; Thurston et al. 1980), whereas structural vascular changes take several

weeks to resolve after reversal of hypertension (Lundgren and Weiss 1979; Watt and Thuston 1990)

1.4. Changes in Contractile Response in Mesenteric Vasculature of 2K1C Hypertensive Rats

It is well accepted that the renin-angiotensin system participates in the pathophysiology of the 2K1C hypertension (Carretero and Gulati 1978; Martinez-Maldonado 1991). It is known that dramatically parallel increases in BP, plasma renin activity and circulating angiotensin II (Ang II) concentrations occur in the period immediately following constriction of the renal artery, whereas over the course of 6 weeks, plasma Ang II levels tend to fall back to near normal but BP remains elevated or continues to increase (Morton and Wallace 1983). It has been shown that blockade of the renin-angiotensin system with antibody to Ang II, a specific competitive antagonist of Ang II or an angiotensin-converting enzyme inhibitors, lowers the blood pressure of 2K1C hypertensive rats in the early phase (Bennett and Thurston 1996; Bing et al. 1981; Brunner et al. 1971; Pals et al. 1971; Tokioka et al. 2000). In addition, an increase in vascular responsiveness to Ang II has been demonstrated in the rat isolated perfused mesenteric arterial bed (McGregor and Smirk 1968) and perfused untouched kidney (Collis and Vanhoutte 1978), and in isolated rabbit aorta, renal, and iliac strips in both early and/or later phases of 2K1C hypertension (Yoshida et al. 1987). Moreover, studies in whole animals showed an increase in the pressor response to low levels of Ang II in the chronic phase (Melaragno and Fink 1995) or after the constricting clip had been removed from the renal artery, which leads BP fall (Skulan et al. 1974). By contrast, vascular reactivity to Ang II was reported to be decreased in rat mesenteric arteries in the early phase of 2K1C hypertension (Benedetti and Linas 1987). Another study also

showed that the pressor response to Ang II was decreased in both early and chronic 2K1C hypertensive rats (Marks et al. 1979). In addition, inhibition of the renin-angiotensin system with Ang II antagonists or by angiotensin-converting enzyme (ACE) inhibition only produced a partial fall in blood pressure in early-phase hypertension (Benetos et al. 1986), and only the converting enzyme inhibition reduced the blood pressure in rats with chronic 2K1C hypertension (Bing et al. 1981; Dickinson and Yu 1967). The latter phenomenon has been attributed to the fact that during ACE inhibition, tissue bradykinin levels increase, causing vasodilation and the subsequent lowering of BP (Benetos et al. 1986; Lindsey et al. 1983). Thus, other factors may also contribute to sustained BP in both early and chronic renovascular hypertension.

An increased vascular reactivity to NE, either in *vivo* (Carvalho et al. 1997; Fortes et al. 1992; Fortes et al. 1990) or *in vitro* (Carvalho et al. 1997; Fortes et al. 1992; Haeusler and Haefely 1970; McGregor and Smirk 1968; Tsuda et al. 1989), in the rat mesenteric arterial bed from the early phase (3-6 weeks after surgery) in 2K1C hypertensive rats has been reported. The increased responsiveness to NE was also observed in perfused rat hindquarters (Baum and Shropshire 1967; McQueen 1961; Mistry et al. 1983) and isolated rabbit renal and iliac arterial strips (Yoshida et al. 1987) from 2K1C models. In addition, Collis (Collis and Alps 1975) found a significant positive correlation between BP and vascular reactivity to NE in the mesenteric arterial bed from renal hypertensive rats, as did McQueen (McQueen 1961) in hindquarters. Furthermore, it was found that intravenous injection of nanonmolar concentrations of neuropeptide Y or NE caused a greater dose-dependent pressor response in anesthetized 2K1C hypertensive rats as compared to normotensive controls (Mezzano et al. 1998). The mechanism that produced exaggerated responsiveness to NE in mesenteric

vasculature from 2K1C rats has not been thoroughly studied. Apart from the structural alterations (see above), whether or not there is altered peripheral adrenergic function is not clear. One study showed that the enhanced superior mesenteric resistance to flow in 2K1C hypertensive rats was largely ascribable to a sympathetic neural mechanism (Shimamoto and Iriuchijima 1989). Schiffrin (Schiffrin 1984;) reported that the density of α_1-adrenergic receptors was significantly decreased, while the affinity was significantly increased in the mesenteric vascular bed of renal hypertensive rats (both 2K1C and 1K1C), and suggested this might be secondary to increased sympathetic nervous activity. In contrast, Tsuda *et al* (Tsuda et al. 1989) showed that NE release and pressor responses during periarterial nerve stimulation were unchanged in isolated mesenteric vasculature during the acute phase (3 weeks after surgery) of hypertension, and were rather reduced during the chronic phase (7-8 weeks after surgery) in 2K1C compared to sham normotensive control rats, while responses to exogenous NE were significantly increased. In contrast, Cauvin and Pegram (Cauvin and Pegram 1983) found that in isolated mesenteric small resistance arteries, responses to exogenous NE were comparable in 2K1C hypertensive and sham-operated normotensive rats after a 2-week period of clipping. However, few studies have been carried out to clarify the post-adrenergic receptor alterations, including calcium handling, in mesenteric arteries from 2K1C hypertensive rats.

Impaired endothelial function could also contribute to the abnormal vasoconstriction. It has been reported that endothelium-dependent relaxation to ACh was attenuated in aorta (Heitzer et al. 1999; Van de Voorde and Leusen 1986) or mesenteric arteries from 2K1C hypertensive rats (Bennett et al. 1993; Carvalho et al. 1997; Cauvin, Pegram 1983; Fortes et al. 1992). In mesenteric arteries, most of the studies found that incubation with the COX

inhibitor indomethacin restored the ACh relaxation, suggesting that the abnormal endothelial-dependent relaxation is due to an increased release of an EDCF (Bennett et al. 1993; Fortes et al. 1992), probably TxA_2, as mentioned above (Carvalho et al. 1997). These *in vitro* results are consistent with studies in whole animals with NOS inhibitors, which have suggested that there was no deficiency in NO but rather that NO serves as an important buffer mechanism by counterbalancing COX-sensitive vasoconstriction, thereby lessening renal artery clipping-induced blood pressure elevation (Huang et al. 2000; Sigmon and Beierwaltes 1995; Sigmon and Beierwaltes 1998). However, the contribution of COX pathway products to NE-induced responses is uncertain. It has been reported that incubation with indomethacin had no effect on the increased reactivity to NE either *in vivo* or *in vitro* in the mesenteric arterial bed (Fortes et al. 1992), while TxA_2 receptor antagonists inhibited the potentiated response to NE *in vitro*, but not *in vivo* in mesenteric arterial bed (Carvalho et al. 1997).

2. Hypertension and Hyperinsulinemia /Insulin Resistance

2.1. Insulin Hypothesis of Hypertension

An independent association of hypertension with hyperinsulinemia in insulin resistance states is well established from epidemiological studies (He et al. 1999; Lissner et al. 1992; Manicardi et al. 1986; Masuo et al. 1997; Modan et al. 1985; Salonen et al. 1998; Skarfors et al. 1991; Tsuruta et al. 1996). Primary insulin resistance is defined as a reduced ability of insulin to stimulate glucose uptake, principally in skeletal muscle. Insulin resistance is an abnormal state and a common feature of type 2 diabetes mellitus and obesity, that also share an association with hypertension (Manicardi et al. 1986; Modan et al. 1985; Reaven 1988). Compensatory hyperinsulinemia is a signal of the presence of insulin resistance, and

is also found in essential hypertension regardless of obesity and glucose intolerance (Ferrannini et al. 1987). It has been reported that high BP is positively correlated with fasting plasma insulin levels independently of the effect of age, obesity, fasting glycaemia and antihypertensive medications (Denker and Pollock 1992; Modan et al. 1985; Salonen et al. 1998; Tsuruta et al. 1996). In addition, hyperinsulinemia occurs in the young normotensive offspring of patients with essential hypertension (Ferrari et al. 1991; Grunfeld et al. 1994), and is associated with an increased incidence of hypertension in men (Salonen et al. 1998; Skarfors et al. 1991) and women (Lissner et al. 1992), in both African Americans and whites (He et al. 1999), as well as in non-obese and non-diabetic Japanese (Tsuruta et al. 1996). Furthermore, several genetically and experimentally hypertensive animal models, such as SHR and fructose fed hypertensive rats, also demonstrate insulin resistance and hyperinsulinemia (Hwang et al. 1987; Mondon and Reaven 1988). Interestingly, McNeill' s group has repeatedly reported that chemically diverse drugs that have the common property of attenuating hyperinsulinemia also lower BP in both SHR and fructose hypertensive rats, and the antihypertensive effects of these drugs could be reversed by simply restoring the plasma insulin levels in the drug-treated rats to those that existed before drug treatment (Verma and McNeill, 1999 and references therein). These findings have led to the hypothesis that insulin may be of primary pathophysiological importance in the development of hypertension.

2.2. Possible Mechanisms of Association Between Hyperinsulinemia/Insulin Resistance and Hypertension: Vascular Action of Insulin

A number of mechanisms may contribute to the development of hypertension associated with hyperinsulinemia/ insulin resistance. Hyperinsulinemia and insulin resistance

may independently alter vascular reactivity of arterial blood vessels, and thereby BP, although it is not known yet if this relationship is causal.

To ascribe a causal role to hyperinsulinemia in the pathogenesis of essential hypertension, sensitivity to the possible blood pressure elevating actions of insulin should be preserved despite resistance to the glucose-lowering action of the hormone. It has been demonstrated that insulin can promote renal tubular sodium reabsorption (DeFronzo 1981; DeFronzo et al. 1975), stimulate sympathetic nerve activity and increase catecholamine levels both in humans and in animals (Dornfeld et al. 1987; Liang et al. 1982; Rowe et al. 1981; Sowers et al. 1982). Thus, hyperinsulinemia could increase vascular resistance and arterial pressure (Landsberg 1986; Reaven 1988). However, evidence against this notion also exists (Anderson et al. 1992; Anderson et al. 1991).

Recently, it has been suggested that direct interaction of insulin with blood vessels may be a potentially important link to hyperinsulinemia/insulin resistance to hypertension (Brands et al. 1998; Yki-Jarvinen and Utriainen 1998). In the isolated rat mesenteric arterial bed, insulin at physiological concentrations was shown to significantly increase pressor responses to exogenous NE (Townsend et al. 1992; Verma and McNeill, 1999), and to potentiate arginine vasopressin (AVP)-induced vasoconstriction (Wu et al. 1994). The insulin-induced potentiation of the response of MAB to NE has been reported to be further augmented in arteries from fructose hypertensive rats, suggesting chronic hyperinsulinemia may serve to increase peripheral vascular resistance (Verma and McNeill 1999). Insulin has also been shown to enhance proliferation of vascular smooth muscle cells (Ridray 1995; Stout et al. 1975) and to increase ET-1 gene expression (Oliver et al. 1991) and ET-1 release (Hattori et al. 1991; Hu et al. 1993). In rat femoral arteries, an insulin-mediated increase in

contraction to KCl was significantly reduced in the presence of ET-1 receptor antagonists, suggesting a role for ET-1 (Nava et al. 1997). In addition, Verma et al have demonstrated that the MAB from fructose hypertensive rats contained greater absolute amounts of ET-1 than the control rats (Verma et al. 1995). They also reported that chronic ET-1 receptor blockade completely prevented the rise in BP in these rats and proposed the possibility that hyperinsulinemia may serve as a continual stimulus for ET-1 synthesis, leading to increased peripheral resistance and raised BP. Furthermore, insulin may also exert a vascular action through regulating production of COX pathway metabolites (Axelrod and Levine 1983; Axelrod et al. 1986; Keen et al. 1997; Rebolledo et al. 1998; van Veen and Chang 1997). It has been reported that inhibition of TxA_2 synthesis attenuated hypertension induced by chronic insulin infusion in SD rats (Keen et al. 1997). Thus, interplay between EDCF and insulin may be important in regulating vascular reactivity, and thereby peripheral resistance and blood pressure.

In addition to its pressor action, insulin at physiological concentration has been shown to produce vasodilation of skeletal muscle vascular beds in humans (Laakso et al. 1990). This vasodilatory action has been shown to be impaired in states of insulin resistance, such as obesity and type 2 diabetes (Laakso et al. 1990; Laakso et al. 1992). It has been suggested that impairment of insulin-mediated vasodilation may contribute to the increase in peripheral resistance, the characteristic of hypertension (Feldman and Bierbrier 1993). The insulin-induced increase in blood flow could be abolished by L-NMMA (Scherrer et al. 1994) and this *in vivo* observation is supported by a study in isolated skeletal muscle arterioles (Chen and Messina 1996). Insulin attenuation of NE-induced vasoconstriction by stimulation of NO release was also observed in isolated rat mesenteric resistance arteries

(Walker et al. 1997b). Recently, insulin was reported to directly increase NO production in cultured human umbilical vein endothelial cells (Zeng and Quon 1996). Insulin may also regulate NO production by increasing availability of the cofactor BH_4 for activation of NO synthas (Verma et al. 1998). Insulin may also exert a modulatory effect on local vasodilator responses by increasing Na^+-K^+ ATPase and Ca^{2+}-ATPase activity (Sowers et al. 1991; Tirupattur et al. 1993).

Taken together, these data suggested that the link between hyperinsulinemia/ insulin resistance and hypertension is likely to be complex and multifactorial. However, it has been suggested that interplay between insulin and endothelial factors may be an important factor in regulating vascular reactivity, and thereby peripheral resistance and blood pressure (Baron 1999; Brands et al. 1998; Nava et al. 1999; Yki-Jarvinen, Utriainen 1998). Hence in hyperinsulinemia/insulin-resistant states a blunted vasodilator and/or an exaggerated pressor effect of insulin may cause an increase in peripheral resistance leading to hypertension.

2.3. An Animal Model of Hypertension with Insulin Resistance and Hyperinsulinemia: the Zucker Obese Rat

2.3.1. Characteristics of Zucker obese rats

The Zucker strain of obese rats represents an animal model that combines obesity heredity with insulin resistance, hyperinsulinemia and hyperlipidemia. By contrast, lean Zucker rats are normal in this regard.

Zucker obese rats were first described by Zucker and Zucker (Zucker and Zucker 1961). The obesity is transmitted through an autosomal recessive gene. By 5 weeks of age, animals that are homozygous for this trait (*fa/fa*) show visible differences in body fat content and in body shape. Hyperinsulinemia appears as early as 3 to 4 weeks of age (Bray

1977). Circulating insulin levels have been shown to be 3 to 10 fold higher, while concentrations of plasma glucose are normal as compared with age-matched lean Zucker rats (Bray 1970; Ionescu et al. 1985; York et al. 1972). The major site of insulin resistance in obese Zucker rats appears to be skeletal muscle (Crettaz et al. 1980; Kemmer et al. 1979; Smith and Czech 1983) and involves both receptor and post-receptor abnormalities (Slieker et al. 1990; van de Werve et al. 1987). Hyperlipidemia is uniformly present in obese Zucker rats (Witztum and Schonfeld 1979). Although total cholesterol levels were higher in obese than in lean Zucker rats, the most striking abnormality was found in triglycerides (TG). The abnormalities in lipids occur at an early age and the levels of TG and cholesterol increase with age (Bray 1977; Kasiske et al. 1988; Kasiske et al. 1991).

There is evidence that obese Zucker rats develop a modest hypertension at an older age than the metabolic changes (Cox and Kikta 1992; Kurtz et al. 1989). Cox and Kikta (Cox and Kikta 1992) measured systolic BP of Zucker rats indirectly on a weekly basis from the age of 6 weeks up to 36 weeks. They found a significantly higher arterial pressure developing in the obese group between 24 and 36 weeks than lean littermates. However, the data in the literature in this regard are not consistent. Some studies were unable to find a difference in blood pressure in obese Zucker rats relative to lean littermates (Auguet et al. 1989; Bunag and Barringer 1988; Pawloski et al. 1992). The variable findings could result from different measurement techniques (Bunag 1983), from differences in the age and or sex of rats studied, or from differences in the animal colonies. Recently Alonso-Galicia *et al* measured BP continuously 24 h per day in conscious chronically instrumented, age-matched lean and obese Zucker rats, with carefully controlled NaCl intake, and found that the obese Zucker rats were hypertensive at age 13 to 14 weeks of age (Alonso-Galicia et al. 1996).

Zemel and coworkers also reported an increased blood pressure (both systolic and diastolic pressure) by direct measurement in 10-week old obese Zucker rats (Zemel et al. 1990). The increase in MAP was about 14 to 20mmHg in the studies that reported a greater BP in obese Zucker rats as compared with lean Zuckers (Alonso-Galicia et al. 1996; Bohlen and Lash 1995; Kam et al. 1996; Paulson and Tahiliani 1992; Wu et al. 1996). It has been suggested that the mild hypertension that develops in the obese rats is not dependent on increased body weight *per se* since moderate caloric restriction, achieved by pair-feeding with lean rats, decreased weight gain but did not attenuate hypertension (Kurtz et al. 1989).

Thus, the obese Zucker rat may be a useful animal model for detailed and controlled investigation into the abnormalities of smooth muscle and endothelium function in hypertension associated with hyperinsulinemia/insulin resistance, and how hyperinsulinemia /insulin resistance may be linked to the pathogenesis of hypertension.

2.3.2. Changes in vascular reactivity

The evidence that the hypertension observed in obese Zucker rats may be dependent on exaggerated vascular reactivity comes from the observation that the obese Zucker rats exhibited greater pressor sensitivity to both Ang II and NE during ganglionic blockade (Zemel et al. 1992).

Studies with the conduit vessel, aorta, revealed an enhanced sensitivity to vasoconstrictors, which was independent of endothelium function and structural changes (Cox and Kikta 1992; Ouchi et al. 1996; Turner et al. 1995; Zemel et al. 1991; [Hopfner, 1998 #319]). In contrast, Kam *et al* reported that there was no significant difference in the sensitivity and maximum response to NE, methoxamine or serotonin in isolated small

mesenteric arteries from obese and lean Zucker rats at age of 22 weeks, when the obese rats were hypertensive (Kam et al. 1996). In agreement with this, Wu *et al* did not find a difference in the contractile sensitivity to NE between the isolated perfused mesenteric arterial beds from 25 week-old hypertensive obese Zucker rats and lean controls (Wu et al. 1996). An unchanged reactivity to PE or NE was also observed in isolated small mesenteric arteries from young (12 week-old) pre-hypertensive (Walker et al. 1997a) and in perfused mesenteric arterial beds from older (32 week-old) hypertensive (Turner et al. 1995) obese Zucker rats. Interestly, it has been reported that the maximum tension development for ET-1- and methoxamine-evoked vasoconstrction in perfused MAB was slightly but not significantly lower in 12-week old hypertensive obese compared with lean Zucker rats (Hopfner et al. 1999). The results of the studies of the endothelium function in mesenteric vasculature are not consistent. Endothelium-dependent relaxation in response to ACh, ADP or methacholine were reported to be normal in intestinal arteriole, small mesenteric arteries and mesenteric arterial bed of obese Zucker rats as compared to lean Zucker rats (Bohlen and Lash 1995; Kam et al. 1996; Turner et al. 1995; (Hopfner et al. 1999). In other studies, the relaxation-induced by ACh was attenuated in obese mesenteric resistant arteries (Walker et al. 1997a; Wu et al. 1996; Zanchi et al. 1995), while the responses to ADP (Wu et al. 1996) and A23187 (Walker et al. 1997a) were not significantly different from those in lean Zucker rats. Endothelium-independent relaxation of mesenteric arteries to sodium nitroprusside (SNP) was not impaired in obese Zucker rats as compared with lean Zucker rats (Kam et al. 1996; Turner et al. 1995). Furthermore, there were no significant structural changes in the resistance vessels from obese Zucker rats when the passive tension-

circumference relationships and morphological characteristics were evaluated (Bohlen and Lash 1995; Kam et al. 1996).

The effects of insulin on the reactivity of mesenteric vasculature from Zucker obese rats have not been well characterized. Two studies examined the influence of exogenous insulin on vasoconstriction induced by α-adrenoceptor agonists. Insulin was reported to have no effect in either lean or hypertensive obese Zucker rats in one study (Turner et al. 1995), and to have a small inhibitory effect (8 to 13% inhibition in the presence of 50 to 5000 mU/l insulin) in lean but not pre-hypertensive obese Zucker rats in the other (Walker et al. 1997a). The latter observation suggested that insulin-induced attenuation of NE-mediated vasoconstriction is impaired in the obese Zucker rat, and that this defect precedes, and therefore could contribute to, the development of hypertension in this insulin-resistant animal model (Walker et al. 1997a).

VI. SUMMARY

The mesenteric arterial bed plays an important role in the maintenance and control of peripheral resistance. The sympathetic neuronal control of mesenteric vascular tone appears to predominate along the mesenteric arterial tree, and is mainly mediated by the neurotransmitter NE acting on α_1-adrenoceptors. A particular feature of the excitation-coupling properties of the mesenteric artery smooth muscle, especially the smooth muscle of small arteries, appears to be the dependence of tone on voltage-operated Ca^{2+} channels and, in turn on the membrane potential. Endothelium-derived factors interact with neuronal, humoral and myogenic determinants to help maintain the normal resistance, or change it in response to metabolic demand.

Further investigation into the factors determining these characteristics and the mechanisms that regulate smooth muscle tone, and how they are altered in hypertension may be expected to provide important information as regards our understanding of the control of the cardiovascular system in health and disease.

This dissertation work examined some cellular mechanisms that regulate smooth muscle reactivity and endothelium functionality in rat mesenteric vasculature, as well as the abnormalities in hypertensive states. The topics of the three parts of the study focus on: 1) whether agonist-induced Cl^- current contributes functionally to the VSM response to α_1-adreoceptor activation, and the possible functional changes in Cl^- channels in renovascular hypertensive rats; 2) the role of Cl^- and K^+ channels in ACh-induced endothelium-dependent vasorelaxation and the factors that mediate the responses; and 3) how endothelium-derived relaxing and contracting factors regulate vascular reactivity to catecholamines and how abnormal release of these vasoactive factors contributes to the vascular abnormalities in hypertensive rats with hyperinsulinemia and insulin resistance. The hypotheses and specific research objectives for each part of the study are described in the respective Chapters.

PART 1. THE CONTRIBUTION OF CHLORIDE CHANNELS TO ALPHA$_1$-ADRENOCEPTOR MEDIATED VASOCONSTRICTION IN RAT MESENTERIC ARTERY

I. RATIONALE

α_1-adrenergic receptors play an important role in the control of vascular smooth muscle contraction and thereby, in regulation of peripheral resistance, blood flow and blood pressure.

The contraction mediated by the α_1-adrenoceptor depends mainly on an increase of free intracellular calcium concentration that results from Ca^{2+} release from intracellular organelles (i.e. the sarcoplasmic reticulum) and/or influx from extracellular fluid. It is clear that in smooth muscle cells, activation of α_1-adrenoceptors causes formation of inositol 1,4,5,-triphosphate which promotes Ca^{2+} release from intracellular stores. The mechanism by which the receptor activation opens cell surface Ca^{2+} channels is still an interesting topic attracting many researchers' attention (Minneman 1988; Clapham 1995; Fasolato et al. 1994; Mironneau and Macrez-Lepretre 1995)

In vascular smooth muscle, α_1-adrenoceptor-mediated contraction is usually accompanied by a depolarization and an increase in membrane conductance (Bolton et al. 1984; Byrne and Large 1987; Casteels et al. 1977; Mekata and Niu 1972; Takata 1980). Since it is well known that Cl^- is concentrated inside the smooth muscle cell (Aickin and Brading 1982; Chipperfield et al. 1993; Davis 1992; Davis et al. 1991; Gerstheimer et al. 1987) and its equilibrium potential is more positive than the resting membrane potential (see Introduction), the Cl^- conductance must represent a potentially important depolarizing

mechanism. In rat portal vein, it was demonstrated that NE greatly increased Cl^- efflux with a smaller effect on K^+ efflux and no influence on Na^+ flux. This indicates that α_1-adrenoceptor activation increased Cl^- permeability (Wahlstrom 1973b). The involvement of Cl^- ions in NE-induced depolarization was also confirmed in rat mesenteric arteries where NE increased Cl^- efflux when producing depolarization without altering the rate of K^+ efflux or Na^+ influx (Videbaek et al. 1990). In addition, microelectrode recording from guinea pig mesenteric veins showed that the reversal potential of NE-stimulated current is the same as E_{Cl}. Lowering external Cl^- concentration suppressed the rapid depolarization produced by NE. This implies that an increased Cl^- conductance is responsible for the NE-induced depolarization (Van Helden 1988). Although membrane depolarization may result from either an influx of cation or efflux of anion, current evidence in the literature favors the latter mechanism for α-adrenoceptor-mediated depolarization.

A calcium activated chloride channel ($I_{Cl(ca)}$) has now been identified in several types of blood vessels, and can be activated by a number of vasoconstrictor agonists (Amedee et al. 1990b; Byrne and Large 1988b; Droogmans et al. 1991; Klockner 1993; Pacaud et al. 1989a; Van Renterghem and Lazdunski 1993; Wang and Large 1993). The properties of $I_{Cl(Ca)}$ have been intensively studied in single VSM cells. The whole cell patch pipette recording technique has given the most convincing data, which demonstrated that pharmacological agonists utilize intracellular Ca^{2+} stores to evoke $I_{Cl(Ca)}$, while extracellular Ca^{2+} is not essential for activation of $I_{Cl(Ca)}$. (Amedee et al. 1990b; Droogmans et al. 1991; Pacaud et al. 1992; Pacaud et al. 1989b; Wang and Large 1993). In addition, it was shown that activation of the Cl^- channels by NE could depolarize the membrane (see above references) and that the depolarization brought the membrane potential to between -20 and -30 mV. At these values,

the open-state probability of Ca^{2+} channels is high (Pacaud et al. 1989b). Based on the evidence obtained from electrophysiological studies, it was suggested that in vascular smooth muscle, α_1-adrenoceptor-mediated calcium release from intracellular stores activates the Cl^- channels leading to changes in membrane potential. The resulting depolarization could then stimulate calcium entry through voltage-dependent calcium channels (Amedee et al. 1990b; Hogg et al. 1993; Pacaud et al. 1991; Pacaud et al. 1992; Pacaud et al. 1989b). Thus, the most likely role of $I_{Cl(Ca)}$ in vascular smooth muscles is to produce membrane depolarization and subsequently Ca^{2+} entry and sustained vasoconstriction, especially in response to excitatory agonists.

The lack of a potent selective antagonist has been an obstacle in evaluating the physiological role of $I_{Cl(Ca)}$ (Doughty et al. 1998; Large and Wang 1996). Recently, electrophysiological studies have demonstrated that NFA, a nonsteroidal anti-inflammatory agent, is a potent reversible blocker of $I_{Cl(Ca)}$ (White and Aylwin 1990). In some vascular smooth muscle cells, NFA seemed to block $I_{Cl(Ca)}$ when the channels were open (Hogg et al. 1994a). Unlike other Cl^- channel blockers, it inhibits agonist-evoked $I_{Cl(Ca)}$ at concentrations in the micromolar range (Hogg et al. 1994a; Lamb et al. 1994; Pacaud et al. 1989b). At concentrations up to $5x10^{-5}$ M, NFA did not (1) reduce the NE-evoked non-specific cation current (Hogg et al. 1994a), (2) inhibit voltage-dependent Ca^{2+} channels (Hogg et al. 1994a; Lamb et al. 1994); or (3) evoke a K^+ current (Greenwood and Large 1995; Ottolia, Toro 1994; Xu et al. 1994). It was also suggested that NFA did not inhibit at the α_1-adrenoceptor recognition site or NE-induced release of Ca^{2+} from the intracellular stores, since NFA did not inhibit NE-evoked Ca^{2+}-activated K^+ current (Greenwood and Large 1995; Hogg et al.

1994a). Therefore, NFA seems to be a potentially useful tool for evaluation of the role of $I_{Cl(Ca)}$ in α_1-adrenoceptor-induced contraction.

At the time this project was started, no studies on the functional role of Cl^- channels in α_1-adrenoceptor-mediated vasoconstriction in mesenteric arteries were available. However, during the course of the investigation, Criddle et al (Criddle et al. 1996) demonstrated that at a concentration of 10μM, NFA produced a comparable attenuation of a component of the NE-evoked contraction when compared with the Ca^{2+} channel antagonist nifedipine in rat isolated aorta. Later, they also reported that NFA (30 μM) could reduce a component of NE-induced pressor responses in rat mesenteric arteries (Criddle et al. 1997). However, they did not examine in either study whether the reduction of the mechanical response in blood vessels by NFA could be attributed to the specific blockade of $I_{Cl(ca)}$. Moreover, there is no evidence available in the literature to indicate whether NFA can affect α_1-adrenoceptor-mediated vasoconstriction *in* vivo, nor any study on its effects in hypertension.

The 2K1C renovascular hypertensive rat has been widely used in investigations of the mechanisms producing and maintaining hypertension. Hemodynamic studies in the 2K1C rat indicate that the 2K1C hypertensive rat is associated with increased peripheral resistance and the main increase in resistance lies in resistance vessels, especially in the mesenteric vascular bed (Meininger et al. 1984; Russell et al. 1983; Teranishi and Iriuchijima 1985). Experiments in 2K1C rats showed that the sensitivity of mesenteric vasculature to NE is increased (Carvalho et al. 1997; Fortes et al. 1990; McGregor and Smirk 1968). There is no unequivocal explanation of the molecular mechanism(s) that mediate the increased sensitivity to NE in 2K1C hypertensive rats, and little is known about changes in the signal transduction

pathways of the α_1-adrenoceptor that are responsible for membrane depolarization, and thereby Ca^{2+} influx in this type of hypertension (see Introduction for more details).

II. WORKING HYPOTHESES AND SPECIFIC RESEARCH OBJECTIVES

The major aim of this part of the study was to obtain further information about the possible physiological role of the $I_{Cl(Ca)}$ in the process of α_1-adrenoceptor-mediated vasoconstriction, and therefore in regulation of blood flow and blood pressure. In these experiments, we have used NFA as a tool to analyze the functional role of the $I_{Cl(Ca)}$ in α_1-adrenoceptor-mediated vasoconstriction both *in vitro* and *in vivo* in mesenteric resistance arteries. The change in the function of $I_{Cl(Ca)}$ that mediates α_1-adrenoceptor-mediated contraction in hypertensive rats was also examined. The following working hypotheses and specific objectives were addressed.

Working Hypotheses

A. Blockade of calcium-activated chloride channels with niflumic acid (NFA) inhibits α_1-adrenoceptor-induced vasoconstriction in rat mesenteric artery both *in vitro* and *in vivo*. The inhibitory effect of NFA may be greater in hypertensive rats due to an increased functional contribution by Cl^- channels.

B. The decrease in α_1-adrenoceptor-induced contraction due to chloride channel inhibition with NFA, in rat mesenteric artery, results from an indirect inhibition of voltage-gated nifedipine-sensitive Ca^{2+} channels.

In other words:

A. Niflumic acid (NFA), a putative selective calcium-activated chloride channel antagonist, inhibits α_1-adrenoceptor-induced vasoconstriction in rat mesenteric artery both *in vitro* and *in vivo*. The inhibitory effect of NFA may be greater in hypertensive rats than that in normotensive rats.

B. NFA, in rat mesenteric artery, inhibits α_1-adrenoceptor-induced contraction by blocking a chloride channel, leading to an indirect inhibition of voltage-gated nifedipine-sensitive Ca^{2+} channels.

Specific Objectives:

Functional studies:

1) To examine the influence of NFA and Cl^- free solution (propionate ions as substitute) on the vasopressor response to cirazoline, a selective α_1-adrenoceptor agonist, in rat isolated perfused mesenteric arterial beds (MAB).

2) To investigate the effects of NFA on cirazoline-induced changes in vascular conductance in the superior mesenteric artery in pentobarbital-anaesthetized rats.

3) To compare the vascular effects of NFA on α_1-adrenoceptor-stimulated vasoconstriction in two kidney one-clip (2K1C) hypertensive rats to those in normotensive rats both *in vitro* and *in vivo*.

To rule out that NFA has a direct effect on cirazoline-evoked Ca^{2+} release or Ca^{2+} entry and to confirm that NFA indirectly blocks cirazoline-induced Ca^{2+} influx in smooth muscle of MAB, the following specific objectives were proposed:

4) To examine the effects of NFA on cirazoline-induced vasoconstriction in low Ca^{2+} and Ca^{2+}-free, EGTA-containing solution in rat isolated perfused MAB.

5) To investigate the influence of nifedipine, an L-type calcium channel blocker, alone and in combination with NFA on cirazoline-induced vasoconstriction in rat isolated MAB.

Ion efflux study:

1) To assess the action of cirazoline on Cl^- ion efflux (^{125}I efflux was measured as an index of membrane Cl^- conductance) in absence or presence of prazosin in rat isolated small mesenteric arteries (2^{nd} or 3^{rd} order branches of the superior mesenteric artery) Only preliminary experiments were done.

2) To examine the effect of NFA on cirazoline-induce Cl^- ion efflux (using ^{125}I as substitute) in rat isolated small mesenteric arteries. Only preliminary experiments were done.

III. METHODS AND MATERIALS

1. Surgical Preparation of Hypertensive Rats

Goldblatt hypertension (2K1C) was induced as described previously by Goldblatt (1934). Briefly, male Sprague-Dawley rats (180 - 230g) were anaesthetized with halothane (5% in 100% oxygen for induction; 1% in 100% oxygen for maintenance). After a retroperitoneal flank incision, the left renal artery was dissected free, and a U-shape silver clip with an internal diameter of 0.22 ± 0.01 mm was placed around the renal artery, close to its junction with the aorta. The wound was closed and bupivacaine (1%) and Cicatrin were applied topically to the site of incision. Sham-operated rats underwent renal artery isolation but no clip was placed on the renal artery. Animals were housed individually with 12 h light/dark cycle and free access to normal food (Purina rat chow) and tap water. Animals were then randomly selected for experiments.

Four weeks after renal artery clipping or sham operation, animals were anaesthetized with halothane (5% mixed with 100% oxygen for induction; 1% mixed 100% oxygen for maintenance), and catheters (Polyethylene tubing I.D. 0.58 mm, O.D. 0.965 mm) were inserted into the left femoral artery for measurement of arterial blood pressure and removal of blood samples, and the left femoral vein for administration of drugs. The catheters were filled with heparinized saline (25 IU/ml in 0.9% NaCl) and tunneled subcutaneously to the back of the neck, exteriorized and secured. Bupivacaine (1%) was applied topically to the site of incision and animals were allowed to recover for 24 hr. On the following day, blood pressure was recorded using a pressure transducer (PD23ID Gould Statham, CA, USA) and Grass polygraph (Model 79D Grass Instruments, MA, USA) and the heart rate was measured using a tachograph (Model 7P4G Grass Instruments, MA, USA) continuously for 30-45 minutes in free-moving conscious rats. After 30-45 minutes, a blood sample was taken for

measurement of renin activity. 2K1C rats with diastolic blood pressure of >100 mmHg were used, and animals with malignant phase hypertension, as evidenced by the onset of weight loss, were excluded from the study.

2. Measurement of Plasma Renin Activity

Renin-dependent hypertension was verified by determination of plasma renin activity. Blood (1 ml) was collected into a pre-chilled syringe containing EDTA to yield a final concentration of 1 mg/ml. After centrifugation, the plasma was frozen and stored at -20° C until it was assayed. Plasma renin activity was determined as angiotensin I generated under control conditions in which converting enzyme and angiotensinase activities were inhibited by use of EDTA, dimercaprol and 8-hydroxyquinoline. The amount of generated angiotensin I was measured by radioimmunoassay using a commercial polyclonal antiserum against angiotensin I (Du Pont, Ont., Canada) and a double antibody determination system.

Function Study:

3. Perfused Isolated Mesenteric Artery Preparation

Each animal was anaesthetized with sodium pentobarbital (35 mg/kg, iv). The abdominal cavity was opened and mesenteric artery was cannulated through an incision at the confluence with the dorsal aorta and then isolated as previously described by McGregor (McGregor 1965). The mesenteric artery and its branches were flushed with heparinized physiological salt solution, and then the MAB was transferred into a warmed organ chamber, and perfused with Krebs-bicarbonate (normal Krebs) buffer maintained at 37° C and gassed with 95% O_2: 5% CO_2. The Krebs-bicarbonate buffer used was of the following composition (in mM): NaCl 120, KCl 4.6, glucose 11, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH2PO4 1.2, $NaHCO_3$

25.3. The pH of the buffer following saturation with a 95%O_2: 5%CO_2 gas mixture was 7.4. The other perfusion buffers used in the experiments were: 1) Cl^--free buffer of the following composition (in mM): C_2H_5COONa 120, C_2H_5COOK 3.5, glucose 11, $MgSO_4$ 1.2, Ca $(C_6H_{11}O_7)_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25; 2) Low Ca^{2+} buffer: Ca^{2+} was decreased to 0.5 mM in normal Krebs; 3) Ca^{2+}-free EGTA containing buffer: Ca^{2+} was omitted from and 1mM EGTA was added to normal Krebs. The perfusion rate was kept constant at 5 ml/min using a polystaltic peristaltic pump (Buchler Instruments, Buchler Fort Lee, NJ, USA). Changes in perfusion pressure were measured and recorded using a pressure transducer (PD23ID Gould Statham, CA, USA) and Grass polygraph (Model 79D Grass Instruments, MA, USA). The perfused blood vessels were allowed to stabilize for 1 hr before the start of each experiment.

4. Experimental Protocols in Perfused MAB

4.1. Effects of Vehicle or NFA on the Vasoconstrictor Responses to Cirazoline in Perfused MAB

Series 1. This procedure was performed using normal Krebs buffer. The MABs from 2K1C and sham rats were initially exposed to a submaximal dose of cirazoline (9 nmol) to check the viability and responsiveness of the preparations, and then were allowed to further equilibrate for 1 hr. A control dose-response curve for cirazoline was constructed by injection of 6 separate bolus doses of cirazoline (0.09 - 30 nmol). Perfusion pressure was allowed to return to baseline between each injection of agonist. The second and the third dose-response curve to cirazoline were determined in the presence of vehicle (0.03 or 0.1% alcohol), or NFA (3 or 10 μM) in the perfusion media. Blood vessels were perfused with buffer containing either vehicle or NFA for 20 min and thereafter dose-response curves for

the agonist were determined. After the completion of each dose-response curve for cirazoline, a single bolus injection of KCl (60 μmol) was also made.

Series 2. Effects of vehicle and NFA were also evaluated in Cl^--free buffer. A control dose-response curve to cirazoline in mesenteric arteries perfused with normal Krebs was obtained as described before. The tissues were then allowed to stabilize for 40 min. while being perfused with normal Krebs solution. The solution was then changed to Cl^--free buffer and 20 min. was allowed to elapse before a dose-response curve to cirazoline was determined. After the completion of the second dose-response curve, the tissues were perfused again with normal Krebs for 40 minutes. The perfusion solution was then changed to Cl^--free buffer containing vehicle (0.1% alcohol) or NFA (3 or 10 μM), with which blood vessels were perfused for 20 min and thereafter the determination of the final dose-response curve to cirazoline. The perfusion time is long enough to greatly decrease $[Cl^-]_i$ in smooth muscle cells (Aickin and Brading 1982). Separate tissues were used for each concentration of NFA.

4.2. Effects of NFA on Cirazoline-Induced Vasoconstriction in MAB Perfused with Low Ca^{2+} and Ca^{2+}-free Solution, and Compared with Effect of Nifedipine.

In the presence of nifedipine The MAB from 2K1C and sham rats were initially exposed to a submaximal dose of cirazoline (9 nmol), and then were allowed to further equilibrate for 1 hr. Three consecutive dose-response curves for cirazoline were determined by injection of 6 separate bolus doses of cirazoline (0.09 - 30 nmol). Perfusion pressure was allowed to return to baseline between each injection of agonist. The first dose-response curve served as control. The second dose-response curve to cirazoline was performed with nifedipine (3μM) in the perfusion media, while the third dose response curve was determined in the presence of

nifedipine (3μM) plus NFA (3 or 10 μM) in the perfusion media. Inhibitors were added 20 min before and until dose-response curves for the agonist were determined. After the completion of each dose-response curve for cirazoline, a single bolus injection of KCl (60 μmol) was also made.

Low Ca^{2+} solution The protocol was the same as the above except that the second dose-response curve to cirazoline was obtained in a perfusion medium containing 0.5 mM Ca^{2+}, while the third dose response curve was determined in the presence of 0.5 mM Ca^{2+} plus NFA (3 or 10 μM) in the perfusion buffer.

