CHANGES IN VASCULAR FUNCTION IN HYPERTENSION: ROLE OF CHLORIDE IN ALTERED ELECTROMECHANICAL COUPLING IN SALT HYPERTENSION

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Changes in Vascular Function in Hypertension:
Role of chloride in altered electromechanical coupling in salt hypertension

by

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ABSTRACT

According to the working hypothesis put forth, the role of Cl^- in excitation-contraction coupling is altered in salt hypertension, in part, due to altered nitric oxide (NO)/NO synthase pathways. To test this hypothesis, an assessment was made of the hemodynamic, mechanical, electrical and morphological properties in mesenteric blood vessels from Dahl salt-resistant normotensive (SRN) and salt-sensitive hypertensive (SSH) rats. Female Dahl salt-resistant and salt-sensitive rats were placed on a 4% salt diet for 7 weeks. The Dahl SSH rats had significantly higher blood pressure, heart rate, and body weight compared to SRN. In addition, the SSH rats had left ventricular and renal hypertrophy, but the plasma and total blood volume were not different compared to SRN. Morphological examination revealed an increase in media thickness and lumen diameter in mesenteric arteries from SSH, but the ratio of the two was not different between the strains.

The α_1-adrenoceptor agonist, cirazoline, produced a dose-dependent increase in blood pressure, decrease in heart rate, mesenteric blood flow, and mesenteric vascular conductance in anesthetized SRN and SSH rats. The nitric oxide synthase inhibitor N^{ω} nitro-L-arginine methyl ester (L-NAME) increased blood pressure, decreased mesenteric blood flow and conductance in SRN but not in SSH. Niflumic acid-induced attenuation of cirazoline-mediated decreases in mesenteric blood flow and conductance was more pronounced in SRN than in SSH rats. This difference in the inhibitory actions of niflumic acid was abolished by pretreatment with L-NAME.

In isolated perfused mesenteric blood vessels, replacement of Cl^- with propionate ions significantly inhibited cirazoline-mediated vasoconstriction in SRN but not in SSH. Exposure to L-NAME potentiated cirazoline-induced vasoconstriction in SRN but not in SSH rats. The absence of Cl^- significantly inhibited the potentiating effect of L-NAME at the higher doses of cirazoline in SRN without any effects in SSH. The presence of niflumic acid significantly inhibited cirazoline-mediated vasoconstriction, the inhibition being more pronounced in SRN than SSH. Niflumic acid did not produce any additional inhibitory effect in Cl^--free medium.

The resting E_m of smooth muscle cells in superior mesenteric artery was not different in SRN and SSH. Cirazoline did not produce a membrane depolarization in smooth muscle cells from either strain. Removal of Cl^- from the extracellular space produced a significant depolarization which was significantly larger in SSH than SRN. Addition of L-NAME in normal Krebs resulted in significant depolarization in tissues from SRN but not in SSH.

There was a lack of increased reactivity and magnitude in vasoconstrictor response to cirazoline in mesenteric blood vessels in SSH. The absence of increased reactivity in blood vessels from SSH can be attributed to receptor desensitization, morphology, and resting E_m. Cl^- handling and Ca^{2+}-dependent Cl channel activity seem to undergo modification as a consequence of salt-induced hypertension. The dysfunction of the NO system and Cl^- channels could contribute to the hypertension observed in the SSH model.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATP: adenosine 5-triphosphate
BP: blood pressure
Ca^{2+}: calcium ion
CaM: calmodulin
cAMP: cyclic adenosine 3,5-monophosphate
cGMP: cyclic guanosine 3,5-monophosphate
Cl^-: chloride ion
CO: cardiac output
DAG: diacylglycerol
DR: Dahl salt-resistant
DS: Dahl salt-sensitive
EBD: Evans blue dye
E_m: membrane potential
GDP: guanine nucleotide diphosphate
GTP: guanine nucleotide triphosphate
Hct: hematocrit
IP_3: inositol 1,4,5-triphosphate
K^+: potassium ion
K_{ATP}: ATP-sensitive K channels
K_{Ca}: Ca^{2+} sensitive K channels
L-NAME: N^{ω} nitro-L-arginine methyl ester
L-NMMA: N^{ω}-monomethyl-L-arginine
L-NNA: N^{ω}-nitro-L-arginine
MAP: mean arterial blood pressure
MLCK: myosin light chain kinase
Na^+: sodium ion
NO: nitric oxide
NOS: nitric oxide synthase
PKA: cAMP-dependent protein kinase
PKC: protein kinase C
PKG: cGMP-dependent protein kinase
PLC: phospholipase C
PLV: plasma volume
SEM: standard error of mean
SMAC: superior mesenteric arterial conductance
SMAF: superior mesenteric arterial blood flow
SRN: salt-resistant normotensive
SSH: salt-sensitive hypertensive
STIC: spontaneous transient inward current
TBV: total blood volume

TPR: total peripheral resistance

α: alpha
β: beta
γ: gamma

1. INTRODUCTION

The cardiovascular system is composed of the heart and the blood vessels. The heart is a pump that supplies the body with blood, which is distributed throughout the body by a network of blood vessels. The cardiovascular system provides a blood supply that maintains an optimal environment for the body tissues by supplying oxygen and nutrients and removing waste products. Any disease condition such as hypertension can cause abnormalities in this system. Blood pressure in a vessel is generated by, and is proportional to, the output of the heart (cardiac output) and the resistance to flow in the system. Blood pressure can be raised by increasing cardiac output and/or total peripheral resistance.

The dynamics of blood flow through blood vessels can be best illustrated using Ohm's law. Essentially, Ohm's law states that potential difference (ΔP) between two points is equal to the products of current (flow of electrons) (I) and resistance to flow (R) ($\Delta P = I \times R$). Thus, by comparison, flow of fluids (F) through a pipe (blood vessel) is determined by two factors: the pressure difference (gradient) (ΔP) between the two points in the vessels, which is the force that pushes the fluid through the pipe, and the resistance (R) to that flow, as defined by the equation $R = \Delta P/F$ (Tabrizchi and Iida, 2004). Thus the flow through a blood vessel is dependent on the blood pressure and/or total peripheral resistance.

1.1. Blood Vessels

The following is a textbook summary (Seeley *et al.*, 1989; Tortora and Grabowski, 1993). Blood vessels are an important component within the circulatory system. There are two main types of blood vessels, namely arteries and veins. The blood vessel walls consist of three relatively distinct layers (from the lumen to the outer wall of the blood vessels), which are tunica intima, tunica media and tunica adventitia. The tunica intima which lines the internal surface of the vessel, usually the thinnest constituent layer, consists of a single layer of endothelial cells mounted on a basement membrane (or basal lamina) and a layer of elastic tissue called the internal elastic lamina. The tunica media contains predominantly smooth muscle cells and elastin fibers. Circumferentially arranged smooth muscle cell layers tend to be more highly organized in larger arteries owing to the function that these vessels play in the movement of large volumes of blood. At the outer border of the tunica media is an external elastic lamina, which separates the tunica media from the tunica adventitia and also provides structural support. The last and outermost layer of the blood vessel is the tunica adventitia that is composed of connective tissues and collagen. Both lymphatic and nerve plexus are observed in the adventitia (for review see Burnstock *et al.*, 1984).

Arteries can be classified into two distinct types: elastic and muscular. Elastic or conducting arteries are present near the heart and other organs that are associated with the movement of large volumes of blood. These types of arteries, such as the aorta, superior mesenteric artery, carotid artery are composed of many layers of perforated elastic

membrane and are therefore particularly adapted to accommodate large changes in blood volume such as those typically associated with ejection from the left ventricle of a beating heart. The highly elastic nature of the walls of these blood vessels provides an important function to the smaller blood vessels – they effectively damp the large oscillations in blood flow and provide for a more continuous movement of blood away from the heart. The function of muscular or distributing arteries, such as the large and small mesenteric arteries, renal arteries, cerebral arteries is to ensure the rapid and complete distribution of blood to all organs and tissues. These arteries contain discontinuous elastic fibers within the large layer of smooth muscle cells. In fine distributing arteries, the number of layers of smooth muscle cells decreases. Terminal arterioles, which have only a single layer of smooth muscle cells, give rise to capillaries that are devoid of smooth muscle (for review see Pugsley and Tabrizchi, 2000). Individual arterial and arteriolar smooth muscle cells have lengths of 40-60 μm and diameters near the nucleus of 4 μm; the cells taper toward their ends (Hua and Cragg, 1980).

1.2. Vascular Smooth Muscle Cells

Smooth muscle is a major component in the wall of all blood vessels. These single (uninuclear), non-striated cells have abundant caveolae and numerous cell-to-cell junctions. Cell-to-cell junctions serve two fundamental functions – mechanical coupling, and excitation coupling (Gabella, 1994). Smooth muscle contains both actin and myosin

protein filaments, similar to skeletal muscle, which plays an important role in excitation-contraction coupling (Schoenberg and Haselgrove, 1974).

1.2.1. Membrane properties

As with all cells, vascular smooth muscle cells maintain large ionic gradients across their membranes (for review see Hirst and Edwards, 1989). The unequal distribution of K^+, Cl^-, Na^+ and Ca^{2+} across the arterial and arteriolar smooth muscle cell membranes results in a resting membrane potential of approximately in the range of –55 to –75 mV (Casteels *et al.*, 1977a; Kuriyama and Suzuki, 1978; Bolton *et al.*, 1984) (Table 1). There is a general agreement that resting vascular smooth muscle membranes have a higher permeability to K^+ than they do to Cl^-, Na^+ or Ca^{2+} (Hirst and Van Helden, 1982). The observed values of resting membrane potential of arteries and arterioles are consistent with their membrane being more permeable to K^+ than to other ions (*i.e.* Na^+, Ca^{2+}).

1.2.2. Receptors

Cellular communication can occur via the interaction of endogenous and/or exogenous chemical mediators with the surface of cells. These endogenous mediators interact with specific sites on the cell membrane known as receptors. The cellular responses to receptor activation vary considerably according to the structure and nature of the receptor. There are four types of mechanisms by which receptor activation transmits the information (*i.e.*, signal transduction). These include guanine nucleotide binding protein

Table 1

Ion concentrations inside $[X]_i$, outside $[X]_o$, and equilibrium potential (E_p) in vascular smooth muscle cell (for review see Hirst and Edwards, 1989)

Ions	$[X]_i$, (mM)	$[X]_o$, (mM)	E_p, (mV)
Sodium (Na^+)	10 – 20	~ 150	+ 50
Potassium (K^+)	130 – 160	3 – 5	– 90
Chloride (Cl^-)	40 – 70	~ 140	– 20
Calcium (Ca^{2+})	10^{-6} – 10^{-8} M	~ 2	> + 150

(G-protein) coupled receptor, DNA-coupled receptor, receptor directly coupled to enzymes, and receptor coupled directly to ion channels (Walker *et al.*, 2002).

G-protein coupled receptors are located in the cell membranes and are composed of seven transmembrane helices (I-VII) (for review see Gilman, 1987). In the resting state, the receptor is bound to a G-protein that holds the receptor in an inactive conformation. The G-protein is composed of three subunits (*e.g.*, α, β, γ). In the resting state, the three subunits are bound together, and guanine nucleotide diphosphate (GDP) is tightly bound to the α-subunit of the G-protein (for review see Kaziro *et al.*, 1991). When an agonist interacts and activates a G-protein coupled receptor, a conformational change in the receptor leads to the activation of the G-protein. Activation of G-proteins involves the release of GDP and binding of guanine nucleotide triphosphate (GTP) to its α-subunit and the dissociation of this subunit from the $\beta\gamma$-subunit heterodimer (Kahn and Gilman, 1984). The α and $\beta\gamma$ subunits are then believed to activate a number of effector molecules. The α subunit then hydrolyzes GTP to GDP, which in turn inactivates the α-subunit, allowing it to reassociate with the $\beta\gamma$ complex, rendering the G-protein inactive. Stimulation or inhibition of G-proteins results in activation of other enzyme systems such as adenylyl cyclase or phospholipase C (PLC) (for review see Gilman, 1987). This initiates the sequence of events that constitutes G-protein-coupled receptor transduction (for review see Birnbaumer, 1990). Examples of G-protein receptors include the β_2- and α_{1A}-adrenoceptors (Shaw and McGrath, 1996).

DNA-coupled receptors are intracellular receptors mainly composed of nuclear proteins. Therefore, agonists have to enter the cell membranes in order to interact with this type of receptor. Agonist binding to the receptor causes a conformational change in the receptor molecule, which results in dissociation of the receptor from the inhibitory molecule. One example of a DNA-coupled receptor is illustrated by a steroid molecule that enters a cell and binds to a cytoplasmic receptor and dissociate the inhibitory molecule, heat shock protein 90, bound to it and thus activate the receptor (Howard and Distelhorst, 1988).

Enzymes that are part of the cell membrane can have sites for agonists to interact. An example of the latter is the tyrosine kinase receptor, which is involved in the regulation of growth, cell differentiation, and responses to metabolic stimuli (Shaw and McGrath, 1996). This receptor consists of an external moiety which contains the agonist recognition site and a cytosolic region which incorporates a tyrosine kinase enzyme. Agonists such as insulin, epidermal growth factor, and platelet-derived growth factor bind to the receptor and induce a conformational change and subsequently activate the tyrosine kinase enzyme (Baron *et al.*, 1990).

Receptors can also be directly linked to ion channels or alternatively be part of an ion channel. This type of receptor is composed of subunits, each of which has four transmembrane domains. Each receptor-coupled channel possesses, as part of their structure, an extracellular ligand-binding site. When an endogenous ligand or certain drugs bind to the receptor, it initiates a conformational change in other parts of the receptor-operated channel entity that leads to the opening of a central ion-selective pore.

Thus, the passage of ions occurs only when the pore is open and the receptor activated. Examples of receptor operated ion channels include K channels, Ca channels, Na channels, and nicotinic receptor (Changeux *et al.*, 1987).

Therefore, the release of neurotransmitters from nerve endings, circulating humoral factors, and/or locally released mediators can bind and activate specific receptors located on the smooth muscle or endothelial cells. Some examples of receptors associated with smooth muscle cells include adrenoceptors, 5-hydroxytryptamine receptors, muscarinic receptors, histamine receptors, angiotensin receptors, vasopressin receptors, and prostanoid receptors (Shaw and McGrath, 1996). Furthermore, endothelial cells also contain several receptors such as angiotensin receptors, adenosine receptors, acetylcholine receptors, purinoceptors, and neuropeptide receptors (for review see Tabrizchi, 2003). Each receptor can be further subdivided depending on the rank order of agonist potency, affinity of an antagonist, pharmacological profile, and/or signaling pathway (Shaw and McGrath, 1996). As a historical example, adrenoceptors were classified as either α or β each being associated with excitation (vasoconstriction) or inhibition (vasorelaxation), respectively (Ahlquist, 1948). α-Adrenoceptors are further subdivided into α_1 and α_2, whereas β-adrenoceptors are subdivided into β_1, β_2, β_3, and β_4 receptors (for review see Minneman, 1988).

Further sub-classification of subtypes of α_1 and α_2 adrenoceptor have been made. These subtypes have been found to be present in vascular smooth muscle cells. At present,

based on pharmacological and biochemical evidence, there appear to be at least three different subtypes of the α_1-adrenoceptor that contribute to contractile responses in smooth muscle. These are α_{1A}, α_{1B}, and α_{1D} adrenoceptors (for review see Minneman, 1988). According to the convention, capital letter subscripts (*e.g.*, α_{1A}, α_{1B}, α_{1D}) are used to denote receptors identified from functional/binding studies whereas small letter subscripts (*e.g.*, α_{1a}, α_{1b}, α_{1d}) are used to denote cloned receptors. It has been suggested that in rat mesenteric artery, mixtures of α_{1A} and α_{1B} adrenoceptors are present (Han *et al.*, 1990). Kong *et al.* (1994) have suggested that in the rat mesenteric vasculature the α_{1A} adrenoceptor subtype is predominant and is highly sensitive to antagonism by 5-methylurapidil, an α_{1A} adrenoceptor-selective antagonist. Williams and Clarke (1995) supported the latter view that vasoconstrictor responses via the activation of α_1-adrenoceptors in rat mesenteric blood vessels are mediated as a result of activation of α_{1A}-adrenoceptor subtypes. In contrast, Hussain and Marshall (2000) have suggested that the contractions of the rat mesenteric artery are mediated via the α_{1D} and possibly α_{1B} adrenoceptor. A study using human cloned α_1-adrenoceptors indicated that cirazoline, an α_1-adrenoceptor agonist, has a higher affinity for the α_{1a} subtype than α_{1b} and α_{1d} subtypes (Horie *et al.*, 1995). Taken together, the current views suggest that cirazoline-induced vasoconstriction is mediated principally via the activation of α_{1A}-adrenoceptor subtype in mesenteric blood vessels (Kong *et al.*, 1994; Horie *et al.*, 1995; Williams and Clarke, 1995).

1.2.3. Membrane ion channels in vascular smooth muscle cells

The vascular smooth muscle cell membrane is believed to be spanned by a number of different ion channels, which act in a co-ordinated fashion to regulate ion influx or efflux, thereby contributing to the control of the intracellular ion concentrations, and hence tension development and vascular constriction and relaxation (Aaronson and Smirnov, 1996).

1.2.3.1. K channels: Most vascular smooth muscle have a resting membrane potential close to the equilibrium K^+ potential, which means that the K^+ permeability is mainly responsible for setting the resting potential (for review see Nelson and Quayle, 1995). Four major classes of K channels/currents have been distinguished in vascular smooth muscle cells, namely voltage-dependent K channels (K_v), Ca^{2+}- and voltage-sensitive K channels (K_{Ca}), ATP-sensitive K channels (K_{ATP}) and inward rectifier K channels (K_{IR}) (Aaronson and Smirnov, 1996). K_v channels open when the membrane potential of the cell is depolarized (Beech and Bolton, 1989). The voltage at which this current is 50% activated is –10 mV and the range of inactivation voltage is –25 to –45 mV (Robertson and Nelson, 1994). 4-Aminopyridine is perhaps the most selective inhibitor of K_v channels in vascular smooth muscle (Robertson and Nelson, 1994). K_{Ca} channel opening was observed at membrane potentials between –60 and –50 mV when intracellular Ca^{2+} was between 10^{-8} and 10^{-7} M, and open probability increased steeply with membrane depolarization (Benham *et al.*, 1986; Gelband *et al.*, 1989). It is known that tetraethylammonium is a relatively selective blocker of K_{Ca} channel (Benham and Bolton,

1986). K_{ATP} channels are inhibited by intracellular ATP and are considered to be a link between cellular metabolism and electrical events (Ashcroft and Ashcroft, 1990). The K_{ATP} channel in vascular smooth muscle is the main target for the vasodilators such as pinacidil and cromakalim (Weston, 1989). Glibenclamide and tolbutamide are proposed to be selective blockers of K_{ATP} channels (Standen *et al.*, 1989). K_{IR} channel in vascular smooth muscle conducts inward current at membrane potential negative to the K equilibrium potential and smaller outward current at membrane potential positive to K equilibrium potential (Edwards *et al.*, 1988). The K_{IR} channel is very sensitive to inhibition by extracellular Ba^{2+} (Quayle *et al.*, 1993).

1.2.3.2. Ca channels: Two types of voltage-operated Ca channels, namely L-type and T-type have been described in a variety of vascular smooth muscle cells (Benham *et al.*, 1987; Aaronson *et al.*, 1988). The L-type Ca channels are characterized by a relatively slow inactivation, a greater permeability to Ba^{2+} than to Ca^{2+}, a high sensitivity to dihydropyridines, a unitary conductance of 15-28 pS in 80-110 mM Ba^{2+}, and a voltage range of activation of –60 to –40 mV (for review see Bean, 1989). T-type Ca channels are characterized by rapid inactivation, a small (6-8 pS) unitary conductance, and voltage ranges of activation and inactivation, which are more negative (–70 to –50 mV) than those of the L-type channels. In addition to the two types of voltage-operated channel, a third type of Ca channel, the so called receptor-operated Ca channel, has been identified in smooth muscle cells. The latter channel has been suggested to be responsible for

causing both direct Ca^{2+} influx and depolarization leading to indirect Ca^{2+} influx via voltage-gated Ca channels (Bolton, 1979).