Ca^{2+} free-EGTA-containing solution Since reproducible dose-response curves for cirazoline could not be obtained in Ca^{2+}-free, EGTA-containing solution (preliminary experiments, data not shown), only a single bolus dose of cirazoline was applied each time. In addition, a single bolus injection of KCl (30 μmol) was made before each cirazoline dose was given. A total of 5 sets of injections of KCl and cirazoline were given in each MAB under different perfusion buffer conditions. The perfusing sequence of the different perfusion buffers was as follows: normal Krebs, Ca^{2+}-free solution, Ca^{2+}-free solution in the presence of NFA, Ca^{2+}-free solution again, and Ca-free solution in the presence of nifedipine. After each injection of cirazoline, tissue was allowed to equilibrate for 40 min by perfusing with normal Krebs to refill the internal Ca^{2+} stores. Antagonists were added in the perfusion buffer 20 min before injection of KCl and were present until the pressor response to cirazoline returned to baseline. The Ca^{2+}-free-EGTA containing buffer was perfused for 10 min before KCl was applied and thereafter until vasoconstriction to cirazoline was measured.

5. *In vivo* Measurement of Blood Flow and Vascular Conductance

Surgical preparation. Each animal was anaesthetized with sodium pentobarbital (35 mg/kg, iv), and an additional catheter (Polyethylene tubing I.D. 0.58 mm, O.D. 0.965 mm) was inserted into the right femoral vein for administration of cirazoline. The abdominal cavity was opened through a ventral midline incision, and the superior mesenteric artery was exposed and dissected free. A transonic flow probe (Model 1RB630, Transonic System Inc. NY, U.S.A.) was placed on the mesenteric artery. Blood flow was measured using the flowmeter (Model T206, Transonic Systems Inc. NY, U.S.A) and displayed on a Grass polygraph (Model 79D Grass Instruments, MA, U.S.A.). Blood pressure and heart rate were continuously monitored. Body temperature in these animals was maintained at 36 ± 1°C using a heating lamp and monitored by a rectal mercury thermometer. After completion of the surgery, each animal was allowed to stabilize for a period of 60 min..

6. Experimental Protocols for *in vivo* Experiments

Effects of NFA on blood pressure, blood flow and mesenteric vascular conductance were examined in four groups of rats. Each animal initially received a cumulative continuous infusion of cirazoline (0.13, 0.34, 1.00 and 2.77 mg/kg/min), and each dose was infused for 6 min. After the completion of the first dose-response curve, animals were allowed to recover for 50 min. This period was sufficient to allow blood pressure, heart rate and mesenteric blood flow to return to the baseline. Each animal then received either vehicle (0.3 ml/kg; $NaHCO_3$ in glucose solution) or NFA (3 mg/kg) as a bolus iv injection, and 10 min. was allowed to elapse before the second cumulative doses-response curve to cirazoline was determined.

Ion Efflux Studies:

7. Isolation of Small Mesenteric Arteries

Male Sprague-Dawley rats (300-400g) were anaesthetized with sodium pentobarbital (65 mg/Kg) i.p. and the mesenteric arterial bed was removed and placed in the Krebs - bicarbonate buffer. The second- or third-order branches from the superior mesenteric artery were dissected free from surrounding tissue and cleaned. The small arteries were cut 0.5 cm in length and mounted on a single stainless steel wire holder.

8. Experimental Protocols for Measurement of ^{125}I Efflux in Small Mesenteric Arteries

^{125}I was chosen as a marker of Cl^- channel activity because: 1) it has higher specific activity than ^{36}Cl and it is transported poorly by the $Na^+/K^+/Cl^-$ cotransporter in vascular smooth muscle cells and by anion exchangers (O'Donnell and Owen 1986; Dalmark and Wieth 1972); 2) the permeability of I^- through calcium-activated Cl^- channels is greater than that of Cl^- (Amedee et al. 1990b); 3) measurement of ^{125}I efflux from cells and tissues has been used by other investigators as an index of membrane Cl^- conductance (White et al. 1995). ^{125}I efflux was measured using a washout method (McMahon and Jones 1988; Smith and Jones 1985). Briefly, isolated arteriolar segments were allowed to equilibrate for 2 hours in Krebs buffer at 37 °C, pH 7.4. Following equilibration, tissues were transferred to fresh Krebs (2 ml) containing 6 μCi ^{125}I to load tissues for 1 hour. For each experiment, four loaded arteriolar segments from the same mesenteric vascular bed were tested in parallel. After a 2-second rinse, each loaded tissue was transferred at 1 min intervals through a series of vigorously gassed (with 95% O_2: 5% CO_2) tubes containing 1 ml non-radioactive Krebs in the absence or presence of cirazoline (1 or 3 or 10 μM) or prazosin (0.3 μM) or NFA (10

μM) or cirazoline (3 μM) plus either of the antagonists. The total washout time was 32 min. Antagonists were added at the beginning (t = 0) and were present throughout. Cirazoline stimulation started at 21 min and was present for the last 12 min. At the end of the washout, tissues were blotted and then the radioactivity in each tissue and each washout tube was counted using a gamma counter.

9. Chemicals

All chemicals were purchased from Fisher Scientific (Richmond, B.C., Canada), Sigma Chemical Co (St. Louis, MO, USA) or Research Biochemical International (Natick, MA, USA). Angiotensin I [^{125}I] radioimmunoassay kits and carrier-free ^{125}I were purchased from Du Pont Company (Mississauga, Ont., Canada). A stock solution of NFA (10^{-1} M) was prepared in 100% ethanol (ETOH) and diluted to the required concentration in perfusate reservoir for experiments in isolated mesenteric vascular beds, or in Krebs buffer for the ion efflux assays. NFA was dissolved in $NaHCO_3$ with 5% glucose (4×10^{-1} M, pH 8.5) and prepared as a stock solution (10 mg/ml) for *in vivo* studies. Cirazoline and prazosin were dissolved in normal saline (0.9% NaCl) or twice distilled water for both *in vivo* and *in vitro* studies. Nifedipine was dissolved in ethanol and the experiments with nifedipine were performed in tissue baths protected from light. All solutions were made freshly each day.

10. Data and Statistical Analysis

For *in vitro* studies, the absolute increases in perfusion pressure following bolus injection of each dose of cirazoline were plotted. Vascular conductance *in vivo* was calculated as flow divided by mean blood pressure (MAP). Conductance was calculated in order to assess active changes in vascular tone (Lautt 1999; Tabrizchi and Pang 1993). MAP was calculated as diastolic blood pressure + 1/3(systolic blood pressure - diastolic blood

pressure). The decreases in conductance were expressed as decreases in percentage of the control conductance obtained just before infusion of cirazoline. Ion efflux is characterized by a simple elimination model. The elimination rate constant equation (Wahlstrom 1973a) is as follows:

$$C = C_0 e^{-kt},$$

where C is radioactivity in the tissue at time t; C was calculated by sequentially back-adding the radioactive counts in each tube to the radioactive counts remaining in the tissue at the end of the experiment; C_0 is the initial radioactivity in the tissue at t=0; k is elimination constant per min (efflux rate). In the ion efflux study, each washout curve was computed by the equation and then the k values in the cirazoline-stimulated portion of the efflux curve were averaged and compared with the averaged k of the control in the absence of any drugs, and the averaged k obtained in the presence of antagonist and cirazoline plus antagonist for the same period, respectively. The effects of drugs on ^{125}I efflux were plotted as percentage of control k.

All data are presented as mean ± SEM. Student's unpaired t test was used for comparisons between two means, and two-way ANOVA was used for multiple comparisons between the two groups of rats (*i.e.* 2K1C and sham). One-way ANOVA was used for multiple comparisons in one group of rats (normal rats). Duncan's multiple range test was used to compare between multiple means. $P < 0.05$ was considered as significant in the analysis.

IV. RESULTS

1. Characteristics of 2K1C Hypertensive Rats

Systolic and diastolic blood pressure and heart rate of conscious 2K1C rats were significantly ($n= 42$; $P < 0.05$) higher than those of sham rats (Table 1.1). Furthermore, the plasma renin activity was significantly ($n = 42$; $P < 0.05$) elevated in 2K1C hypertensive rats when compared to that of sham normotensive rats (Table 1.1).

2. Effect of NFA on Cirazoline-Induced Vasoconstriction in Isolated Mesenteric Arteries Perfused with Normal Krebs.

There was no significant difference in the basal perfusion pressures in isolated MAB perfused with normal Krebs between 2K1C hypertensive and sham normotensive rats, (27.4 ± 0.9 and 27.9 ± 0.9 mmHg, 2K1C vs. sham rats mean ± SEM $n = 12$ $P > 0.05$). Bolus injections of cirazoline (0.09 - 30 nmol) evoked dose-dependent pressor responses in isolated MAB from 2K1C hypertensive and sham normotensive rats. Cirazoline-evoked increases in perfusion pressure in mesenteric arteries obtained from 2K1C hypertensive rats were significantly higher than those in sham normotensive rats (Fig. 1.1 - 1.2). The presence of vehicle (0.03% & 0.1% ethanol) did not influence the dose-response curve to cirazoline (Fig 1.1). While 3 μM NFA inhibited the response at only 0.9 nmol cirazoline, cirazoline-mediated vasoconstriction was significantly ($n = 6$; $P < 0.05$) inhibited at all doses (0.09-30 nmol) in presence of the higher concentration of NFA (10 μM) in MAB from both 2K1C and sham rats (Fig. 1.2). There were no differences in the magnitude of the inhibition of the cirazoline responses by NFA between 2K1C hypertensive and sham normotensive rats. On the other hand, vasoconstriction evoked by bolus injection of KCl (60 μmol) in isolated mesenteric arterial beds perfused with normal Krebs were not affected by the presence of

TABLE 1.1

Characteristics of 2K1C and Sham rats: Blood pressure (mmHg), heart rate (beats/min), plasma renin activity (mg ml^{-1} h^{-1}) and body weight (g) of 2K1C hypertensive and sham normotensive rats.

	2K1C	Sham
Arterial pressure		
Systolic	244±5[a]	134±2
Diastolic	166±4[a]	94±2
Heart rate	417±8[a]	370±5
Plasma renin activity	18.37±2.10[a]	3.03±0.28
Body weight	367±6	392±6

Values are pooled and shown as mean ± SEM n = 42 for each group of rats.

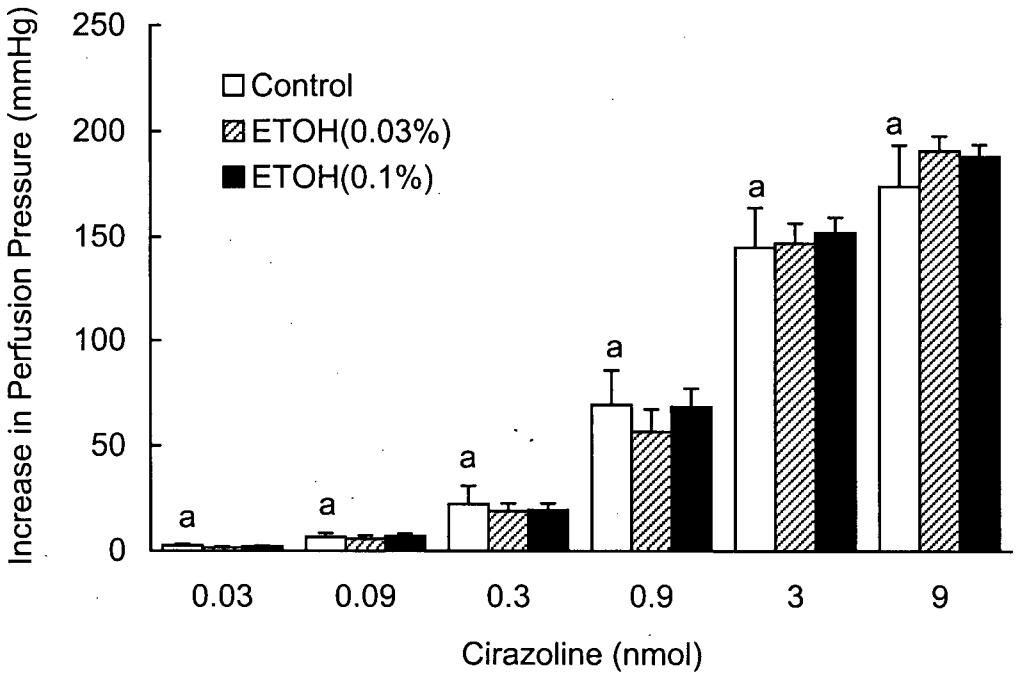
[a]Significantly different from sham, $P < 0.05$ (unpaired t-test).

FIGURE 1.1

Effect of vehicle (ETOH, 0.03% and 0.1%) on vasoconstrictor responses to bolus injection of cirazoline in isolated MAB from either hypertensive (2K1C) or normotensive (Sham) rats perfused with normal Krebs at constant flow.

Data are shown as mean ± SEM, n = 6. [a] $P < 0.05$ vs. sham (two-way ANOVA followed by Duncan's test).

2K1C


Increase in Perfusion Pressure (mmHg)
250
200
150
100
50
0
Control
ETOH(0.03%)
ETOH(0.1%)
a
a
a
a
a
0.03
0.09
0.3
0.9
3
9
Cirazoline (nmol)

Sham

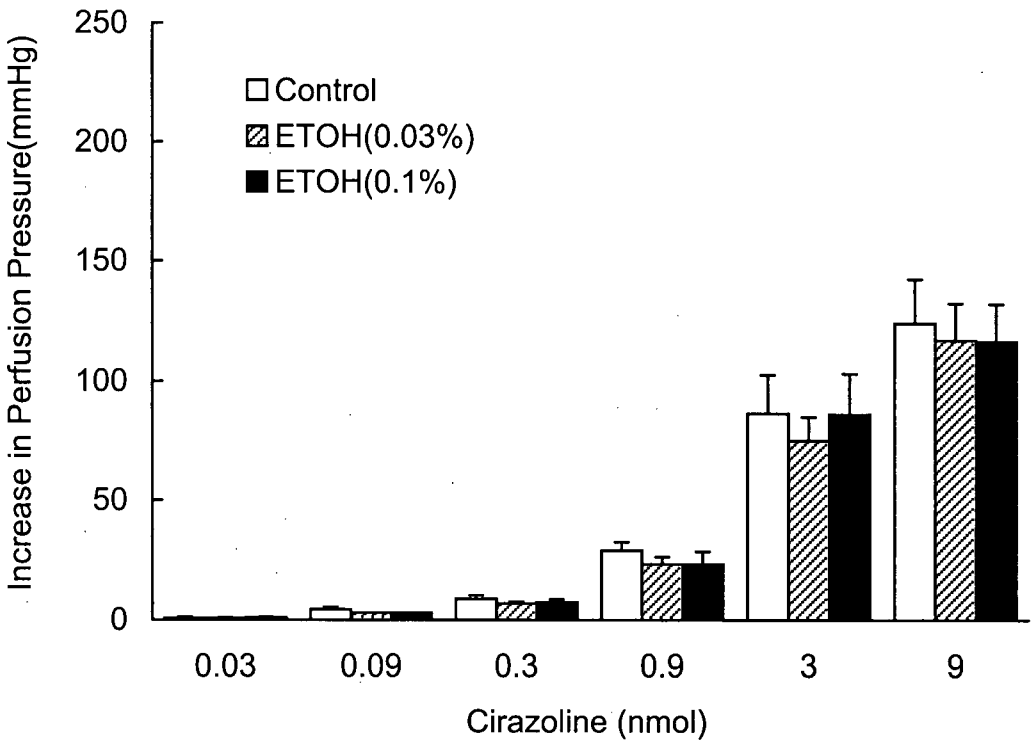
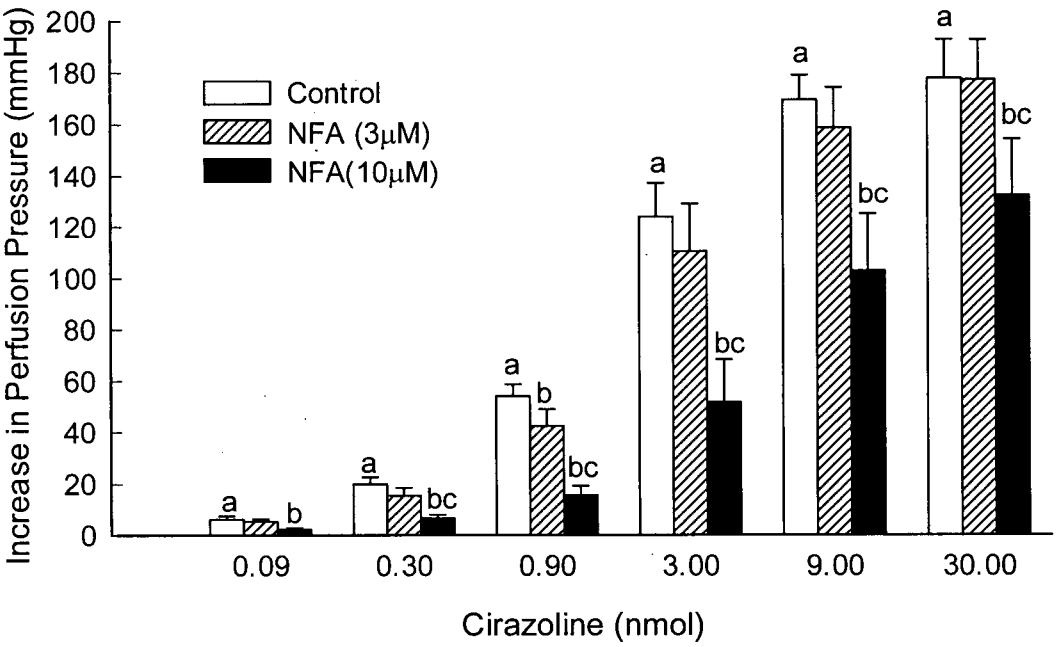

Increase in Perfusion Pressure(mmHg)
250
200
150
100
50
0
Control
ETOH(0.03%)
ETOH(0.1%)
0.03
0.09
0.3
0.9
3
9
Cirazoline (nmol)

FIGURE 1.2

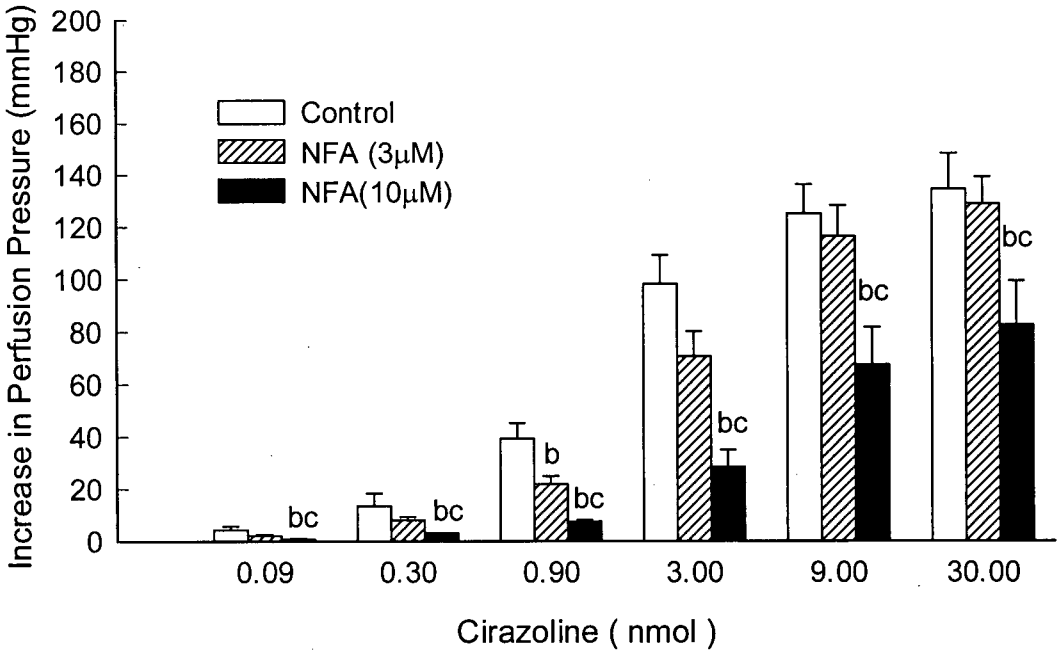
Effect of NFA (3 μM and 10 μM) on pressor responses to cirazoline in MAB from hypertensive (2K1C) and normotensive (Sham) rats perfused with normal Krebs.

Data are shown as mean ± SEM, n = 6. [a] $P < 0.05$ vs. sham, [b] $P < 0.05$ vs. control, [c] $P < 0.05$ vs. NFA (3 μM) (two-way ANOVA followed by Duncan's test).

2K1C


Increase in Perfusion Pressure (mmHg)
200
180
160
140
120
100
80
60
40
20
0
Control
NFA (3μM)
NFA(10μM)
a
b
a
bc
a
b
bc
a
bc
a
bc
a
bc
0.09
0.30
0.90
3.00
9.00
30.00
Cirazoline (nmol)

Sham


Increase in Perfusion Pressure (mmHg)
200
180
160
140
120
100
80
60
40
20
0
Control
NFA (3μM)
NFA(10μM)
bc
bc
b
bc
bc
bc
bc
bc
0.09
0.30
0.90
3.00
9.00
30.00
Cirazoline (nmol)

NFA in the perfusion medium (Fig. 1.3). There was also no difference in the response to KCl between 2K1C hypertensive and sham normotensive rats (87.5 ± 8.8 mmHg and 71.1 ± 9.8 mmHg, respectively).

3. Effect of NFA on Cirazoline-Induced Vasoconstriction in Isolated Mesenteric Arteries Perfused with Cl^--Free Buffer.

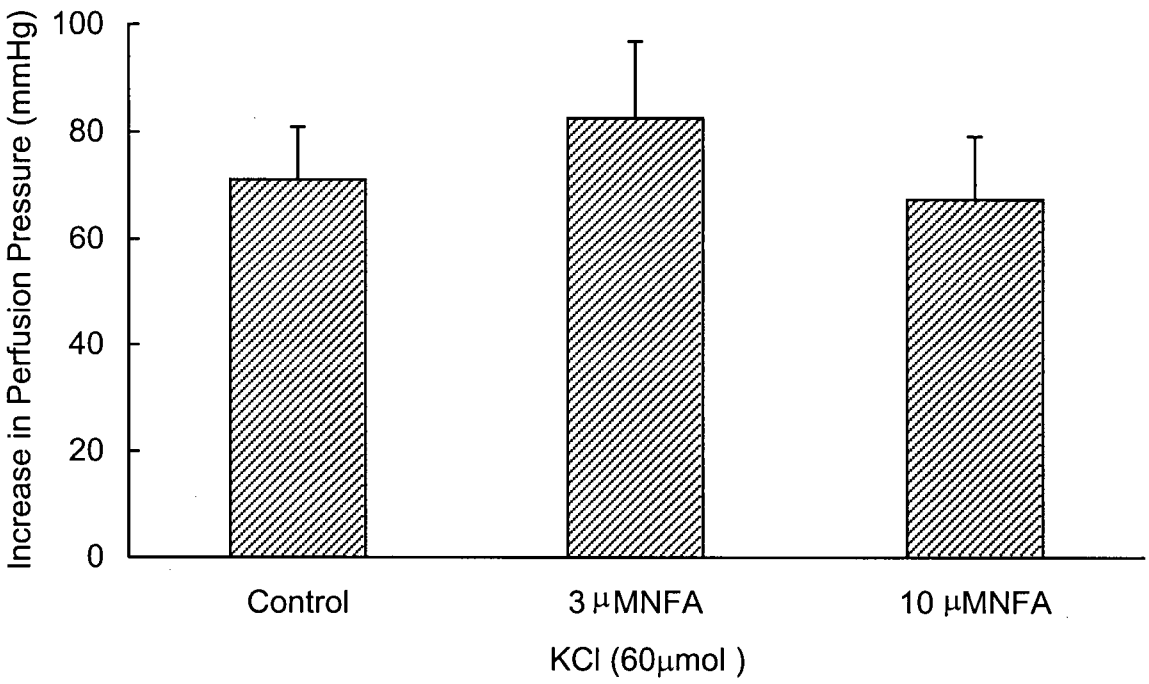
When the perfusion buffer was changed from normal Krebs to the Cl^--free buffer, there was a transient increase in perfusion pressure with the peak being reached in 2 to 3 min, and then the perfusion pressure stabilized again at 28.7 ± 1.1 and 27.3 ± 1.0 mmHg for 2K1C and sham rats respectively. The stabilized basal perfusion pressure obtained in Cl^--free solution did not significantly differ from control value achieved in normal Krebs for 2K1C and sham rats (see above). The transient increase in perfusion pressure in Cl^--free buffer was 18.4 ± 4.4 and 4.9 ± 0.5 mmHg for 2K1C hypertensive and sham normotensive rats, respectively. This increase in perfusion pressure was significantly ($n = 18$; $P < 0.05$) greater in 2K1C hypertensive than that in sham normotensive rats. Cirazoline-induced vasoconstriction in isolated mesenteric beds obtained from 2K1C hypertensive and sham normotensive rats was impaired following perfusion with Cl^--free buffer when compared to normal Krebs (Fig. 1.4, 1.5 & 1.6). The inhibition was significant ($P < 0.05$) at cirazoline doses of 3, 9 and 30 nmol. Perfusion of mesenteric blood vessels with Cl^--free buffer resulted in a significantly ($P < 0.05$) greater inhibition of cirazoline-mediated vasoconstriction in sham normotensive rats than in 2K1C hypertensive rats (Fig. 1.5 & 1.6 *insert*). We did find that in Cl^--free buffer, cirazoline-mediated vasoconstriction was further inhibited by the presence of NFA (Fig. 1.5, 1.6), but not vehicle (Fig. 1.4) in the perfusion media. NFA (3 μM) significantly ($P < 0.05$) inhibited cirazoline-mediated vasoconstriction at doses of 0.9, 3,

FIGURE 1.3

Effect of NFA (3 μM and 10 μM) on KCl-evoked vasoconstriction in MAB from hypertensive (2K1C) or normotensive (Sham) rats perfused with normal Krebs.

Data are shown as mean ± SEM, n = 6. No difference was found in this experiment. (two-way ANOVA)

2K1C


Increase in Perfusion Pressure (mmHg)
100
80
60
40
20
0
Control
3 μMNFA
10 μMNFA
KCl (60μmol)

Sham

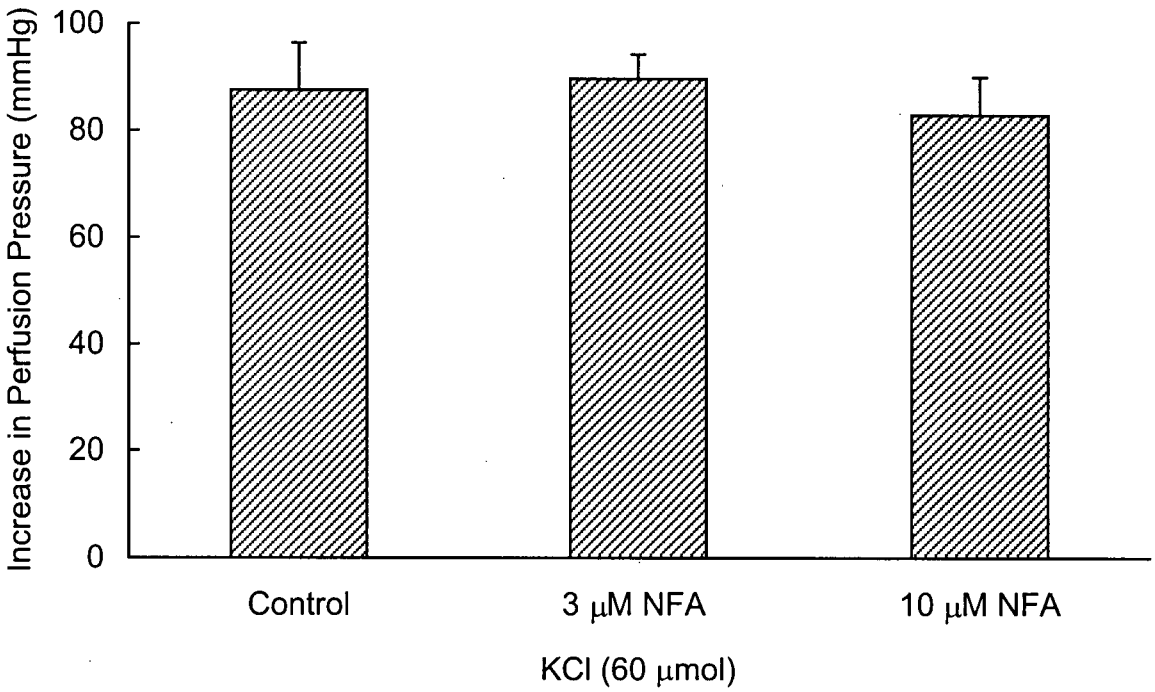
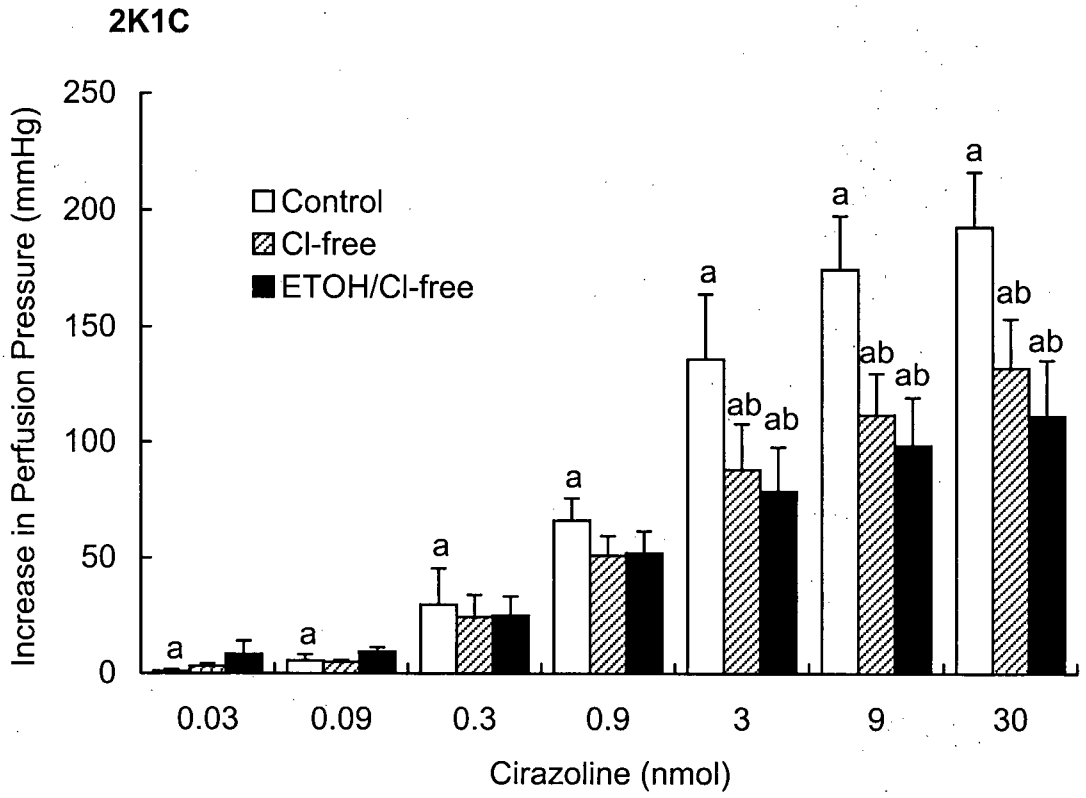

Increase in Perfusion Pressure (mmHg)
100
80
60
40
20
0
Control
3 μM NFA
10 μM NFA
KCl (60 μmol)

FIGURE 1.4

Effects of Cl^--free buffer and vehicle (0.1% ETOH) on pressor responses to bolus injection of cirazoline. Control (in normal Krebs, open bar), Cl^--free buffer alone (hatched bar), vehicle in Cl^--free buffer (solid bar).

Data are shown as mean ± SEM, n = 6. [a] $P < 0.05$ vs. sham, [b] $P < 0.05$ vs. control. (two-way ANOVA followed by Duncan's test).


2K1C
Increase in Perfusion Pressure (mmHg)
250
200
150
100
50
0
Control
Cl-free
ETOH/Cl-free
a
a
a
a
a
a
ab
ab
ab
ab
ab
0.03
0.09
0.3
0.9
3
9
30
Cirazoline (nmol)

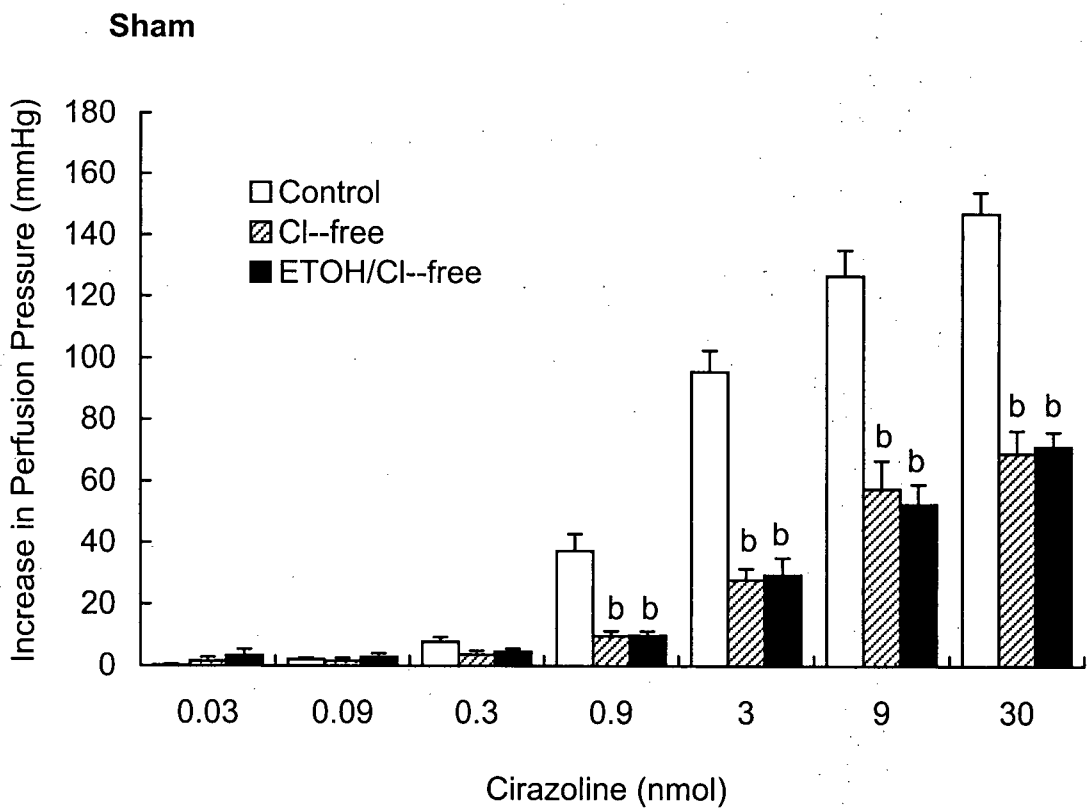

Sham
Increase in Perfusion Pressure (mmHg)
180
160
140
120
100
80
60
40
20
0
Control
Cl--free
ETOH/Cl--free
b
b
b
b
b
b
b
0.03
0.09
0.3
0.9
3
9
30
Cirazoline (nmol)

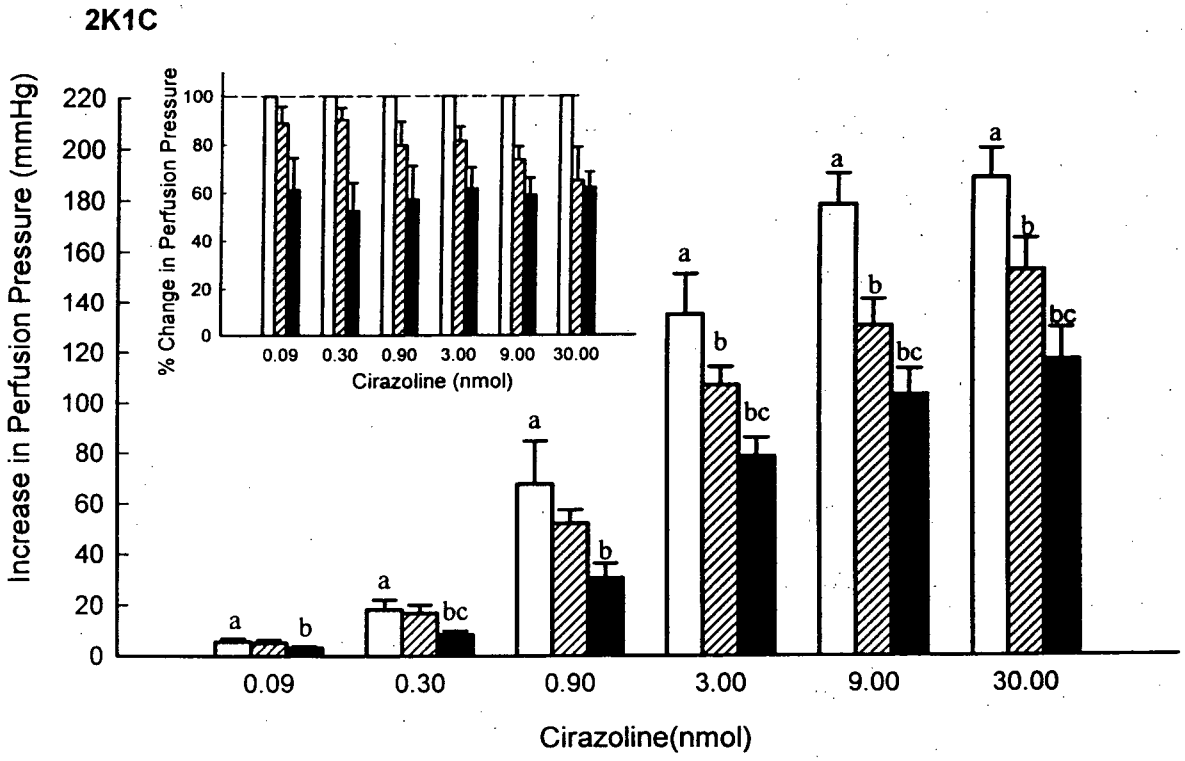
FIGURE 1.5

Effect of NFA (3 μM) on pressor responses to cirazoline in MAB from 2K1C and sham rats perfused with Cl^- -free buffer. Insert: shows % change in perfusion pressure corresponding to the data in (2K1C) and (Sham). Control (normal Krebs, opened columns); Cl^--free buffer alone (hatched columns); NFA (3 μM) in Cl^-- free buffer (solid columns).

Data are shown as mean ± SEM, n = 6.

[a] $P < 0.05$ vs. sham. [b] $P < 0.05$ vs. control, [c] $P < 0.05$ vs. Cl^--free buffer alone (two way ANOVA followed by Duncan's test).

[e] $P < 0.05$ vs. Cl^--free buffer in 2K1C rats, [d] $P < 0.05$ vs. 3 μM NFA+ Cl^--free buffer in 2K1C rats (unpaired student's t-test).


2K1C
Increase in Perfusion Pressure (mmHg)
% Change in Perfusion Pressure
Cirazoline (nmol)
0.09
0.30
0.90
3.00
9.00
30.00
Cirazoline(nmol)

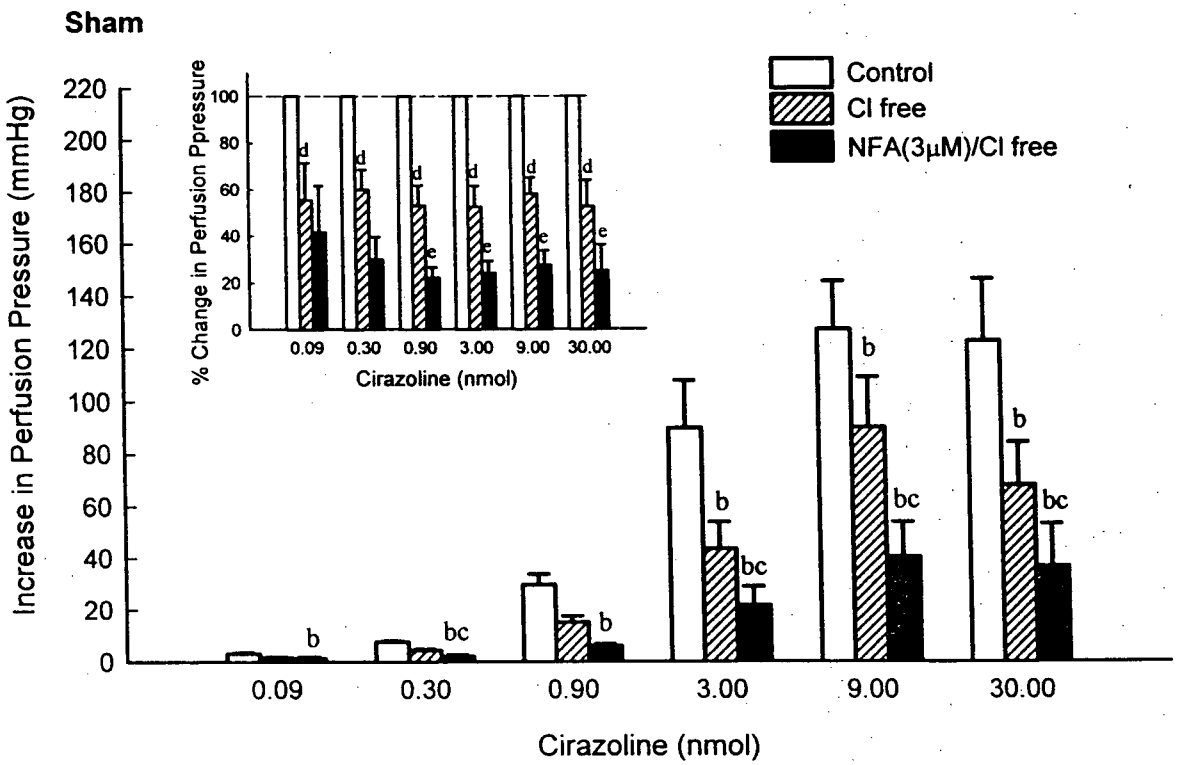

Sham
Control
Cl free
NFA(3μM)/Cl free
Increase in Perfusion Pressure (mmHg)
% Change in Perfusion Ppressure
Cirazoline (nmol)
0.09
0.30
0.90
3.00
9.00
30.00
Cirazoline (nmol)

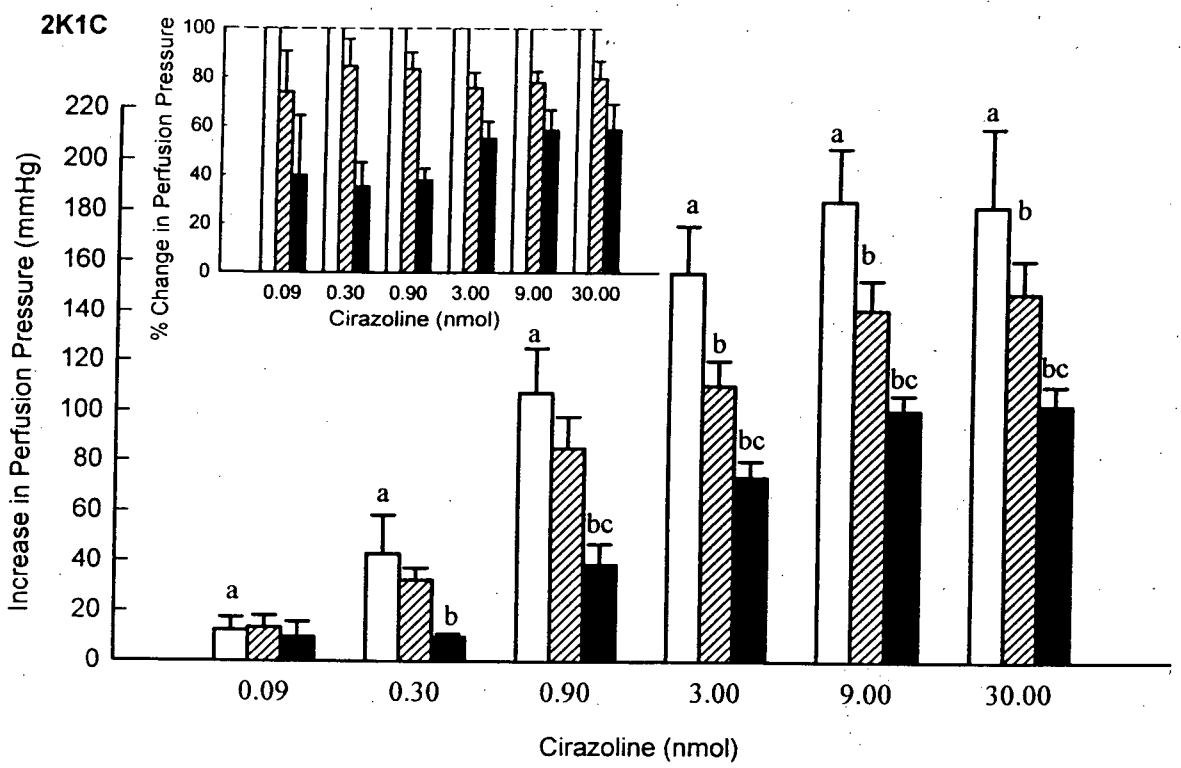
FIGURE 1.6

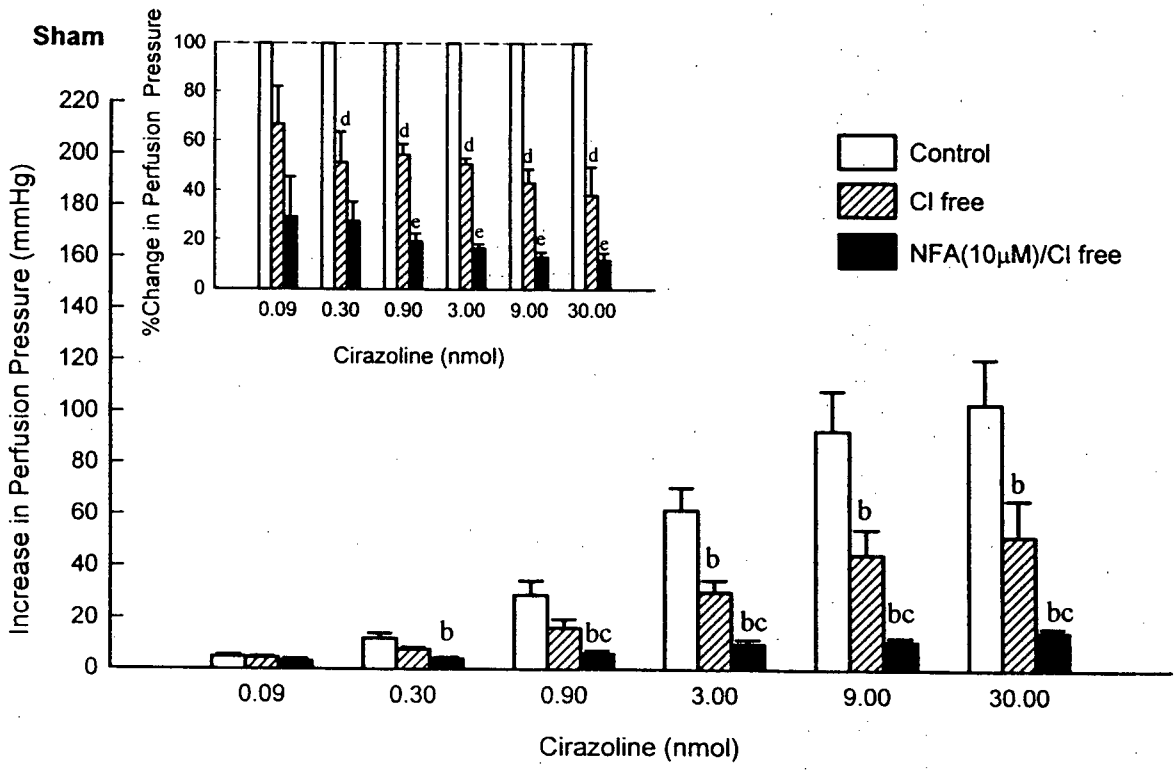
Effect of NFA (10 μM) on pressor responses to cirazoline in MAB from 2K1C and sham rats perfused with Cl^- -free buffer. Insert: shows % change in perfusion pressure corresponding to the data in (2K1C) and (Sham). Control (normal Krebs, opened columns); Cl^--free buffer alone (hatched columns); NFA (10 μM) in Cl^--free buffer (solid columns).

Data are shown as mean ± SEM, n = 6.

[a] $P < 0.05$ vs. sham. [b] $P < 0.05$ vs. control, [c] $P < 0.05$ vs. Cl^--free buffer alone (two way ANOVA followed by Duncan's test).

[e] $P < 0.05$ vs. Cl^--free buffer alone in 2K1C rats, [d] $P < 0.05$ vs. 10 μM NFA + Cl^- free in 2K1C rats (unpaired student's t-test).


2K1C
Increase in Perfusion Pressure (mmHg)
% Change in Perfusion Pressure
Cirazoline (nmol)
0.09
0.30
0.90
3.00
9.00
30.00
a
b
bc


Sham
Increase in Perfusion Pressure (mmHg)
%Change in Perfusion Pressure
Cirazoline (nmol)
Control
Cl free
NFA(10μM)/Cl free
0.09
0.30
0.90
3.00
9.00
30.00
b
bc
d
e

9 and 30 nmol in 2K1C hypertensive rats and sham normotensive rats. The magnitude of blockade produced by NFA of cirazoline-mediated vasoconstriction was significantly ($n = 6$; $P < 0.05$) greater in sham rats than that in 2K1C rats (Fig. 1.5 *insert*). NFA (10 μM) suppressed responses to cirazoline in Cl^--free buffer in a similar manner (Fig 1.6). The presence of a higher concentration of NFA significantly ($n = 6$; $P < 0.05$) inhibited cirazoline-mediated vasoconstriction at doses of 0.9, 3, 9 and 30 nmol in 2K1C hypertensive rats and in normotensive rats. The magnitude of the reduction in the vasoconstrictor response to cirazoline again was significantly ($n = 6$; $P < 0.05$) greater in sham normotensive rats than that in 2K1C hypertensive rats (Fig. 1.6 *insert*).

4. **Influence of NFA on Cirazoline-Induced Change in Mesenteric Vascular Conductance in Anaesthetized 2K1C Hypertensive and Sham Normotensive Rats**

The effect of NFA was tested *in vivo* in anaesthetized rats. There was no significant difference in the baseline values of the superior mesenteric blood flow between 2K1C hypertensive and sham normotensive rats. However, the basal vascular conductance in superior mesenteric artery was significantly ($n = 5$; $P < 0.05$) lower in 2K1C hypertensive rats in comparison to sham normotensive rats (Table 1.2).

Administration of either NFA or vehicle did not alter the baseline values of MAP, superior mesenteric flow or conductance in anaesthetized rats (Table 1.2). Intravenous infusion of cumulative doses of cirazoline caused dose-dependent increases in MAP (Table 1.3), and decreases in superior mesenteric vascular conductance in 2K1C hypertensive and sham normotensive rats (Table 1.4). However, the degree of reduction in the conductance induced by cirazoline in 2K1C hypertensive rats was similar to that in sham normotensive

TABLE 1.2

Mean blood pressure (MAP; mmHg), superior mesenteric artery blood flow (SMAF; ml/min) and conductance (SMAC; ml $mmHg^{-1}min^{-1}$) values before and after injection of vehicle ($NaHCO_3$, 0.3 ml/kg) or NFA (3 mg/kg) in 2K1C hypertensive and sham normotensive rats.

	MAP		SMAF		SMAC	
	2K1C	Sham	2K1C	Sham	2K1C	Sham
Pre-vehicle	164±14[a]	98±3	13.9±0.9	14.1±2.5	0.088±0.012[a]	0.140±0.028
Post-vehicle	148±8[a]	92±3	14.2±0.9	14.5±1.9	0.097±0.009[a]	0.158±0.021
Pre-NFA	162±13[a]	97±5	11.9±1.3	15.5±1.8	0.076±0.012[a]	0.165±0.027
Post-NFA	153±16[a]	95±7	12.4±2.8	16.6±2.4	0.086±0.025[a]	0.180±0.032

Each value represents the mean ± SEM, n = 5

[a]Significantly different from sham rats, $p < 0.05$ (two-way ANOVA followed by Duncan's test).

TABLE 1. 3.

Effects of cirazoline on mean arterial pressure (MAP, mmHg) in anesthetized 2K1C hypertensive and sham normotensive rats before (control) and after treatment with either vehicle ($NaHCO_3$, 0.3 ml/kg) or NFA (3 mg/kg).

	Cirazoline ($\mu g\ kg^{-1}\ min^{-1}$)			
	0.13	0.34	1.00	2.77
2K1C				
Control	171±16[a]	181±17[a]	200±14[a]	250±19[a]
Vehicle-treated	160±9	168±10	188±13	243±15
Control	170±13[a]	183±15[a]	215±14[a]	253±5[a]
NFA-treated	154±15	163±14	188±14	237±5
Sham				
Control	104±3	108±2	127±4	172±3
Vehicle-treated	96±4	103±3	118±4	173±5
Control	104±7	109±4	126±5	167±3
NFA-treated	99±7	106±6	125±5	165±2

Each value represents the mean ± SEM, n = 5

[a]Significantly different from sham rats, $P < 0.05$ (two-way ANOVA followed by Duncan's test).

TABLE 1. 4

Effects of cirazoline on decrease in vascular conductance (% of control) in superior mesenteric artery (SMAC; ml $mmHg^{-1}$ min^{-1}, control conductances were shown in Table 1.2) in anesthetized 2K1C hypertensive and sham normotensive rats before (control) and after treatment with either vehicle ($NaHCO_3$, 0.3 ml/kg) or NFA (3 mg/kg).

	Cirazoline (μg kg^{-1} min^{-1})			
	0.13	0.34	1.00	2.77
2K1C				
Control	8.9±2.6	20.1±3.8	39.8±5.0	65.3±3.7
Vehicle-treated	9.2±3.8	17.6±3.8	36.2±5.0	64.4±3.9
Control	10.8±2.0	22.4±3.2	46.8±3.5	68.4±3.2
NFA-treated	1.3±2.0[a]	2.0±6.0[a]	24.3±8.0[a]	61.0±6.5
Sham				
Control	12.4±1.3	23.2±3.1	42.6±4.4	69.3±3.1
Vehicle-treated	10.4±2.2	19.8±3.9	34.6±4.9	69.1±3.1
Control	12.5±1.6	26.4±3.6	47.7±4.1	72.1±4.0
NFA-treated	3.0±3.0[a]	13.6±4.1[a]	34.3±6.2[a]	67.0±5.0

Each value represents the mean ± SEM, n = 5

[a]Significantly different from control (before treatment with NFA), $P < 0.05$ (two-way ANOVA followed by Duncan's post test).

rats (Table 1.4). Pretreatment with vehicle did not affect cirazoline-induced changes in MAP or conductance when compared to the absence of vehicle (Table 1.3 and 1.4). In addition, in animals that were treated with NFA, cirazoline-mediated pressor responses were not significantly affected when compared to control (Table 1.3). However, pretreatment with NFA significantly ($n= 5$; $P < 0.05$) impaired cirazoline-mediated decreases in vascular conductance at doses of 0.13 to 1.00 mg/kg/min in 2K1C hypertensive and normotensive rats (Table 1.4). There was no significant difference in the magnitude of the attenuation of the decrease in vascular conductance between 2K1C and sham rats ($P > 0.05$)

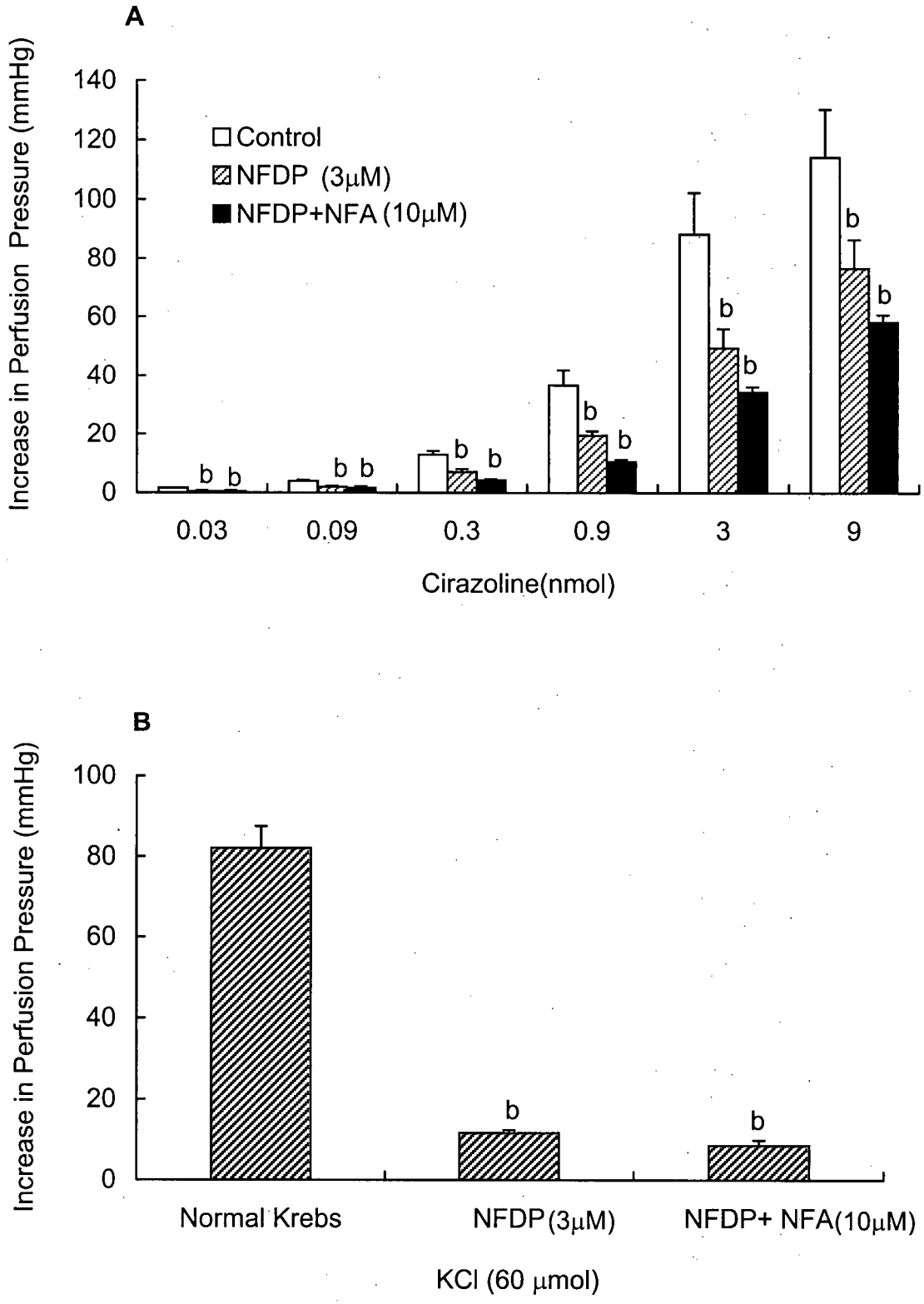
5. Effect of Nifedipine and Nifedipine Plus NFA on Cirazoline- and KCl-Induced Vasoconstriction in Isolated MAB Perfused with Normal Krebs

The effect of the voltage-dependent Ca^{2+} channel blocker, nifedipine and the effect of nifedipine plus NFA were evaluated in isolated MAB from normal SD rats perfused with normal Krebs. The presence of nifedipine (3 μM) in the perfusion media significantly inhibited the vasoconstrictor action of all doses of cirazoline (0.03-9 nmol) (Fig. 1.7A). Nifedipine also markedly decreased KCl (60 μmol)-evoked vasoconstriction (Fig. 1.7B). However, in the presence of nifedipine plus NFA (10 μM), the inhibitory effect on pressor responses of MAB to cirazoline did not statistically differ from that in the presence of nifedipine alone (Fig. 1.7A). The combination of nifedipine and NFA also had no additive inhibitory effect on contractile response to KCl (Fig. 1.7B).

FIGURE 1.7

Effect of nifedipine (NFDP, 3 μM) and NFDP (3μM) plus NFA (10 μM) on vasoconstriction induced by bolus injection of cirazoline (A) or KCl (B) in MAB from normal SD rats perfused with normal Krebs.

Data are shown as mean ± SEM, n = 6. ^{b} $P < 0.05$ vs. control (normal Krebs, in absence of NFDP or NFA) (one-way ANOVA followed by Duncan's test)


A
Increase in Perfusion Pressure (mmHg)
140
120
100
80
60
40
20
0
Control
NFDP (3μM)
NFDP+NFA (10μM)
b b
b b
b b
b
b
b
b
b
b
0.03
0.09
0.3
0.9
3
9
Cirazoline(nmol)
B
Increase in Perfusion Pressure (mmHg)
100
80
60
40
20
0
b
b
Normal Krebs
NFDP (3μM)
NFDP+ NFA(10μM)
KCl (60 μmol)

6. Effect of NFA on Cirazoline- and KCl-Induced Vasoconstriction in Isolated MAB Perfused with Low Ca^{2+} Solution

Lowering Ca^{2+} concentration from 2.5 to 0.5 mM in perfusion buffer did not significantly affect cirazoline-induced vasoconstriction in isolated MAB from normal SD rats (Fig 1.8A & Fig. 1.9 A B), whereas the pressor response to KCl was significantly reduced (Fig, 1.8B & Fig. 1.10A B). Addition of NFA (either 3 or 10 μM) into low Ca^{2+} perfusion buffer had no further inhibitory effect on the response to KCl as compared to perfusion with low Ca^{2+} buffer alone (Fig 1.10A.B). In contrast, the presence of 10 μM, but not 3 μM, NFA, decreased the responses to cirazoline significantly (Fig. 1.9A B). In low Ca^{2+} solution, repetition of the dose-response curve in the presence of vehicle did not affect the pressor responses to cirazoline (Fig. 1.8A). Similarly, the second response evoked by repeated application of KCl, in the presence of vehicle, was not significantly different from the first in isolated MAB perfused with low Ca^{2+} solution alone (Fig. 1.8B).

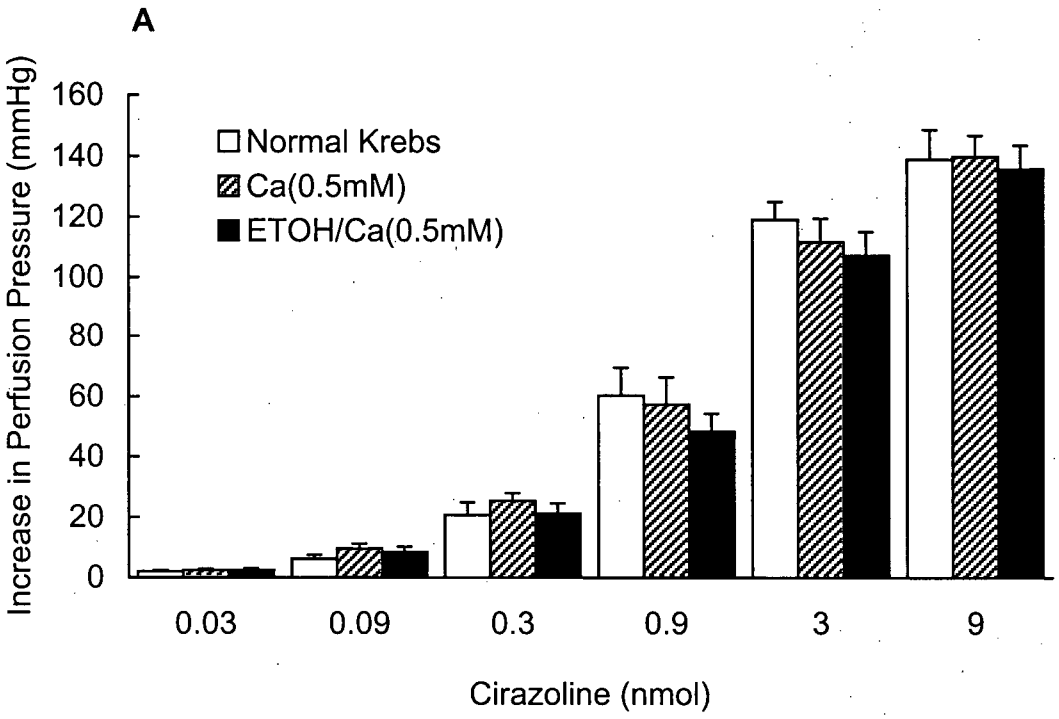
7. Effect of NFA on Cirazoline-Induced Vasoconstriction in Isolated MAB Perfused with Ca^{2+}-free-EGTA Solution

Cirazoline, at 0.3 nmol, induced an initial transient peak followed by a sustained increase in perfusion pressure in isolated MAB perfused with normal Krebs containing 2.5 mM Ca^{2+} (Fig 1.11A:a). Perfusion with Ca^{2+}-free solution containing 1 mM EGTA abolished the sustained plateau of pressor response, while leaving the initial transient peak intact (Fig. 1.11A:b). The amplitude of the initial transient peak response to cirazoline with Ca^{2+}-free solution did not differ from that obtained with normal Krebs (Fig. 1.11B). In contrast, perfusing with Ca^{2+}-free EGTA-containing solution totally abolished KCl-evoked vasoconstriction (Fig. 1.11A). Thus, the initial contractile response to 0.3 nmol cirazoline

FIGURE 1.8

Effect of low Ca^{2+} (0.5 mM) buffer and vehicle (0.1% ETOH) on the pressor response to bolus injection of cirazoline (A) and KCl (B) in MAB from normal SD rats.

Data are shown as mean ± SEM, n = 6. ^{b} $P < 0.05$ vs. normal Krebs. (one-way ANOVA followed by Duncan's test)


A
Increase in Perfusion Pressure (mmHg)
160
140
120
100
80
60
40
20
0
Normal Krebs
Ca(0.5mM)
ETOH/Ca(0.5mM)
0.03
0.09
0.3
0.9
3
9
Cirazoline (nmol)

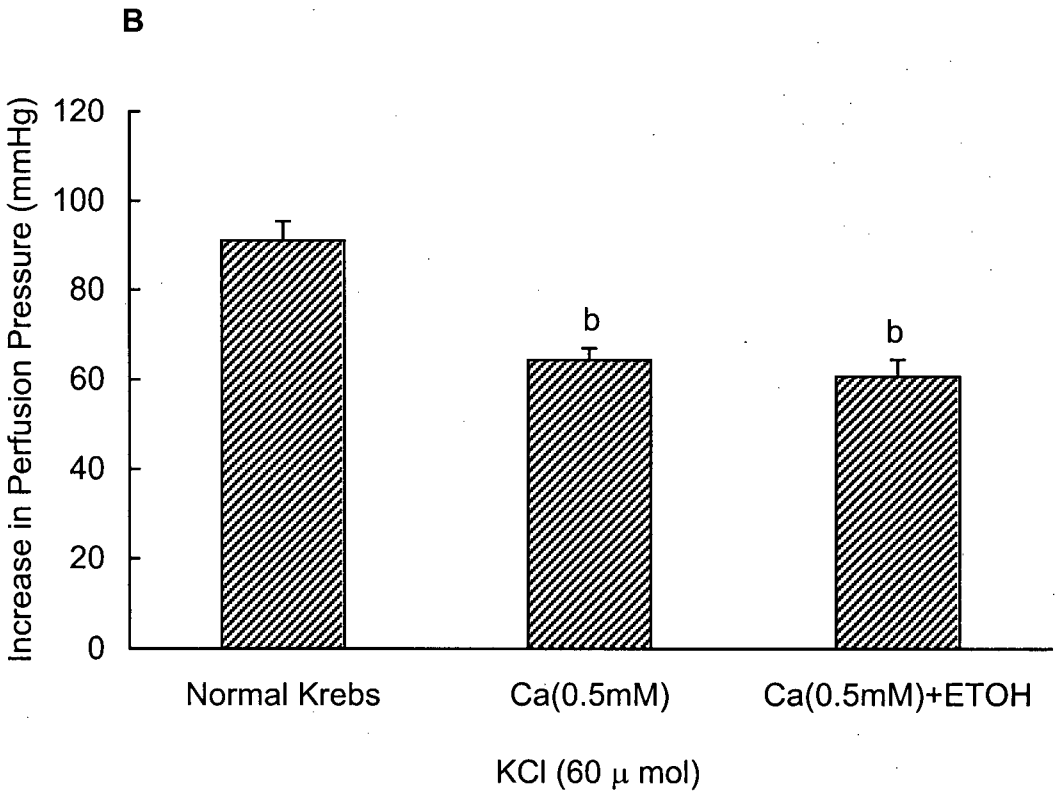
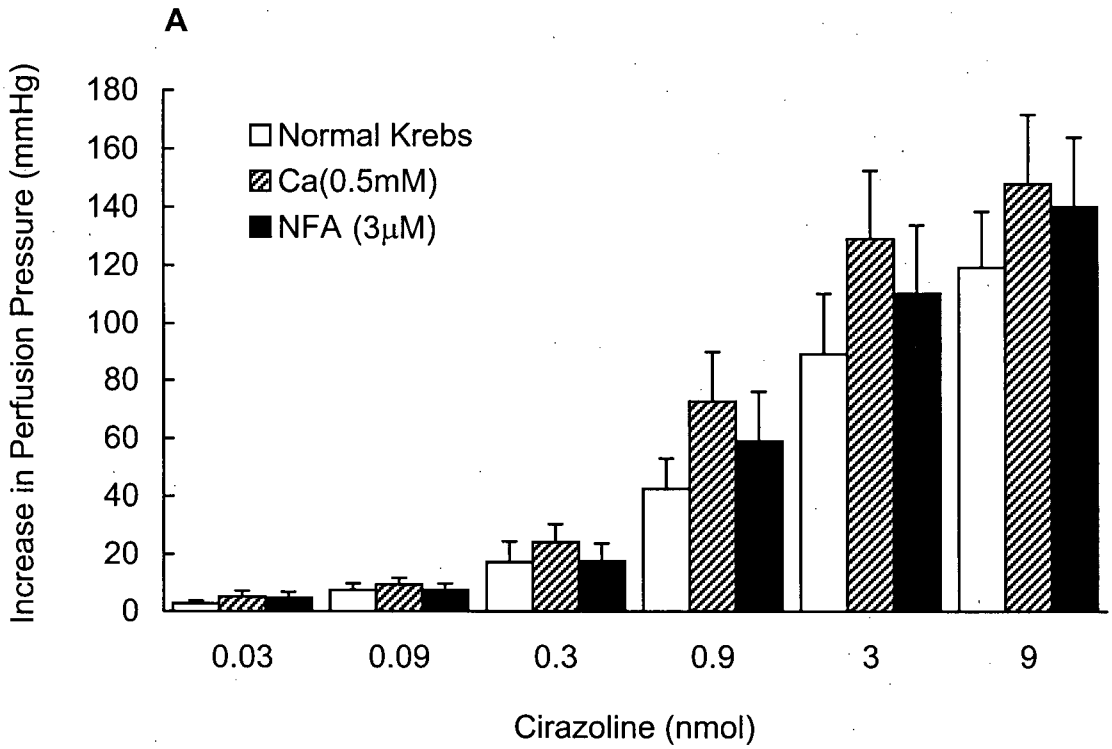

B
Increase in Perfusion Pressure (mmHg)
120
100
80
60
40
20
0
b
b
Normal Krebs
Ca(0.5mM)
Ca(0.5mM)+ETOH
KCl (60 μ mol)

FIGURE 1.9

Effect of NFA (3 μM in A, 10 μM in B) on pressor response to bolus injection of cirazoline in MAB from SD rats perfused with low Ca^{2+} buffer (0.5 mM).

Data are shown as mean ± SEM, n = 6. [b] $P < 0.05$ vs. normal Krebs , [c] $P < 0.05$ vs. low Ca^{2+} (0.5 mM) buffer (one-way ANOVA followed by Duncan's test)


A
Increase in Perfusion Pressure (mmHg)
180
160
140
120
100
80
60
40
20
0
Normal Krebs
Ca(0.5mM)
NFA (3μM)
0.03
0.09
0.3
0.9
3
9
Cirazoline (nmol)

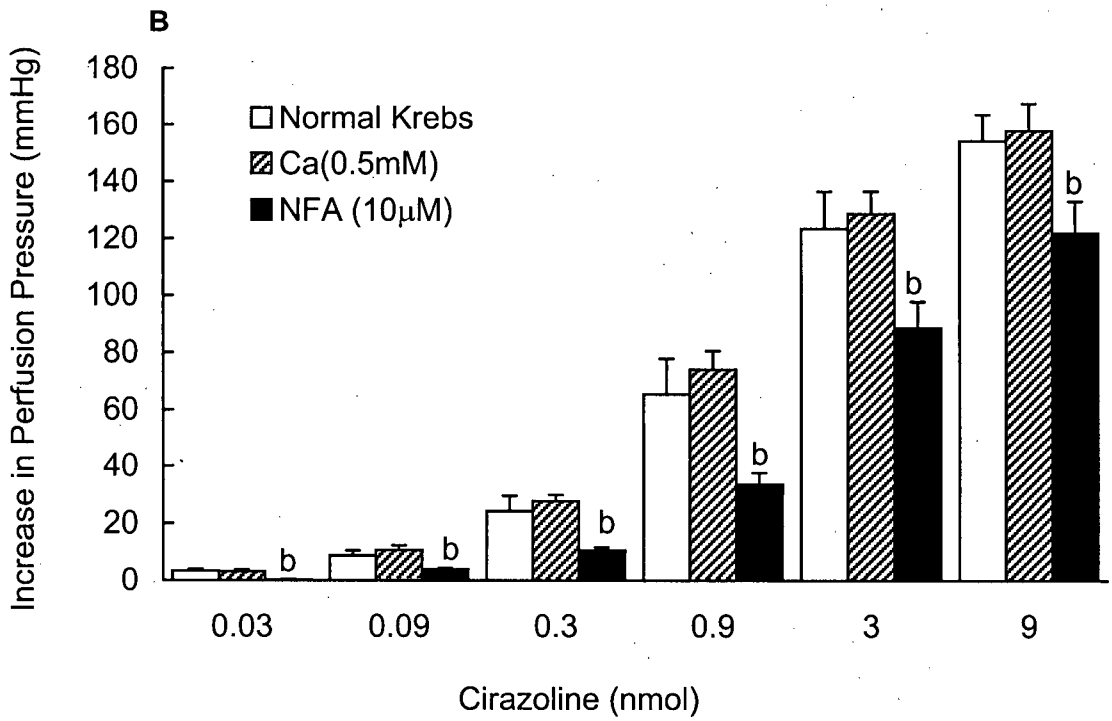
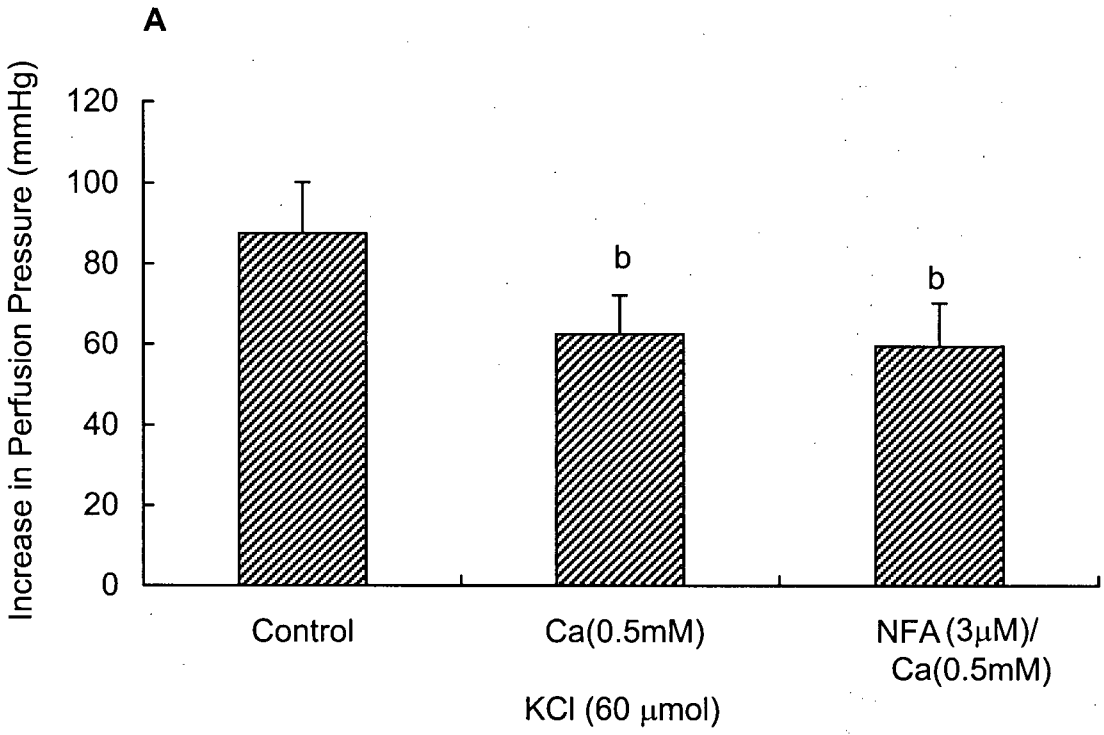

B
Increase in Perfusion Pressure (mmHg)
180
160
140
120
100
80
60
40
20
0
Normal Krebs
Ca(0.5mM)
NFA (10μM)
b
0.03
0.09
0.3
0.9
3
9
Cirazoline (nmol)

FIGURE 1.10

Effect of NFA (3 μM in A, 10 μM in B) on KCl evoked contraction in MAB from normal SD rats perfused with low Ca^{2+} (0.5 mM) buffer.