1.2.3.3. Ca^{2+}-dependent Cl channels: An increase in intracellular Ca^{2+} concentration is an essential prerequisite for activation of Ca^{2+}-dependent Cl currents (for review see Large and Wang, 1996). It was reported that in rat lacrimal gland cells, the threshold concentration of intracellular Ca^{2+} for activation of Ca^{2+}-dependent Cl current was 100 nM to 1μM (Marty *et al.*, 1984). Single Cl channel activity was recorded (in response to caffeine) from myocytes of human mesenteric artery, and their single channel conductance was estimated to be 2.8 pS (Klöckner, 1993). The half-duration of spontaneous transient inward currents (STICs), which are believed to be a Ca^{2+}-dependent Cl current, in guinea-pig mesenteric vein was reported to be 390 ms at –70 mV (Van Helden, 1991). However, Hogg *et al.* (1993) has reported that the time constant of decay of STICs was 86 ms at –50 mV and that this time constant increases with depolarization in rabbit portal vein. The opening of Ca^{2+}-dependent Cl channels is believed to be responsible for membrane depolarization and thus further activates voltage-operated Ca channels (for review see Large and Wang, 1996). This has been further discussed in detail in section 1.5.

1.3. Excitation-Contraction Coupling of Vascular Smooth Muscle

Many aspect of excitation-contraction coupling in smooth muscle have been studied. It is recognized that smooth muscle cell contain actin and myosin filaments (regulatory subunits), and that these filaments are responsible for the contractile process (for review see Horowitz *et al.*, 1996). The contractile process is activated by Ca^{2+}, and it is an energy-dependent process requiring the degradation of adenosine 5-triphosphate (ATP). The process of excitation-contraction in smooth muscle can be initiated by interaction of endogenous mediators with membrane-bound receptors located on the cell surface (Sneddon and Burnstock, 1984). In addition, processes such as shear stress can also initiate contraction or relaxation of vascular smooth muscle cells (for review see Bevan, 1997).

Vascular smooth muscle contraction is mediated, in part, by electromechanical and/or pharmacomechanical coupling (Somlyo and Somlyo, 1968). Electromechanical coupling is associated with membrane depolarization, which ultimately leads to contraction, whereas pharmacomechanical coupling is believed to occur independent of changes in membrane potential (Bolton, 1976; for review see Bolton and Large, 1986). Sympathetic nerve stimulation releases various neurotransmitters, which can initiate contraction by three different mechanisms. Neurally released noradrenaline is believed to activate the α-adrenoceptors, which are coupled to G-proteins, and subsequently activate the enzyme PLC. PLC subsequently hydrolyses the membrane bound inositol phosphates which

eventually leads to the formation of inositol triphosphate that releases Ca^{2+} by acting on the receptor on sarcoplasmic reticulum (Van Helden, 1991). This mechanism does not rely on a change in the membrane potential of the vascular smooth muscle cells. Alternatively, sympathetic nerve stimulation may activate a pathway which does not involve α-adrenoceptors (Hirst and Lew, 1987). Sympathetic nerves releases a cotransmitter, probably ATP (Sneddon and Burnstock, 1984). ATP then activates a set of purinoceptors, which are linked to cation-selective channels, so producing a membrane depolarization. This depolarization triggers the opening of the voltage-dependent Ca channels present in the membranes of vascular smooth muscle cells (for review see Hirst and Edwards, 1989). Thirdly, sympathetic nerves may release vasoactive peptides (*e.g.*, neuropeptide Y), which produce persistent vasoconstriction of vascular smooth muscle cells (for review see Wharton and Gulbenkian, 1987).

1.3.1. Structure of sympathetic axons

The nerves supplying blood vessels are unmyelinated and form a plexus of branching varicose axons. The plexus in most blood vessels is situated in the outermost layer of the vessel wall, the adventitia, outside of the layer of contractile smooth muscle cells (the media) (Appenzeller, 1964; Burnstock and Costa, 1975). Hence, in vessels with several layers of smooth muscle cells only the muscle cells at the medio-adventitial border can be in direct contact with the axons. There are some exceptions where axons have been reported to ramify the outer two or three layers of smooth muscle cells of some arteries and veins. The axon plexus around blood vessels is predominantly composed of

sympathetic postganglionic axons. Most sympathetic axons are grouped in bundles (Burnstock and Costa, 1975). Each axon bundle is surrounded by a layer of basal lamina, which consists of types IV and V collagens, laminin, entactin, and heparan sulfate (for review see Bunge *et al.*, 1986). Sympathetic varicosities contain numerous vesicles, one or two mitochondria, and some smooth endoplasmic reticulum. Vesicles within the varicosities contain neurotransmitter and consequently are believed to be sites for neurotransmitter release (for review see Geffen and Livett, 1971). Three types of vesicles are found in sympathetic varicosities, many granular and agranular small vesicles (30-60 nm in diameter) and a few dense-cored vesicles (60-150 nm in diameter) (for review see Geffen and Livett, 1971). There is substantial evidence to show that noradrenaline, ATP, and neuropeptide Y are cotransmitters in sympathetic nerves (for reviews see Burnstock, 1976, 1990). Numerous studies have also demonstrated that cotransmission of noradrenaline and ATP also occurs in many different blood vessels in a variety of species (for review see Burnstock, 1990). Following release from sympathetic nerve terminals, noradrenaline and ATP elicit vasoconstriction of the smooth muscle via an action at α-adrenoceptors and purinoceptors, respectively (Hirst and Jobling, 1989). The evidence in the literature supports the view of the existence of neuromuscular junction between the varicosities and vascular smooth muscle cells (Luff *et al.*, 1987).

1.3.2. Neuromuscular transmission involving the activation of α-adrenoceptors

In pulmonary arteries and veins, sympathetic nerve stimulation produces membrane depolarizations that last for many seconds (Suzuki, 1981, 1983). When triggered by only

a few stimuli, the membrane potential changes occur after a long latency (1-2 sec) and are of small amplitude (2-3 mV). Repetitive nerve stimulation causes larger more sustained depolarizations but the latency to onset remains unchanged (Suzuki, 1981). In both types of vessels the potential change becomes complex, often a transient component occurs at the start of the response, and it is followed by the sustained component. Both of the components are blocked by α-adrenoceptor antagonist, phentolamine (Suzuki, 1981). Suzuki (1981) has demonstrated that phentolamine suppressed the depolarizing action of noradrenaline in mesenteric vein from guinea-pigs. The process of neuromuscular transmission has been analyzed in detail in mesenteric veins (Van Helden, 1988). In these vessels an ongoing discharge of spontaneous transient depolarizations are detected. Spontaneous transient depolarizations increase in frequency shortly after a period of sympathetic nerve stimulation, thus summing to give the slow excitatory junction potential. However, spontaneous transient depolarizations do not result from the spontaneous release of quanta of transmitter from sympathetic nerve terminals; rather they appear to result from the irregular pulsatile release of Ca^{2+} from internal stores (Van Helden, 1991). After release from the internal stores, Ca^{2+} activates a set of Cl selective channels present in the membranes of these cells (Van Helden, 1991). This results in an increase in Cl conductance which leads to depolarization. Taken together, activation of α-adrenoceptors seems to release Ca^{2+} from intracellular stores which leads to contraction of vascular smooth muscle cells via (a) activating a contractile system, and (b) activating Cl channels, which results in depolarization and further increases Ca^{2+} influx.

1.3.3. Neuromuscular transmission involving the activation of purinoceptors

In all mammalian arteries sympathetic nerve stimulation triggers a sequence of membrane potential changes. A single sympathetic nerve stimulus initiates a membrane depolarization, termed an excitatory junction potential (e.j.p.) (Bell, 1969; Hirst, 1977). E.j.ps by themselves do not initiate contraction, rather several e.j.ps must sum up to give a depolarization of some 15-20 mV before threshold is reached for voltage-dependent Ca channels to be activated (Hirst, 1977; for review see Hirst and Edwards, 1989). When this occurs a contraction ensues. If the stimulus is applied close to the recording point, e.j.ps are seen to have short latencies, 10-20 ms. Typically such e.j.ps have a total duration of about 1 s. For example, an e.j.p. recorded from a submucosal arteriole has a time to peak potential of 100 ms and then decays with a time constant of 500 ms (Hirst and Neild, 1978). The time course of the excitatory junctional current, underlying an e.j.p. is brief. When measured directly from submucosal arterioles using a single electrode voltage-clamp technique the peak current occurred after 10 ms and with a total duration of about 200 ms (Finkel *et al.*, 1984). After the peak, the decay of current could be described by a single exponential with a time constant of 50 ms. These observations indicate that the long time course of an e.j.p. reflects the long membrane time constant of arteriolar smooth muscle cell. Clearly, since one frequently has to sum together successive e.j.ps to reach threshold for the initiation of voltage-dependent Ca^{2+} entry, any agent which makes the membranes of arteries more depolarized will make it more unlikely that this threshold is reached. The amplitudes of excitatory junctional currents depend upon the membrane potential at which they are recorded. They increase linearly with hyperpolarization and decrease linearly with depolarization (Finkel *et al.*, 1984).

Their reversal potential, the membrane potential where inward current just equals outward current, is about 0 mV (Finkel *et al.*, 1984).

Most muscle cells in an arteriole are directly innervated and hence may be directly activated by action of transmitters upon them. However in larger vessels most cells are not directly innervated with the innervation being restricted to the medio-adventitial border (Burnstock and Costa, 1975). For the membrane potentials of all cells to be changed during sympathetic neuromuscular transmission, some of the junctional current generated on the outer most layer of muscle cells must flow to the deeper non-innervated cells. This occurs because individual vascular smooth muscle cells are electrically connected via gap junctions to their neighboring cells (Brink, 1998). Such electrical coupling has been demonstrated in many preparations (*e.g.*, coronary artery, mesenteric artery) (Beny and Pacicca, 1994; Yamamoto *et al.*, 1999). The analyses show that many arterial preparations behave as simple linear cables with electrical length constants in the range of 0.3 – 1.5 mm (Bolton, 1974; Casteels *et al.*, 1977a). Electrical coupling between arteriolar cells has been demonstrated more directly using two independent intracellular electrodes, one to pass current and the other to record differences in the potential (Hirst and Neild, 1980). It should be noted that as individual cells have circumferential organization, the two electrodes could not have been in the same cell. When a small current was passed through one electrode; it caused a membrane potential change at the second electrode. Again some of the injected current must have flowed to the recording point via gap junctions between cells (Smeda and King, 1998; Brink, 1998).

1.3.4. Changes in membrane potential

Vascular smooth muscle cell contraction may or may not be associated with membrane depolarization. There are a number of observations reported where there were significant depolarizations associated with contraction after the application of an α-adrenoceptor agonist (Suzuki, 1981; Mulvany *et al.*, 1982; Haeusler and De Peyer, 1989). For example, the membrane potential of smooth muscle cells from guinea-pig mesenteric vein became significantly depolarized by the application of noradrenaline (from –64.2 ± 2.3 to –50.2 ± 2.5 mV) (Suzuki, 1981). In addition, Mulvany *et al.* (1982) have also demonstrated that membrane potentials (–59.2 ± 0.4 mV) from rat small mesenteric arteries (second or third order vessels) were depolarized (–33.7 ± 1.0 mV) following the addition of noradrenaline (10 μM). Thus, it has been suggested that activation of α-adrenoceptor in vascular smooth muscle produces depolarization of a magnitude close to ~14 mV.

Interestingly, several other studies have shown that smooth muscle contraction could be mediated without depolarization (Su *et al.*, 1964; Somlyo and Somlyo, 1968; Matthews *et al.*, 1984; Frieden and Beny, 1995). For example, Su *et al.* (1964) using glass microelectrodes have demonstrated that there was no change in membrane potential during contraction by sympathetic nerve stimulation or noradrenaline application in rabbit pulmonary artery. Somlyo and Somlyo (1968) using the sucrose-gap method, have also reported that the contraction produced by noradrenaline in rabbit pulmonary artery is not associated with depolarization. Evidence suggest that noradrenaline can cause

contraction of pulmonary arteries without depolarization (Casteels *et al.*, 1977b). Therefore, it seems that in some types of arteries, certain agonist-mediated contractions of vascular smooth muscle can occur independent of changes in membrane potential. This process is known as pharmacomechanical coupling. Therefore, based on the evidence it can be concluded that electromechanical and pharmacomechanical coupling may act synergistically or they may be complementary to each other in promoting vasoconstriction.

1.3.5. Cell signaling pathways

A vast array of agonists regulate smooth muscle function by interacting with specific receptors located on the cell membrane. The resultant effect is the activation of intracellular signaling pathways and the formation of a number of key second messengers. In addition, other endogenous molecules such as nitric oxide can alter smooth muscle cell tone by initiating cell signaling directly through non-receptor-mediated mechanisms. In Figure 1 the current and recognized signaling pathways in smooth muscle cells have been summarized.

In the late 70s and early 80s Berridge and coworkers identified the hydrolysis of inositol-containing phospholipids as a universal signaling pathway activated in response to Ca^{2+} mobilizing agonists (Fain and Berridge, 1979; Irvine *et al.*, 1982; for review see Berridge and Irvine, 1989). Following receptor activation, membrane-bound inositol lipid, phosphatidylinositol 4,5-biphosphate is hydrolysed by a phosphoinositide-specific PLC to generate two second messengers, inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol

Figure 1: Simplified schematic diagram summarizing some of the receptors and various transduction mechanisms which regulate vascular smooth muscle tone.

$[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; AC, adenylyl cyclase; ATP, adenosine 5-triphosphate; C, contraction; CaATPase, calcium ATPase enzyme; CaM, calmodulin; cAMP, cyclic adenosine 3,5-monophosphate; cGMP, cyclic guanosine 3,5-monophosphate; DAG, diacylglycerol; GC, guanylyl cyclase; G_p, G-protein; G_s, stimulatory G-protein; GTP, guanine nucleotide triphosphate; $InsP_2$, phosphatidylinositol 4,5-biphosphate; IP_3, inositol 1,4,5-triphosphate; K_{ATP}, ATP-sensitive K channels; MLCK–, inactivated myosin light chain kinase; MLCK+, activated myosin light chain kinase; NO, nitric oxide; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PLC, phospholipase C; R, receptor; ROC, receptor-operated Ca channel; SR, sarcoplasmic reticulum; VOC, voltage-operated Ca channel.

Solid lines represent pathways mediating contraction and *dotted* lines represent pathways leading to relaxation.

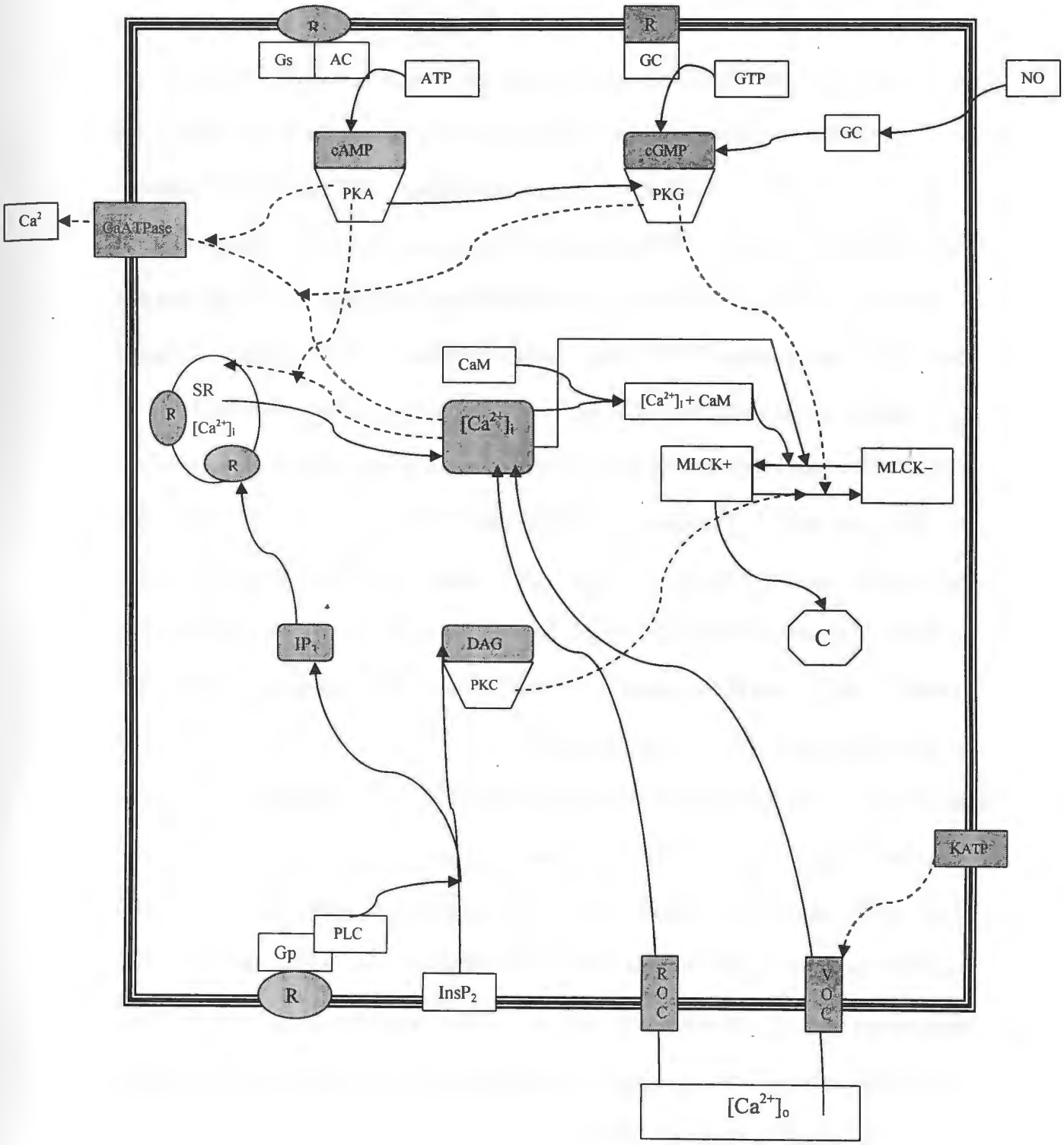

R
Gs
AC
ATP
cAMP
PKA
R
GC
GTP
NO
GC
cGMP
PKG
Ca2
CaATPase
CaM
SR
R
[Ca2+]i
R
[Ca2+]i
[Ca2+]i + CaM
MLCK+
MLCK−
C
IP3
DAG
PKC
KATP
PLC
Gp
R
InsP2
ROC
VOC
[Ca2+]o

Figure 1

(DAG). These messengers are generated by membrane transduction processes where the agonist-receptor complex is transduced via the activation of a heterotrimeric G-protein (for review see Gilman, 1987). IP_3 stimulates the release of Ca^{2+} from intracellular Ca^{2+} stores through interaction with specific IP_3 receptors located on the sarcoplasmic reticulum (Berridge, 1993). After release in response to IP_3, Ca^{2+} binds to calmodulin (CaM) inducing a conformational change, which allows the Ca^{2+}-CaM complex to bind to and activate myosin light chain kinase (MLCK) (Dabrowska *et al.*, 1978). The active kinase (Ca^{2+}-CaM-MLCK) catalyzes the phosphorylation of myosin at Ser-19 of the two 20-kDa light chain subunits of myosin. This triggers cross-bridge cycling and the development of force or shortening of the muscle (for reviews see Walsh, 1994; Arner and Malmqvist, 1998). Relaxation is achieved by extrusion of Ca^{2+} from the cell or uptake into the sarcoplasmic reticulum, where upon Ca^{2+} dissociates from CaM and the inactive apoenzyme of MLCK is regenerated. Myosin is dephosphorylated by myosin light chain phosphatase and the muscle relaxes (for review see Walsh, 1994). In agonist-stimulated (angiotensin II, vasopressin) smooth muscle cells it was observed that IP_3 formation is transient while DAG formation is sustained (Griendling *et al.*, 1986; Sunako *et al.*, 1990). It has been established that phosphatidylcholine (PC) hydrolysis provides the majority of DAG formed (Lassegue *et al.*, 1991; Plevin and Wakelam, 1992). DAG may be derived from PC by two main routes, (1) a phospholipase D catalysed pathway, which generates phosphatidic acid which can then be converted to DAG by phosphatidic acid phosphohydrolase, and (2) a route that directly generates DAG via the putative PC-PLC enzyme, which is distinct from phosphoinositide-specific PLC (Lassegue *et al.*, 1991, 1993). The main physiological target of DAG is a family of phosphatidyl serine-

dependent serine/threonine-directed kinases collectively called protein kinase C (PKC) (Lassegue *et al.*, 1993). Thus DAG is believed to play a role in contraction by activating PKC enzyme which initiates signal transduction mechanisms.