Data are shown as mean ± SEM, n = 6. ^{b} $P < 0.05$ vs. normal Krebs. (one-way ANOVA followed by Duncan's test)


A
Increase in Perfusion Pressure (mmHg)
120
100
80
60
40
20
0
b
b
Control
Ca(0.5mM)
NFA (3μM)/
Ca(0.5mM)
KCl (60 μmol)

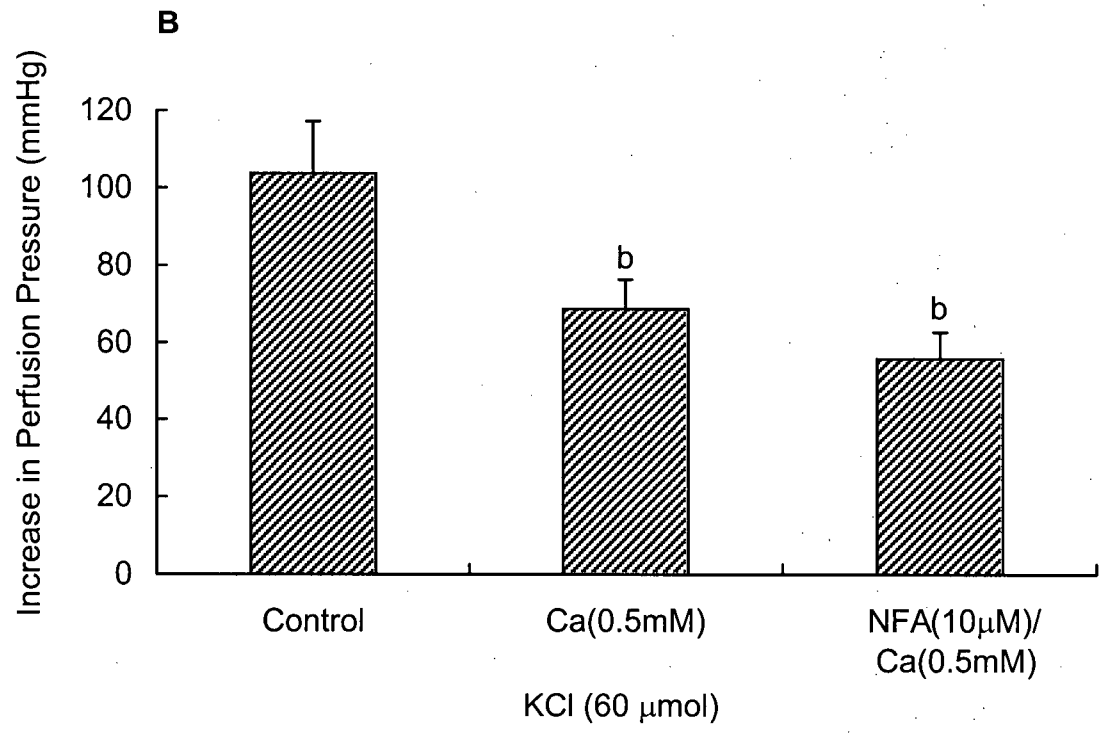
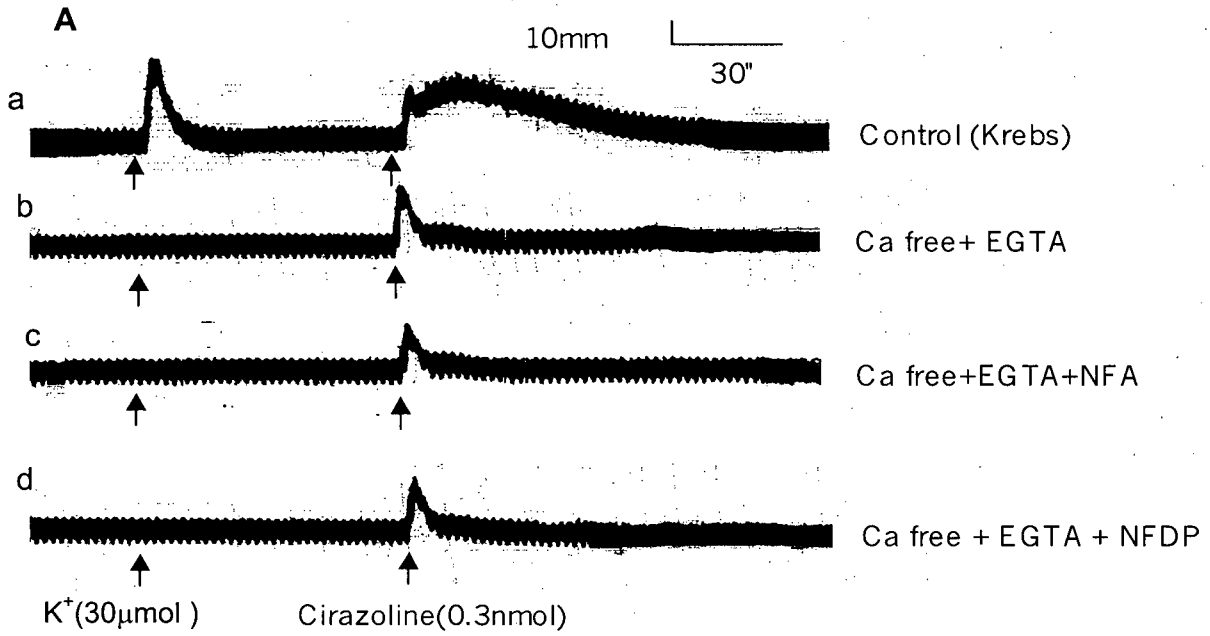
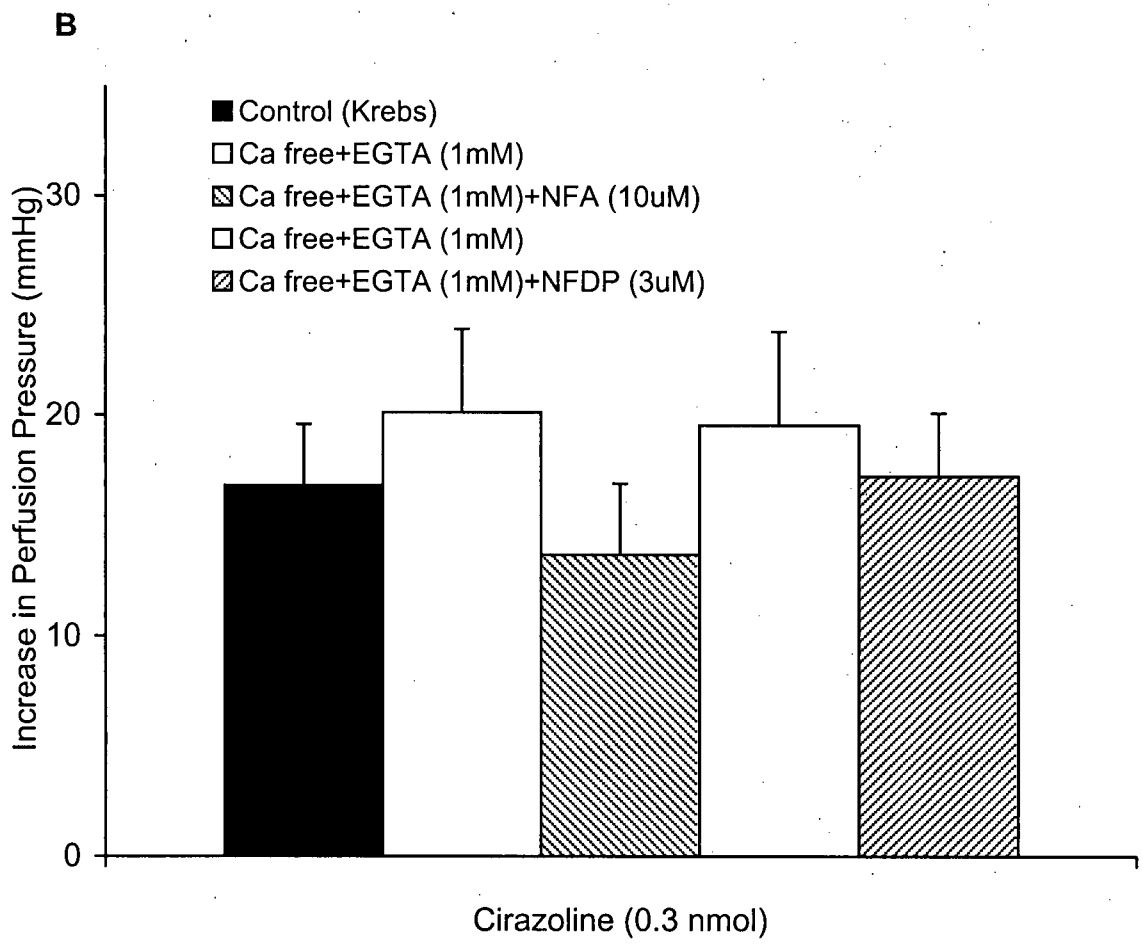

B
Increase in Perfusion Pressure (mmHg)
120
100
80
60
40
20
0
b
b
Control
Ca(0.5mM)
NFA(10μM)/
Ca(0.5mM)
KCl (60 μmol)

FIGURE 1.11

Effect of NFA (10 μM) on the pressor response to bolus injection of cirazoline (0.3 nmol) in MAB from SD rats perfused with Ca^{2+} free-EGTA (1 mM) solution (B). Upper panel (A) is a representative trace obtained from one of the four MAB tested.

Data are shown as mean ± SEM, n=4. (one-way ANOVA)


A
10mm
30"
a
Control (Krebs)
b
Ca free+ EGTA
c
Ca free+EGTA+NFA
d
Ca free + EGTA + NFDP
K+(30μmol)
Cirazoline(0.3nmol)


B
Control (Krebs)
Ca free+EGTA (1mM)
Ca free+EGTA (1mM)+NFA (10uM)
Ca free+EGTA (1mM)
Ca free+EGTA (1mM)+NFDP (3uM)
Increase in Perfusion Pressure (mmHg)
30
20
10
0
Cirazoline (0.3 nmol)

seems not to include the component that induced by Ca^{2+} influx from extracellular space. Therefore, it allows assessing the effect of NFA on the cirazoline-induced vasoconstrictor response that mediated by Ca^{2+} released from intracellular store independently of the influence of Ca^{2+} influx via VOCs. To confirm this, nifedipine was used as a positive control. Neither niflumic acid (10 μM) nor nifedipine (3 μM) had a significant inhibitory effect on the vasoconstrictor response to cirazoline in MAB perfused with Ca^{2+}-free-EGTA containing solution (Fig 1.11A:c.d & B)

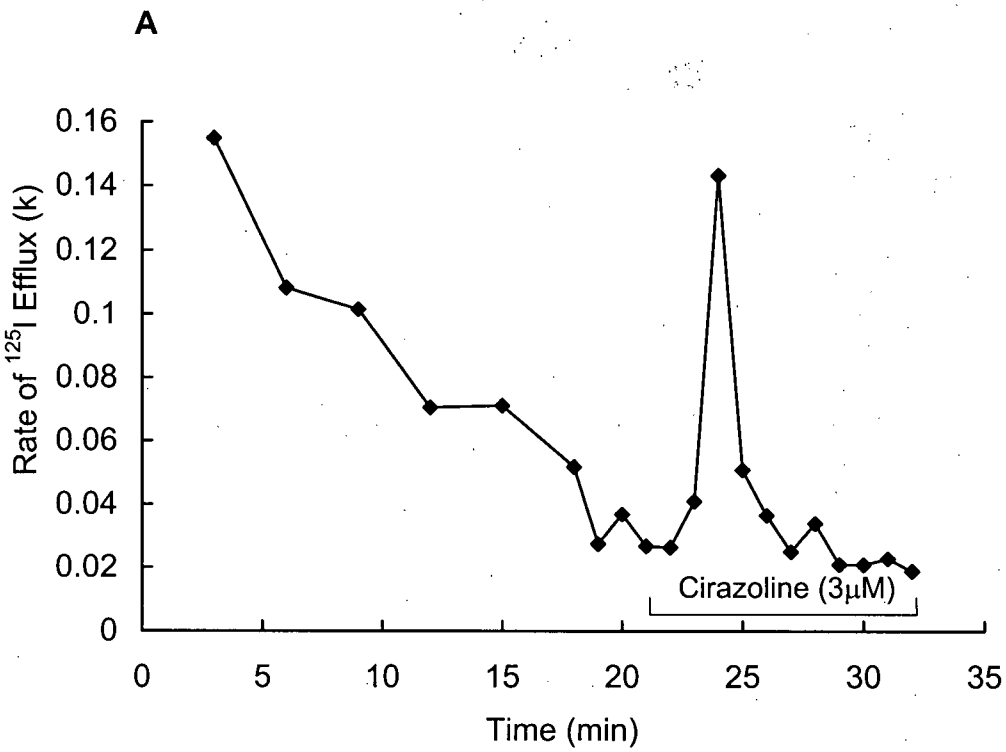
8. ^{125}I Efflux from Small Mesenteric Arteries

The effect of cirazoline on ^{125}I efflux was evaluated, and preliminary experiments on the effects of prazosin and NFA were carried out. As illustrated in Fig. 1.12, cirazoline caused an increase in ^{125}I efflux. Fig. 1.12A shows the ^{125}I efflux curve from an individual vessel; the effect of cirazoline took 2-3 min to reach its peak, and then fell rapidly. However, it took another 2-3 min to return from the peak to basal value. The effect of cirazoline was concentration-dependent, 1, 3, 10 μM cirazoline increasing ^{125}I efflux by 141 ± 2%, 187 ± 20% and 168 ± 49%, respectively (Fig. 1.12 B). Prazosin (0.3 μM) as well as NFA (10 μM) did not affect the basal ^{125}I efflux, but inhibited cirazoline-induced increase in ^{125}I efflux (Table 1.5).

FIGURE 1.12

A. Representative ^{125}I efflux curve in isolated small mesenteric arteries of SD rats. Cirazoline (3 μM) was added at 21 min.

B. Effects of cirazoline on ^{125}I efflux in isolated small mesenteric arteries of SD rats (n = 4). Data are shown as mean ± SEM, n = 4


A
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Rate of ^{125}I Efflux (k)
Cirazoline (3μM)
0
5
10
15
20
25
30
35
Time (min)

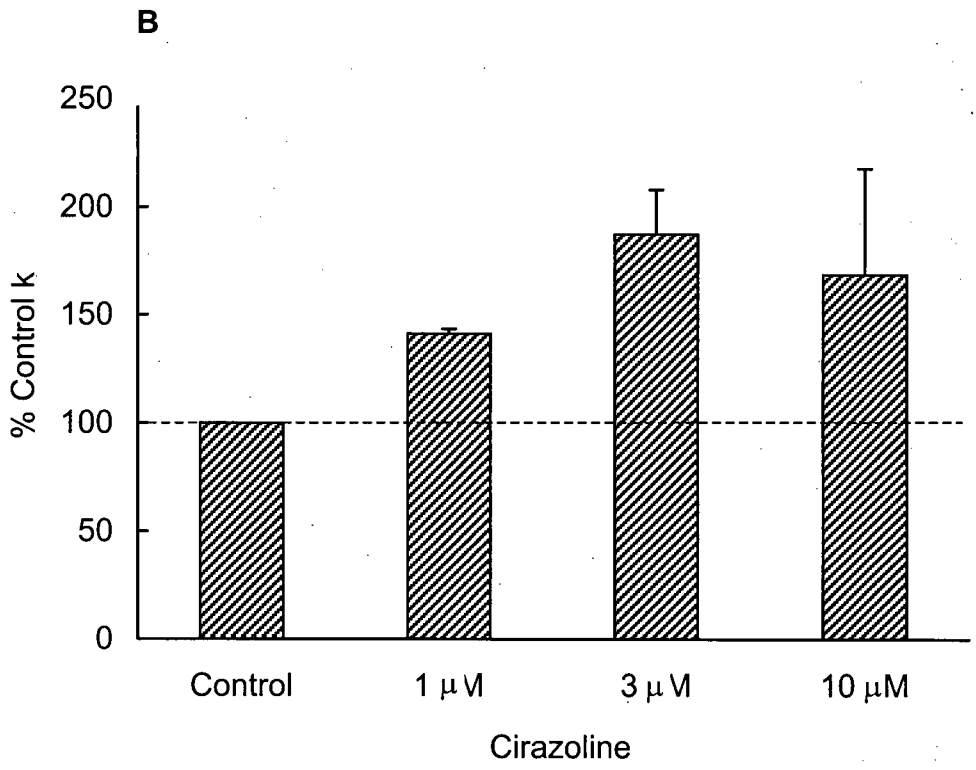

B
250
200
150
100
50
0
% Control k
Control
1 μM
3 μM
10 μM
Cirazoline

TABLE 1.5

A. Effect of Prazosin on cirazoline-induced ^{125}I efflux (n=2)

	Control	Cirazoline (3μM)	Prazosin (0.3μM)	Cirazoline+Prazosin (3μM) (0.3μM)
k (min^{-1})	0.098±0.0016	0.129±0.0095	0.095±0.00115	0.072±0.0010
%Control	100	145.1±12.1	101.2±4.1	75.6±1.8

B. Effect of NFA on cirazoline induced ^{125}I efflux (n=2)

	Control	Cirazoline (3μM)	NFA (10μM)	Cirazoline+NFA (3μM) (10μM)
k (min^{-1})	0.096±0.00076	0.154±0.0020	0.076±0.0037	0.085±0.0082
%Control	100	160.1±16.4	88.3±2.3	95.9±8.6

V. DISCUSSION

The main objective of this part of the work was to examine the importance of Cl^- ions in α_1-adrenoceptor-mediated vasoconstriction in rat mesenteric arteries. To test this we employed NFA, an anti-inflammatory agent that has been characterized as a potent $I_{Cl(Ca)}$ blocker with fewer nonselective actions in rabbit portal vein as compared with other known $I_{Cl(Ca)}$ blockers, such as DIDS, A-9-C IAA (Hogg et al. 1994a; Large and Wang 1996). We found that NFA was capable of inhibiting cirazoline-induced vasoconstriction both *in vitro* as well as *in vivo*. It failed to produce additive inhibition in the presence of the Ca^{2+} channel inhibitor nifedipine, which also attenuated the cirazoline-induced contraction significantly. We also found that removal of Cl^- could suppress the cirazoline-induced contraction. In addition, we showed that cirazoline induced ^{125}I efflux from rat small mesenteric arteries. This effect of cirazoline was inhibited by both prazosin and NFA. These data suggest that chloride ions play an important role in vasoconstrictor responses that are mediated via the stimulation of α_1-adrenoceptor in rat mesenteric arteries. Based on our results, it also seems that the role of Cl^- in cirazoline-mediated vasoconstriction is less important in blood vessels obtained from 2K1C hypertensive compared to normotensive rats.

The Role of Cl^- Channels in α_1-Adrenoceptor-Induced Vasoconstriction.

Opening of Cl^- channels depolarizes VSM. This is because Cl^- is transported into VSM cells against its electrochemical gradient, resulting in an intracellular Cl^- concentration that is much higher than that predicted by passive distribution (Davis 1992). Based on the evidence obtained from electrophysiological studies, a role for agonist-induced $I_{Cl(Ca)}$ has been identified in a number of blood vessels, such as rabbit and rat portal veins (Byrne and Large

1988a; Pacaud et al. 1989b), rabbit ear artery (Amedee et al. 1990b), human mesenteric artery (Klockner 1993; Klockner and Isenberg 1991) and rat renal resistance artery (Gordienko et al. 1994). In rat portal veins as well as in rabbit ear arteries, it has been repeatedly reported that NE-mediated calcium release from intracellular stores preferentially produces an increase in Cl^- conductance leading to changes in membrane potential (Amedee et al. 1990a; Pacaud et al. 1991; Pacaud et al. 1989b) and the opening of voltage-gated calcium channels (Pacaud et al. 1991; Pacaud et al. 1989b). Calcium activated Cl^- current in some vascular smooth muscles has been reported to be blocked by drugs such as 4',4'-diisothiocyanostilbene-2,2-disulfonic acid (DIDS) and NFA (Hogg et al. 1994a; Hogg et al. 1994b; Kirkup et al. 1996a; Lamb et al. 1994; Large and Wang 1996; Pacaud et al. 1989a). Our current findings demonstrated that both NFA and removal of Cl^- inhibited α_1-adrenoceptor-mediated vasoconstriction in perfused mesenteric blood vessels. In addition, as expected, nifedipine, a specific blocker of voltage-gated Ca^{2+} channels (Kuriyama et al. 1995), also significantly inhibited cirazoline-induced contraction; there was however no additional reduction in the contraction of mesenteric arteries by the combination of NFA and nifedipine, a phenomenon that was also observed on NE-induced contraction in rat aorta (Criddle et al. 1996). Norepinephrine has been reported to increase Cl^- efflux while producing depolarization in rat mesenteric arteries without altering either the rate of K^+ efflux or Na^+ influx (Videbaek et al. 1990). Moreover, responses that are mediated via the α_1-adrenoceptors have been shown to be sensitive to the actions of calcium channel antagonists (Chen and Rembold 1995). In rat mesenteric microvessels, the calcium-entry blocker nitrendipine was found to reduce NE-mediated constriction (Chen et al. 1996). Therefore, the most likely mechanism that mediates the relaxant action of NFA in rat

mesenteric arteries is an inhibition of Cl^- current evoked by cirazoline, thereby indirectly preventing Ca^{2+} from entering via voltage-gated Ca^{2+} channels.

The role of Cl^- channels in mediating α_1-adrenoceptor-induced contraction was further confirmed by experiments in which extracellular Cl^- was removed. It has been shown that reduction of extracellular Cl^- concentration decreases $[Cl^-]_i$ in smooth muscle cells (Aickin and Brading 1982). In addition, the probability of opening of certain Cl^- channels is dependent on the intracellular and extracellular concentrations of Cl^-, and the Cl^- current is much more affected by changes of the intracellular Cl^- concentration than predicted simply from the change in Cl^- driving force (Chesnoy-Marchais 1983; Dinudom et al. 1993; Jackson et al. 1996). Furthermore, it has been demonstrated that removal of Cl^- or lowering the extracellular Cl^- concentration ($[Cl^-]_0$) suppresses α_1-adrenoceptor-induced Cl^- currents and membrane depolarization in several types of smooth muscles including VSM (Bulbring and Tomita 1987; Large 1984; Van Helden 1988; Van Renterghem and Lazdunski 1993). Van Helden has studied the kinetics of the effect of low-Cl^- on changes in Cl^- conductance induced by NE in smooth muscle of guinea pig mesenteric veins (Van Helden 1988). He observed that the currents increased in amplitude during about the first 40s exposure to low-Cl^- solution and decreased afterward. However, complete suppression was not always observed even after a 15min exposure to low-Cl^- solution. He also found that the initial increase in amplitude of the Cl^- current was consistent with an increase in the driving force for Cl^-. He suggested that the decrease in the response must be related to an inactivation of the Cl^- conductance mechanism itself, because when the response was significantly suppressed in low Cl^- solution, the reversal membrane potential remained more positive than the control values. Thus, we expected that prolonged perfusion with Cl^--free buffer would

decrease the level of intracellular Cl^-, thereby inhibiting agonist-activated Cl^- channels and attenuating cirazoline-induced vasoconstriction in the mesenteric arterial bed. Our results confirmed this notion and showed that the pressor responses to cirazoline were significantly inhibited after perfusing with Cl^--free buffer for 20 min. It was also found that in Cl^--free solution not only 10 μM but also 3 μM NFA significantly inhibited cirazoline-induced vasoconstriction. There can be a number of explanations for this observation, which could account for the further inhibitory actions of NFA in Cl^--free buffer. First, it is possible that NFA inhibited the efflux of residual Cl^- that remained inside the vascular smooth muscle cells in Cl^--free buffer. This speculation is supported by observations in a number of articles. McMahon and Jones demonstrated that in Cl^--free propionate substituted solution, the reduction of $[Cl^-]_i$ was slower than the loss of Cl^- from the extracellular site in rat aorta strips (McMahon and Jones 1988). Others showed that the relationship of $[Cl^-]_i$ to $[Cl^-]_0$ in smooth muscle cells measured with a Cl^--sensitive microelectrode was hyperbolic (Aickin and Brading 1982). Total removal of extracellular Cl^- (gluconate as substitution) caused a rapid fall in $[Cl^-]_i$, but measurement of $[Cl^-]_i$ in Cl^--free solution one hour later still showed a positive value. The value of $[Cl^-]_i$ agrees well with $[Cl^-]_i$ estimated from the Cl^- efflux study with three other Cl^- substitutes (Aickin and Brading 1982; Casteels 1971). An alternative explanation is that either propionate or bicarbonate anions made a small contribution to the process of agonist-induced depolarization via efflux through the Cl^- channels, while in the presence of NFA this effect was blocked. It has been shown that Cl^- channels are rather unselective for many anions, although with varying permeability (Amedee et al. 1990b; Franciolini and Petris 1990; Large and Wang 1996).

^{125}I efflux from vascular smooth muscle cells has been used as an indicator of Cl^- movements to study the properties of agonist-induced Cl^- channels, owing to its high selectivity for conductive channels (White et al. 1995). It has known that I^- has a higher permeability than Cl^- via agonist-activated Cl^- channels in blood vessels (Amedee et al. 1990b; Large and Wang 1996). In addition, it has been demonstrated that ^{125}I is transported poorly by various anion carriers, such as $Na^+/K^+/Cl^-$ cotransporters and Cl^-/HCO_3 exchangers (Dalmark and Wieth 1972; O'Donnell and Owen 1986), and that these transporters do not affect agonist-stimulated ^{125}I efflux in vascular smooth muscle cells (White et al. 1995). The results of ^{125}I efflux measurement in the present study suggest that rat small mesenteric arteries contain conductive Cl^- channels that are activated in response to cirazoline. The cirazoline-induced increase in ^{125}I efflux was antagonized by prazosin in a concentration-dependent manner, which suggests that the Cl^- channels are opened by cirazoline through stimulation of α_1-adrenoceptors. Moreover, like prazosin, NFA also inhibited the ^{125}I efflux induced by cirazoline. This result, together with the data from contractile experiments in perfused mesenteric arterial bed, suggests that Cl^- ions play a direct role in the α_1-adrenoceptor-mediated excitation-contraction coupling.

Current evidence in the literature supports the view that vasoconstrictor responses arising from the activation of α_1-adrenoceptor in rat mesenteric arterial bed are mediated by the activation of α_{1A}-adrenoceptor sub-types (Chen et al. 1996; Kong et al. 1994; Williams and Clarke 1995). Studies using rat aorta indicate that cirazoline has a higher affinity for the α_{1A} and α_{1D} subtypes than for the α_{1B} sub-type (Buckner et al. 1996). However, a previous study using human cloned α_1-adrenoceptors had indicated that cirazoline had a higher affinity for the α_{1a} subtype rather than the α_{1b} and α_{1d} subtypes (Horie et al. 1995).

Contraction in vascular smooth muscle mediated via the activation of α_1-adrenoceptors is dependent upon both an influx of Ca^{2+} and Ca^{2+} release from intracellular stores (Cauvin and Malik 1984; Minneman 1988; Chen and Rembold 1995). An elevation in the concentration of intracellular Ca^{2+} is believed to produce an increase in Cl^- conductance (Amedee et al. 1990b; Pacaud et al. 1992; Large and Wang, 1996). Based on our present findings, it would seem that vasoconstriction mediated via the stimulation of α_{1A}-adrenoceptors in the mesenteric arterial bed depends in part on the presence of intracellular Cl^-.

The Selectivity of NFA

Because of the relatively low degree of selectivity of most Cl^- channel blockers, alternative explanations, i.e. actions unrelated to inhibition of Cl^- channels, for the ability of NFA to inhibit the contractile responses to cirazoline in mesenteric arteries must be considered.

NFA did not affect KCl-evoked contraction in normal Ca^{2+} containing buffer. Second, NFA remained ineffective in low Ca^{2+} solution in which the response to KCl was impaired. However, NFA significantly inhibited pressor responses to cirazoline under both conditions. These observations suggest that the inhibitory effect of NFA on cirazoline-induced vasoconstriction is not due to a direct blockade of voltage-gated Ca^{2+}channels, or to non-specific effects on the contractile apparatus. Additionally, NFA did not affect cirazoline-induced vasoconstriction in the absence of extracellular Ca^{2+}, so it is unlikely that NFA interfered with the contraction induced by Ca^{2+} release from SR or had an inhibitory effect on the contractile proteins. It has been demonstrated that NFA has no effect on spontaneous transient current produced by $I_{K(Ca)}$, but it can enhance NE-induced $I_{K(Ca)}$ in rabbit portal vein.

It was thus suggested that NFA increases Ca^{2+} release from SR (Hogg et al. 1994a). In our experiments, in which rat mesenteric arteries were employed, this seems not to be the case. We found that NFA alone neither affected basal perfusion pressure in the perfused mesenteric arterial bed nor inhibited mesenteric blood flow or vascular conductance in anaesthetized rats. Therefore, it seems unlikely that the inhibitory effect of NFA on cirazoline-induced vasoconstriction is due to an activation of K^+ channels.

Altered Function of Cl^- Channels in mediating α_1-Adrenoceptor-Induced Vasoconstriction in MAB from 2K1C Hypertensive Rats.

In a previous study, McGregor and Smirk (McGregor and Smirk 1968) reported that mesenteric arteries from renal hypertensive rats (2K1C) exhibited a higher vasoconstrictor response to NE. In the present study, it was found that the cirazoline-induced increase in perfusion pressure was greater in blood vessels from hypertensive rats. The higher vasoconstrictor responses observed in renal hypertensive tissues have been suggested to be the result of increased resistance to flow (Russell et al. 1983). Significant reductions in external diameter and increased media-to-lumen ratio have been reported to be responsible for increased vascular reactivity in 2K1C hypertensive rats (Deng and Schiffrin 1991). In the present study, we also found that basal vascular conductance *in situ* was lower in 2K1C hypertensive rats when compared to sham normotensive rats, which is consistent with the proposal that morphological changes may account for changes in the function of blood vessels in 2K1C hypertensive rats (Bennett and Thurston 1996; Li et al. 1996). However, this may not entirely account for the altered behavior of blood vessels in 2K1C hypertensive *versus* normotensive rats, since no difference in vasoconstrictor responses evoked by KCl was observed in the present study.

The results of our *in vitro* and *in vivo* studies indicate that NFA had a similar efficacy at inhibiting cirazoline-mediated vasoconstriction in both normotensive and hypertensive rats. However, this was not the case when Cl^- ions were replaced with propionate ions. In Cl^--free solution, NFA was more effective at inhibiting cirazoline-mediated vasoconstriction in normotensive than in hypertensive rats. Moreover, removal of Cl^- affected cirazoline-induced vasoconstriction to a greater extent in sham than in hypertensive rats. This may reflect a diminished role of Cl^- channels in 2K1C hypertensive rats, which could be caused by a decreased channel number or impaired channel activity. However, the interpretation of these results may not be straightforward. Differences in membrane potential between blood vessels from 2K1C hypertensive and those of sham normotensive rats in Cl^--free buffer may have been responsible for the increased ability of NFA to inhibit cirazoline-induced vasoconstriction in sham when compared with 2K1C hypertensive rats. The possibility exists that adaptive changes in ion content and /or permeability of vascular smooth muscle in 2K1C hypertensive rats may have occurred. Certainly, lowering Cl^- concentration of physiological salt solution has been found to result in changes in resting membrane potential in vascular smooth muscle secondary to changes in E_{Cl} (Davis et al. 1991; Harder and Sperelakis 1978). It has been reported that removal of $[Cl^-]_0$ accompanied an initial transient depolarization of smooth muscle membrane due to initial net efflux Cl^- from cells (Aickin and Brading 1982; Harder and Sperelakis 1978). In agreement with this, we found that a small transient contraction developed shortly after changing to Cl^--free buffer in mesenteric arterial beds from both groups of rats, suggesting that removal of $[Cl^-]_0$ produces a small depolarization sufficient to reach the threshold for activation of VOCs and thereby smooth muscle contraction. We also found that the magnitude of the spontaneous contraction was greater in

2K1C hypertensive rats than that in sham rats, implying that the depolarization produced upon removal of Cl^- is greater in MAB from 2K1C hypertensive rats than normotensive rats. On the other hand, the greater spontaneous response could also be due to a decreased resting membrane potential (V_m) in MAB from 2K1C rats. It has been shown that in one-kidney, one-clip (1K1C) hypertension, the resting tail artery was depolarized by about 7 mV. This depolarization may be caused by a humoral substance since plasma supernatants from hypertensive rats also depolarized the muscle cells in control animals (Pamnani et al. 1985). However, V_m of mesenteric smooth muscle in 2K1C hypertensive rats may be not changed under control conditions as compared with normotensive control rats because the vasoconstrictor responses to KCl in MAB were similar between the two groups of rats in our experiments. Thus, the greater transient contraction induced by initial removal of Cl^- may be due to a higher $[Cl^-]_i$ that could shift E_{Cl} to more positive direction, and/or a greater permeability in hypertensive mesenteric smooth muscle than normotensive ones, leading to more depolarization as the net efflux Cl^- contributes more to V_m. It has been demonstrated that deoxycorticosterone acetate (DOCA)/salt-induced hypertension in the rat is associated with a significant rise in intracellular Cl^- in arterial smooth muscle and this difference in $[Cl^-]_i$ can be attributed to an increase in activity of the $Na^+/K^+/Cl^-$ cotransporter (Davis et al. 1993). Data regarding the changes in Cl^- handling and membrane properties in 2K1C hypertensive rats are limited. Not until quite recently was there direct evidence to support our speculation. However, recently Goecke et al reported that there was a significant increment in the Na^+/K^+ pump and $Na^+/K^+/Cl^-$ cotransporter in aortic rings from two kidney-Goldblatt hypertensive rats (Goecke et al. 1998). This evidence supports our assumptions. Since the Na^+/K^+ pump is electrogenic, activation of Na^+/K^+ pump would hyperpolarize the membrane

(Cheung 1989). On the other hand, the $Na^+/K^+/Cl^-$ cotransporter accumulates intracellular Cl^- above equilibrium. Although it is electroneutral (Aickin and Brading 1990; Chipperfield 1986), by raising $[Cl^-]_i$, it has a depolarizing influence on V_m (Davis 1992; Davis et al. 1991). Thus, the V_m of mesenteric smooth muscle in 2K1C hypertensive rats may not change under control conditions, although there are increased activities of the Na^+/K^+ pump and $Na^+/K^+/Cl^-$ cotransporter. However, the levels of $[Cl^-]_i$ may be higher in smooth muscle of the hypertensive MAB perfused with physiological salt solution than that of normotensive ones due to the high activity of $Na^+/K^+/Cl^-$ cotransporter. If this is true, the greater spontaneous contraction shown in the present study in 2K1C MAB upon changing the perfusion solution to Cl^--free was most likely due to an elevated $[Cl^-]_i$ (see above discussion). The elevated $[Cl^-]_i$ could also explain why an impaired function of Cl^- channels in MAB from 2K1C was only shown in Cl^--free buffer. It is known that the magnitude of ion currents flowing through open channels is determined by the number of ion channels, the single channel current and the channel activity (or P_{open}) (Nelson and Quayle 1995). The single channel current depends on the ion concentration and the driving force for the ions (Aidley 1998). Therefore, in normal physiological buffer, the elevated $[Cl^-]_i$ could enhance the efficacy of Cl^- channels when they are open, owing to the enhanced driving force for Cl^- in MAB from 2K1C hypertensive rats. Thus, the effect caused by decreased channel number or/and impaired channel activity could be masked by an increased single channel current. However, in Cl^--free buffer, the fall in $[Cl^-]_i$ due to removal of Cl^- would be greater in 2K1C MAB than in sham control rats because of the greater driving force and increased $Na^+/K^+/Cl^-$ cotransporter activity. Prolonged perfusion with Cl^--free buffer could eventually abolish or greatly decrease the differences in $[Cl^-]_i$ between mesenteric smooth muscles from 2K1C hypertensive and

normotensive rats, thereby revealing a decreased function of Cl^- channels. The diminished function of Cl^- channels could account for the smaller reduction in contractile responses and the lesser inhibitory effect of NFA in hypertensive MAB when challenged with cirazoline in Cl^--free buffer. Whether the above explanations are correct can be clarified with the help of studies that combine measurement of membrane potential, $[Cl^-]_i$ and channel activity. In addition, a future study involving blockade of $Na^+/K^+/Cl^-$ cotransport may help to resolve the involvement of the cotransporter in the changes observed in the present study. The impaired Cl^- function might reflect an adaptive change due to the enhanced reactivity to α_1-adrenoceptor stimulation in 2K1C hypertension. Yoshida et al (Yoshida et al. 1989) had previously reported that an increase in intracellular calcium content of vascular smooth muscle in 2K1C hypertensive rabbits did occur. However, the possibility that an increase in the availability of intracellular calcium may be responsible for a diminished role for Cl^- during agonist-mediated pharmacomechanical processes in 2K1C hypertensive rats cannot be determined from our present study. Nevertheless, the results presented here are the first indication of altered function of Cl^- channels in blood vessels in experimental hypertension.

VI. SUMMARY

1. In *vivo,* cirazoline induced a dose-dependent reduction in superior mesentery vascular conductance. The extent of the reduction in the conductance in 2K1C hypertensive rats was similar to that in normotensive rats. NFA attenuated the reduction in the vascular conductance induced by cirazoline to a similar degree in both normotensive and hypertensive rats.

2. *In vitro,* cirazoline induced a concentration-dependent increase in perfusion pressure in rat isolated perfused mesenteric arterial bed. The pressor responses to cirazoline in MAB from 2K1C hypertensive rats were significantly greater than those in sham rats. NFA suppressed the cirazoline-induced vasoconstriction in sham and 2K1C MAB, but had no effect on KCl-evoked pressor responses.

3. Removal of Cl^- from the perfusion buffer also impaired the pressor responses to cirazoline. The inhibition was greater in normotensive rats than that in hypertensive rats. NFA caused a further inhibition of cirazoline-mediated vasoconstriction in Cl^--free buffer. The inhibitory effect of NFA was smaller in hypertensive rats than that in normotensive rats.

4. Nifedipine also inhibited cirazoline-induced vasoconstriction. The magnitude of the inhibitory effect of NFA plus nifedipine on cirazoline-induced contraction in perfused MAB was similar to that seen with nifedipine alone. In the presence of nifedipine, NFA had no additive effect on pressor responses to KCl.

5. In low Ca^{2+} solution, in which the response to KCl was already attenuated, NFA reduced cirazoline-induced contraction with no effect on KCl-evoked contraction.

6. Cirazoline elicited contraction in Ca^{2+}-free, EGTA-containing solution. The contraction was transient in response to 3 nmol of cirazoline, and was not inhibited by either 10 μM NFA or 3μM nifedipine. However, in the Ca^{2+}-free, EGTA-containing solution, responses to KCl were abolished.

7. In small mesenteric arteries, cirazoline caused a dose-dependent increase in ^{125}I efflux. The increased ^{125}I efflux was inhibited by prazosin and blocked by NFA.

VII CONCLUSIONS

Overall, our results demonstrated that NFA is capable of inhibiting α_1-adrenoceptor-mediated vasoconstriction of rat mesenteric artery both *in vitro* and *in vivo*. The mechanism of this action of NFA in our experimental system appears to involve a decreased Cl^- efflux, but not direct inhibition of Ca^{2+} influx or release from intracellular stores. Our observations suggest that Cl^- plays an important role in α_1- adrenoceptor-mediated vasoconstriction in rat mesenteric vessels, probably by producing membrane depolarization that leads to opening of voltage-gated Ca^{2+} channels, and consequently, a sustained contraction will be maintained though Ca^{2+} influx. This contribution of Cl^- in blood vessels from hypertensive rats appears to be reduced.

PART 2. THE MECHANISMS OF THAT ACETYLCHOLINE-INDUCED RELAXATION IN RAT MESENTERIC ARTERY: A COMPARISON WITH AORTA

I. RATIONALE

In rat mesenteric arteries, endothelium-dependent relaxation to ACh has been demonstrated to be mediated by NO, and an unidentified EDHF that elicits NO/PGI_2-independent hyperpolarization by activation of K^+ channels (Chen and Cheung 1997; Fukao et al. 1995). However, there are conflicting results regarding the relative contribution of these EDRFs and the mechanisms that mediate ACh-induced relaxation by these factors in rat mesenteric arteries. The types of K^+ channels activated by EDHF have not yet been definitely identified. In addition, the role of PGI_2 in ACh-induced relaxation in mesenteric arteries is not clear (Adeagbo and Triggle 1993; Chen and Cheung 1997; Garland and Plane 1996; Hansen and Olesen 1997; Hwa et al. 1994; Waldron and Garland 1994; Weidelt et al. 1997; White and Hiley 1997; Wu et al. 1997) (and please see Introduction).

The release of NO, EDHF and PGI_2 by ACh acting at muscarinic receptors has been studied (Fukao et al. 1997c; Luckhoff and Busse 1990). There is evidence that an agonist-induced increase Ca^{2+} influx from the extracellular space is essential for the maintained production of EDRFs, and consequently, the full magnitude of agonist-evoked smooth muscle relaxation (Fukao et al. 1997c; Kruse et al. 1994; Luckhoff and Busse 1990). However, the dependence on extracellular Ca^{2+} for their synthesis seems to be different among these EDRFs (Luckhoff 1988; Luckhoff and Busse 1990; White and Martin 1989). In endothelial cells, opening of Ca^{2+} entry channels is believed to require agonist binding to membrane receptor and / or depletion of intracellular Ca^{2+} stores (Putney 1991; Wang and

van Breemen 1997). Unlike in smooth muscle cells, opening Ca^{2+} entry channels in endothelial cells is not voltage-dependent (Colden-Stanfield et al. 1987; Johns et al. 1987). However, the driving force for Ca^{2+} entry depends on the transmembrane potential, which is controlled by a variety of ion channels. Membrane hyperpolarization augments the electrochemical driving force promoting Ca^{2+} entry (Adams et al. 1989; Johns et al. 1987; Laskey et al. 1990; Luckhoff and Busse 1990).

It has been reported that in freshly isolated rabbit aortic endothelial cells and in native endothelium from intact rat aorta, stimulation of muscarinic receptors with ACh activates Ca^{2+}-dependent K^+ channels leading to transient membrane hyperpolarization, which is sensitive to tetraethylammonium (TEA) and/or charybdotoxin (CTX) (Busse et al. 1988; Marchenko and Sage 1994; Sakai 1990; Wang et al. 1995a; Wang et al. 1996). ACh activation of K^+ channels leading to endothelium hyperpolarization was also demonstrated in freshly isolated guinea pig coronary artery (Chen and Cheung 1992) and intact guinea pig carotid artery (Quignard et al. 2000). Thus, K^+ channels present on endothelium seem to have an important role in hyperpolarizing endothelium, and facilitating Ca^{2+} entry (Usachev et al. 1995). Indeed, in native freshly isolated rat aortic endothelial cells, high K^+ blocked the increase in $[Ca^{2+}]_i$ and greatly inhibited NO release in response to ACh (Luckhoff and Busse 1990), while in intact rabbit aorta, TEA was capable of inhibiting ACh-induced Ca^{2+} elevation and EDRF synthesis/release as well as vasorelaxation based on results of bioassay and fura-2 spectrofluorimetry techniques (Demirel et al. 1994). These results suggest that endothelial K^+ channels can modulate ACh-induced relaxation by controlling membrane potential, and subsequent Ca^{2+} influx and EDRF synthesis/release.

Recently, Cl^- channels have been identified in various endothelial cells (Groschner and Kukovetz 1992; Nilius et al. 1996; Nilius et al. 1997a; White et al. 1995). Some researchers have demonstrated that lowering the extracellular Cl^- concentration or exposing cells to Cl^- channel antagonists (NFA or N-phenylanthranilic acid) inhibits sustained Ca^{2+} signaling stimulated by agonists such as ATP and histamine and triggered by Ca^{2+} store depletion with thapsigargin and cyclopiazonic acid in human aortic endothelial cells (Hosoki and Iijima 1994; Hosoki and Iijima 1995; Yumoto et al. 1995). Wang and van Breemen (Wang and van Breemen 1999) have reported that in freshly isolated rabbit aortic endothelial cells, ACh activated a slowly developing Cl^- current, which was blocked by Cl^- channel antagonists. In addition, removal of extracellular Cl^- ions abolished the ACh-induced sustained Ca^{2+} signal as well as divalent cation entry. After clamping the membrane potential at a hyperpolarizing level close to K^+ equilibrium potential, Cl^- removal had no effect on ACh- induced Ca^{2+} entry, indicating that Cl^- current modulates Ca^{2+} influx by maintaining a polarized membrane potential after ACh activation. Thus, Cl^- channels may act in conjunction with K^+ channels on regulating ACh-induced EDRF synthesis, thereby causing vasorelaxation. However, there is no evidence yet for a functional role of Cl^- channels in contributing to agonist-induced relaxation.

Based on the evidence mentioned above, we conducted a functional study to investigate the mechanisms mediating ACh-induced relaxation, especially to see whether besides K^+ channels, Cl^- channels in vascular endothelial cells could also modulate ACh-induced relaxation of underlying smooth muscle; if so, whether the relative contribution of Cl^- as well as K^+ channels is different in elastic vs. muscular arteries. We also looked at how NO, PGI_2 and EDHF differentially contribute to ACh-induced relaxation in the different

arteries. These experimentally observed differences in the relative contribution of EDRFs and the mechanisms that mediate endothelium-dependent relaxation between large and small arteries may have some physiological significance; in particular, since it may be clinically important for vascular diseases, such as atherosclerosis and hypertension.

To test this, we compared the effects of NFA, which is a potent calcium-activated Cl^- channel blocker in endothelium (Nilius et al. 1997a), TEA, and the combination of these two drugs on ACh-induced relaxation in isolated rings of rat aorta and superior mesenteric artery with intact endothelium. L-NMMA, the NO synthase inhibitor, and indomethacin, the COX inhibitor, were used alone or in combination to functionally separate the relaxation mediated by different factors, namely, NO, PGI_2 and EDHF. In addition, to better understand the mechanisms of ACh-mediated relaxation in the rat mesenteric vascular bed, we compared the characteristics of the muscarinic receptor-induced and the receptor-independent calcium ionophore A23187-induced relaxation in mesenteric arteries to that in rat aorta. We also further analyzed EDHF-mediated ACh-induced relaxation using different K^+ channel blockers in mesenteric arteries. The following research hypotheses and specific experimental objectives were addressed:

II. WORKING HYPOTHESES AND SPECIFIC RESEARCH OBJECTIVES:

Working Hypotheses:

A. The relative importance of endothelial Cl^- channels and K^+ channels in modulating ACh-induced relaxation is different in muscular mesenteric arteries vs. elastic aorta.

B. The relative contributions of NO, EDHF, and PGI_2 to ACh-induced relaxation are different in rat mesenteric artery and aorta.

Specific Objectives:

1). To examine the effects of niflumic acid (NFA), tetraethylammonium (TEA), a relatively selective large conductance Ca^{2+}-activated K^+ channel antagonist and the combination of these two drugs on ACh- induced relaxation in intact rat aortic and mesenteric rings.

2). To test the influence of L-NMMA, a NO synthase inhibitor, on ACh-induced relaxation in the absence or presence of TEA or NFA plus TEA in isolated rat aorta and mesenteric arteries.

3) To examine the effects of NFA, TEA, and the combination of these two drugs on Ca^{2+} ionophore A23187-induced relaxation in intact rat aortic and mesenteric rings.

4) To compare the influence of L-NMMA in combination with indomethacin on ACh-induced relaxation in the absence and presence of TEA and the combination of NFA plus TEA in isolated rat aorta and mesenteric arteries.

5) To examine the effects of L-NMMA and isotonic high K^+ buffer on A23187- induced relaxation in isolated rat aorta and mesenteric arteries.

6) To investigate the effects of isotonic high K^+ buffer, K^+ channel blockers and their combinations {including charybdotoxin (CTX), a intermediate and large conductance Ca^{2+}-activated K^+ channel blocker, apamin (AMP), a small conductance Ca^{2+}-activated K^+ channel blocker, the combination of CTX and AMP, the combination of TEA and AMP} on ACh-induced relaxation resistant to L-NMMA/indomethacin in isolated rat mesenteric arteries.

III. METHODS AND MATERIALS

1. Isolated Artery Ring Preparation for Isometric Tension Measurement

Male Wistar rats (250-350g) were anaesthetized by intraperitoneal injections of sodium pentobarbitone (65mg/kg). The thoracic aorta and superior mesenteric arteries were removed and placed in Krebs solution of composition (in mM): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2 $NaHCO_3$ 35 and dextrose 11.5, at room temperature. The arteries were carefully cleaned of connective tissues and fat, and were then cut into rings of 4 mm and 3 mm in length for aorta and mesenteric arteries, respectively. In some rings, the endothelium was removed by inserting a wire or the tip of a forceps and gently rolling the rings back and forth on a finger moistened with Krebs buffer. Each ring was suspended horizontally between two triangular-shaped stainless steel hooks in individual organ baths containing 20 ml Krebs solution maintained at 37° C and gassed with 95% O_2 –5% CO_2 resulting in pH 7.4. Rings of aorta and mesenteric arteries were placed under resting tension of 2.0 and 1.0 g, respectively. These tensions were determined in preliminary studies to be optimal. Isometric tension was measured and recorded using a Grass FT 0.3 force displacement transducer and a Grass 7E polygraph (Grass Instrument Quincy Mass). Rings were allowed to stabilize for 90 min, during which the bathing solution was changed every 20 min, before the start of each experiment.

2. Experimental Protocols

The rings were initially stimulated with a submaximal concentration (ED_{80}) of phenylephrine (PE, 10^{-7} M and 3x 10^{-6} M for aorta and mesenteric arteries, respectively, except where stated). After the responses to PE had stabilized, tissues were relaxed with ACh

(10 μM) to ascertain endothelial integrity. The tissue then was allowed to re-equilibrate for 60 min with washing every 20 min. The rings with intact endothelium were then contracted with PE (10^{-7} M and 3 x 10^{-6} M for aorta and mesenteric arteries, respectively) again. When a stable contraction was obtained, ACh (10^{-8} to 10^{-4} M) or A23187 (3 x10^{-9} to 3 x10^{-6}M) was added to the bath cumulatively. In experiments in which the inhibitors were used, they were added to the tissue bath before the tone was raised with PE. The pre-incubation time was 30 min with NFA (30 μM), TEA (3 mM) and indomethacin (20 μM), 15 min with CTX (0.1 μM) and apamin (0.3 μM), and 1h with L-NMMA (300μM). Control experiments with vehicle were performed in the same manner. The effects of each inhibitor alone and in combination were examined in separate tissues. When the effect of K^+ (30 mM) was tested, the bath solution was exchanged with Krebs solution containing 30 mM KCl immediately before addition of PE. The high K^+ solution was prepared by isotonic substitution of NaCl by KCl. In some experiments, indomethacin (10 μM) was present throughout to prevent formation of prostanoid by COX.

3. Chemicals

Acetylcholine chloride, the calcium ionophore A23187, (-)-phenylephrine hydrochloride, NFA, TEA, CTX, apamin, and indomethacin were purchased from Sigma (St. Louis, MO, USA). L-NMMA monoacetate was obtained from Calbiochem (San Diego, CA, USA). Stock solutions of NFA (0.1 M) and indomethacin (0.1 M) were prepared in ethanol. A23187 (10^{-2} M) was dissolved in dimethyl sulphoxide (DMSO) and diluted with ethanol. The final concentration in the bath was < 0.06 (vol)% for ethanol, < 0.03 (vol)% for DMSO. ACh, PE, TEA, L-NMMA, CTX and apamin were dissolved in twice-distilled water. All solutions were made fresh every day.

4. Statistical Analysis

Relaxation is expressed as the percent decrease in PE-induced tone. pD_2 values {defined as -log (ED_{50})} and percentage of maximum relaxation (R_{max}) as determined from individual curves were fitted according to the following logistic equation:

$$R = \frac{R_{max} \cdot A^{n_H}}{ED_{50}{}^{n_H} + A^{n_H}}$$

where R is the relaxation (%), A is the concentration of vasorelaxant, nH is the Hill coefficient and ED_{50} is the molar concentration of vasorelaxant which causes 50% of the maximum relaxation. All data are presented as mean ± SEM. One-way ANOVA was used for multiple comparisons between control and treated tissue values for pD_2 and R_{max}. Two-way ANOVA was used to compare data between two groups (as stated). Duncan's multiple range post test was used to compare between multiple means. Unpaired Student's t-test was used for comparison between two means. $P < 0.05$ was considered significant.

IV. RESULTS

1. ACh- Induced Relaxation

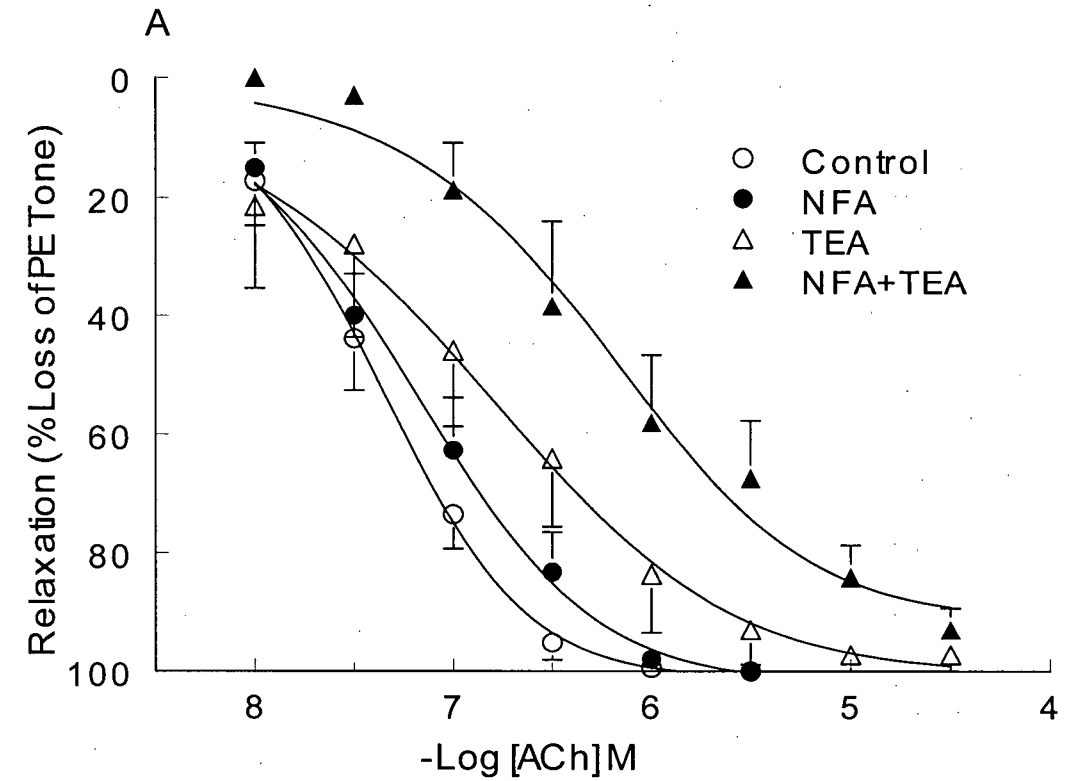
1.1. The Effect of NFA and TEA on ACh-Induced Relaxation in Rat Aorta and Mesenteric arteries.

ACh induced a concentration-dependent relaxation of intact rings of aorta and mesenteric arteries precontracted with PE (Fig. 2.1). This relaxation effect was endothelium-dependent since removal of endothelial cells abolished the relaxation in both aorta and mesenteric arteries (data not shown). Addition of 30 μM NFA did not affect baseline or PE-induced tension in either arteries (legend to Fig. 2.1). Incubation of aorta or mesenteric rings with 3 mM TEA also had no effect on the basal tone, but potentiated PE-evoked tone to a similar degree in the two arterial rings in terms of percentage of their respective control (165 ± 13%, n = 6 and 150 ± 14%, n = 5 for aorta and mesenteric arteries, respectively, $P > 0.05$). In intact aorta, neither NFA nor TEA alone had a significant effect on the response to ACh; however, in the presence of NFA (30 μM) plus TEA (3mM), the ACh concentration-relaxation curve was shifted to the right (Fig. 2.1A, Table 2.1). Combined use of NFA and TEA significantly decreased the sensitivity (pD_2) to ACh without changing the maximum relaxation (Table 2.1). In intact mesenteric arteries, as in aorta, NFA alone had no effect on the response to ACh (Fig. 2.1B, Table 2.1). In contrast to aorta, TEA alone resulted in a rightward displacement of concentration-response curve to ACh in mesenteric arteries (Fig. 2.1B). TEA significantly decreased the ACh pD_2 value without affecting the maximum relaxation (Table 2.1). The concomitant application of NFA and TEA did not further inhibit the relaxation to ACh as compared to TEA alone (Table 2.1).

FIGURE 2.1

A: Effect of NFA (30 μM) and TEA (3 mM) or NFA (30 μM) plus TEA (3 mM) on relaxation response to ACh in intact rat aorta rings. Corresponding PE-induced maximum tensions were 1.02 ± 0.11g (Control), 1.03 ± 0.10g (NFA), 1.62 ± 0.11g^{ab} (TEA), 1.51 ± 0.10g^{ab} (NFA +TEA) (^{a} $P < 0.05$ vs. control, ^{b} $P < 0.05$ vs. NFA). n= 6.

B: Effect of NFA (30 μM), TEA (3 mM) or NFA (30 μM) plus TEA (3 mM) on ACh-induced relaxation in intact rat mesenteric artery rings. Corresponding PE-induced maximum tensions were 0.61 ± 0.10g (Control), 0.62 ± 0.09g (NFA), 0.88 ± 0.15g^{ab} (TEA), 0.85 ± 0.10g^{ab} (NFA +TEA) (^{a} $P < 0.05$ vs. control, ^{b} $P < 0.05$ vs. NFA). n = 5.


A
0
20
40
60
80
100
Relaxation (%Loss of PE Tone)
Control
NFA
TEA
NFA+TEA
8
7
6
5
4
-Log [ACh] M

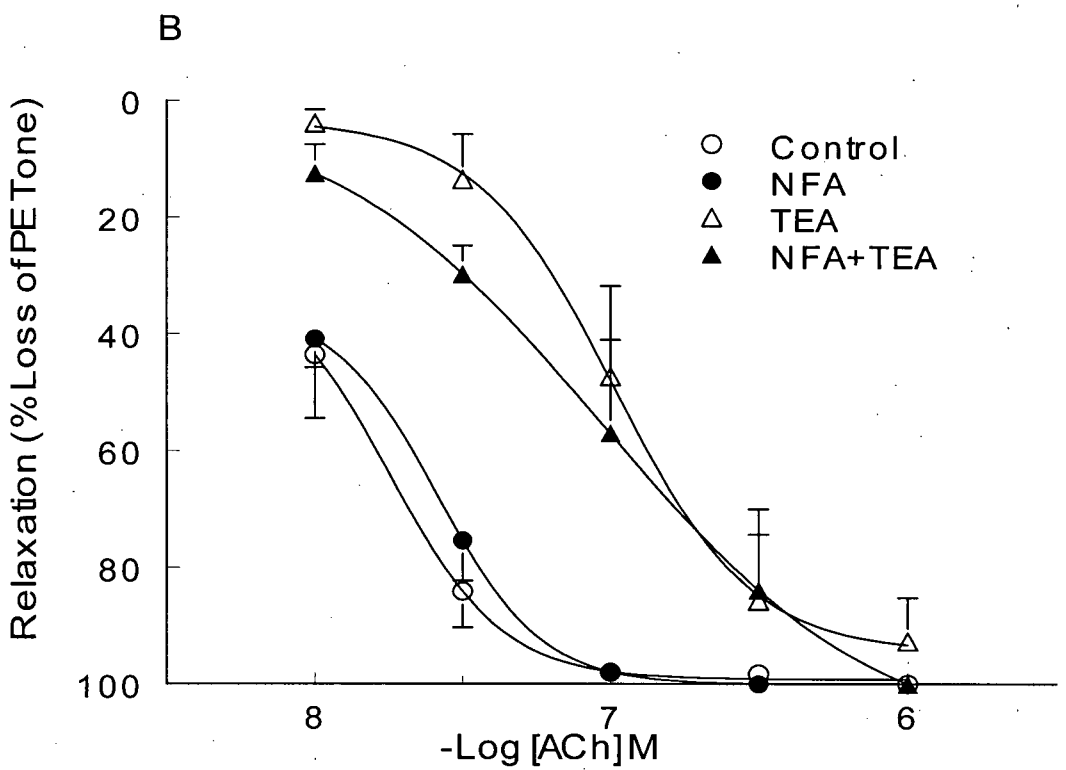

B
0
20
40
60
80
100
Relaxation (%Loss of PE Tone)
Control
NFA
TEA
NFA+TEA
8
7
6
-Log [ACh] M

TABLE 2.1

Potency (pD_2) and maximum relaxation (R_{max}, % loss of PE tone) to ACh or A23187 (aorta only) in the absence (Control) and in the presence of NFA (30 μM), TEA (3 mM) or NFA (30 μM) plus TEA (3 mM) in isolated aortic and mesenteric artery rings with intact endothelium. Arteries were precontracted with PE (10^{-7} M and 3 x 10^{-6}M for aorta and mesenteries, respectively).

	Aorta				Mesentery	
	ACh		A23187		ACh	
	pD_2	R_{max} (%)	pD_2	R_{max} (%)	pD_2	R_{max}(%)
Control	7.41±0.13	102±1	7.88±0.06	86±4	7.94 ± 0.09	102±2
NFA	7.11±0.14	105±1	7.55±0.13	80±4	7.84 ± 0.59	107±5
TEA	7.06±0.41	102±1	7.63±0.10	72±12	7.07±0.16 [a b]	111±6
NFA+TEA	6.11±0.29[a b c]	100±10	7.73±0.15	83±5	6.94±0.17 [a b]	101±1

Each value represents the mean of six (aorta) and five (mesentery) experiments ± SEM. ([a] $p < 0.05$ vs. control, [b] $p < 0.05$ vs. NFA, [c] $p < 0.05$ vs. TEA).

1.2. Effect of L-NMMA on ACh-Induced Relaxation of PE-Evoked Tension

In order to elucidate the nature of the inhibition by NFA in combination with TEA of the response to ACh in rat aorta, and to further explore the different mechanisms that may be responsible for ACh mediated endothelium-dependent relaxation in aorta and mesenteric arteries, the effect of a NO synthase inhibitor, L-NMMA, on ACh-induced relaxation under the experimental conditions tested above (excepted for NFA alone, because of it's lack of effect in both arteries) was investigated. 300 μM L-NMMA alone slightly raised the basal tone in aorta (by 0.17 ± 0.03g tension, n = 7), but not in mesenteric arteries (n = 9). Pre-contractions produced by ED_{80} of PE were augmented by L-NMMA (n = 6 and 5, for aorta and mesenteric arteries, respectively, $P < 0.05$-see legend to Fig. 2.2). Neither TEA (3 mM) alone nor NFA (30 μM) plus TEA (3 mM) further increased the PE tone in the presence of L-NMMA in either artery (Fig. 2.2 legend). In aorta, L-NMMA significantly attenuated the relaxation response induced by ACh (Fig. 2.2A). It decreased the pD2 value and also reduced the maximum relaxation (Table 2.2). In the presence of L-NMMA, TEA alone, as well as NFA plus TEA, further inhibited ACh-induced relaxation; they caused a greater reduction in both pD_2 value and maximum relaxation in comparison with L-NMMA alone (n=6, $P < 0.05$) (Fig 2.2A, Table 2.2). However, there was no difference between the inhibitory effect of TEA alone and NFA plus TEA in the presence of L-NMMA (n = 6, $P > 0.05$) (Table 2.2). In mesenteric arteries, the ACh concentration-response curve was shifted to the right in the presence of L-NMMA (300 μM) (Fig. 2.2B). L-NMMA significantly decreased the ACh pD_2 value (n = 5, $P < 0.05$), but the maximum relaxation to ACh was not affected (Table 2.2). Pretreatment with L-NMMA plus TEA (3 mM) further reduced the ACh pD_2 value (n = 5, P

FIGURE 2.2

A: Effect of L-NMMA (300 μM) on the relaxation response to ACh in intact rat aorta, in the absence and presence of TEA (3 mM) or NFA (30 μM) plus TEA (3 mM).

The initial tensions induced by PE were 0.90 ± 0.19g (Control); 1.66 ± 0.38g[a] (L-NMMA); 1.74 ± 0.29g[a] (TEA+L-NMMA); 1.75± 0.21g[a] (NFA+TEA+L-NMMA) ([a]$P < 0.05$ vs. control). n = 6

B: Effect of L-NMMA (300 μM) on the relaxation response to ACh in intact mesenteric artery rings, in the absence and presence of TEA (3 mM) or NFA (30 μM) plus TEA (3 mM).

The initial tensions induced by PE were 0.62 ± 0.14g (Control); 1.11 ± 0.12g[a] (L-NMMA); 1.15 ± 0.07g[a] (TEA+L-NMMA); 1.12 ± 0.11g[a] (NFA+TEA+L-NMMA) ([a]$P < 0.05$ vs. control). n = 5.

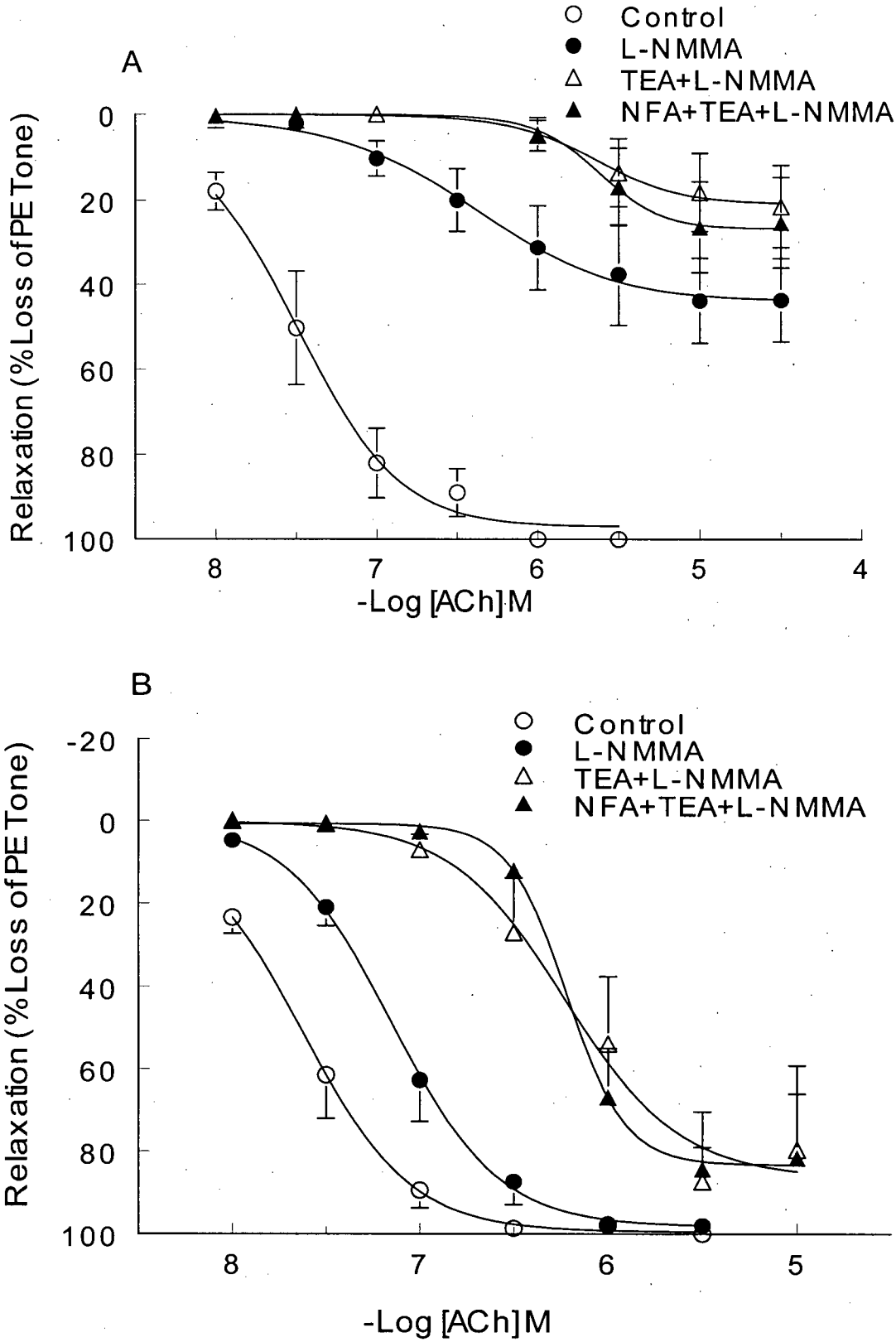

A
Control
L-NMMA
TEA+L-NMMA
NFA+TEA+L-NMMA
Relaxation (%Loss of PE Tone)
0
20
40
60
80
100
8
7
6
5
4
-Log [ACh] M
B
Control
L-NMMA
TEA+L-NMMA
NFA+TEA+L-NMMA
Relaxation (%Loss of PE Tone)
-20
0
20
40
60
80
100
8
7
6
5
-Log [ACh] M

TABLE 2.2

Effects of L-NMMA (300 μM) (A) and L-NMMA plus indomethacin (Indo, 20μM) (B) on potency (pD_2) and maximum relaxation (R_{max}) to ACh in the absence and in the presence of 3 mM TEA or 30 μM NFA plus 3 mM TEA in intact rat aortic and mesenteric arterial rings precontracted with PE (10^{-7} M and $3x10^{-6}$ M, respectively). The ACh curve without L-NMMA (A) or L-NMMA+Indo (B) served as a control.

	ACh (Aorta)		ACh (Mesentery)	
	pD_2	R_{max} (%)	pD_2	R_{max} (%)
A.				
Control	7.44 ± 0.12	98 ± 3	7.57 ± 0.04	111 ± 5
L-NMMA	6.40 ± 0.25 [a]	40 ± 9 [a]	7.09 ± 0.14 [a]	103 ± 6
TEA+L-NMMA	5.67 ± 0.28 [ab]	21 ± 13 [ab]	5.97 ± 0.23 [ab]	101 ± 2
NFA+TEA+L-NMMA	5.64 ± 0.30 [ab]	26 ± 9 [ab]	6.20 ± 0.06 [ab]	90 ±14
B.				
Control	7.47 ± 0.22	106 ± 7	7.75 ± 0.12	101 ± 2
L-NMMA ± Indo	6.83 ± 0.24 [a]	34 ± 7 [a]	6.99 ± 0.15 [a]	91 ± 5
TEA+L-NMMA ± Indo	6.06 ± 0.26 [ab]	23 ± 4 [a]	5.71 ± 0.19 [ab]	102 ± 2
NFA+TEA+L-NMMA ± Indo	/	/	5.85 ± 0.21 [ab]	85 ±14

Values represent the mean ± SEM. from six (aorta) and five (mesenteric arteries) experiments. ([a] $P<0.05$ vs. control, [b] $P<0.05$ vs. L-NMMA in A or vs. L-NMMA+Indo in B; there are no significant differences between A and B, $P > 0.05$; two-way ANOVA)

< 0.05). However, the maximum response to ACh was still unchanged (Table 2.2). In the presence of L-NMMA, NFA plus TEA shifted the concentration-relaxation curve for ACh to the right to a similar degree as TEA alone did.

1.3. Effect of Indomethacin on ACh-Induced Relaxation of PE-Evoked Tension

The above experiments were repeated in the presence of indomethacin. Application of indomethacin in combination with other inhibitors namely L-NMMA, TEA or TEA plus NFA had no additional effect on basal tone as compared with the corresponding controls in either mesenteric arteries or aorta (data not shown) There was no significant difference in pD_2 values or the maximum relaxations to ACh (n = 5 for each artery, P > 0.05) between the curves obtained in the absence or in the presence of indomethacin under each of the conditions (Table 2.2). Indomethacin alone had also no effect on the response of control to ACh obtained in absence of any inhibitor (pD_2: control, 7.47 ± 0.22, indomethacin, 7.40 ± 0.11; R_{max}: control, 106 ± 7%, indomethacin, 107 ± 6% n = 5 for aorta, P > 0.05; pD_2: control, 7.63 ± 0.08, indomethacin: 7.58 ± 0.02; R_{max}: control: 102 ± 3%, indomethacin: 105 ± 6% n = 5, for mesenteric arteries, P > 0.05). Thus, the contribution of PGI_2 to ACh-induced relaxation in both arteries seems to be negligible.

2. A23187-Induced Relaxation

2.1. The Effect of NFA and TEA on A23187-Induced Relaxation in Rat Aorta and Mesenteric Arteries.

Relaxation to A23187 was examined as a comparison with ACh. A23187 induced a concentration-dependent relaxation of endothelium-intact rings of rat aorta and mesenteric arteries precontracted with PE (Fig. 2.3A, B). In aorta, pretreatments with NFA (30 μM) or

TEA (3 mM), or a combination of NFA (30 μM) and TEA (3 mM) had no effect on the A23187-induced relaxation (Table 2.1). In mesenteric arteries, pretreatment with TEA (3mM) (n = 5, Fig. 2.3B) reduced the maximum relaxation of PE tone to 38 ± 11 %. Since NFA had no effect on ACh-induced relaxation in mesenteric arteries, we did not further test the effect of the Cl^- channel blocker on responses to A23187.

2.2. Effect of L-NMMA and K^+ on A23187-Induced Relaxation of PE-Evoked Tension

When the tissues were incubated with 300 μM L-NMMA, the responses to A23187 were almost abolished in both arteries. The maximum relaxations to A23187 in the presence of L-NMMA were 12.3±7.3 % (n = 6) and 6.0 ± 6.0 % (n = 5) for aorta and mesenteric artery, respectively (Fig. 2.3A, B). In addition, pretreatment with 30 mM K^+ also greatly inhibited the relaxation induced by A23187 in both arteries (Fig. 2.3A, B). The maximum relaxations of PE tension were 10.7 ± 6.6 % (n = 6) and 21 ± 12 % (n = 5), respectively. Interestingly, as the representative traces in Fig. 2.4 show, application of ACh (30μM) did not further relax the aorta in the presence of either L-NMMA (Fig. 2.4A(b)) or K^+ (Fig. 2.4A(c)), while when no further relaxation to A23187 was observed, subsequent addition of ACh (30 μM) caused full relaxation in the presence of L-NMMA (Fig. 2.4 B (b)) and a small further decrease (to 41 ± 12 %, n = 5) in tension in the presence of K^+ (Fig. 2.4 B(c)) in the mesenteric arteries.

3. Effect of KCl and K^+ Channel Blockade with Apamin, CTX, TEA and Their Combinations on ACh-Induced NO- Independent Relaxation

The ACh-induced relaxation of rat mesenteric arteries that was resistant to a L-NMMA and TEA was further investigated in mesenteric arteries (Fig. 2.5). In the presence

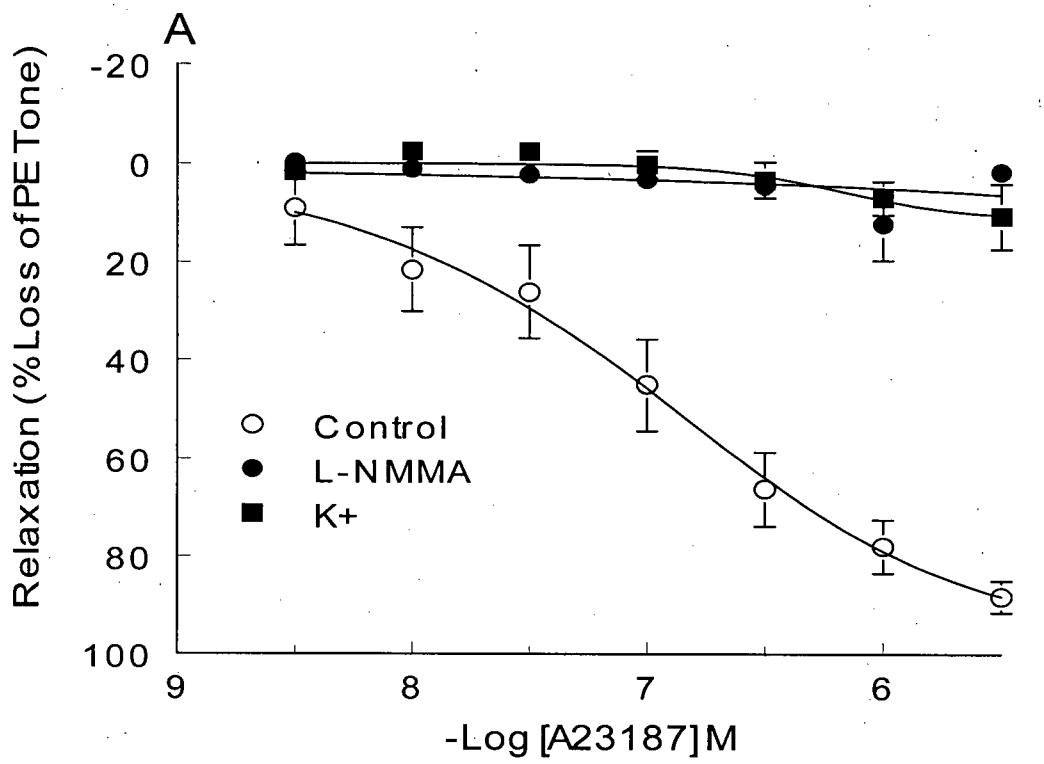
FIGURE 2.3

A: Effects of L-NMMA (300 μM) and KCl (30 mM) on A23187-induced relaxation in intact rat aorta.

The initial maximum tensions induced by PE were 1.18 ± 0.29g (Control), 1.72 ± 0.14g [a] (L-NMMA) and 1.70 ± 0.18g [a] (K^+) ([a] $P < 0.05$ vs. control). n = 6

B: Effect of TEA (3 mM), L-NMMA (300 μM) and KCl (30 mM) on A23187-induced relaxation in intact rat mesenteric arteries.

The initial maximum tensions induced by PE were 0.49 ± 0.09g (Control), 0.88 ± 0.09g [a] (L-NMMA), 0.89 ± 0.09 [a] (K^+), and 0.90 ± 0.12 [a] (TEA) (B) ([a] $P < 0.05$ vs. control). n = 5


A
Relaxation (%Loss of PE Tone)
-20
0
20
40
60
80
100
9
8
7
6
-Log [A23187]M
Control
L-NMMA
K+

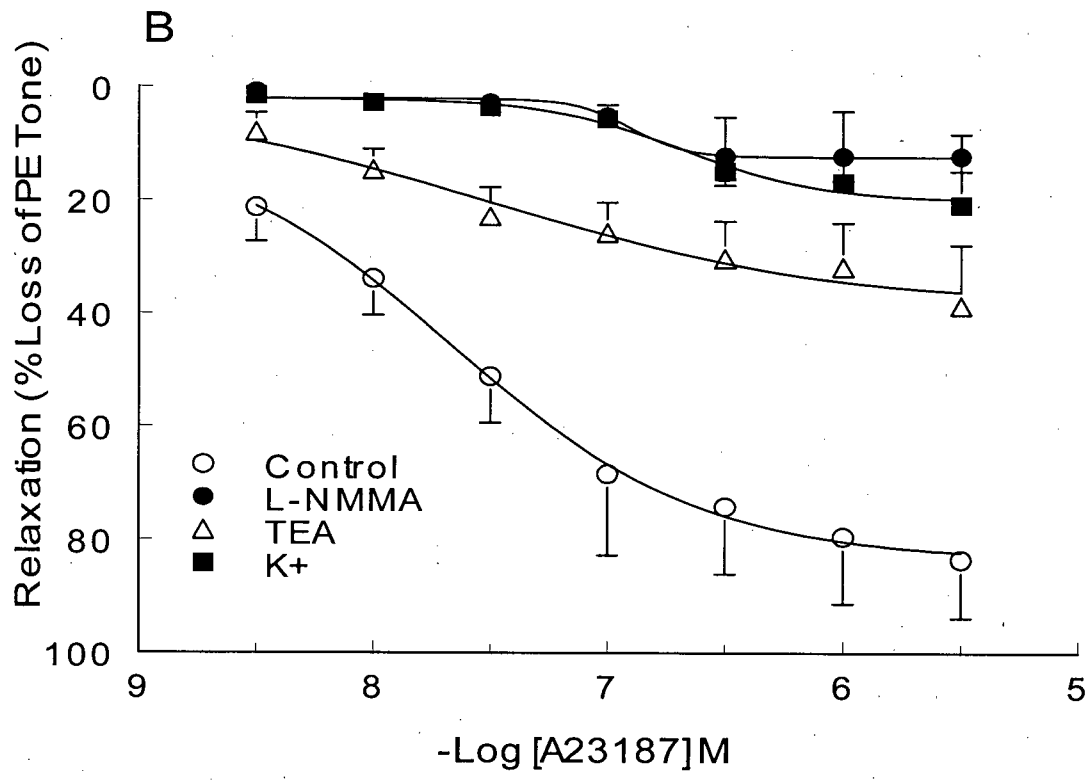
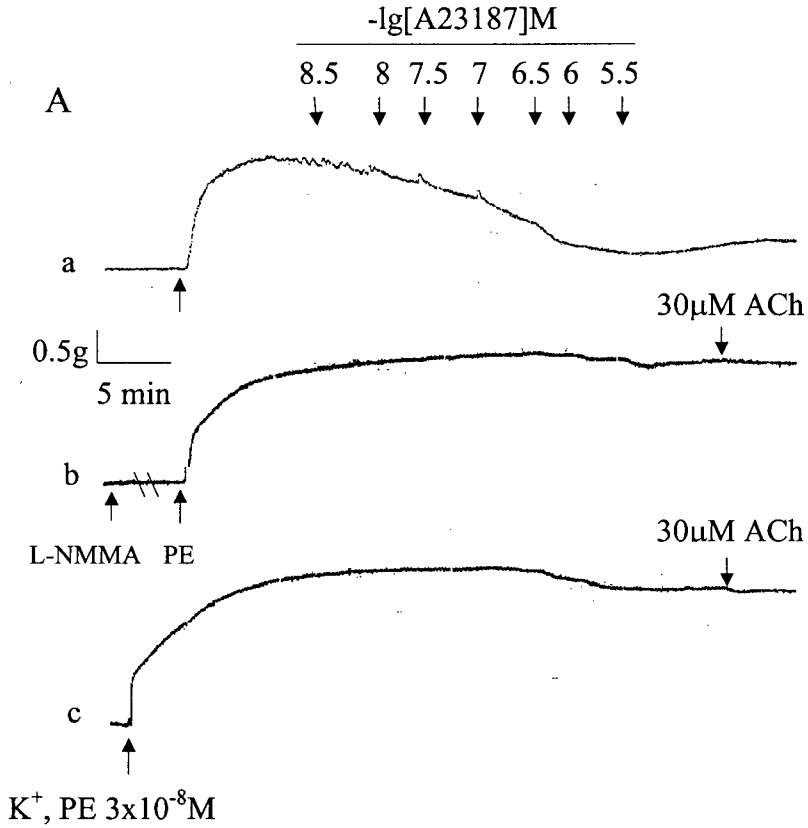
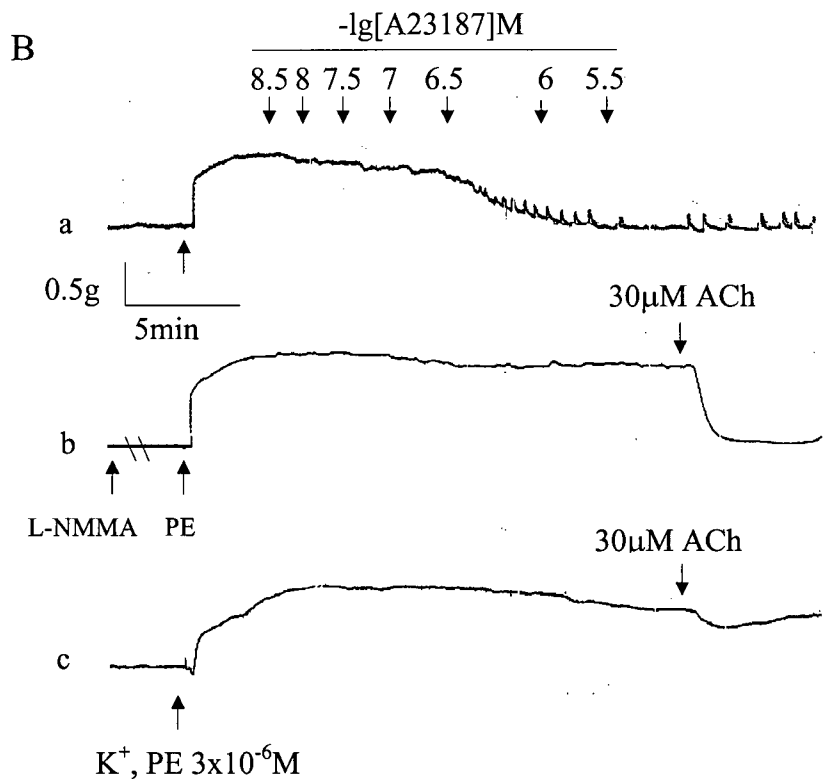

B
Relaxation (%Loss of PE Tone)
0
20
40
60
80
100
9
8
7
6
5
-Log [A23187]M
Control
L-NMMA
TEA
K+

FIGURE 2.4

Representative traces showing the relaxation responses to A23187 in intact rings from aorta (A) and mesenteric artery (B).