Sensitization of contractile elements in vascular smooth muscle cells can result in an enhanced contraction at a given intracellular Ca^{2+} concentration. This can be achieved through an inhibition of myosin light chain phosphatase (Somlyo and Himpens, 1989) and involves the activation of a small molecular weight G-protein (Kitazawa *et al.*, 1991; Hirata *et al.*, 1992). These monomeric proteins are derived from Ras-related low molecular mass GTPases of the Rho subfamily (Pfitzer and Arner, 1998). PKC appears to play a role in the G-protein-mediated sensitization of smooth muscle contraction (Sato *et al.*, 1994). It has been suggested that PKC is involved in activation of G-protein by exchanging GTP for GDP (Antonietta De Matteis *et al.*, 1993). This activated small G-protein might be involved in phosphorylating myosin light chain phosphatase and thus causing sensitization of the contractile element. Thus it can be summarized that although Ca^{2+} is the major physiological regulator of smooth muscle contraction, the Ca^{2+} sensitivity of the regulatory/contractile apparatus can be modified by other mechanisms.

1.4. Vascular Smooth Muscle Relaxation

It is now generally accepted that cyclic adenosine 3,5-monophosphate (cAMP) and cyclic guanosine 3,5-monophosphate (cGMP) are intimately involved in initiating relaxation in vascular smooth muscle (for review see McDaniel *et al.*, 1994). The intracellular levels

of cAMP and cGMP are regulated by the enzymes, adenylyl cyclase and guanylyl cyclase, respectively, as well as by the cyclic nucleotide phosphodiesterases, a family of isoenzymes which degrade these cyclic nucleotides (for review see Polson and Strada, 1996). A major role for the adenylyl cyclase/cAMP signaling system in relaxation is implicated by agonists such as adrenaline, prostacyclin, adenosine, and vasoactive intestinal peptide, which raise intracellular cAMP levels and evoke relaxation of vascular smooth muscle (Holzmann *et al.*, 1980; Ganz *et al.*, 1986; Hessen and De Mey, 1990). The effects of cAMP are thought to be mediated through the activation of a family of cAMP-dependent protein kinases designated as PKA (Parks *et al.*, 1987). The proposed mechanisms of cAMP-induced relaxation include (i) reduction in myoplasmic Ca^{2+} by a decrease in Ca^{2+} influx (Chen and Rembold, 1992), (ii) increase in Ca^{2+} efflux (Hoshi *et al.*, 1988), (iii) sequestration of Ca^{2+} into internal stores (Tawada *et al.*, 1988), (iv) activation of PKA, which could alter the Ca^{2+} sensitivity of phosphorylation by phosphorylation of either MLCK (Adelstein *et al.*, 1978, 1981) or myosin light chain phosphatase, and/or (v) activation of cGMP-dependent protein kinase (PKG) which could mediate the relaxation (Lincoln, 1989).

A number of studies have demonstrated that cAMP may relax smooth muscle by mechanisms that decrease the intracellular Ca^{2+} (Abe and Karaki, 1989; Kimura *et al.*, 1982). In porcine carotid artery, relaxations induced by low concentrations of forskolin were associated with significant decreases in myoplasmic Ca^{2+} without changes in the relationship between Ca^{2+} and myosin phosphorylation (McDaniel *et al.*, 1991). De Lanerolle *et al.* (1984) reported that MLCK could be phosphorylated by PKA.

Phosphorylation of MLCK would decrease the affinity of MLCK for the Ca^{2+}-CaM complex, resulting in lower levels of myosin kinase activity for a given level of Ca^{2+} (*i.e.*, a decrease in the Ca^{2+} sensitivity of phosphorylation). The net effect is to reduce the Ca^{2+} sensitivity of the contractile apparatus and favor relaxation (Adelstein *et al.*, 1978). Thus cAMP can mediate vascular smooth muscle relaxation by either reducing intracellular Ca^{2+} concentration, decreasing Ca^{2+} sensitivity of the contractile elements, or a combination of the two.

1.5. Chloride Channels

In skeletal muscle Cl^- is distributed across the cell membranes according to the Donnan equilibrium (Hodgkin and Horowicz, 1959). In contrast, it seems that in the smooth muscle cells Donnan distribution of Cl^- does not exist (Casteels, 1965; Aickin and Brading, 1982). It would appear that the intracellular Cl^- concentration is considerably higher in smooth muscle (34-51 mM) compared to skeletal muscle (2.4 mM) than would fit the theory of a passive distribution (Casteels and Kuriyama, 1966). In Table 2 the estimated values of intracellular Cl^- concentration in various smooth muscle cells have been given. It has been suggested that the high intracellular Cl^- concentration might be playing an essential role in smooth muscle cell function and, needless to say, this has been of considerable interest for the past four decades. Over this time span many suggestions have been made as to the role of Cl^- in function of smooth muscle. Casteels (1971) has suggested that the high intracellular Cl^- results from the movement of Cl^- into

Table 2

Estimates for intracellular Cl^- concentration in smooth muscle cell

	N	$[Cl]_i$ (mM)	E_m (mV)	E_{Cl} (mV)	Reference
Guinea-pig taenia coli	30	61[a]	–57 ± 0.3	–21	Casteels and Kuriyama (1966)
Guinea-pig vas deferens	79	41.2 ± 6.7[b]	–67.6 ± 7.8		Aickin and Brading (1982)
Guinea-pig ureter	13	51.1 ± 4.0[b]	–48.7 ± 5.4	–18.6	Aickin and Vermüe (1983)
Rabbit aorta	8	32.6[c]	–60.1 ± 2.9		Gerstheimer *et al.* (1987)
Rat femoral artery	4	43.8 ± 3.5[d]	–61.5 ± 1.3		Davis (1992)
Rat saphenous artery	18	31.0 ± 0.5[d]	–63.7 ± 2.4	–42.6	Davis *et al.* (1997)

All data are given as mean ± SEM

[a]Intracellular Cl^- ion concentration is given in mmoles $liter^{-1}$ cell water

[b]Using double barreled ion-selective microelectrode, and the value is given as activity (estimated Cl_i expressed as mmoles $liter^{-1}$ cell water was assumed to equal $[Cl]_i$, and this value was multiplied by the calculated activity coefficient (0.764) of the normal Krebs solution)

[c]By uptake studies using ^{36}Cl

[d]Using double barreled ion-selective microelectrode, and the value is given as concentration

the cells against the electrochemical gradient. Thus an active transport system was thought to be involved in accumulating Cl^- inside the cells (Casteels, 1971; Aickin and Brading, 1982). The three mechanisms by which Cl^- is thought to be accumulated inside smooth muscle cells are (a) chloride-bicarbonate (Cl^-/ HCO_3^-) exchange, (b) sodium-potassium-chloride ($Na^+ + K^+ + Cl^-$) cotransport, and (c) pump III (for review see O'Donnell and Owen, 1994).

Chloride-bicarbonate exchange: The Cl^-/ HCO_3^- exchanger is reversible and hence in Cl^--free medium, HCO_3^- influx and Cl^- efflux was observed and this caused pH to increase from 7.1 to pH 7.8 (Aickin and Brading, 1984). Upon readmission of Cl^- into the extracellular compartment, pH changed from 7.8 to pH 7.1. According to Vaughan-Jones (1979), HCO_3^- is not at equilibrium due to the action of Na^+/H^+ exchange, which holds the intracellular pH at a more alkaline level than it would be if H^+ were in equilibrium with membrane potential. This makes the intracellular HCO_3^- concentration higher and the outward HCO_3^- gradient provides the driving force for Cl^- accumulation (for review see Chipperfield and Harper, 2000). Thus, this exchanger is believed to play an important role in regulation of intracellular Cl^- concentration.

Sodium-potassium-chloride cotransport: $Na^+ + K^+ + Cl^-$ cotransport is a symport system present in vascular smooth muscle cells. It has an apparent stoichiometry of 1:1:2, respectively (Owen, 1984). Although the cotransporter is bidirectional, the sum of the electrochemical gradients for the three transported ion species determines the direction of

net flux of ions but it mediates a modest net uptake of the transported ions (for review see Chipperfield, 1986). It was proposed by Davis *et al.* (1993) that the smooth muscle cells of hypertensive rats have an elevated ($Na^+ + K^+ + Cl^-$) cotransport activity that elevates the intracellular concentration of Cl^-. This elevation in the intracellular Cl^- concentration in turn was suggested to act as a depolarizing influence in the cells that contributes to the elevated resting tension in these cells (Davis *et al.*, 1993). Therefore, alteration in intracellular concentration of Cl^- has been implicated as a factor influencing smooth muscle function in state of hypertension.

Pump III: It has been demonstrated in conditions where neither ($Na^+ + K^+ + Cl^-$) cotransport or Cl^-/HCO_3^- exchange could function, the intracellular Cl^- concentration was still above equilibrium. This has led to the suggestion that there must be a third process capable of driving the Cl^- inwards. Its existence was demonstrated by first blocking ($Na^+ + K^+ + Cl^-$) cotransport and Cl^-/HCO_3^- exchange and then reducing the outward leak with a Cl channel blocker, with the result that intracellular Cl^- increased due to action of pump III (Chipperfield *et al.*, 1993). Based on the current published evidence in the literature it can be suggested that these three transport systems together are responsible for the accumulation of Cl^- inside the cells.

1.5.1. Functions of intracellular Cl^-

1.5.1.1. Membrane potential

The membrane potential (E_m) is given by Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949):

$E_m = RT/F \log_e (P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i) / (P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o)$

E_m is given by the weighted mean of E_K, E_{Na}, and E_{Cl}, weighted according to the relative conductances of the ions. According to the Nernst equation

$E_{Cl} = RT/F \log [Cl]_i/[Cl]_o = 61.5 \log [Cl]_i/[Cl]_o$ at 37°C

According to this equation it is evident that even if intracellular Cl^- concentration is at equilibrium, the E_m will be greatly depolarized from its resting value of –70 mV. Thus it has been suggested that if intracellular Cl^- concentration is at or above equilibrium it will exert a depolarizing influence on the membrane potential (for review see Chipperfield and Harper, 2000). Also the Cl^- conductance reflects the degree of Cl channel opening and it is well established that the opening of Cl channels produces depolarization of the E_m (for review see Large and Wang, 1996). Davis *et al.* (1997) has suggested that the activation of Cl^- accumulation serves two purposes, which include (a) prevention of a fall in intracellular Cl^- concentration which would have attenuated the depolarization, and (b) potentiation of the depolarization secondary to opening of Cl channels. Thus E_m must depend on the activity of the Cl^--accumulating systems and there is evidence that their activity can be influenced by agonists which open Cl channels (Davis *et al.*, 1997).