A
-lg[A23187]M
8.5 8 7.5 7 6.5 6 5.5
a
30μM ACh
0.5g
5 min
b
L-NMMA PE
30μM ACh
c
K+, PE 3x10-8M


B
-lg[A23187]M
8.5 8 7.5 7 6.5 6 5.5
a
0.5g
5min
30μM ACh
b
L-NMMA PE
30μM ACh
c
K+, PE 3x10-6M

of L-NMMA (300 μM), APM (0.3 μM), a small conductance Ca^{2+}-dependent K channel blocker, and CTX (0.1 μM), a large- and intermediate- conductance Ca^{2+}-dependent K channel blocker had no effect on baseline tension or PE ($3x10^{-6}$ M) tone (Fig. 2.5, legend). APM (n = 5) alone did not affect the response to ACh ($P > 0.05$, Table 2.3). CTX (n = 6) shifted the ACh concentration-response curve to the right without altering the maximum relaxation of PE tension (Fig. 2.5). The ACh pD_2 value was significantly decreased by CTX as compared to control, but the magnitude of the reduction was smaller ($P < 0.05$) than that obtained in the presence of TEA (3 mM, n = 5) alone (Table 2.3). When APM (0.3 μM) was applied in combination with CTX (0.1 μM, n = 7) or TEA (3 mM, n = 5), the relaxations induced by ACh were further attenuated. The maximum relaxation was significantly ($P < 0.05$) decreased to 64 ± 8% and 69 ± 5%, respectively. The degree of reduction in pD_2 value and maximum relaxation was similar ($P > 0.05$) between the two combinations (Table 2.3). Changing Krebs buffer to high K^+ (30 mM) solution caused a small contraction of artery rings (0.40 ± 0.06g, n = 5). However, the initial maximum tension, after addition of PE ($3x10^{-6}$ M), was not significantly ($P < 0.05$) different from L-NMMA alone (Fig. 2.5 legend). 30 mM K^+ further reduced the maximum relaxation to 38 ± 4 %. The blockade was greater than that of co-application of TEA and APM ($P < 0.05$), as well as CTX plus APM ($P < 0.05$).

FIGURE 2. 5

Effects of KCl (30 mM) and $K^+_{(ca)}$ channel blockers on L-NMMA/indomethacin-resistant response to ACh in intact rat mesenteric artery rings. Corresponding PE-induced maximum tensions were 0.96 ± 0.09g (Control), 1.04 ± 0.12g (APM), 1.01 ± 0.07g (CTX), 1.31 ± 0.27g (TEA), 1.08 ± 0.14g (CTX + APM), 1.38 ± 0.09g [a] (TEA + APM), 1.11 ± 0.15g (K^+) ($P > 0.05$, one-way ANOVA). n = 5-7.

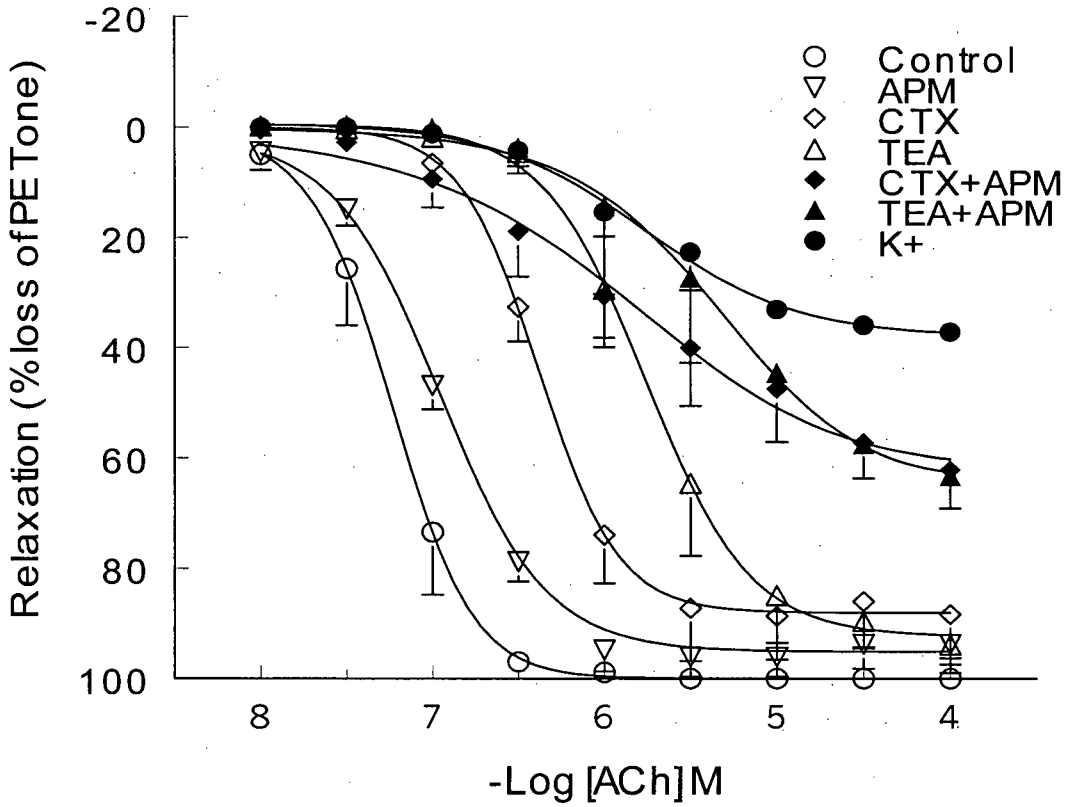

-20
0
20
40
60
80
100
Relaxation (%loss of PE Tone)
8
7
6
5
4
-Log [ACh] M
Control
APM
CTX
TEA
CTX+APM
TEA+APM
K+

TABLE 2.3

Potency (pD_2) and maximum relaxation (R_{max}, % loss of PE tone) to ACh in the absence (Control) and in the presence of APM (0.3 μM), CTX (0.1 μM), TEA (3 mM), APM (0.3 μM) plus CTX (0.1 μM), APM (0.3 μM) plus TEA (3 mM) or KCl (K^+, 30 mM) in isolated mesenteric artery rings with intact endothelium and precontracted with PE ($3x10^{-6}$ M). The experiments were performed in the presence of L-NMMA (300 μM) plus indomethacin (10 μM).

	ACh	
	PD_2	R_{max} (%)
Control	7.27 ± 0.11	100 ± 1
APM	6.98 ± 0.07	96 ± 5
CTX	6.37 ± 0.07 [a]	91 ± 9
TEA	5.85 ± 0.24 [ab]	95 ± 4
CTX +APM	5.88 ± 0.33 [ab]	64 ± 8 [ac]
TEA + APM	5.48 ± 0.25 [ab]	69 ± 5 [ac]
K^+	5.60 ± 0.15 [ab]	38 ± 4 [a]

Each value represents the mean of five to seven experiments ± SEM. ([a] $P<0.05$ vs. Control; [b] $P<0.05$ vs. CTX; [c] $P<0.05$ vs. K^+).

V. DISCUSSION

The aim of the second part of the thesis was to understand how the endothelium differentially regulates smooth muscle relaxation in rat mesenteric arteries as compared to aorta. Using isometric tension measurements, we examined the function of Cl^- and K^+ channels in muscarinic receptor-mediated relaxation in both arteries, and compared the relative importance of NO and PGI_2 as well as EDHF in endothelium-dependent smooth muscle relaxation. We also examined the component of ACh-induced relaxation that is resistant to inhibition of NO and PGI_2, in mesenteric artery. The effects of A23187 were examined as a comparison.

Aorta

Effect of NFA and TEA

Addition of either 30 μM NFA or 3 mM TEA alone to aorta did not significantly affect ACh-induced endothelium-dependent vasorelaxation. However, pretreatment with a combination of the two compounds decreased the potency of ACh approximately 10 fold, although it had no effect on the magnitude of the maximal relaxation. NFA is a potent calcium-activated Cl- channel blocker in both endothelium and smooth muscle (Hogg et al. 1994a; Nilius et al. 1997a). TEA at the concentration used in the study has been shown to selectively inhibit large conductance calcium-activated K^+ channels in arteries (Beech and Bolton 1989; Farley and Rudy 1988; Langton et al. 1991; Nelson and Quayle 1995). Thus the results suggest that in rat aorta both calcium-activated Cl^- and K^+ channels are involved in the response to ACh, and activation of either channel is sufficient to produce a full relaxation. In contrast to ACh, we found that NFA plus TEA did not affect the endothelium-dependent relaxation induced by A23187. Like ACh, A23187 evokes relaxation by releasing

NO (White and Martin 1989) and also induces endothelium-dependent hyperpolarization (Chen and Suzuki 1990). A23187 is a receptor-independent Ca^{2+} ionophore, which increases $[Ca^{2+}]_i$ by causing Ca^{2+} entry from the extracellular space via electroneutral exchange of one Ca^{2+} for two H^+ ions (Reed and Lardy 1972). On the other hand, ACh activates the muscarinic receptor, and elevates $[Ca^{2+}]_i$ by releasing Ca^{2+} from IP_3-sensitive intracellular stores (Wang et al. 1995b) and stimulating transmembrane Ca^{2+} influx through receptor- and/or store-operated Ca^{2+} channels (Putney 1991; Wang and van Breemen 1997). The elevation of $[Ca^{2+}]_i$ in endothelial cells is believed to trigger the release of NO, PGI_2 and EDHF that evoke endothelium-dependent smooth muscle relaxation (Busse et al. 1989; Fukao et al. 1997c; Whorton et al. 1984). Activation of Cl^- or K^+ channels by ACh in aortic endothelial cells has been shown to facilitate Ca^{2+} influx by providing a constant driving force for Ca^{2+} entry, as well as by preventing the depolarization-mediated inactivation of ROC (Usachev et al. 1995; Wang and van Breemen 1999). Blocking endothelial Cl^- and K^+ channels would inhibit Ca^{2+} influx (Demirel et al. 1994; Hosoki and Iijima 1994; Hosoki and Iijima 1995; Luckhoff and Busse 1990; Wang and van Breemen 1999; Yumoto et al. 1995), whereas Ca^{2+} entry produced by A23187 should not be affected. These data suggest that the inhibitory effect of the combination of NFA and TEA on ACh -induced relaxation in aorta is due to an action of the two channel blockers on endothelial cells, rather than an action of blocking the activity of these EDRFs on smooth muscle cells. Considering the fact that the presence of either of the channel blockers alone had no effect on the ACh relaxant response, we speculate that when Cl^- channels are inhibited, EDRF release by ACh may elicit vasodilation through a compensatory pathway involving activation of K^+ channels and vise versa in aorta.

Activation of agonist-induced Cl^- channels on smooth muscle evokes smooth muscle depolarization (Amedee and Large 1989; Byrne and Large 1988a; Pacaud et al. 1989b; Van Helden 1988), which is excitatory. We have reported that NFA inhibited α_1-adrenoceptor-induced vasoconstriction in isolated perfused rat mesenteric arterial beds (He and Tabrizchi 1997). However, NFA did not inhibit PE-induced contraction in this study since the initial PE-induced tension in the presence of NFA was not different from that in the absence of NFA in either aorta or superior mesenteric artery. In preliminary experiments (data not shown), we did find that NFA, at the concentration used in the present study, significantly inhibited cirazoline-induced tone in superior mesenteric artery but had little effect on PE-induced tension. In some preparations, we also found that the efficacy of cirazoline, a specific α_{1A}-adrenoceptor agonist, is apparently less than that of PE, a nonselective α_1-adrenoceptor agonist. Therefore, we chose PE to precontract the arterial segments in these experiments.

Among the EDRFs, NO is the one whose synthesis most critically relies on extracellular Ca^{2+} (Fukao et al. 1997c; Luckhoff and Busse 1990; White and Martin 1989). We assessed whether activation of Cl^- and K^+ channels could contribute specifically to NO synthesis/release by employing 300 μM L-NMMA. L-NMMA, an L-arginine analogue, is a specific NO synthase inhibitor that causes reversible inhibition of NO synthesis due to the competitive inhibition of L-arginine metabolism (Mayer et al. 1989; Moncada et al. 1991; Rees et al. 1989). It has been reported that 300 μM L-NMMA functioned the same as 1000 μM L-NMMA, causing maximum inhibition of endothelium-dependent relaxation to ACh in rat aorta (Rees et al. 1990). Furthermore, 100 μM L-NMMA could completely inhibit ACh-induced NO release measured by chemiluminescence in a bioassay system (Rees et al. 1989).

In the preliminary experiments, we confirmed that application of a higher concentration of LNMMA had no further effect on relaxation in response to ACh as compared with 300 μM L-NMMA when incubated for 60 min before segments were subjected to agonist challenge. We assumed that using this protocol NO synthesis would be blocked, and that if the Cl^- and K^+ channels do specifically contribute to stimulation of NO synthesis, the combination of NFA and TEA would no longer have any inhibitory effect on ACh-induced relaxation. In the presence of L-NMMA, the maximum relaxation to ACh was decreased nearly 60% and the pD_2 was reduced by approximately 10 fold, and TEA caused a further significant reduction in maximum relaxation and sensitivity to ACh, whereas NFA plus TEA had no further inhibitory effect as compared to TEA alone. These results imply that the inhibitory effect of NFA plus TEA on ACh-induced relaxation seen in the absence of L-NMMA is in part due to an inhibition of NO synthesis. Since the results also revealed that the inhibition of NO synthesis unmasked a relaxation component sensitive to TEA, our observations thus suggest that Ca^{2+} activated Cl^- and K^+ channels are involved, at least in part, in ACh-induced NO-dependent relaxation, while K_{Ca} channels, but not Cl^- channels mediate the NO-independent relaxation response to ACh in rat aorta.

NO-Mediated and NO-Independent Relaxation

In the presence of L-NMMA, ACh- induced endothelium-dependent relaxation was greatly but not completely inhibited, suggesting that vasorelaxation to muscarinic agonist in aorta has at least two components; one of which is mediated via NO, while the other is probably mediated via EDHF because it is sensitive to K_{Ca} blocker TEA. Although the chemical nature of EDHF has not been defined, it has been suggested that EDHF relaxes

vascular smooth muscle cells through hyperpolarization via opening of K^+ channels. That ACh could induce an endothelium dependent hyperpolarization of smooth muscle owing to an increase in K^+ conductance has been found in rat aorta (Taylor and Weston 1988). NO may stimulate smooth muscle hyperpolarization in some vessels (Bolotina et al. 1994; Mistry and Garland 1998), but ACh-induced vasorelaxation may also be associated with NO-independent hyperpolarization (Garland et al. 1995; Komori and Vanhoutte 1990). The existence of an EDHF distinct from NO, which may also contribute to ACh-induced relaxation in aorta, was first tested by Chen and Suzuki (Chen and Suzuki 1989; Chen et al. 1988). They found that in rat aorta ACh caused an endothelium dependent relaxation that was reduced but not blocked by methylene blue. They also found that ACh produced an endothelium-dependent hyperpolarization of smooth muscle cells that was not blocked by methylene blue but could be abolished by raising the external K^+ concentration. These observations have been confirmed in later studies using NO synthase inhibitors. It was shown that in the rat aorta, application of ACh or carbachol evoked an endothelium-dependent hyperpolarization that contains an initial peak component and is followed by a sustained component. NOS inhibitor had no effect on the magnitude of the first transient peak although it diminished the second component of the endothelium-dependent hyperpolarization (Vanheel et al. 1994). ACh-induced endothelium-dependent relaxation was only partially reduced by NOS inhibitor, and this NOS-resistant relaxation was blocked by high K^+ solution in the aorta (Hatake et al. 1995; Zygmunt et al. 1994a; Zygmunt et al. 1995). Our data confirm and extend the results of those functional studies, by suggesting that K_{Ca} is, at least in part, responsible for the relaxation mediated by the EDHF.

Previous studies have reported that the NOS inhibitor-resistant relaxation in rat aorta amounted to 30 % to 40 % of the unblocked response to ACh (Chen and Suzuki 1989; Zygmunt et al. 1995) and appeared only when carefully titrated the precontactile response to a certain lower level (Hatake et al. 1995; Zygmunt et al. 1994a). Therefore, it has been suggested that in rat aorta EDHF may play a minor role in the relaxation response to ACh in the absence of NOS inhibitor (Chen and Suzuki 1989; Hatake et al. 1995). The different effects of precontractile responses on ACh-induced NOS inhibitor-resistant relaxation observed by Zygmunt (Zygmunt et al. 1994a) and Hatake (Hatake et al. 1995) may also reconcile with earlier reports that in the presence of a NOS inhibitor the ACh-induced relaxation was completely abolished in aorta (Nagao et al. 1992; Rees et al. 1990; Thomas and Ramwell 1991; Vargas et al. 1991). Other factors may also account for the different degree of relaxation response to ACh in the presence of NOS inhibitor, such as the strains of rats and the anatomical location of the vessel segments used by these researchers. It has been reported that endothelium-derived nitric oxide (NO)-dependent relaxation to ACh in the thoracic aorta precontracted with NE was significantly greater in the middle and distal segments than in the proximal segments, suggesting that there are regional variations in the ACh-induced release of endothelium-derived NO in the rat thoracic aorta (Honda et al. 1997). Nevertheless, evidence in the literature is basically consistent, i.e. in rat aorta NO may be a main EDRF mediating ACh-induced relaxation. In our study, the L-NMMA-resistant, TEA sensitive component was revealed only after NO synthesis was inhibited since TEA had no effect on ACh-induced relaxation in the absence of L-NMMA, and it was responsible for only a small part of ACh-induced relaxation (in the presence of L-NMMA TEA reduced the maximum relaxation to ACh from 40% to 20%), consistent with reports that NO is a major

mediator of ACh-induced relaxation, while EDHF may be a back up mechanism when NO pathway is impaired in rat aorta.

Mesenteric Artery

Effect of NFA and TEA

Most electrophysiological data on endothelium hyperpolarization in the literature were obtained from large conduit blood vessels due to the difficulties in isolating the endothelial cells from small vessels. Recently, endothelium membrane potentials have been recorded in isolated intact smaller arteries with intracellular microelectrodes. It was reported that stimulation with ACh induced endothelium hyperpolarization which was also reduced by K^+ channel blockers in resistance arteries from hamster gracilis muscles (Bolz et al. 1999). ACh hyperpolarization of endothelial cells by activating Ca^{2+}-activated K^+ channels which are sensitive to CTX was also recorded in endothelial cells using patch clamp technique in multicellular preparations from guinea pig mesenteric arterioles (Yamamoto et al. 1999). However, to our knowledge there are no electrophysiological data available so far for the effect of Cl^- channels on endothelium membrane potential and Ca^{2+} handling in muscular and resistance vessels.

In contrast to the large elastic aorta, superior mesenteric artery is a small muscular conduit artery that directly transfers blood flow to the resistance vascular bed. In this artery, NFA alone, as in aorta, did not alter the relaxation caused by ACh, but in contrast to aorta, TEA alone decreased ACh pD2 by approximately 8.7 fold without reducing the maximum relaxation. Furthermore, NFA plus TEA had no further inhibitory effect as compared to TEA alone. These observations suggest that in contrast to aorta, Cl^- channels are not involved in mediating endothelium-dependent relaxation to ACh in mesenteric arteries. The lack of the

involvement of Cl^- channels has been further confirmed by the observation that there was no difference between the inhibitory effects of NFA plus TEA and TEA alone on responses to ACh when L-NMMA was present. The existence of an inhibitory effect of TEA in both the absence and presence of L-NMMA implicates K^+ channels in ACh-induced relaxation in superior mesenteric arteries.

NO-Mediated and NO-Independent Relaxation

Heterogeneous distribution of endothelium-dependent relaxations resistant to NOS in rats has been reported (Nagao et al. 1992). In contrast to aorta, we found that inhibition of NO synthase with L-NMMA decreased the potency of ACh by 4.8 fold, but did not affect the maximal relaxation to ACh in mesenteric arteries. This suggests that the contribution of NO is less, while the NO-independent relaxation in response to ACh is greater in mesenteric arteries than that in aorta. Our results are similar to previous reports of a large NOS inhibitor-resistant component of ACh-induced relaxation in Wistar rat superior mesenteric arteries (Chen and Cheung 1997; Fukao et al. 1995). In contrast, two other studies, one using Sprague-Dawley (Hwa et al. 1994), and the other using young female Wistar (Van de Voorde and Vanheel 1997) rats showed a greater inhibition of ACh-induced relaxation of mesenteric arteries by NOS blockers. The difference may be attributed to the different strain, age or sex of rats used.

As in aorta, it has been suggested that the NO-independent relaxation to ACh is mediated by EDHF, which hyperpolarizes the smooth muscle through K^+ channel activation, in the superior mesenteric arterial circulation (Adeagbo and Triggle 1993; Chen and Cheung 1997; Fukao et al. 1997a; Garland and McPherson 1992). From the data with TEA alone, we could not distinguish whether TEA had an inhibitory effect on K_{Ca} channels in the

endothelium, which would interfere with NO/EDHF synthesis, and/or in the smooth muscle cells, which would directly inhibit NO (see discussion below)/EDHF action. Nevertheless, the fact that the inhibitory effect of TEA alone was greater than that of L-NMMA alone, and that in the presence of L-NMMA, TEA further decreased potency of ACh by about 10 fold, suggests that besides NO, EDHF is also involved in ACh-induced relaxation, and that its effect is greater than that of NO in the mesenteric arteries. In addition, since L-NMMA and TEA alone produce significant inhibition of the ACh response and their effect in combination is additive, it can be postulated that NO and EDHF may be released at the same time and act in parallel to cause relaxation in response to ACh in mesenteric artery via different mechanisms.

Although TEA significantly decreased the sensitivity of the NO-independent response to ACh, it had no effect on the maximal relaxation. Thus, other K^+ channels present in arteries both in smooth muscle (Nelson and Quayle 1995) and endothelial cells (Marchenko and Sage 1996) may also be involved. Using specific $K_{(Ca)}$ channel blockers, we found that in the presence of L-NMMA and indomethacin, apamin alone did not significantly affect relaxation to ACh; CTX, like TEA, attenuated the response to ACh, but did not reduce the maximal relaxation; a combination of CTX and apamin significantly inhibited the L-NMMA/indomethacin-resistant relaxation induced by ACh, and the maximal relaxation was reduced to an extent similar as that following pretreatment with the combination of TEA and apamin. Similar results were reported by Chen and Cheung (Chen and Cheung 1997). These investigators simultaneously measured smooth muscle membrane potential and tension in rat superior mesenteric arteries and found that in the presence of a NOS inhibitor, apamin was effective in inhibiting ACh-induced hyperpolarization in resting arteries, but less effective in

NE-contracted arteries. Furthermore, TEA significantly inhibited the hyperpolarization to ACh to a similar extent in both the resting and NE-stimulated arteries, as did CTX, although the effect of CTX was smaller. However, in their study, the combination of apamin and CTX completely abolished the both hyperpolarization and relaxation in response to ACh, while in our study the maximal relaxation ACh was reduced only to 57% in the presence of these two toxins. Nevertheless, these results suggest that in superior mesenteric arteries ACh simultaneously activates both SK_{Ca} and BK_{Ca}, and that combined inhibition of both channels is necessary to inhibit EDHF.

Elevation of the extracellular K^+ concentration $[K^+]_0$ to above 25 mM (25, 30 or 60 mM) abolishes the NO/PGI_2-independent hyperpolarization and relaxation induced by ACh in rat mesenteric arteries (Adeagbo and Triggle 1993; Fukao et al. 1995; Garland and McPherson 1992; McCulloch et al. 1997; Randall et al. 1997; Waldron and Garland 1994). Generally, increasing the $[K^+]_0$ above 20 mM will decrease the K^+ equilibrium potential to the extent low enough to prevent hyperpolarization to K^+ channel activation (Nelson and Quayle 1995), thereby preventing relaxation to EDHF. In the present study, application of 30 mM KCl with PE together further reduced the maximal relaxation to ACh to 38% of the control, indicating that besides K_{Ca} channels other K^+ channels may be also involved. However, ACh-induced vasorelaxation was not completely inhibited by the combination of L-NMMA and KCl. Therefore, it would appear that ACh may produce another EDRF (or more than one) in mesenteric artery that mediates ACh-induced vasorelaxation independent of NO and K^+ channel activation. Previous studies have suggested that ACh-induced endothelium-dependent relaxation is mediated by a relaxing factor that is not NO, PGI_2 or EDHF in rat superior mesenteric artery (Shimokawa et al. 1996; Wu et al. 1993). In addition,

cyclopiazonic acid, a Ca^{2+} mobilizing compound like ACh, as well as A23187, induced an endothelium-dependent relaxation that was affected by neither 60 mM K^+ nor the combination of K^+ and NO pathway inhibitors in the rat mesenteric arterial bed (Kamata et al. 1996b). However, the actual characteristics of the novel relaxing factor(s) need to be further investigated.

Aorta and Mesenteric Arteries

Effect of PGI_2 in ACh - Induced Relaxation in Aorta and Mesenteric Arteries

Prostacyclin (PGI_2), the principal metabolite of arachidonic acid, is produced by COX in endothelium of most blood vessels including aorta and mesenteric arteries (Moncada et al. 1977; Peredo et al. 1997). It mediates endothelium-dependent relaxation, probably via the cAMP pathway and evokes membrane hyperpolarization of smooth muscle sensitive to glibenclamide in some blood vessels and species (Gryglewski et al. 1991; Jackson et al. 1993; Moncada and Vane 1978a; Murphy and Brayden 1995b; Parkington et al. 1995; Triggle et al. 1999; Zygmunt et al. 1998). Data available in the literature have demonstrated that inhibition of COX with indomethacin either alone or in the presence of a NOS inhibitor does not interfere with the relaxing effect of ACh on rat aorta and mesenteric arteries, suggesting that PGI_2 is not involved (Adeagbo and Triggle 1993; Chen et al. 1988; Hatake et al. 1995; Shimokawa et al. 1996). However, recent studies showed that COX-dependent, indomethacin-sensitive relaxation and hyperpolarization to ACh were revealed after inhibition of both NO and EDHF pathway in rat hepatic and rabbit mesenteric arteries, and raised concerns that the role of PGI_2 may have been overlooked (Murphy and Brayden 1995b; Zygmunt et al. 1998). In this study, we systemically compared the effects of indomethacin on ACh-induced relaxations. Indomethacin alone or in combination with L-

NMMA or L-NMMA plus TEA did not alter either the sensitivity or the magnitude of the maximum relaxation to ACh obtained in the absence of indomethacin. Our results confirmed the previous observations in the aorta and mesenteric arteries and further excluded the role for a COX product after inhibition with L-NMMA and TEA in these two arteries.

Endothelium-dependent relaxation to A23187 in aorta and mesenteric arteries

In the present study, A23187 also induced an endothelium- concentration-dependent relaxation in both aorta and mesenteric arteries. We found that in the presence of L-NMMA the relaxations induced by A23187 were completely inhibited in both aorta and mesenteric arteries, suggesting the relaxations were exclusively NO dependent. It was surprising that increasing K^+ to 30 mM in the bathing solution also totally abolished the relaxation by A23187 in aorta as well as in mesenteric arteries. This indicates that activation of K^+ conductance(s) in smooth muscle was responsible for the full relaxation of the arteries, since responses to EDRF release by calcium ionophore will be insensitive to K^+ channel blockade at the level of the endothelium.

Thus, it appears that NO-mediated endothelium-dependent relaxation to A23187 was mediated via K^+ channel on smooth muscle in both aorta and mesenteric arteries. The results with A23187 are apparently different from those obtained with ACh (discussed above), which induced both L-NMMA-sensitive and insensitive relaxations although the latter was less prominent in aorta. The lack of L-NMMA-insensitive response to A23187 could be due to the influence of the degree of precontraction with PE. It has been shown that the degree of inhibition of ACh-induced relaxation by NOS inhibitors depends on the level of precontraction in rat aorta (Hatake et al. 1995; Zygmunt et al. 1994a) (see above discussion). However, under the same conditions as the A23187 response was obtained, ACh did induce a

NO-independent relaxation in both aorta and mesenteric arteries. Therefore, the initial tension seems not to be a factor that would affect only the A23187-induced relaxation. This was further supported by experiments in mesenteric arteries, where application of ACh in the presence of A23187 fully reversed the inhibitory effect of L-NMMA, but had only small relaxant effect on the PE tone in the arterial rings challenged with K^+. Addition of ACh did not stimulate further relaxation in the presence of L-NMMA or KCl in aorta. The effects of ACh in these experiments were consistent with those obtained in the absence of A23187 in mesenteric arteries. The lack of further relaxation to ACh in the presence of L-NMMA in aorta is perhaps due to the minimal contribution of EDHF in this vessel. In this situation, the presence of A23187, which could interfere with the initial Ca^{2+} profile, may have an effect on the synthesis/release of EDHF. The results supported our contention that: 1) NO accounts fully for A23187-induced relaxation in both aorta and mesenteric arteries, 2) NO is also a major mediator in ACh-induced relaxation in aorta, 3) EDHF plays a predominant role in mesenteric artery, and 4) activation of K^+ conductance is involved in regulating both NO-dependent and NO-independent relaxation.

That NO mediated vasorelaxation may involve hyperpolarization of vascular smooth muscle by activation of K^+ channels has been proposed. The contribution of NO to ACh- or carbachol-induced hyperpolarization of smooth muscle has been observed in guinea pig uterine artery (Tare et al. 1990), rat aorta (Vanheel et al. 1994), rabbit carotid artery (Cohen et al. 1997) and rat small mesenteric arteries (Weidelt et al. 1997). NO has been reported to directly stimulate CTX-sensitive K^+ channels in the rabbit aorta, rat mesenteric artery and rabbit carotid artery (Bolotina et al. 1994; Mistry and Garland 1998; Plane et al. 1998; Weidelt et al. 1997). NO has also been reported to activate K_{ATP} channels in rabbit and rat

mesenteric arteries (Murphy and Brayden 1995a; Weidelt et al. 1997), K_{Ca} channels in rabbit middle cerebral arteries (Dong et al. 1998) and voltage-gated K^+ channels in rat pulmonary artery (Yuan et al. 1996) through either guanylate cyclase- cGMP-dependent or independent pathways. In addition, NO donor SIN-evoked relaxation can be fully accounted for by activation of a CTX-sensitive pathway with little or no contribution from a pathway activated by increased levels of cyclic GMP in rat mesenteric arteries (Plane et al. 1996).

In this study we did not attempt to characterize the specific type of K channels implicated in NO action on smooth muscle cells. We did find that in the absence of L-NMMA, TEA did not affect A23187-induced relaxation in aorta, but greatly inhibited the response in mesenteric arteries. This result at least indicates that: 1) there are different K^+ channels that mediate NO-dependent response to A23187 in mesenteric artery and aorta, and 2) Ca^{2+}-activated K^+ channels contribute to the process in mesenteric arteries but not in aorta. In addition, the differential effects of TEA in aorta and mesenteric arteries also eliminated the possibility that TEA could exert a nonspecific effect on membrane potential rather than a specific effect on K_{Ca} channels.

Different abilities of A23187 and ACh to release NO and/or EDHF in the same preparations have been suggested in other studies. It has been suggested that A23187 only evokes the release of NO from the endothelium in rabbit carotid artery preconstricted with PE, whereas ACh can induce release of both NO and EDHF (Dong et al. 1997). In rabbit femoral artery, the relaxation to A23187 of NE-induced tension seems to be mediated predominantly via EDHF, while ACh-induced relaxation has been explained solely in terms of NO release (Plane et al. 1995). Based on our observations and those of others, we speculate that A23187 acting as an ionophore, and ACh, releasing endoplasmic reticulum

(ER) Ca^{2+} and opening Ca^{2+} channels, might facilitate an increase $[Ca^{2+}]_i$ into different regions of endothelial cells, leading to activation of different enzymes that are responsible for synthesis of NO and EDHF.

The question of cellular compartmentalization with respect to the synthesis of NO, PGI_2 and EDHF, and also colocalization of ion channels and kinases has been raised by Triggle *et al* (Triggle et al. 1999) based on the high variability of the cellular mechanisms that mediate vasodilation in response to these factors in different tissues and species (Gambone et al. 1997; Garland and McPherson 1992; Triggle et al. 1999; Waldron et al. 1999). It has also been suggested that the nature of the contractile agonist can determine the release and/or effects of NO and/or other endothelial-derived mediators (Plane and Garland 1996). Although there is very little in the literature that addresses this question, it was reported recently that the synthesis of PGI_2 *versus* 6-oxo-$PGF_{1\alpha}$ was different in porcine endothelial cells stimulated chemically with A23187 compared with cell stimulated mechanically (Erdbugger et al. 1997).

Our study did not explore the chemical nature of the EDHF that mediated NO-independent relaxation in response to ACh. It is possible that either a diffusible factor(s) (Campbell et al. 1996; Chen et al. 1991; Popp et al. 1996) or direct electric connection between VSM and endothelium (Chaytor et al. 1998; Edwards et al. 1999; Yamamoto et al. 1999) is involved. Endothelium-dependent hyperpolarization is produced by a humoral substance, EDHF, in the coronary arteries of guinea pig (Chen et al. 1991). In porcine coronary arteries, EDHF may be eicosatrienoic acids metabolized from arachidonic acid (Campbell et al. 1996; Popp et al. 1996). However, electrical coupling between endothelial and smooth muscle cells through gap junctions has also been demonstrated in the rat aorta

(Marchenko and Sage 1996), porcine coronary arteries (Beny 1997; von der Weid and Beny 1993) and guinea pig submucosal arterioles (Iwase et al. 1998). Recently, it has been reported that blocking of the myoendothelial junction with a specific inhibitory gap junction peptide abolished ACh-induced hyperpolarization in guinea pig internal carotid artery (Edwards et al. 1999) and mesenteric arterioles (Yamamoto et al. 1999), and inhibited ACh-induced, NO/PGI_2-independent relaxation in rabbit aorta and superior mesenteric artery (Chaytor et al. 1998). These findings suggest that endothelium-dependent hyperpolarization of smooth muscle is produced by an electrotonic spread of potentials from the endothelial cells. It was also reported that inhibiting the gap junction blocked ACh-, but not A23187-evoked hyperpolarization of the rabbit mesenteric artery, and it was concluded that A23187-mediated endothelium-dependent relaxation requires chemical transmission through the extracellular space, whereas relaxation to ACh involves gap junction communication (Hutcheson et al. 1999). If the NO-independent relaxation to ACh was mediated through myoendothelial gap junction in our preparations, that A23187 failed to conduct endothelial hyperpolarization response to smooth muscle cells through myoendothelial junctions may also account for its differential ability to release NO and EDHF.

VI. SUMMARY

1. ACh induced a concentration- and endothelium-dependent relaxation of PE-induced tone in both isolated aorta and mesenteric arteries.

2. In intact aorta, neither NFA nor TEA alone had a significant effect on the response to ACh. However, in the presence of NFA plus TEA, the concentration-relaxation curve (CRC) to ACh was shifted to the right without a change in maximum relaxation (R_{max}). In intact mesenteric arteries, the presence of TEA alone resulted in a rightward displacement of the CRC to ACh. The combination of NFA and TEA did not further inhibit the relaxation to ACh as compared to TEA alone. NFA alone also had no effect on response to ACh.

3. In aorta, L-NMMA greatly attenuated the relaxation response induced by ACh, decreasing both the pD_2 value and R_{max}. In the presence of L-NMMA, TEA further shifted the CRC for ACh to the right without change in R_{max} as compared to L-NMMA alone. In the presence of L-NMMA, NFA plus TEA had no additive effect as compared to TEA alone. In contrast, in mesenteric arteries, L-NMMA displaced the CRC for ACh to the right without altering the R_{max}. L-NMMA plus TEA further shifted the CRC to the right as compared to L-NMMA alone, but the R_{max} to ACh was still unchanged. In the presence of L-NMMA, NFA plus TEA had no additional effect as compared with TEA alone.

4. When indomethacin was used in combination with L-NMMA or with L-NMMA plus TEA, the relaxation responses to ACh were not different as compared to L-NMMA alone or L-NMMA plus TEA only in either aorta or mesenteric arteries.

5. A23187 also induced an endothelium-dependent relaxation in both aorta and mesenteric arteries. In intact aorta, the pretreatment with NFA or TEA or NFA plus TEA had no effect on the response to A23187. In intact mesenteric arteries, pretreatment with TEA reduced the R_{max} to A23187. In the presence of L-NMMA or KCl (30 mM) the response to A23187 was abolished in both arteries. The PE response resistant to A23187 in the presence of L-NMMA was completely reversed by subsequent addition of ACh in mesenteric arteries but not in aorta. ACh had no further effect on the tension remaining in the presence of KCl (30 mM) in aorta, but slightly decreased it in mesenteric arteries

6. Apamin alone had no effect on ACh-evoked, NO-independent relaxation in mesenteric arteries. CTX and TEA displaced the ACh CRC to the right without altering the R_{max}. Apamin plus CTX or TEA further inhibited the relaxation to ACh with a reduction in the R_{max}, while high K^+ (30mM) buffer had a greater inhibitory effect on the R_{max} than the combinations in mesenteric arteries.

VII. CONCLUSIONS

Our results indicate that both NO and EDHF-like factors mediated ACh-induced endothelium-dependent relaxation in both aorta and mesenteric arteries. However, the mechanisms by which ACh-induces endothelium-dependent relaxation are different in these two arteries. ACh-induced relaxation appears to be primarily mediated by NO in aorta, and Cl^- channels and K^+ channels together may regulate the NO-dependent, ACh-induced relaxation in this artery. In contrast, in mesenteric artery, EDHF played a more important role in ACh-mediated relaxation. K^+ channels, but not Cl^- channels, contributed to ACh-induced relaxation. Other endothelium-dependent relaxing factor(s) may also be involved. Ca^{2+} ionophore A23187-induced relaxation is solely NO dependent, which is mediated by K^+ conductance in both aorta and mesenteric artery. Blockade of Cl^- channels had no effect on A23187-mediated relaxation in rat aorta. The results suggest that K^+ conductance regulates both NO-dependent and -independent relaxation in both aorta and mesenteric arteries. Both small- and large- conductance $K^+_{(Ca)}$ channels play a role in ACh-induced NO-independent relaxation in mesenteric artery. Other K^+ channels may be also involved. Cl^- channels in the endothelium are only involved in NO-dependent ACh-induced relaxation in rat aorta, probably by participating in maintaining endothelial membrane potential compatible for Ca^{2+} influx, thus ensuring a sustained NO synthesis and release.

VIII PHYSIOLOGICAL SIGNIFICANCE

The physiological importance of the relative contribution by NO and EDHF released by a variety of stimuli in vasculature is still unclear. From our study and many others (Garland et al. 1995) (Clark and Fuchs 1997; Triggle et al. 1999; Woodman et al. 2000), it seems that the relative contribution depends on the function of blood vessels. In some arteries, mainly large conducting arteries such as aorta, and also some resistance arteries such as coronary beds (Clark and Fuchs 1997), which assume primary importance in some disease states such as atherosclerosis, NO is the major mediator under normal conditions, while EDHF may be of a secondary importance. However, in the majority of small arteries, such as mesenteric arteries, skeletal beds (Clark and Fuchs 1997; Woodman et al. 2000), which are mainly responsible for regulating peripheral resistance, EDHF appears to be a major determinant of vascular caliber, while NO and possibly other endothelium-dependent relaxing factors may act together with EDHF to achieve the optimal relaxation. However, many questions still need be answered in mesenteric artery and other blood vessels, including the nature of EDHF and other EDRF(s), the cellular target of the EDHF, and the interaction between these endothelium-dependent relaxing factors.

PART 3. NOREPINEPHRINE-INDUCED VASOCONSTRICTION IN ISOLATED PERFUSED MESENTERIC ARTERIAL BED FROM OBESE ZUCKER RATS: THE EFFECT OF INSULIN

I. RATIONALE

The hemodynamic hallmark of most forms of hypertension is an increase in peripheral vascular resistance, which is largely ascribed to abnormalities in the reactivity of small resistance vessels to neurotransmitters and circulating hormones, such as NE. In addition, altered regulation of vascular tone by endothelium-derived vasoactive products has been implicated. An abnormal release of endothelial-derived relaxing factors such as NO and also contracting factors such as endothelin (ET) and COX pathway metabolites have been observed in several types of hypertension (Luscher et al. 1993b; Mistry and Nasjletti 1988; Purkerson et al. 1986; Wilcox et al. 1996).

In states of insulin resistance, hyperinsulinemia has been found to be associated independently with hypertension (Modan et al. 1985; Salonen et al. 1998). However, whether there is a causal relationship between hyperinsulinemia/insulin resistance and hypertension remains controversial (Brands et al. 1998; Yki-Jarvinen and Utriainen 1998). Insulin is known to exert many actions that may directly affect vascular reactivity at the levels of both the endothelium and smooth muscle. These include, on one hand, increasing NO synthesis/release (Chen and Messina 1996; Steinberg et al. 1994; Zeng and Quon 1996), and enhancing Na^+-K^+-ATPase and Ca^{2+}-ATPase gene expression and activity (Sowers et al. 1991; Tirupattur et al. 1993), and on the other, promoting ET-1 gene expression (Oliver et al. 1991) and increasing ET-1 release (Hu et al. 1993; Nava et al. 1997), elevating sympathetic

activity and increasing NE release (Lembo et al. 1992; Liang et al. 1982) and promoting vascular smooth muscle cell growth (Ridray 1995). In addition, the effects of insulin on vascular reactivity may also involve modulation of COX pathway metabolism (Axelrod 1991; Keen et al. 1997; Rebolledo et al. 1998; van Veen and Chang 1997; Yanagisawa-Miwa et al. 1990). Studies *in vitro* in intact resistance vessels from control animals have revealed both inhibitory and potentiating effects of insulin on the pressor responses to vasoactive substances (Alexander and Oake 1977; Townsend et al. 1992; Walker et al. 1997b; Wu et al. 1994). Therefore, either impaired vasodilator and/or exaggerated vasoconstrictor effects of insulin could contribute to an increased vascular reactivity leading to hypertension in insulin-resistant states.

The genetically obese Zucker rat (fa/fa) is an insulin-resistant animal model with early onset severe hyperinsulinemia, hyperlipidemia and normal plasma glucose (York et al. 1972). These rats usually develop a modest hypertension at an older age (Cox and Kikta 1992). The obese Zucker rat thus represents a model in which the effects of insulin resistance/hyperinsulinemia associated with hypertension on vascular reactivity can be examined. However, although the reactivity of arteries from obese Zucker rats has been investigated in a number of studies (e.g. Bohlen and Lash 1995; Kam et al. 1996; Turner et al. 1995; Walker et al. 1997a; Wu et al. 1996; Zanchi et al. 1995), the results are not all in agreement. Furthermore, the effect of insulin on vascular reactivity in Zucker rats has not yet been defined (Turner et al. 1995; Walker et al. 1997a).

Thus, the purpose of the present study was to investigate whether altered vascular reactivity to NE could be detected, in the absence and/or presence of a pathophysiologically relevant concentration of insulin, in obese Zucker rats with established hypertension. In

addition, the contribution of endogenous vasoactive substances including NO, prostanoids and ET-1 to NE responses and to the vascular actions of insulin was investigated. The whole mesenteric arterial bed (MAB) was chosen for this study. The following research hypotheses were proposed and specific experimental objectives were undertaken.

II. WORKING HYPOTHESIS AND SPECIFIC RESEARCH OBJECTIVES

Working Hypotheses:

A. NE-induced vasoconstriction is altered in Zucker obese rat MAB compared to their lean littermate controls.

B. Insulin, at a concentration similar to that to which obese Zucker rats are exposed *in vivo*, alters pressor responses to NE to a different extent in MAB from obese Zucker rats compared to their lean littermates.

C. Release of endothelium-derived vasoconstrictors (such as ET-1 or PGH_2/TxA_2) and vasodilators (such as NO) and their interaction influence the pressor responses to NE as well as the effects of insulin in MAB from Zucker rat. The impact of these endothelial factors is different in Zucker obese rat MAB than in their lean littermates

Specific Objectives:

1). To compare the vasoconstrictor responses to NE in isolated perfused MAB from obese Zucker rats and their lean littermates.

2). To compare the effect of insulin, at a concentration similar to the circulating level of insulin in obese Zucker rats, on the pressor response to NE in isolated perfused MAB from obese Zucker rats and their lean littermates.

3). To investigate the effects of N^G- monomethyl-L-arginine (L-NMMA), a nitric oxide synthase inhibitor, alone and in the presence of insulin on the vasoconstriction to NE in MAB from Zucker rats.

4). To examine the effects of indomethacin, a COX inhibitor, alone and in the presence of insulin, on NE-induced contraction in MAB from Zucker rats.

5). To test the effect of SQ 29,548, a TxA_2/PGH_2 receptor antagonist, alone and in the presence of insulin, on pressor response to NE in MAB from Zucker rats.

6). To evaluate the effects of L-NMMA plus indomethacin and L-NMMA plus indomethacin plus insulin on pressor responses to NE in MAB from Zucker rats.

7). To investigate the effects of bosentan, a non-selective endothelin receptor (both ET_B, and ET_A) antagonist, alone and in the presence of insulin on vasoconstriction to NE in MAB from Zucker rats.

8). To examine the effect of BQ 788, a selective ET_B receptor antagonist, or BQ 123, a selective ET_A receptor antagonist, alone and BQ 788 or BQ 123 in the presence of insulin on pressor responses to NE in MAB from Zucker rats

III METHODS AND MATERIALS

1. General Methodology

Animals Male obese (fa/fa) Zucker rats and their lean (Fa/?) littermate controls were obtained at age 8 to 10 weeks from the Department of Physiology, University of British Columbia (Vancouver, Canada). They were treated according to the guidelines of the Canadian Council on Animal Care. Animals were pair-housed under a 12 h light/dark regime and given free access to normal food (Purina rat chow) and tap water, until they were 25 weeks old.

Blood pressure measurement Systolic blood pressure (SBP) was measured by the tail-cuff method in animals randomly selected from those used in *in vitro* studies, one week before the animals were used in experiments. Rats were placed in restrainers and pre-warmed for 30 min at 27°C. SBP was measured with an inflatable cuff and a sensor placed around the tail and coupled to a blood pressure analyzer (IITC model 179, IITC Inc./Life Science Instruments, Woodland Hills, CA, U.S.A). The inflated cuff pressure was 250 mmHg and pressure was released by 500 mmHg min^{-1}. To accustom them to the setting, rats were placed in the apparatus once each day for 3 days prior to the actual day of measurement. Blood pressure was recorded and calculated as the mean of five to six measurements.

Biochemical analysis of blood samples Blood samples from a tail tip cut were collected into heparinized capillary tubes. The blood was centrifuged at 10,000 x g for 15 min. and the plasma was collected immediately, frozen and stored at –70° C until it was assayed. Plasma glucose and triglyceride levels were determined by enzymatic colorimetric methods using

commercial kits obtained from Boehringer Mannheim (Laval, Quebec, Canada). Plasma insulin levels were determined by radioimmunoassay using kits obtained from Linco Research, Inc. (St. Charles, Missouri, U.S.A.)

Perfused isolated MAB preparation On each day of experiments, one obese rat and a lean littermate were anaesthetized with sodium pentobarbital (120 mg kg^{-1}, subcutaneously over the back and thighs in four equivalent dosages). The abdominal cavity was opened, the mesenteric artery was cannulated through an incision at the confluence with the dorsal aorta and then the MAB was isolated as described by McGregor (McGregor 1965). The MAB was flushed with heparinized physiological salt solution (25 IU/ml), transferred into a warmed organ chamber and perfused with Krebs-bicarbonate (normal Krebs) buffer maintained at 37° C and gassed with 95% O_2: 5% CO_2. The Krebs-bicarbonate buffer was of the following composition (in mM): NaCl 113, KCl 4.7, glucose 11.5, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0. The pH of the buffer following saturation with a 95% O_2: 5% CO_2 gas mixture was 7.4. The perfusion rate was kept constant at 3 ml/min using a polystaltic peristaltic pump (Buchler Instruments, Buchler Fort Lee, NJ, USA). Changes in perfusion pressure were measured and recorded using a pressure transducer (PD23ID Gould Statham, CA, U.S.A) and Grass polygraph (Model 79D Grass Instruments, MA, USA). The perfused MAB was allowed to stabilize for 1 hr before the start of the experiment.

2. Experiment Protocols

The tissues were initially treated with a maximal concentration of KCl (120 μmol) by bolus injection 4 times. Perfusion pressure was allowed to return to baseline between each

injection of KCl. The MABs were then allowed to equilibrate for a further 40 min following which two or three consecutive dose-response curves (DRCs) to NE were constructed from 5 separate bolus injections of NE (0.9-90 nmol). Perfusion pressure was allowed to return to baseline between the injections of each dose of agonist. The first NE DRC served as a control. The second DRC for NE was constructed in the presence of insulin (200 mU/l) or indomethacin (20 μM) or SQ 29,548 (0.3 μM) or L-NMMA (300 μM) or L-NMMA plus indomethacin or bosentan (3 μM) or BQ 788 (0.3 μM) or BQ 123 (0.3 μM) in the perfusion buffer. The third curve was constructed in the presence of a combination of insulin plus the inhibitor(s) used in the 2nd DRC. The MAB was pre-perfused with insulin for 2 hrs. Bosentan, BQ 788, BQ 123, L-NMMA, indomethacin and SQ 29,548 were added into perfusion buffer 5 min, 15 min, 15 min, 30 min, 30min and 30min, respectively, before the DRC was constructed or before insulin was added. After the completion of each DRC for NE, a single bolus injection of KCl (60 μmol) was made. The three DRCs were constructed at fixed time intervals. A time control experiment that consisted of three DRCs for NE without the addition of insulin or any inhibitors was also done. To confirm the effect of insulin on the NE response, another set of experiments was performed, in which the first two NE DRCs were obtained in untreated tissues, followed by insulin infusion for 2 hrs and thereafter the 3rd DRC was constructed.

3. Chemicals

(-)-Norepinephrine hydrochloride and indomethacin were obtained from Sigma Chemical Co. (St. Louis MO, U.S.A.). L-NMMA and BQ 788 were purchased from Calbiochem Corporation (La Jolla, CA, U.S.A.). Bosentan (R047-0203) was a gift from

Hoffmann-La Roche Ltd. (Bazel Switzerland). BQ 123 and SQ 29,548 were purchased from Research Biochemical International (Natick, MA, USA). Insulin (Humulin R, Eli Lily Co., St. Louis) was purchased from a local pharmacy. A stock solution of NE was prepared daily in distilled water containing 1 mg ml^{-1} ascorbic acid. The volume of NE for each injection was 30 µl. Indomethacin and bosentan were dissolved in 100% ethanol and prepared as stock solutions of 0.1 M and 0.01 M, respectively. The solutions were made fresh each day. L-NMMA (0.1 M) was made in distilled water. All inhibitors and insulin were diluted to the required concentration in the perfusate reservoir. The final ethanol concentrations (0.03% and 0.02%, v/v) in the perfusion buffer were without effect on contractile responses.

4. Statistical Analysis

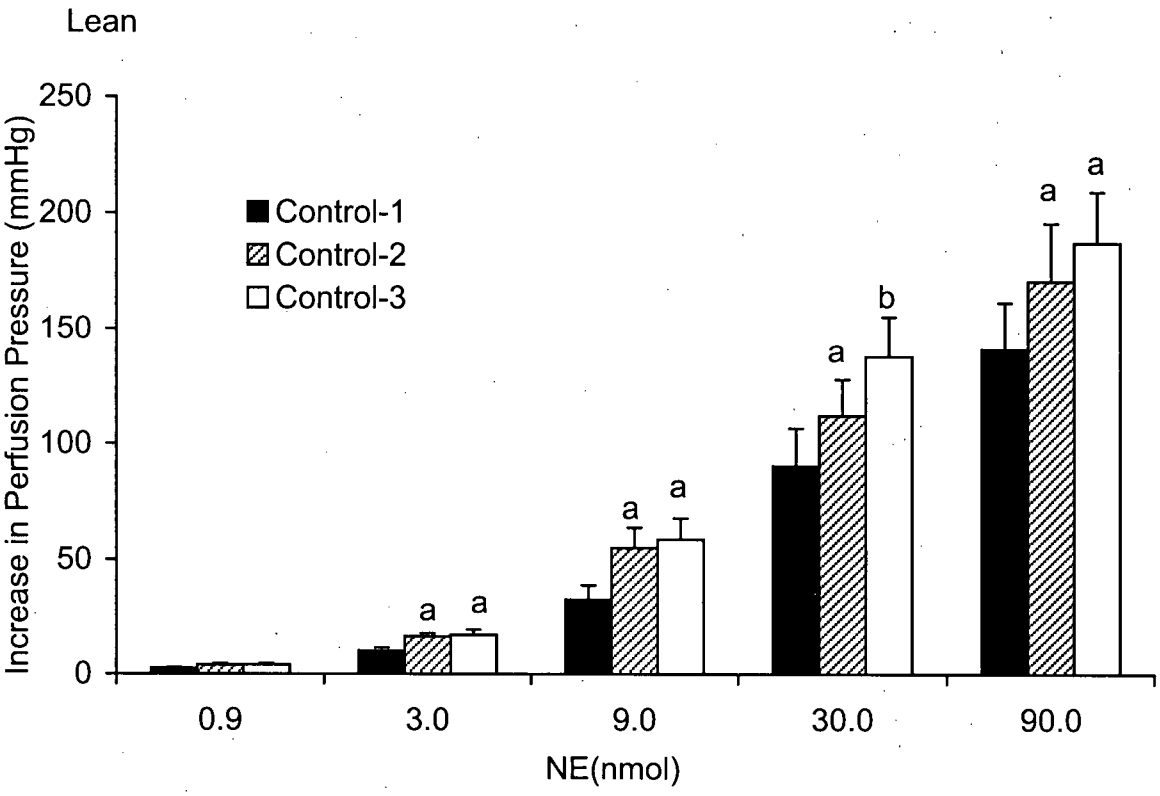
To compare the reactivity of MAB from lean and obese rats to NE and KCl, vasoconstrictor responses were expressed as the absolute increase in perfusion pressure. In control experiments, NE-induced pressor responses were found to increase on the second and third exposure to 3 to 90 nmol NE in untreated MAB from both obese and lean rats (Fig. 3.1). Therefore, to allow evaluation of the effects of the inhibitors, alone and in the presence of insulin, NE responses in the second and third DRC were expressed as a percent of the maximum response of the initial NE DRC. The responses to NE at each time point in the control experiments served as the control for the responses in the presence of the inhibitors, alone or in combination with insulin.

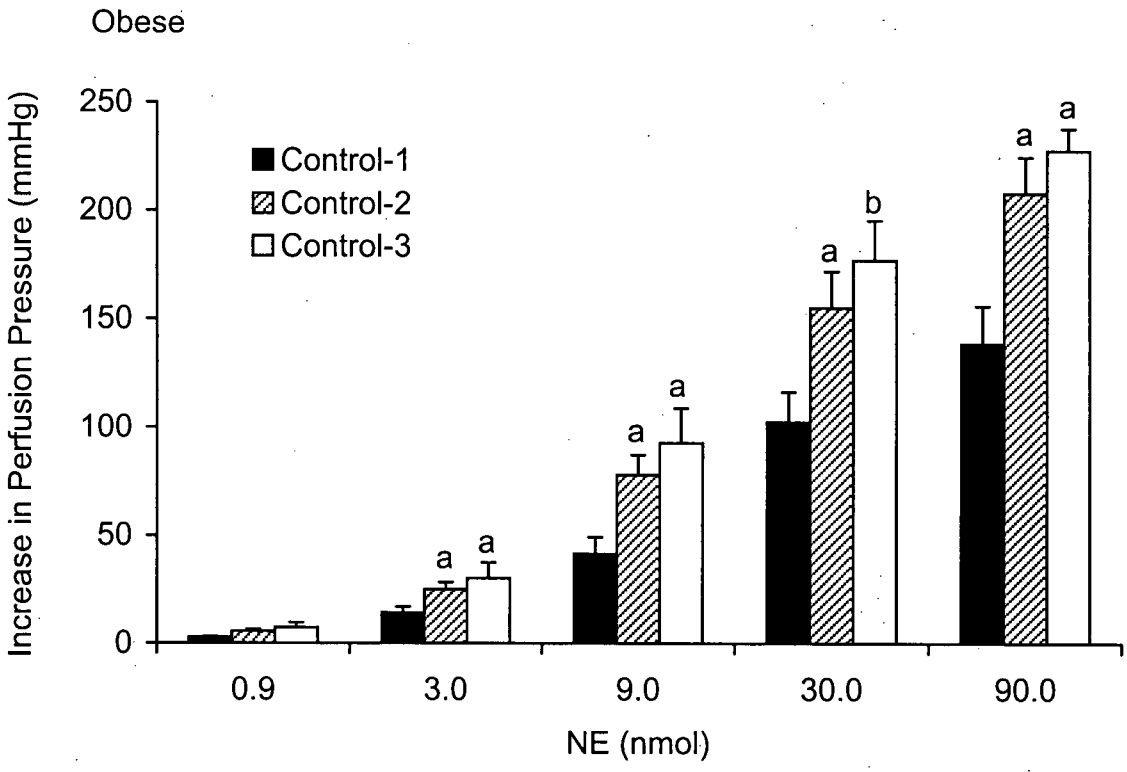
All data are presented as mean ± SEM. Student's unpaired t-test was used for comparisons between two means. Two-way ANOVA using the general linear model approach (repeated measurements) followed by Newman-Keul's test was used for multiple

comparisons between obese and lean rats. One-way ANOVA followed by the Bonferroni post-hoc test was used for within-group comparison of multiple means. $P < 0.05$ was considered statistically significant.

FIGURE 3.1

Control experiments for responses to NE in isolated mesenteric arterial bed obtained from lean or obese Zucker rats, perfused with normal Krebs at constant flow. The responses at each concentration of NE (control-1, control-2 and control-3, respectively) were obtained 2.5 h apart. Data represent the mean ± SEM of seven (lean) and six (obese) experiments. [a] $P < 0.05$ vs. control-1; [b] $P<0.05$ vs. control-2.


Lean
Increase in Perfusion Pressure (mmHg)
250
200
150
100
50
0
Control-1
Control-2
Control-3
a
a
a
a
a
b
a
a
0.9
3.0
9.0
30.0
90.0
NE(nmol)


Obese
Increase in Perfusion Pressure (mmHg)
250
200
150
100
50
0
Control-1
Control-2
Control-3
a
a
a
a
a
b
a
a
0.9
3.0
9.0
30.0
90.0
NE (nmol)

IV. RESULTS

1. General Characteristics of Zucker Rats

At 25 weeks of age, systolic blood pressure was significantly higher and body weight was significantly greater in obese rats than in their lean littermates (Table 3.1). Plasma insulin and triglyceride levels were also significantly elevated in obese as compared to lean rats. However, plasma glucose concentrations were not significantly different between the two phenotypes (Table 3.1).

2. NE-Induced Vasoconstriction in Isolated Perfused MAB from Obese and Lean Zucker Rats

The basal perfusion pressures in isolated MAB of obese and lean rats were 7.1 ± 0.9 and 6.6 ± 0.7 mmHg (mean ± SEM, n = 28, $P > 0.05$), respectively. Bolus injection of NE (0.9 to 90 nmol) produced a concentration-dependent increase in perfusion pressure that was significantly lower at 90 nmol NE in MAB from obese than from lean rats (Fig. 3.2A). Vasoconstrictor responses to KCl were also significantly smaller in MAB from obese than from lean rats (Fig. 3.2B).

3. Effect of NOS and/or COX Inhibition on NE-Induced Responses

Infusion of L-NMMA (300μM) or indomethacin (20 μM) alone or in combination for 30 min did not alter the basal perfusion pressure of MAB from either obese or lean rats (data not shown). However, L-NMMA significantly potentiated vasoconstrictor responses to NE in MAB from both groups of animals (Fig 3.3). L-NMMA appeared to produce a leftward shift in the NE dose-response curve, since there was no increase in the maximum response to NE in MAB from lean rats (Fig. 3.3). In contrast, responses of MAB from obese

TABLE 3.1

Physiological characteristics of lean and obese Zucker rats

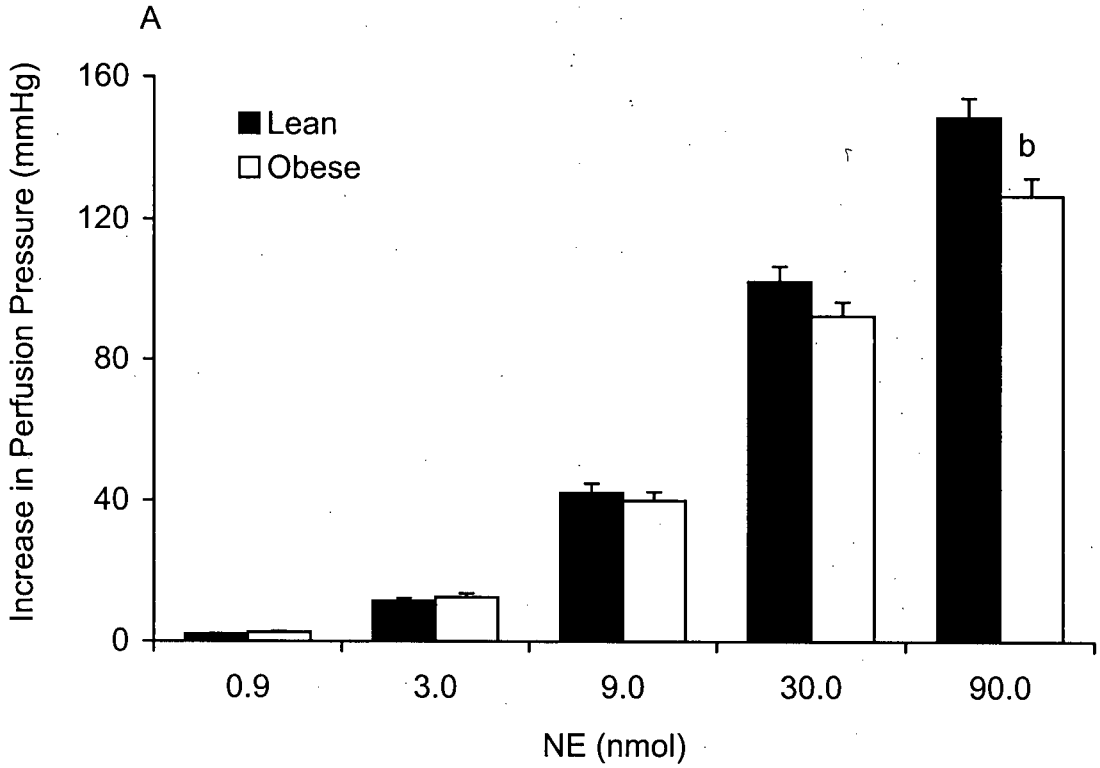
	Lean	Obese
Body weight (g)	418 ± 5 (50)	592 ± 7 (45)[a]
Systolic BP (mmHg)	128 ± 2 (16)	157 ± 1 (15)[a]
Plasma insulin (mU/l)	64 ± 6 (32)	267 ± 27 (31)[a]
Plasma triglyceride (mmol/l)	1.48 ± 0.14 (25)	36.93 ± 0.20 (27)[a]
Plasma glucose (mmol/l)	7.34 ± 0.27(5)	6.92 ± 0.31 (7)

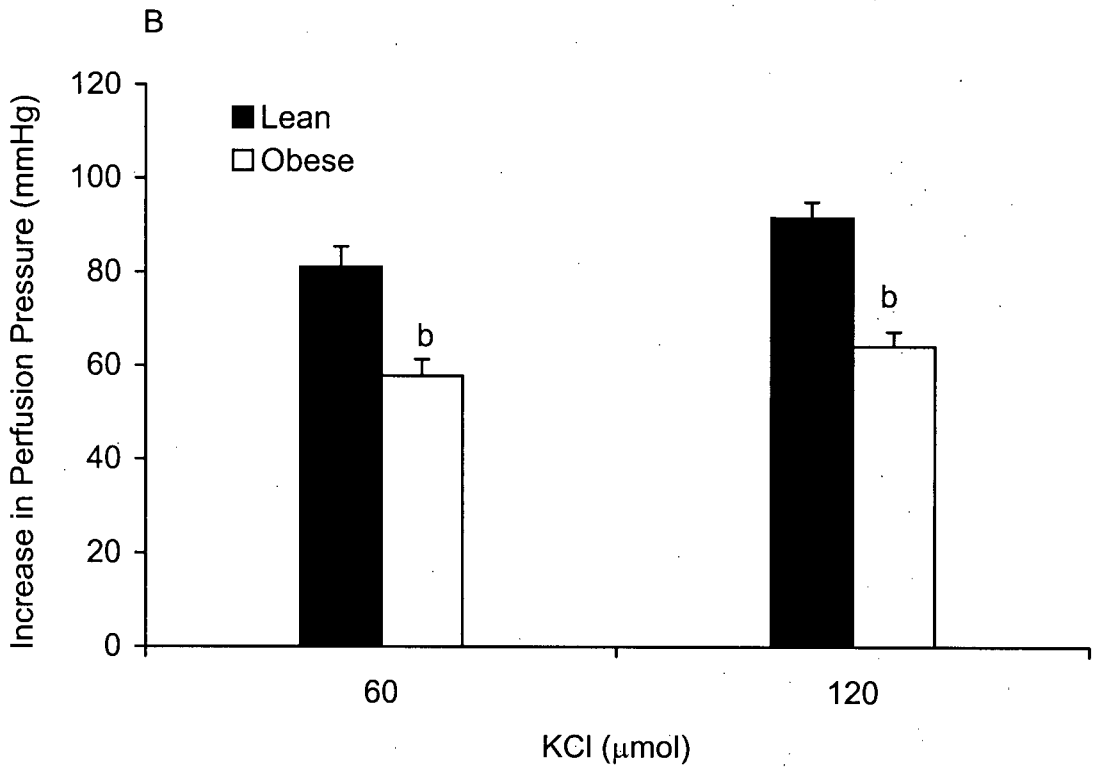
Values are shown as mean ± SEM (number of rats in parentheses).

[a] $P < 0.05$, vs. lean (Student unpaired t-test).

FIGURE 3.2

Initial concentration-response curve to NE (A) and responses to KCl (B) in isolated perfused MAB obtained from lean (■) and obese (□) Zucker rats, perfused with normal Krebs at constant flow. Data represent the mean ± SEM. pooled from 48 experiments. [a] $P < 0.05$ vs. lean (Student unpaired t-test).


A
Increase in Perfusion Pressure (mmHg)
160
120
80
40
0
Lean
Obese
b
0.9
3.0
9.0
30.0
90.0
NE (nmol)


B
Increase in Perfusion Pressure (mmHg)
120
100
80
60
40
20
0
Lean
Obese
b
b
60
120
KCl (μmol)

rats to all concentrations of NE, including the maximal, were significantly increased in the presence of L-NMMA (Fig. 3.3). On the other hand, the effects of indomethacin on NE responses were opposite to those of L-NMMA, in that it inhibited pressor responses to NE in MAB from both lean and obese rats (Fig. 3.3).

Interestingly, in the presence of the combination of indomethacin plus L-NMMA, responses of MAB from lean and obese rats to NE were similar to those in the absence of inhibitors (Fig. 3.3). The only difference noted was that the response of obese MAB to 0.9 nmol NE was significantly potentiated in the presence of L-NMMA and indomethacin compared to in their absence (Fig. 3.3).

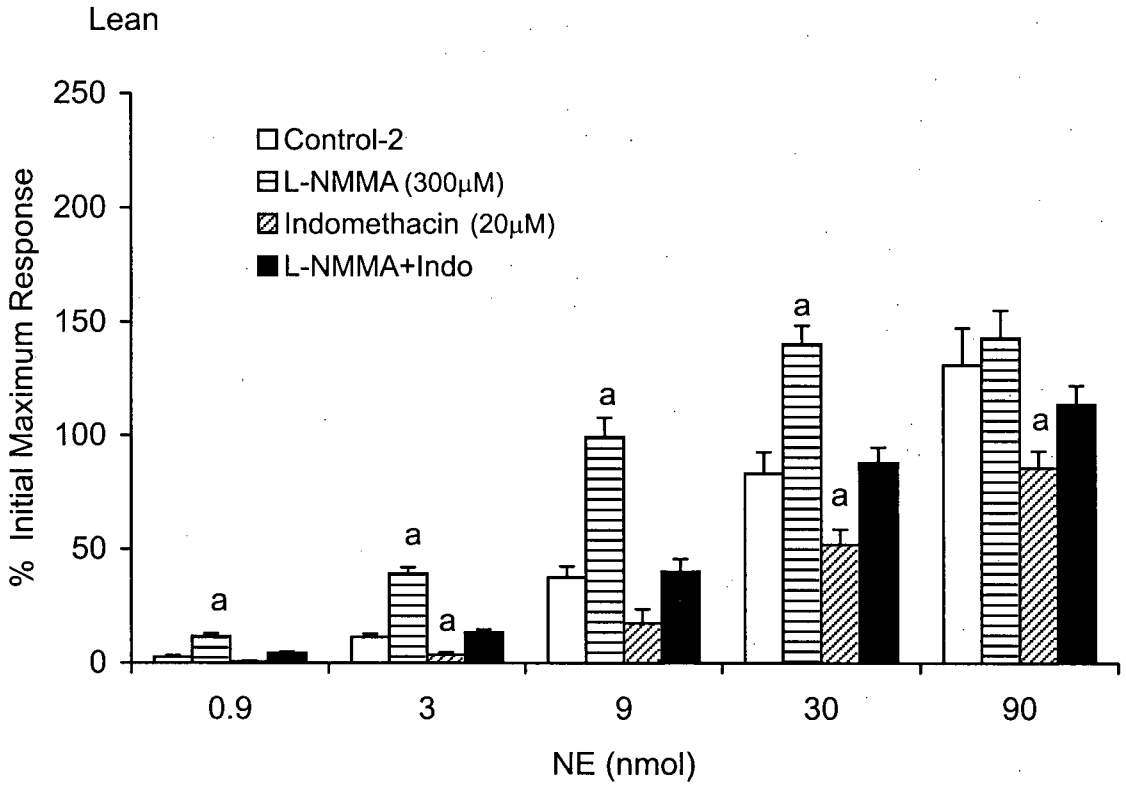
To elucidate whether the inhibitory effect of indomethacin alone on responses of lean and obese rats to NE is due to reduction in the release of the COX pathway contracting factor PGH_2/TxA_2, the effect of SQ 29,548, a PGH_2/TxA_2 receptor antagonist was examined. However, although SQ 29,548 (0.3 μM) tended to inhibit the responses to 9, 30 and 90 nmol NE in both obese and lean MAB, the inhibition was significant only at 30 nmol NE in the obese MAB (113 ± 4% in the absence vs. 92 ± 8% in the presence of SQ 29,548, $P < 0.05$).

4. Effect of Insulin on NE-Induced Vasoconstriction in Isolated Perfused MAB.

To investigate the influence of hyperinsulinemia on reactivity of the MAB to NE, tissues were pre-perfused with 200 mU/l insulin, a concentration close to that which the obese rats were exposed to *in vivo* (Table 3.1). Perfusion with insulin for two hours had no detectable effect on responses of MAB from lean rats to any concentration of NE, when compared to NE responses obtained at the same time in the absence of insulin (Fig. 3.4). Similarly, insulin had no significant effect on the maximum pressor responses to NE of MAB

FIGURE 3.3

Contractile response to NE in the absence (control, n=6) (□) and presence of 300 μM L-NMMA (n=6) (▤), or 20 μM indomethacin (n=6) (▨) or 300 μM L-NMMA plus 20 μM indomethacin (n=5) (■) in isolated MAB from lean or obese Zucker rats, perfused with Krebs solution at constant flow. n represents the number of the experiments. Data are expressed as mean ± SEM. [a] $P < 0.05$ vs. control (one-way ANOVA followed by Bonferroni post test: compare all column vs. control column).


Lean
250
200
150
100
50
0
% Initial Maximum Response
Control-2
L-NMMA (300μM)
Indomethacin (20μM)
L-NMMA+Indo
a
0.9
3
9
30
90
NE (nmol)

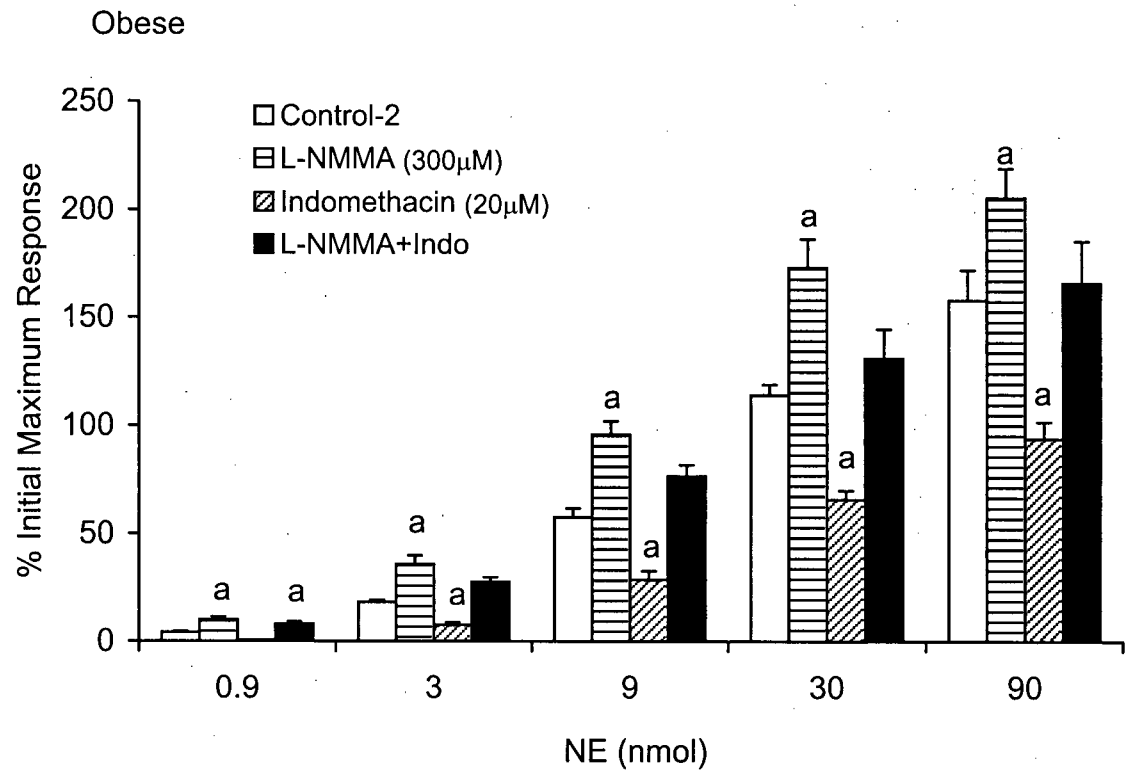
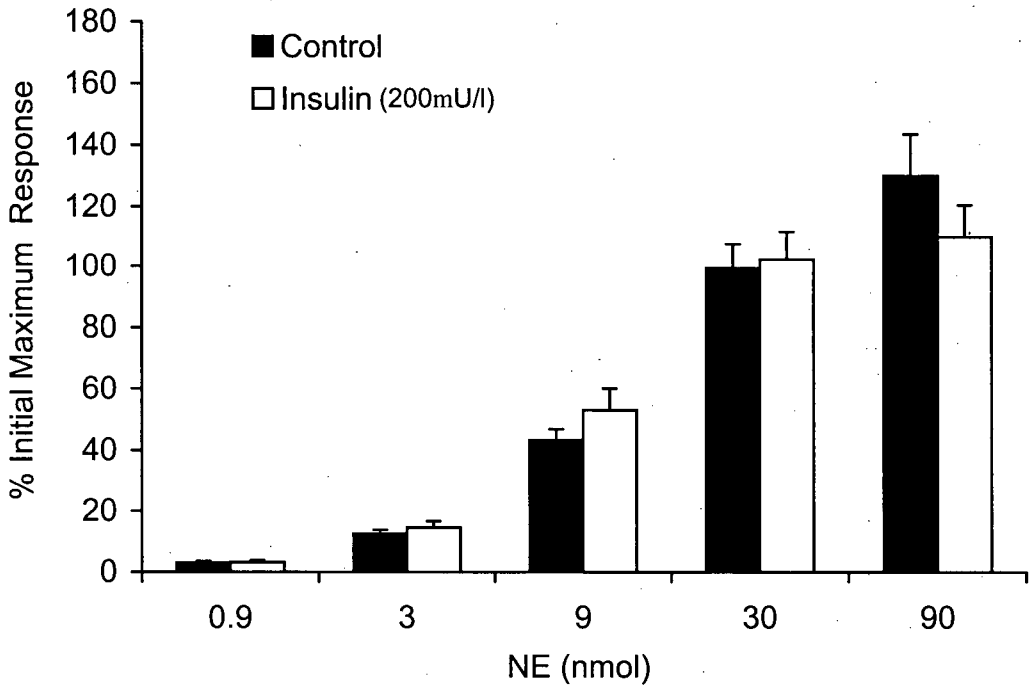

Obese
250
200
150
100
50
0
% Initial Maximum Response
Control-2
L-NMMA (300μM)
Indomethacin (20μM)
L-NMMA+Indo
a
0.9
3
9
30
90
NE (nmol)

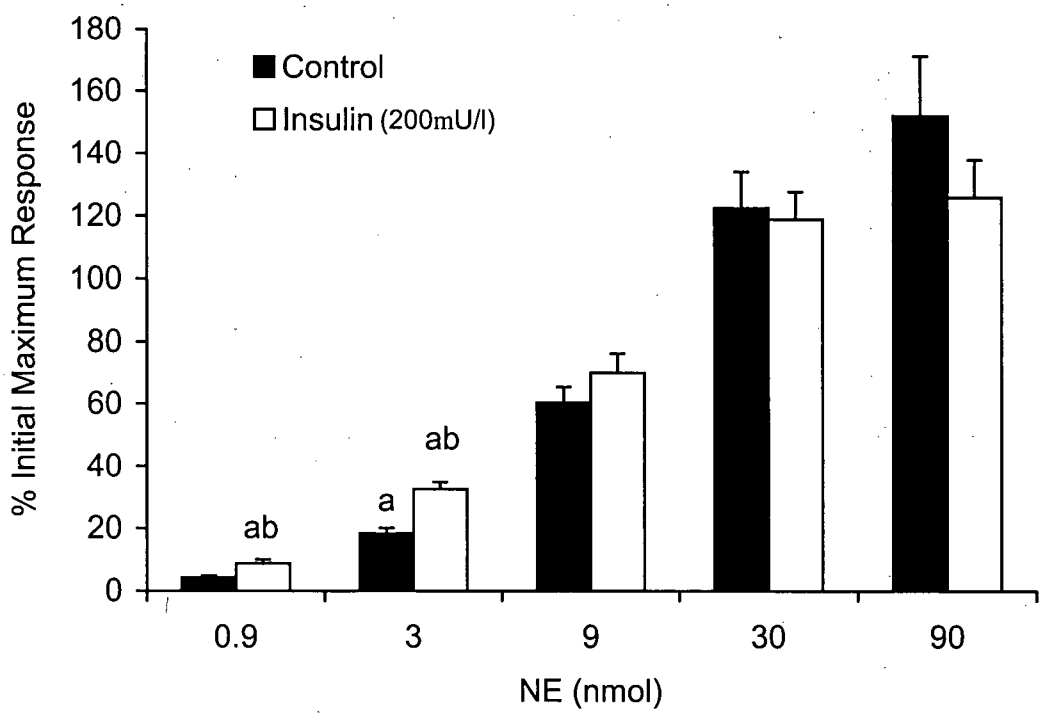
FIGURE 3.4

Concentration-response curves to NE in the absence (control, ■) and presence of 200 mU/l insulin (□) in isolated MAB from lean or obese Zucker rats, perfused with normal Krebs solution at constant flow. Data represent the mean ± SEM from 8 obese, 9 lean and 10 control experiments. [a] $P < 0.05$ vs. lean; [b] $P < 0.05$ vs. control (two-way ANOVA followed by Newman Keuls post test)

Lean


Control
Insulin (200mU/l)
% Initial Maximum Response
180
160
140
120
100
80
60
40
20
0
0.9
3
9
30
90
NE (nmol)

Obese


Control
Insulin (200mU/l)
% Initial Maximum Response
180
160
140
120
100
80
60
40
20
0
ab
a
ab
0.9
3
9
30
90
NE (nmol)

from obese rats. However, responses of the latter preparation to the two lowest concentrations of NE tested (0.9 and 3 nmol) were significantly enhanced in the presence of insulin (Fig. 3.4). Insulin had no effect on either basal perfusion pressure or on the KCl response in MAB from lean or obese rats (data not shown).