1.5.1.2. Intracellular pH

Smooth muscle cells maintain an intracellular pH which is about one unit more alkaline than it would be if H^+ were distributed based on E_m. This might be attributed to the Cl^-/ HCO_3^- exchange process where HCO_3^- accumulation derives the equilibrium towards H_2CO_3 and thus neutralizes H^+ (*i.e.*, $H^+ + HCO_3^- \leftrightarrow H_2CO_3$) (Aickin, 1984; for review see Chipperfield and Harper, 2000).

1.5.2. Role of Cl^-

There is both physiological and pharmacological evidence in support of the view that Ca^{2+}-dependent Cl currents play a pivotal role in influencing changes in membrane potential in vascular smooth muscle cells (for reviews see Large and Wang, 1996; Kitamura and Yamazaki, 2001). There is now ample evidence to suggest that agonist-induced stimulation of α-adrenoceptors causes a release of Ca^{2+} from intracellular stores, probably the sarcoplasmic reticulum (Byrne and Large, 1988; Klöckner, 1993). Consequently, the rise in intracellular Ca^{2+} leads to the opening of Ca^{2+}-dependent Cl channels (Amédéé *et al.*, 1990; Baron *et al.*, 1991). The resulting change in Cl conductance produces membrane depolarization, which leads to the opening of voltage-dependent Ca channels (Van Helden, 1988). Opening of voltage-dependent Ca channels leads to Ca^{2+} influx, which is a key factor in the process involving contractions in blood vessels (Criddle *et al.*, 1996; Lum Min *et al.*, 1999).

1.5.3. Cl channel antagonists

Ca^{2+}-activated Cl channels are believed to be widely distributed in vascular smooth muscle cells and are involved in important physiological processes such as agonist-induced depolarization (for review see Large and Wang, 1996). A number of studies have revealed that selective antagonism of Ca^{2+}-dependent Cl channels results in inhibition of adrenoceptor-mediated contractions in functioning blood vessels (Baron *et al.*, 1991; Criddle *et al.*, 1996, 1997). To assess the physiological role of Ca^{2+}-dependent Cl channels in vascular smooth muscle cells, Criddle *et al.* (1996) examined the effects of niflumic acid on both K^+- and agonist-induced contractions. They showed that niflumic acid (1-30 μM) selectively inhibited the agonist-induced contraction without changing the high K^+-induced contraction in both the rat aorta and rat mesenteric artery (Criddle *et al.*, 1996, 1997). Evidence in the literature appears to indicate that niflumic acid is capable of selectively inhibiting Ca^{2+}-dependent Cl channels by blocking the open state of Cl channels (Hogg *et al.*, 1994). In addition, niflumic acid has been found to antagonize α_1-adrenoceptor-mediated vasoconstriction in isolated perfused mesenteric blood vessels (He and Tabrizchi, 1997; Remillard *et al.*, 2000). Taken together, it can be suggested that niflumic acid appears to be the most potent and selective inhibitor of Ca^{2+}-dependent Cl channels (Greenwood and Large, 1995; for review see Large and Wang, 1996).

A comparative study was conducted recently on the effects of Cl channel blockers on mesenteric vascular conductance in anesthetized rats (Parai and Tabrizchi, 2002). The effects of three putative Cl channel blockers, niflumic acid, indanyloxyacetic acid 94

(IAA-94) and diphenylamine -2- carboxylic acid (DPC), on cirazoline-mediated vasoconstriction in mesenteric blood vessel was examined *in vivo*. The arterial blood pressure, heart rate, and mesenteric blood flow was measured in anesthetized rats. Infusion of α_1-adrenoceptor agonist, cirazoline produced a dose-dependent increase in arterial blood pressure, and a decrease in heart rate, superior mesenteric blood flow, and mesenteric vascular conductance. It was observed that niflumic acid and IAA-94 did not have any impact on cirazoline-induced changes in blood pressure, whereas DPC accentuated the pressor effect of cirazoline. Neither agent affected cirazoline-mediated reflex reduction in heart rate. In addition, niflumic acid and DPC were found to be most effective in attenuating α_1-adrenoceptor mediated decreases in mesenteric blood flow and mesenteric vascular conductance, whereas IAA-94 had a negligible effect. Based on the profile of the actions of these compounds, it was suggested that IAA-94 did not appear to act as a selective inhibitor of Ca^{2+}-activated Cl channels when compared to niflumic acid in the mesenteric blood vessels. In addition, while DPC seems to be as effective as niflumic acid in its effects on mesenteric blood vessels, its actions may be attributed to other pharmacological effects (Parai and Tabrizchi, 2002). Taken together, it would appear that niflumic acid is capable of inhibiting receptor-mediated contraction in blood vessels by affecting Ca^{2+}-dependent Cl channels (Greenwood and Large, 1995; Criddle *et al.*, 1996; He and Tabrizchi, 1997; Parai and Tabrizchi, 2002).

1.5.4. Cl^- and endothelial cells

In blood vessels there seems to be an interesting relationship between Cl^- and endothelial cells. A report by Lamb and Barna (1998a,b) has suggested that the endothelium may regulate vascular tone via an effect on vascular smooth muscle Cl channels. These studies have demonstrated that noradrenaline-induced contractions of rat aorta from normotensive rats were potentiated in low Cl^- buffer (Lamb and Barna, 1998a,b). In addition, it was suggested that the magnitude of this potentiation is dependent on endothelial nitric oxide (Lamb and Barna, 1998b). Therefore they had concluded that nitric oxide could inhibit the opening of voltage-dependent Ca channels by an agonist. This inhibition may be direct (via cGMP inhibition) or indirect (by preventing the activation of depolarizing Cl currents) (Lamb and Barna, 1998b). In another study, Tabrizchi and Duggan (2000) have also supported a close link between Cl^- handling and endothelial cell function in blood vessels (Tabrizchi and Duggan, 2000). Thus, the relationship between the role of Cl^- and endothelial cells needs to be developed better.

1.6. Nitric Oxide

Furchgott and Zawadzki (1980) first recognized the fundamental importance of endothelial cells in the control of vascular tone. They found that the addition of acetylcholine to rabbit intact aortic strips, contracted with noradrenaline, caused concentration-dependent relaxation, but had little effect in those in which the endothelial cells had been removed (Furchgott and Zawadzki, 1980). Since the relaxation to

acetylcholine was also obtained in a helical strip without endothelium following its close apposition to an intact strip with endothelial cells, they concluded that endothelial cells release a labile factor that causes blood vessels to dilate (Furchgott and Zawadzki, 1980). This factor was termed endothelium-derived relaxing factor and has since been identified as nitric oxide (NO) (Palmer *et al.*, 1987). Endogenous NO is enzymatically produced by the conversion of the amino acid L-arginine to L-citrulline, a reaction catalyzed by the enzyme NO synthase (NOS) (Palmer *et al.*, 1988). This reaction also requires cofactors such as molecular oxygen, nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide, heme, flavin adenine mononucleotide, calmodulin and tetrahydrobiopterin for optimal function (Palmer *et al.*, 1987; Moncada and Higgs, 1993). NOS belong to a family of oxidoreductases that bear close sequence homology to cytochrome P450 enzymes. Three different NOS isoforms have been cloned and characterized: endothelial NOS (eNOS) (Forstermann *et al.*, 1991), inducible NOS (iNOS) (Hevel *et al.*, 1991), and neuronal NOS (nNOS) (Bredt *et al.*, 1991). In the endothelial cell, eNOS is activated by a number of agonists acting on G-protein-coupled receptors and by physical stimuli such as shear stress (for review see Schini-Kerth and Vanhoutte, 1995). The physiology and pharmacology of NO have been greatly aided by the availability of potent and apparently selective inhibitors of NOS, the most commonly used being the substrate analogues of L-arginine (for review see Andrews *et al.*, 2002). For example, N^{ω}-monomethyl-L-arginine (L-NMMA), N^{ω}-nitro-L-arginine (L-NNA), N^{ω} nitro-L-arginine methyl ester (L-NAME) (for review see Andrews *et al.*, 2002).

1.6.1. Chemistry of NO

NO is a paramagnetic and a free radical species, since it carries a single unpaired electron on its outer shell (in total 11 electrons) (for review see Andrews *et al.*, 2002). The solubility of NO in water is quite low ~1.9×10^{-3} M at 25°C (Feelisch, 1991). Due to its low water solubility, it can diffuse rapidly across the phospholipid membranes into the adjacent smooth muscle cells. The half-life of NO under bioassay conditions have been reported to be 3-5 sec (for review see Moncada *et al.*, 1989; Feelisch, 1991). NO reacts with oxygen and produces various free radicals such as nitrite, nitrate, nitroxyl, nitrosonium, peroxynitrite (Beckman *et al.*, 1990). Peroxynitrite oxidizes protein, DNA and lipids and consequently, may be cytotoxic to potential pathogens. The biological actions of NO are terminated when they react with the scavenger molecules such as hydroquinone, circulating oxyhemoglobin (HbO), and hydroxycobalamin. HbO reacts with NO to form the complex nitrosylHbO, which is then oxidized to metHbO and NO^{-3} (Ignarro *et al.*, 1993). Hydroxycobalamin also has a high affinity for NO, which it combines to form nitrosocobalamin (Rajanayagam *et al.*, 1993). Thus due to the unique chemistry of NO it can react with many different cellular constituents.

1.6.2. Mechanisms of NO action

NO plays an important role in the regulation of myocardial contractility, platelet aggregation, vascular tone, and cytostasis. Although NO appears to be the major vasodilator released by endothelial cells in a vast majority of blood vessels, other substances, such as prostacyclin and the endothelium-derived hyperpolarizing factors also play a role (for review see McGuire *et al.*, 2001). These endothelial factors, and

particularly NO, are also involved in other regulatory functions such as inhibition of leucocyte, monocyte and platelet adhesion, vascular smooth muscle proliferation, vascular permeability and inflammatory mechanisms (for review see Boulanger, 1999). Thus they contribute to the protective role of the endothelium. Moncada *et al.* (1991) had proposed "the cardiovascular system is in a constant state of active vasodilation dependent on the generation of NO". This statement represents the importance of NO in endothelium-dependent vasodilator mechanisms. It has also been suggested that NO can directly activate Ca^{2+}-dependent K channels and voltage-gated K channels leading to endothelium-dependent hyperpolarization of vascular smooth muscle cells (Bolotina *et al.*, 1994; Yuan *et al.*, 1996). NO release activates soluble guanylyl cyclase in smooth muscle cells, platelets, neurons and other cells and thereby increasing the levels of intracellular messenger cGMP (Katsuki *et al.*, 1977; Murphy and Walker, 1998). Elevation of cGMP, which is often transient and varies with tissue type is believed to activate the enzyme PKG (for review see Lincoln and Cornwell, 1993), which is correlated with relaxation by an undefined mechanism. Proposed sites of action include (i) reduction of myoplasmic Ca^{2+}, (ii) alteration of Ca^{2+} sensitivity of phosphorylation, and/or (iii) alternative phosphorylation of other contractile or cytoskeletal proteins (Draznin *et al.*, 1986). Cell suspensions from rabbit or pig coronary arteries treated with acetylcholine followed by nitroglycerin demonstrated increased Ca^{2+} efflux (Itoh *et al.*, 1983). Twort and van Breemen (1988) demonstrated increase uptake of Ca^{2+} into cellular stores in saponin-skinned cultured rat aortic cells treated with cGMP. Chen and Rembold (1992) demonstrated decreased Ca^{2+} influx in the intact swine carotid artery in agonist-stimulated preparations treated with nitroglycerin. Therefore, NO/cGMP-mediated

pathway can cause a reduction in intracellular Ca^{2+} concentration which could be produced through decreased Ca^{2+} influx, increased efflux or increased uptake into the intracellular stores (for review see McDaniel *et al.*, 1994). Thus, the belief that NO could be a mediator that initiated relaxation via the generation of cGMP was born.

Furthermore, evidence suggests that eNOS and nNOS are Ca^{2+}/calmodulin-dependent enzymes and they produce NO in low concentrations (nM) for physiological purposes such as relaxation of smooth muscle (for review see Albrecht *et al.*, 2003). In contrast, expression of iNOS results in the production NO in high concentration (μM) (for review see Albrecht *et al.*, 2003). The eNOS is the major NOS isoform expressed in the cardiovascular system mainly in vascular endothelium and plays an important role in regulation of vascular tone (for review see Mungrue *et al.*, 2003). To investigate the role of different NOS enzymes, NOS knockout (–/–) mice have been utilized. Huang *et al.* (1995) first demonstrated that the absence of eNOS led to a decrease in acetylcholine-dependent relaxation in intact aortic rings from eNOS–/– mice, and that these mice were hypertensive, supporting a role for eNOS derived NO in the regulation of blood pressure. Most studies have shown that eNOS–/– mice have higher blood pressure than wild-type mice, although the magnitude of hypertension reported by different laboratories varies (Kurihara *et al.*, 1998; Stauss *et al.*, 1999; Van Vliet *et al.*, 2003). In addition, eNOS–/– mice were subsequently shown to have age-dependent left ventricular hypertrophy associated with hypertension (Barouch *et al.*, 2002). This evidence certainly supports a role for NO, generated primarily from eNOS, in the regulation of vascular tone, and

consequently blood pressure homeostasis and tissue perfusion. However, it is clear that the magnitude of the role of NO in the development of hypertension remains to be determined.

1.7. Hypertension

Hypertension is a state in which the systemic arterial pressure is inappropriately elevated. This is a prevalent cardiovascular condition in Westernized communities, and is a major risk factor for stroke, coronary heart disease, and heart failure (Chobanian *et al.*, 2003). In almost 95% of the affected patients, the cause of the blood pressure elevation is unknown and the condition is classified as *primary* or *essential* hypertension. High blood pressure attributed to a definable cause is termed *secondary* hypertension. The blood pressure is classified based on the mean of two or more properly measured blood pressure readings obtained in the seated position during two or more office visits. The severity of hypertension is classified on the basis of the systolic and/or diastolic blood pressures measured (see Table 3, Chobanian *et al.*, 2003).

A simple definition of blood pressure (BP) based on hemodynamic parameters of the circuit is equal to the product of cardiac output (CO) and total peripheral vascular resistance (TPR) (Deshmukh *et al.*,1998).

i.e., $BP = CO \times TPR$

Table 3

Blood pressure classification* based on systolic and diastolic pressure.

BP classification	Systolic BP, mmHg		Diastolic BP, mmHg
Normal	< 120	and	< 80
Prehypertension	120 – 139	or	80 – 89
Stage 1 hypertension	140 – 159	or	90 – 99
Stage 2 hypertension	≥ 160	or	≥ 100

*According to the US Joint National Committee report VII.

In sustained hypertension, CO is usually normal and the increase in BP can usually be attributed to increase in TPR. The determinants of BP are multifactorial and the relationship of BP = CO × TPR is used to provide a standardized comparison under different conditions. Abnormalities in the structure and function of the vasculatures are recognized as factors contributing to the hypertensive state that increase total peripheral resistance. Increased peripheral resistance in experimental hypertension is the result of an imbalance between vasoconstriction and vasorelaxation. Structural adaptation of the cardiovascular system such as blood vessel narrowing and wall thickening associated with hypercontractility also plays an important role in increasing peripheral resistance to flow (for review see Folkow, 1982).

1.7.1. Structural and functional changes associated with hypertension

Changes in vascular architecture also play an important role in modifying blood vessel function during hypertension. Lee and Smeda (1985) have suggested that morphological changes in muscular arteries can be primarily involved in the initiation of hypertension. Other studies have also shown that the remodeling of large and small arteries contributes to the development and complications of hypertension (for review see Intengan and Schiffrin, 2001). Remodeling can be either hypertrophic or eutrophic in nature (for review see Zervoudaki and Toutouzas, 2003). Hypertrophic remodeling is associated with a same or smaller lumen, increased wall mass, and an increase in wall to lumen ratio. Eutrophic remodeling is characterized by a smaller lumen, same wall mass, and an increase in wall to lumen ratio (for review see Zervoudaki and Toutouzas, 2003). Two

important parameters for the study of vessel remodeling are the "wall thickness/lumen diameter" ratio and the cross-sectional wall area (for review see Mulvany, 1984). The calculation of the "media thickness/lumen diameter" ratio, according to Laplace's law, provides information about the ability of the vessels to contract against intravascular pressure (for review see Zervoudaki and Toutouzas, 2003). The knowledge of wall cross-sectional area indicates the amount of material within the vascular wall and thus provides information about the process of growth or regression. Collectively, it seems that morphological changes can contribute to greater resistance to flow, which by extension can manifest as high systemic pressure.

Folkow and his colleagues (1973, 1982) have also suggested that structural changes lead to a fixed hyperresponsiveness of the arterial vascular system and cause sustained hypertension. They have suggested that in some forms of essential hypertension the elevated resting tone of the blood vessel could be explained entirely by structural modification of the vessel (Folkow *et al.*, 1970; Folkow, 1982). Finch and Hauesler (1974) using a variety of perfused preparations showed that while the responsiveness to noradrenaline was elevated in hypertension, the response to Ca^{2+} in depolarized mesenteric artery preparations was not altered. Hamilton (1975) has demonstrated that the change in perfusion pressure produced by noradrenaline differed both quantitatively and qualitatively from that produced by serotonin in perfused mesenteric bed from spontaneously hypertensive rats. Likewise, Lokhandwala and Eikenburg (1983) demonstrated differential sensitivity changes to noradrenaline, vasopressin and barium chloride in isolated perfused mesenteric bed from spontaneously hypertensive rats. These

studies point out that while structural adaptation could explain some of the characteristics of dose-response curves in perfusion studies from hypertensive rats, the magnitude of contractions differs depending on the agonist used. These difference in sensitivity would be intrinsic to the properties of the vessels from hypertensive animals.

In general, vasoconstrictor drugs are reported to show a greater potency in blood vessels from hypertensive subjects or animals compared with those from matched normotensive controls (McGregor and Smirk, 1968; 1970; Lüscher *et al.*, 1987). It has been demonstrated that noradrenaline, angiotensin, and 5-hydroxytryptamine caused a greater degree of vasoconstriction in isolated perfused mesenteric arteries in rats with experimental renal hypertension than in the normotensive controls (McGregor and Smirk, 1968, 1970). Also endothelium-dependent relaxations are reported to be impaired in arteries from spontaneously hypertensive rats (Konishi and Su, 1983). Alteration in the biochemical signaling pathways have been reported at almost every step in the transduction of agonist-induced contractile activity. Increased activation of PLC has been shown to be responsible for increased accumulation of the second messengers IP_3 and DAG in aortas in spontaneously hypertensive rat (Kato *et al.*, 1992).

1.7.2. NO and hypertension

As NO became recognized as an important endothelial mediator of relaxation, studies investigated the relationship between high blood pressure and altered endothelial function. During the development of hypertension, the balance between the production of endothelial vasoconstrictor and vasodilator compounds is altered (for review see Lüscher,

1990). A study conducted by Rees *et al.* (1989) demonstrated that the L-arginine analogue, L-NMMA (which inhibits endothelial NO formation) increased blood pressure in a dose-dependent manner in anesthetized rabbits. Furthermore, L-NMMA was found to produce hypertension in animals indicating the potential possibility that endothelial NO participated in arterial blood pressure regulation (Moncada *et al.*, 1991). In aorta and mesenteric resistance arteries of spontaneously hypertensive rats, L-NMMA itself produces very little contraction but enhances the contraction produced by noradrenaline to a greater extent in Wistar-Kyoto rats versus spontaneously hypertensive rats (Dohi *et al.*, 1990). Tschudi *et al.* (1996) demonstrated that during hypertension rat mesenteric arteries exhibit an increase in NO metabolism, and unaltered NO release. African-Americans who are more susceptible to high blood pressure development demonstrate both endothelial dysfunction and increased renal vasoconstriction in response to salt loading (Cardillo *et al.*, 1999). Evidence suggest that L-arginine supplementation can lower blood pressure in humans (Nakaki *et al.*, 1990; Siani *et al.*, 2000). Taken together, both experimental and clinical evidence supports the view that the alteration of NO release can play a role in hypertension development.