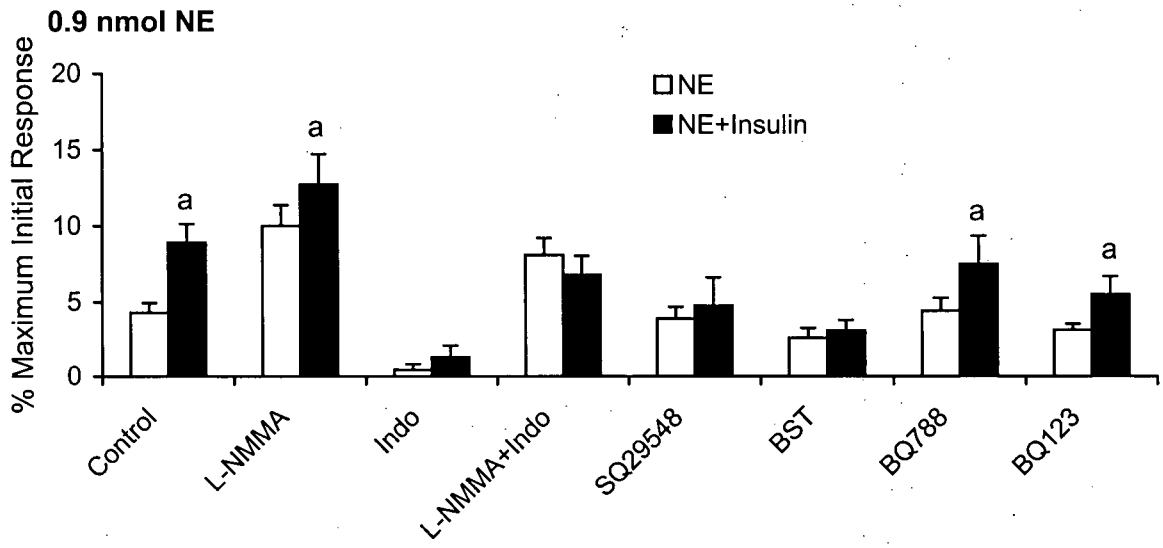
5. Influence of NOS, COX, PGH_2/TxA_2 Receptor and ET Receptor Inhibition on Insulin-Potentiation of NE Responses

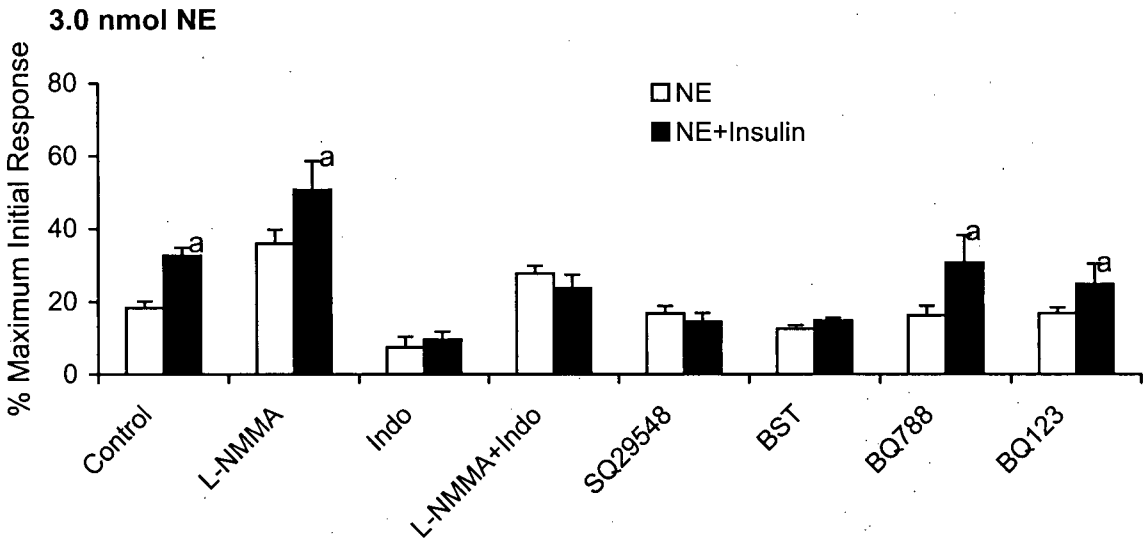
To investigate the pathway mediating insulin potentiation of the responses of MAB from obese rats to 0.9 and 3 nmol NE, the influence of various inhibitors on responses to 0.9, 3.0 and 9 nmol NE in the absence and presence of insulin were determined. None of the inhibitors tested (L-NMMA, indomethacin, SQ 29,548, the selective ET_B antagonist, BQ 788, the selective ET_A receptor antagonist, BQ 123, and the non-selective ET-1 receptor antagonist, bosentan) had any effect on the basal perfusion pressure, either alone or in combination with insulin. As shown in Fig 3.5, insulin further enhanced the L-NMMA-induced potentiation of responses to 0.9, 3 but also 9 nmol NE. In contrast, insulin no longer had effect on responses to NE in the presence of indomethacin. In addition, indomethacin prevented insulin from further increasing the responses to NE in the presence of L-NMMA. Although SQ 29,548 alone did not significantly alter responses to NE, like indomethacin, it blocked the potentiation by insulin of the NE responses.

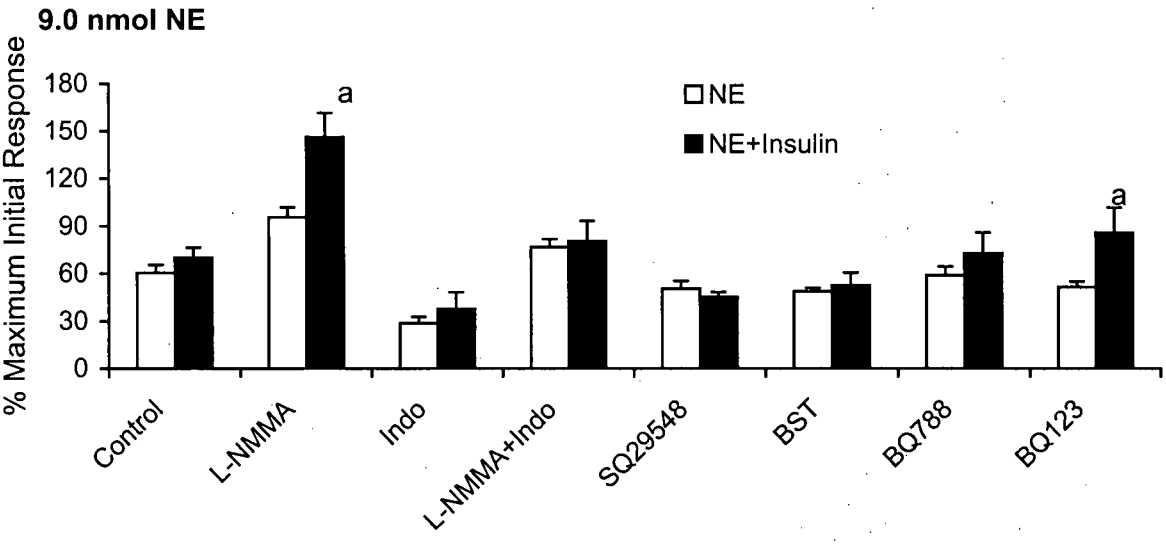
The effects of bosentan were similar to those of SQ 29,548, in that it blocked the potentiating effect of insulin on the responses of the obese preparations to 0.9 and 3 nmol NE, although it had no effect on NE responses in the absence of insulin. In contrast, insulin still produced significant potentiation of the NE responses in the presence of the selective ET receptor antagonists BQ 788 and BQ 123.

FIGURE 3.5

Influence of various inhibitors on potentiating effect of insulin on NE responses in isolated MAB from obese Zucker rats. Responses to 0.9, 3.0 and 9.0 nmol NE were compared in the absence (■) and presence of 200 mU/l insulin (□) under control condition without any inhibitors (n=8) or in the presence of L-NMMA (300μM) (n=6); 20 μM indomethacin (Indo, n=6); 300μM L-NMMA plus 20 μM indomethacin (n=6); 0.3 μM BQ 788 (n=5); 0.3 μM BQ 123 (n=5); 3 μM bosentan (BST, n=5); or 0.3 μM SQ 29,548 (n=6). n represents the number of the experiments. Data are expressed as mean ± SEM. [a] $P < 0.05$ with insulin vs. paired without insulin (one-way ANOVA followed by Bonferroni comparing selected pairs of columns).


0.9 nmol NE
% Maximum Initial Response
20
15
10
5
0
NE
NE+Insulin
a
a
a
a
Control
L-NMMA
Indo
L-NMMA+Indo
SQ29548
BST
BQ788
BQ123


3.0 nmol NE
% Maximum Initial Response
80
60
40
20
0
NE
NE+Insulin
a
a
a
a
Control
L-NMMA
Indo
L-NMMA+Indo
SQ29548
BST
BQ788
BQ123


9.0 nmol NE
% Maximum Initial Response
180
150
120
90
60
30
0
NE
NE+Insulin
a
a
Control
L-NMMA
Indo
L-NMMA+Indo
SQ29548
BST
BQ788
BQ123

V. DISCUSSION

There are two main findings in this part of the thesis work. The first is that NE-induced vasoconstriction is markedly influenced by both NO release and vasoconstrictor COX pathway products in MAB from both obese and lean Zucker rats. A balance between the suppressive effect of NO and the potentiating effect of vasoconstrictor COX product(s) apparently contributes to the net pressor responses to NE. The second finding is that insulin, at a pathophysiological concentration that obese Zucker rats are exposed to *in vivo*, had a potentiating effect on pressor responses to low concentrations of NE in obese MAB, which was mediated by COX metabolites and was enhanced after inhibition of NO synthesis and release.

Characteristics of Zucker Obese Rats

The Zucker obese rats used in this study were moderately hypertensive at 25 weeks of age; the SBP of these animals was on average 29 mmHg greater than that of their lean littermates. The obese rats also had severe hyperinsulinemia, with a plasma insulin levels about 4 times higher than those in the lean rats. The mechanism(s) of hypertension development in Zucker obese rats are not clear. It has been hypothesized that hyperinsulinemia in the insulin resistance state may contribute to hypertension through stimulating sympathetic activity (Anderson et al. 1991; Dornfeld et al. 1987; Sowers et al. 1982) and renal sodium retention (Baum 1987; DeFronzo 1981). However, obese Zucker rats were shown to retain less sodium than lean rats (Kurtz et al. 1989). In addition, renal injury has been found in obese Zucker rats, but it has been reported that mild hypertension preceded the development of progressive focal glomerulosclerosis (Kasiske et al. 1985). Furthermore,

ganglion blockade did not lower *in vivo* blood pressure of conscious obese rats and during the ganglion blockade the obese rats still exhibited greater pressor sensitivity to Ang II and NE (Zemel et al. 1992). Based on these results, it has been proposed that an enhanced pressor sensitivity, independent of sympathetic neural activity, appears to supports hypertension in Zucker obese rats (Zemel et al. 1992). Thus, local regulatory mechanisms including smooth muscle contractility and endothelial function, especially in resistance vessels, may be important determinants of elevated blood pressure in these animals. In addition, in view of the vascular actions of insulin as described in the introduction, hyperinsulinemia itself is still a factor in need of considerable research.

NE-Induced Vasoconstriction in MAB of Zucker Rats

1. Reactivity to NE and KCl in isolated perfused MAB

Previous studies of vascular reactivity in mesenteric resistance vessels have found no marked differences in pressor responses to α-adrenoceptor agonists such as PE and NE (Kam et al. 1996; Turner et al. 1995; Walker et al. 1997a; Wu et al. 1996) in obese *versus* lean rats. Endothelium-dependent relaxations in mesenteric resistance vessels from obese rats were reported to be either impaired (Walker et al. 1997a; Wu et al. 1996; Zanchi et al. 1995) or preserved (Bohlen and Lash 1995; Kam et al. 1996; Turner et al. 1995) as compared to age–matched lean rats. In agreement with those contractile studies, we did not find any major differences in vasoconstrictor responses to NE rats in perfused mesenteric arterial beds from obese compared to lean rats, except for a small decrease in responsiveness to the highest concentration of NE tested (90 nmol). However, we did find that pressor responses to KCl were attenuated in obese MAB. In these experiments, we did not further explore the

mechanisms of the decreased responsiveness to KCl in MAB from obese rats. However, it is known that challenge with KCl stimulates endogenous NE release from peripheral sympathetic nerve endings (Vanhoutte et al. 1981) and it has been shown that inhibition of the endogenous NE with phentolamine decreases KCl-evoked tension. Therefore, it is possible that the reduced response to KCl in MAB reflects the diminished sympathetic nervous system activity that has been reported in obese Zucker rats (Levin et al. 1980).

*2. **Blockade of the NO synthesis enhanced vasoconstrictor responses to NE.***

The augmentation of pressor responses to NE in both obese and lean MAB by L-NMMA suggests a modulatory role for NO in NE-mediated vasoconstriction. Since we found L-NMMA had no effect on basal perfusion pressures, NO may not be spontaneously released or the release may be too low to attenuate perfusion pressure under the basal perfusion conditions (perfusion rate of 3ml/min). Thus, the enhancement of NE-induced vasoconstriction by L-NMMA could be due to an agonist-stimulated NO release. This finding is consistent with those reported by most authors using rat isolated MAB perfused at rates under 10ml/min. In these studies, it has been shown that NOS inhibition augmented α-adrenoceptor mediated vasoconstriction in an endothelium-dependent manner (Adeagbo et al. 1994; Amerini et al. 1995; Tatchum-Talom and Atkinson 1997), but lacked an effect on the basal tone (Adeagbo et al. 1994; Amerini et al. 1995; Baisch et al. 1994; Ebeigbe et al. 1990; Tatchum-Talom and Atkinson 1997). In contrast, in *in vivo* studies in intact (Gardiner et al. 1990) or in ganglion-blocked (Fozard and Part 1991) rats, infusion of L-NMMA induced a 50% reduction of mesenteric vascular conductance, indicating a physiological role of NO in the control of the tone in the mesenteric vascular bed and even in the absence of functional

sympathetic activity. Recently, Hori et al (Hori et al. 1998) have directly measured changes in NO metabolite (NOx) concentration in the perfusate outflow during changes in flow and shear stress in isolated rat mesenteric arterial beds. Their data showed that basal NOx concentration (at perfusion rate of 4ml/min) in control rats was very low as compared with background values and did not significantly increase until the perfusion rate reached 48 ml/min. In addition, after treatment with L-NMMA, the amount of NOx released only decreased significantly at a flow rate of 48 ml/min. We have demonstrated in another study that *in vivo*, the blood flow through the superior mesentery is approximately 14 ml/min in control rats, much higher than the rate we used in this study (He and Tabrizchi 1997). Thus, the lack of effect of L-NMMA on basal perfusion pressure observed in our study is probably due to the low perfusion rate plus the very low viscosity of the Krebs solution so that the shear stress is too low to evoke a significant NO release.

The mechanisms that mediate the release of NO in the presence of NE in blood vessels are not clear. It may be shear stress-dependent and/or adrenoceptor-coupled. It has been reported that an increase in shear stress by a vasoconstriction at constant flow enhances the release of NO, as well as PGI_2 from perfused rabbit femoral arteries (Hecker et al. 1993); the NO release in response to short shear exposure due to decrease in vessel diameter by vasoconstrictors was Ca^{2+}/ camodulin-dependent (Busse et al. 1993; Kuchan and Frangos 1994). This concept was supported by a study in Wistar rat perfused mesenteric arterial bed, in which the inhibition of NO synthesis or endothelium denudation was able to comparably potentiate the response to either receptor-mediated (NE) or receptor-independent (KCl) vasoconstriction, but had no effect on basal tone, suggesting that NO release can be triggered by active tone (Amerini et al. 1995). This shear stress/active tone-induced NO release is

unlikely in the present study since NOS inhibition dramatically enhanced the pressor response to NE, but had little effect on contraction evoked by KCl in mesenteric preparations from either obese or lean Zucker rats (n= 6 for each group of rats, data not shown). Both α_1 and α_2-adrenoceptors have been implicated in the endothelium-dependent NO-mediated depression of NE contraction in rat aorta (Kaneko and Sunano 1993). NE-induced endothelium-dependent relaxation was first shown in pig and dog isolated coronary arteries (Cocks and Angus 1983) and was suggested to be mediated by αv α_2-adrenoceptor on the endothelium (Angus et al. 1986; Vanhoutte and Miller 1989). The partial α_2-adrenoceptor agonist clonidine induced relaxation via NO release in porcine coronary resistance artery (Tschudi et al. 1991), rat aorta (Kaneko and Sunano 1993) and perfused mesenteric arterial bed (Kamata et al. 1994). However, α_2-adrenergic, endothelium-dependent responses are much less pronounced in mesenteric arteries compared to femoral, carotid, and coronary arteries of dog (Angus et al. 1986). This may reflect a lower density of endothelial α_2-adrenoceptors in this vascular bed. In addition, there still is an uncertainty with regard to α_2-adrenoceptor mechanisms in rat mesenteric arteries since clonidine does not increase the cGMP level in rat mesenteric arteries with intact endothelium (MacLeod et al. 1987). Recently, NO release as an endothelial response secondary to vasoconstriction evoked by sympathetic nerve stimulation has been demonstrated in perfused rat MAB (Boric et al. 1999). Both the electrically evoked vasoconstriction and NO release were abolished by prazosin, supporting the involvement of α_1-adrenoceptors and making any possible direct effect of α_2-adrenoceptor unlikely. It is known that in rat mesenteric vasculature, NE causes contraction predominantly, if not exclusively, via α_1-adrenoceptors (Chen et al. 1996; Colucci et al. 1980; McPherson et al. 1984; Nielsen et al. 1991; Pipili 1986). However, there

is no evidence so far to demonstrate the presence of α_1-adrenoceptors on vascular endothelium of rat mesenteric arteries. On the other hand, it has been demonstrated that endothelial cells and smooth muscles are electrochemically coupled through myoendothelial junctions (Chaytor et al. 1998; Yamamoto et al. 1999), and the existence of bi-directional communication between endothelial and smooth muscle cells has been reported (Beny and Pacicca 1994). Recently, Dora et al (Dora et al. 1997) have shown that during PE-induced vasoconstriction, a signal can originate in smooth muscle cells and act on the endothelium to cause synthesis of NO in arterioles from hamster cheek pouch. Thus, α_1-adrenoceptor stimulation of vascular smooth muscle may result in activation of eNOS via intercellular communication, and it may explain the NE responses to L-NMMA observed in the present study.

3. Blockade of COX pathway suppresses pressor responses to NE.

In contrast to effect of L-NMMA, the application of the COX inhibitor indomethacin to mesenteric arterial beds induced a significant suppression of pressor responses to NE in both lean and obese rats. Because indomethacin did not affect the responses to KCl in preparations from either group of rats (data not shown), the suppressive effect of indomethacin on NE responses is unlikely to be non-specific. Thus, our observations strongly suggest that activation of the COX pathway is necessary for NE to exert its full vasoconstrictor effect in rat mesenteric vasculature from Zucker rats. Cyclooxygenase pathway metabolites of arachidonic acid (AA) have previously been found to be released under basal and NE-stimulated conditions in rat perfused MAB (Desjardins-Giasson et al. 1982; Pipili et al. 1988), and to modulate the NE response (Coupar 1980; Malik et al. 1976). The mechanisms that mediate prostanoid release by NE are not known but could include

either a transient increase in shear stress/active tone (Hecker et al. 1993) and/or activation of adrenergic receptors (Pipili et al. 1988). In the present study, we found that indomethacin had no effect on basal perfusion pressure in Zucker mesenteric arterial bed. This may be due to the removal of a balanced basal release of vasodilator and vasoconstrictor prostaglandins (PGs). Indeed, unbalanced release of vasodilator and vasoconstrictor PGs in rat mesenteric resistance vessels in SHR rats has been reported (Matrougui et al. 1997; Soma et al. 1985). Prostanoid modulation of the NE response in rat mesenteric vascular beds was reported repeatedly in the late 1970s and early 1980s. It has been shown that the structurally different COX inhibitors indomethacin (Coupar 1980; Coupar and McLennan 1978; Malik et al. 1976; Manku and Horrobin 1976), aspirin, mefenamic acid (Manku and Horrobin 1976) and 5, 8, 11, 14-eicosatetraynoic acid (Coupar 1980) caused a significant depression of pressor responses to NE in rat isolated perfused mesenteric blood vessels. In addition, indomethacin reduced an increase in release of a PGE_2-like activity stimulated by NE to below resting values (Coupar 1980), and PGE_2, as well as other prostaglandins restored the indomethacin-depressed response to NE (Coupar 1980; Coupar and McLennan 1978; Malik et al. 1976; Manku and Horrobin 1976). Furthermore, arachidonic acid potentiated NE-induced vasoconstriction, which was abolished by simultaneous infusion of indomethacin (Malik et al. 1976). These results provided strong support to our observations in Zucker rats. Since indomethacin had a pronounced inhibitory effect on the response to NE (the maximum inhibition was around 45% and 63% for lean and obese, respectively) in the present study, NE may have a greater propensity to stimulate the release of contracting factor(s) than relaxing factors such as PGI_2 via the COX pathway in Zucker mesenteric vessels. In addition, since indomethacin blocks all pathways of COX, the pronounced decrease in response to NE

seen in Zucker mesenteric vascular beds may be due not only to the removal of the contracting prostanoid(s), but also to an intact vasodilator effect of NO protected from chemical inactivation by superoxide anion. It has long been known that O_2^- is a byproduct of COX pathway metabolism (Katusic and Vanhoutte 1989; Kukreja et al. 1986; Yokota and Yamazaki 1977) and that indomethacin can inhibit O_2^- release arising from this pathway (Holland et al. 1990; Kontos et al. 1985). O_2^- is known to react with and inactivate NO (Gryglewski et al. 1986; Rubanyi and Vanhoutte 1986). Therefore, blockade of COX with indomethacin may not only block release of prostanoids, but also inhibit O_2^- production, and thus enhance NO activity (Cosentino et al. 1994), resulting in an additional inhibition of NE-induced contractions.

Metabolites of the COX pathway known to produce vascular contractions include PGH_2, TxA_2, $PGF_{2\alpha}$, and in rat, PGE_2. Of these, PGH_2 and TxA_2, which interact with a common receptor, have been proposed to be endothelium-derived contracting factors (Luscher et al. 1992; Vanhoutte 1996) and therefore, may be responsible for indomethacin-induced depressor effect in this study. However, the selective PGH_2/TxA_2 receptor antagonist SQ 29,548 only minimally inhibited NE-induced vasoconstriction in both lean and obese MAB. A possible explanation for the smaller inhibitory effect of SQ 29,548 is that besides PGH_2 and/or TxA_2, other vasoconstrictor prostanoids may be also involved (Quilley et al. 1989)(also as mentioned above). Alternatively, the possibility can not be excluded that an increased O_2^- generated via activation of the COX pathway by NE, that was not inhibited by SQ 29,548, may reduce the biological activity of NO and indirectly enhance the NE-induced tone, resulting in a smaller effect of SQ 29,548 than of indomethacin.

4. Effect of COX inhibition on pressor responses to NE after blocking of NO synthesis

We observed an opposing effect on responses to NE after combined COX and NOS blockade in mesenteric arterial beds from both obese and lean Zucker rats. It then should be considered that the potentiation of responses to NE by L-NMMA alone is the result of the concomitant activation of COX leading to release of predominantly vasoconstrictor prostanoids, while the attenuation of responses to NE with indomethacin alone is due to the concomitant release of NO. In the presence of both indomethacin and L-NMMA, the pressor response to NE was essentially the same as the control response, suggesting in mesenteric arterial bed vasculature, NO and COX-derived contracting factors released by NE stimulation almost completely counteract each other. In obese mesenteric arterial beds, after blockade of NOS and COX, we found that the pressor responses tended to be greater at most concentrations of NE, but it was only statistically significant at 0.9 nmol NE. Thus, a slight imbalance in the release of these factors, favoring vasodilation, may account for the decreased maximum response of MAB from obese rats to NE in the absence of insulin.

5. Lack of influence of endothelin on responses to NE.

Pretreatment with bosentan, a non-selective ET-1 receptor antagonist, or blocking of either the ET_A or the ET_B receptor with selective antagonists BQ 123 or BQ 788, respectively, had no effects on NE induced vasoconstriction. Thus, ET-1 does not seem to be involved in modulating the pressor response to NE in Zucker MAB.

Insulin Effect on Vasoconstrictor Responses to NE in MAB of Zucker Rats

1. Hyperinsulinemia elevated pressor responses to NE in MAB from obese rats

Two studies have previously addressed the effects of insulin on reactivity of the MAB to NE, with differing results. Walker et al (Walker et al. 1997a) showed that insulin (50 and 500 mU/l) slightly attenuated the maximum response to NE in isolated small mesenteric arteries from lean rats, while the action of insulin was impaired in tissues from pre-hypertensive obese rats, suggesting obese mesenteric arteries are resistant to a vasodilator action of insulin. The significance of this observation is uncertain, since the attenuation produced by 50 mU/l insulin was only on the order of 8%, and 500 mU/L insulin is much higher than obese rats would be exposed to *in vivo*. On the other hand, Turner *et al* (Turner et al. 1995) demonstrated that 100 mU/l insulin had no effect on pressor responses to PE or depressor responses to ACh in isolated perfused mesenteric arterial beds from either lean or hypertensive obese rats at 12 months of age. However, this concentration of insulin is lower than that which obese rats are exposed to *in vivo*. In contrast with these reports, we found potentiation of pressor responses to 0.9 and 3.0 nmol NE on MAB from obese rats by a concentration of insulin (200 μU/ml) close to that which obese rats were exposed to *in vivo*. This suggests that exposure to chronic hyperinsulinemia enhances vascular reactivity to concentrations of NE that are within the physiological range for circulating NE in rats (Dargie et al. 1977; Katholi et al. 1982). In contrast, perfusion with the same concentration of insulin had no effect on lean tissues, indicating that acutely raising the insulin level does not affect the reactivity of the MAB under normal conditions.

Verma and McNeill demonstrated that in fructose-induced hypertensive (FH) rats, which are insulin-resistant and hyperinsulinemic, insulin at a pathophysiological

concentration potentiated NE responses in MAB to a greater extent as compared to control rats (Verma and McNeill 1999). In addition, this altered MAB response to insulin was evident prior to the development of hypertension in these rats, which were already hyperinsulinemic (Verma and McNeill 1999). Other in vivo studies reported that the mean blood pressure and total peripheral resistance measured during chronic insulin infusion in rats is elevated (independent of changes in cardiac output and heart rate) (Brands et al. 1991; Brands et al 1996). Furthermore administration of an insulin-sensitizing drug bis(maltolato)oxovanadium, restored plasma insulin levels in the obese Zucker rats tolevels in lean rat and ameliorated the age-dependent increase in blood pressure observed in obese Zucker rats (Yuen et al. 1996). These observations support our results suggesting chronic hyperinsulinemia may be physiologically relevant in promoting hypertension by increasing peripheral vascular resistance via exaggeration of MAB responses in Zucker obese rats.

2. *Blockade of NO synthesis enhanced the vascular effect of Insulin in obese rats*

Previous studies have suggested that insulin can exhibit both vasodilator and vasoconstrictor effects, and that its overall effect in a given vascular bed may depend on the balance of vasodilators and vasoconstrictors it releases. For instance, Baron and Brechtel (Baron and Brechtel 1993) reported that in lean human subjects, a physiological concentrations of insulin caused an approximately 5-fold greater fall in muscle vascular resistance than in systemic vascular resistance. They suggested that insulin preferentially reduced vascular resistance in skeletal muscle beds but may actually increase vascular resistance in other vascular beds (e.g. splanchnic circulation). *In vitro* studies in rats have demonstrated that insulin administration attenuates contractile reactivity to NE and dilates isolated resistance vessels (Alexander and Oake 1977; Chen and Messina 1996; Walker et al.

1997b). However, under physiological conditions, insulin has been repeatedly reported to potentiate the vasoconstriction elicited by several agonists in isolated perfused MAB (Townsend et al. 1992; Wu et al. 1994; Verma and McNeill 1999) and in femoral arteries (Nava et al. 1997). In these studies, the vasodilator effect of insulin appeared to be mediated by the release of NO (Chen and Messina 1996; Steinberg et al. 1994; Walker et al. 1997b), while the vasoconstrictor effect of insulin may be linked to changes in production of COX pathway metabolites (Wu et al. 1994) or increased release of endothelin (Nava et al. 1997). Recently, Schroeder *et al.* (Schroeder et al. 1999) demonstrated that insulin induced a concentration-dependent increase in diameter of endothelium-intact arterioles isolated from male Wistar rat gastrocnemius muscle. However, inhibition of NO synthesis or removal of the endothelium inhibited the insulin-induced arteriolar dilation and revealed an insulin-induced vasoconstriction. Consistent with this, in the present investigation L-NMMA further enhanced the insulin-mediated potentiation of vasoconstriction to NE in MAB from obese rats. The amplification by L-NMMA of the potentiating effect of insulin on vasoconstrictor responses to NE in MAB from Zucker obese rats suggests that insulin-induced potentiation is normally suppressed to some extent by concomitant release of NO.

3. Inhibition of COX blocked insulin effect in MAB

Involvement of prostaglandins in the mechanisms of insulin action has been reported. It was demonstrated that indomethacin treatment caused a metabolic state of insulin resistance in rats (Wasner et al. 1994). In addition, indomethacin markedly decreased the insulin-induced increase in forearm blood flow of healthy humans (van Veen and Chang 1997) and prevented the relaxant effects of insulin on ET-1 and AVP contractions after NOS inhibition in male Wistar rat aorta (Rebolledo et al. 1998). Therefore, it has been suggested

that insulin stimulates production/release of vasodilating prostaglandins. Furthermore, Axelrod and coworkers (Axelrod and Levine 1982; Axelrod and Levine 1983; Axelrod et al. 1986) reported that insulin in physiological concentrations inhibited catecholamine-stimulated PGI_2 and PGE_2 production by adipose tissue both *in vitro* and *in vivo*, and hypothesized (Axelrod 1991) that hyperinsulinemia may increase peripheral vascular resistance and blood pressure by inhibiting the stimulatory effect of adrenergic agonists on the production of these vasodilative eicosanoids in adipose tissue (and perhaps other tissues). Insulin has also been shown to specifically enhance TxA_2-induced vasoconstriction in porcine coronary vascular beds (Yanagisawa-Miwa et al. 1990). Moreover, Keen *et al.* (Keen et al. 1997) recently reported that inhibition of TxA_2 synthase markedly attenuated mean blood pressure increased by chronic insulin infusion in rats, suggesting that TxA_2 is a key component of the chronic hypertensive effect of insulin. In this study, we demonstrated that application of indomethacin and SQ 29, 548 completely inhibited the potentiating effect of insulin on pressor responses to NE in MAB from obese Zucker rats, suggesting that the potentiating effect of insulin is mediated by release of vasoconstrictor COX metabolites, possibly PGH_2 and/or TxA_2. In addition, the lack of further effect of insulin in the presence of indomethacin plus L-NMMA on responses to NE in MAB from obese Zucker rats further confirms the notion in MAB from obese Zucker rats.

4. ET-1 contributing to potentiating effect of insulin on responses to NE in obese rats

In this study, we also examined the role of endothelin in the effect of insulin on vasoconstrictor responses to NE in obese Zucker rats. We found that the non-selective ET receptor antagonist bosentan prevented the potentiating effect of insulin on the pressor responses to NE in obese MAB. The result suggests that insulin-induced, prostanoid-

mediated enhancement of vasoconstrictor responses to NE in obese Zucker MAB also involves endothelin release, at least in part. This result is consistent with those of previous studies, which have implicated the vasoconstrictor ET in the vascular actions of insulin. For instance, insulin was reported to increase ET-1 gene expression (Oliver et al. 1991) and induce ET-1 release (Hattori et al. 1991; Hu et al. 1993) in cultured endothelial cells, while an increase in contractile response induced by insulin in rat femoral arteries has been found to be partially mediated by endothelin (Nava et al. 1997). In addition, in fructose hypertensive rats, insulin-induced exaggerated MAB responses to NE were completely abrogated in the presence of both indomethacin and bosentan, By contrast indomethacin completely prevent the insulin response in MAB from control rats, suggesting a role of ET-1 for the FH rats (Verma and McNeill 1999). Furthermore, insulin has been shown to selectively increase ET_A receptor expression in VSM cells (Hopfner et al. 1998) and both ET_A and ET_B receptor expression have been found to be increased in mesenteric arteries and aorta from obese Zucker rats with hypertension although local ET-1 production is decreased (Wu et al. 2000). Moreover, endothelin activated PLA_2 in blood vessels, leading to the release AA (Resink et al. 1989; Reynolds et al. 1989), and release of both vasoconstrictor and vasodilator prostanoids has also been demonstrated (Matsuda et al. 1993; Tabuchi et al. 1989a; Taddei and Vanhoutte 1993). It has also been shown that subpressor doses of endothelin enhanced pressor responses to NE in human coronary arteries, rabbit aorta and perfused rat mesenteric arterial beds (Henrion and Laher 1993; Kita et al. 1998; Tabuchi et al. 1989b; Yang et al. 1990).

The failure of either of the selective ET receptor antagonists to mimic the effects of bosentan means that the effect of endothelin cannot be attributed to its actions on either of the

receptor subtypes alone. Although the effects of bosentan might have resulted from an action unrelated to antagonism of ET receptors, blockade of both receptor subtypes has been previously shown to be required for antagonism of some actions of ET (Fukuroda et al. 1996; Mickley et al. 1997).

VI. SUMMARY

1. Zucker obese rats were moderately hypertensive and severely hyperinsulinemic at 25 weeks of age.
2. In perfused MAB from both obese and lean Zucker rats, NE induced a concentration–dependent increase in perfusion pressure that was significantly lower at maximum response to NE in MAB from the obese than from the lean rats.
3. Insulin perfusion had no effect on NE responses of the lean MAB, but potentiated the responses of the obese MAB to 0.9 and 3 nmol NE.
4. The NOS inhibitor L-NMMA enhanced the responses of both the obese and the lean MAB to NE. In the presence of L-NMMA, insulin further increased the NE response of MAB from the obese rats.
5. Perfusion with indomethacin alone inhibited the pressor responses to NE in MAB from both the lean and the obese rats In the presence of indomethacin, insulin no longer had any effect on the NE responses in the obese MAB.
6. The presence of indomethacin inhibited the potentiating effect of L-NMMA on the responses to NE in MAB from both the obese and the lean rats. Indomethacin also prevented insulin from further increasing the responses to NE in the presence of L-NMMA in the obese MAB.
7. BQ 123, a selective ET_A receptor antagonist, and BQ 788, a selective antagonist for ET_B, alone had no effect on pressor responses to concentrations of NE in both lean and obese MAB. However, in the presence of either BQ 123 or BQ 788, insulin still potentiated the response of MAB to 0.9 and 3 nmol NE.

8. Bosentan, a non-selective ET-1 receptor inhibitor, alone had no effect on pressor responses to concentrations of NE in both lean and obese MAB. The presence of bosentan abolished the potentiating effect of insulin on vasoconstrictor responses to the lower concentration of NE.

VII. CONCLUSIONS

We present evidence that NE-induced vasoconstriction is normally regulated by release of both NO and vasoconstrictor COX product(s) in isolated perfused MAB from both obese and lean Zucker rats. Insulin, at a concentration close to that obese rats are exposed to *in vivo*, increased the release of contracting COX product(s) and enhanced contractile responses to physiological concentrations of NE in MAB from obese, but not from the lean rats. The effects of insulin in obese rats may be partially mediated by ET-1 and are suppressed to some extent by concomitant release of NO. Taken together, our results suggest that chronic hyperinsulinemia may elevate reactivity of mesenteric resistance arteries and serve to increase peripheral resistance in vivo. Thus, hyperinsulinemia could play a role in development and/or maintenance of hypertension in obese Zucker rats. Whether the coexistent hyperlipidemia will interfere with the bioavailability of NO and exaggerate the insulin effects needs to be further investigated. To our knowledge, the present study is the first time an altered action of insulin leading to the release of contracting factor (s) in insulin-resistant animals has been demonstrated.

CONCLUDING REMARKS

Mesenteric arteries and arterioles play an important role in the maintenance and control of peripheral resistance, thereby regulating blood flow and blood pressure.

A particular feature of α_1-adrenoceptor-mediated excitation-contraction coupling of mesenteric arterial smooth muscle appears to be the dependence of the contractile response on VOCs, and thus on the membrane potential. Information on the role of Cl^- channels in the membrane depolarization to agonist activation has been obtained largely from electrophysiological and ion efflux studies. In this dissertation, functional evidence is presented that Cl^- channels mediate α_1-adrenoceptor-induced contraction via opening of nifedipine-sensitive Ca^{2+} channels. This contribution of Cl^- channels in mesenteric arteries from 2K1C hypertensive rats appears to be reduced. The diminished role of Cl^- may reflect an adaptive change in response to an enhanced reactivity in hypertensive mesenteric arterial bed.

The endothelium also has an active role in regulating local tone by integrating diverse biochemical and mechanical signals, and by responding to them through the release of vasoactive substances. In the present study, NO and EDHF were demonstrated to be released in response to ACh and to contribute to different extents to the relaxation of the muscular mesenteric artery compared to the elastic aorta. Endothelial-dependent smooth muscle relaxation in the mesenteric arteries appears mainly to reflect the action of EDHF, with NO playing a minor role. In the aorta, NO is the primary relaxant, with EDHF contributing to lesser extent. The underlying mechanisms of ion channel regulation are also different between the two vessels. K^+ channels, but not Cl^- channels mediate the function of ACh in

mesenteric arteries. In contrast, both channels are important for releasing NO from endothelium in aorta. The differences in relative contributions and regulatory mechanisms of these EDRFs may prove to have physiological and/or pathophysiological significance in disease states such as atherosclerosis and hypertension.

The interplay among EDCFs and EDRFs and among these endothelial-derived factors and neurotransmitters and hormones may have a profound impact on vascular homeostasis. I report in this dissertation that NO and vasoconstrictor COX products modulate NE-induced vasoconstriction in MAB of lean and obese Zucker rats. Insulin, at a concentration close to which obese rats are exposed *in vivo*, increases the release of contracting COX product(s) and enhances contractile responses to physiological concentrations of NE in MAB from hypertensive obese, but not from the normotensive lean rats. Another EDCF, ET-1, and EDRF, NO are also involved. The altered insulin action and the imbalanced interaction among the endothelium-derived substances may elevate reactivity of mesenteric resistance arteries and could play a role in hypertension in Zucker obese rats.

Further investigation into the intracellular signaling, which leads to Cl^- channel activation in response to α_1-adrenoceptor stimulation in mesenteric smooth muscle, would necessarily address the interrelationship between Ca^{2+} and membrane potential and in turn the regulation of contractile mechanisms by Ca^{2+} and membrane potential in these blood vessels. In addition, further research on identification of the EDHF and other substances that released from endothelium would advance the study of mechanisms that mediate relaxation of smooth muscle in the mesenteric arteries. Finally, elucidation of these mechanisms will lead to a greater understanding of the regulation of peripheral resistance under normal and pathological conditions and may have important therapeutic implications.

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