1.7.3. Animal models of hypertension

The predominant form of hypertension (essential/primary) in humans is of an unknown etiology. Inbreeding of animals with naturally occurring high blood pressure has led to the production of several strains of hypertensive rats that are thought to represent models of essential hypertension in humans. Sir Horace Smirk first conceived the idea of selectively breeding rats for hypertension and developed the New Zealand strain of

genetically hypertensive rats (Smirk and Hall, 1958; Phelan *et al.*, 1976). This was followed by the development of other strains such as the Wistar-Kyoto spontaneously hypertensive rat (Okamoto and Aoki, 1963; Yamori and Okomoto, 1976), the Dahl salt-sensitive rats (Dahl *et al.*, 1962), the Milan strain (Bianchi and Baer, 1976) and the Brunus Edwardii strain (Blackmore *et al.*, 1972) which demonstrated inherited hypertension associated with unknown etiology.

1.7.4. Clinical hypertension

While the etiology of primary hypertension is unclear, it is supposed that the cause is multifactorial in nature involving genetic, dietary, and environmental factors. In terms of dietary causes, salt consumption can promote the development of high blood pressure. Many hypertensive individuals are considered to be "salt-sensitive" in that their blood pressure is increased by a high NaCl intake and reduced by dietary salt restriction (Dahl, 1972). Depending on the exact definition of salt sensitivity, 30%-50% of all patients with essential hypertension may be salt sensitive, with this figure approaching 75% in African-Americans (Weinberger, 1996). Since hypertension is maintained by a high peripheral resistance to flow during established phase, one goal of hypertension research has been to define the mechanisms responsible for the development and maintenance of this increased resistance. From a physiological standpoint, it is equally important to understand the effect of a sustained increase in vascular resistance on local blood flow and organ function. Since *in vivo* studies of vasculature resistance and local blood flow often require invasive techniques, most of the information in this subject area has been obtained

from the use of animal hypertensive models as opposed to human hypertensive subjects. One such interesting model is the Dahl salt-resistant and salt-sensitive rat.

1.8. Dahl Rats

1.8.1. Historical background

Effects of a high salt diet on the blood pressure of rat have been studied since the 1950s. Meeneely and Ball (1958) showed that the average blood pressure in rats was positively and linearly related to the dietary NaCl (between 0 and 10% NaCl). In the early 1960s Dr. Lewis K. Dahl showed that chronic excess salt ingestion produced sustained hypertension in some rats (Dahl, 1961). Depending on the observation that not all rats responded to salt with similar changes in blood pressure, Dahl *et al.* (1962) were able to selectively breed Sprague-Dawley rats that were susceptibile (DS rats) or resistant (DR rats) to hypertension development in response to a 8% NaCl diet. This work was carried out on a colony of Sprague-Dawley rats that was presumably maintained by breeding at Brookhaven Laboratory. After three generations of selective breeding, the DS and DR lines were clearly separated. The blood pressures of DR rats were normal and similar on control or high salt diets, but DS rats responded to salt with a pronounced increase in blood pressure (Dahl *et al.*, 1962). Thus, the two strains yielded an interesting model that could be used to assess the interaction of a dietary factor (salt) with genotype. Blood pressure is a polygenic trait, which means that there are multiple genetic loci with effects on blood pressure. Using quantitative genetic techniques it was estimated that approximately 2-4 genetic loci were involved in controlling the blood pressure response

to salt in DS and DR rats (Knudsen *et al.*, 1970). Recently it had been suggested that chromosomes 3,6,7,11 are important in regulating arterial pressure and renal function in female DS rats (Moreno *et al.*, 2003) whereas chromosomes 1,3,19 are responsible for cardiac hypertrophy in DS rats (Siegel *et al.*, 2003). The extreme sensitivity of the DS rat to the salt is a unique trait and the extreme resistance of the DR rat is equally unique (for review see Rapp, 1982).

1.8.2. General characteristics of the strains

DS rats placed on a high salt (8% NaCl) diet at weaning (21-23 days of age) rapidly develop hypertension and all die after 16 weeks of salt feeding, whereas DR rats fed the same diet maintain blood pressure in the normotensive range, and 80% of the animals survive after 48 weeks of high salt feeding (Knudsen *et al.*, 1970). There was no marked retention of Na^+ in DS versus DR rats as measured by whole carcass Na^+, or half-life of ^{22}Na. There was also no depletion of carcass K^+ in DS rats (Schackow and Dahl, 1966). Electron-probe analysis of intracellular Na^+, K^+, and Cl^- concentrations indicated similar levels for DS and DR rats on low- and high-salt diets (Abel *et al.*, 1981). The mechanism of salt-induced hypertension in Dahl salt-sensitive rats involves neural, humoral and renal pathways (for review see Rapp, 1982).

1.8.3. Neural mechanisms in Dahl rats

An early increase in sympathetic nerve activity in salt-induced hypertension has been implicated as an important factor in its pathogenesis (Mark, 1991). Using 6-

hydroxydopamine to induce chemical sympathectomy, Takeshita *et al.* (1979) were able to show that an intact sympathetic nervous system was essential for the development of salt-induced hypertension and increased vascular resistance in the hindquarters of hypertensive DS rats. Increased sympathetic nerve activity in the heart and kidney of DS hypertensive rats was associated with increased noradrenaline turnover (Peular *et al.*, 1989). Surprisingly, this state was usually accompanied by reduced tissue noradrenaline content, reflecting higher noradrenaline utilization in the respective organs (Racz *et al.*, 1987). Using histochemical fluorescent technique, Kunes *et al.* (1991) have shown that a chronic elevation of salt intake reduced the density of visible adrenergic plexus as well as the intensity of catecholamine fluorescence in terminal axons of mesenteric and renal vascular beds in salt hypertensive Dahl rats. They suggested that a reduction of noradrenaline uptake and noradrenaline content might be the result of a less dense adrenergic innervation of mesenteric blood vessels in DS rats (Kunes *et al.*, 1991). Bayorh *et al.* (1998) have reported fourfold increase in plasma arginine-vasopressin, which in turn activates sympathetic nervous system and vascular responses. Altogether it seems that a hyperactive sympathetic nervous system may contribute in maintaining a hypertensive state in salt hypertensive Dahl rats.

Furthermore, DR rats show more exploratory behavior and greater aggressive behavior than DS rats (Welner *et al.*, 1968; Ben-Ishay and Welner, 1969). The relationship of this behavior to blood pressure is obscure, and the difference could have arisen from genetic drift, *i.e.*, chance selection of behavior pattern.

1.8.4. Renal mechanisms in Dahl rats

There is a considerable amount of evidence to indicate a primary defect in renal function in DS rats as a cause of hypertension. Tobian *et al.* (1966) were the first to show that a genetic fault in the kidneys of the DS rat potentially caused hypertension. This work was confirmed later by Dahl *et al.* (1972, 1974) where they found that when kidneys are transplanted from one strain to the other, the influence on blood pressure arrives with the kidney. Therefore, a kidney from DS rat when transplanted into a DR rat elevates the blood pressure of the later and vice versa (Tobain *et al.*, 1966; Dahl *et al.*, 1972, 1974). These transplantation experiments suggested some type of baroreceptor resetting within the kidneys, which by itself was able to regulate blood pressure. It has been reported that the DS rats have 15% fewer glomeruli than DR (Azar *et al.*, 1978). Following 20-30 days of high salt to induce mild hypertension in the DS rats, the DS showed increased single nephron blood flow, increased single nephron glomerular filtration rate, and decreased resistance at the afferent and efferent arteriolar segments which might be the adaptive changes to the reduced number of glomeruli (Azar *et al.*, 1979).

1.8.5. Changes in blood vessel structure in Dahl hypertensive rats

The elevation in blood pressure in salt hypertensive Dahl rats has been suggested to be associated with the development of structural alterations in blood vessels. Lee and Triggle (1986) did an extensive morphometric analysis of mesenteric arteries from DS hypertensive rats. Their results showed that the cross-sectional area of the media was increased in superior, large, and small mesenteric arteries from DS-high salt rats when compared to DS-low salt, DR-high salt, and DR-low salt groups. In the large muscular

arteries, the increase in the size of the vessel wall in hypertensive Dahl rats was produced by a widening of subendothelial space, hyperplasia of medial smooth muscle cells and the presence of a larger adventitial layer. In the small mesenteric arteries of hypertensive DS rats, the increase in size of the medial wall was probably due to hypertrophy of the smooth muscle cells and/or an increase in the extracellular space (Lee and Triggle, 1986). Mesenteric vessels have been noted to undergo medial wall thickening that positively correlates with the increase in blood pressure during salt-induced hypertension (Lee and Triggle, 1986). Therefore, it is inferred that hyperactivity of the mesenteric arteries of Dahl hypertensive rats is either due to increased smooth muscle mass or impaired endothelial cell function (Lee and Triggle, 1986). Increased media thickening has also been noted in carotid artery (Benetos *et al.* 1995), basilar artery (d'Uscio *et al.* 1997), and aorta (Duggan and Tabrizchi, 2002) of Dahl hypertensive rats. Intengan and Schiffrin (1998) have argued that the morphological changes in mesenteric bed of Dahl salt-sensitive hypertensive rats are due to eutrophic remodeling, and have further suggested that this could, in part, be responsible for hyperactivity of the blood vessels in this bed in such animals.

1.8.6. Endothelial dysfunction in Dahl hypertensive rats

During the past decade, it has become increasingly clear that the endothelium, the single innermost layer of blood vessels, is more than a passive barrier between the blood and the vascular smooth muscle (for review see Boulanger, 1999). We now know that the endothelium plays a crucial role in circulatory homeostasis responding not only to humoral and chemical signals, but also to changes in hemodynamics of blood flow such

as shear stress (for review see Fisher *et al.*, 2001). Lüscher *et al.* (1987) have demonstrated that in DS rats, contractions in response to noradrenaline in aortic rings are increased while relaxations in response to acetylcholine are depressed. Evidence suggests that in hypertensive Dahl rats, administration of L-arginine reduces blood pressure and restores endothelium-dependent relaxations (Chen and Sanders, 1991; Patel *et al.*, 1993). Nishida *et al.* (1998) has suggested that the vascular hypercontractile responses to noradrenaline in DS hypertensive rats can, in part, be explained by impairment in endothelial NO production. Clearly, a popular hypothesis for the enhanced vasoconstriction seen in Dahl hypertensive rat is endothelial cell dysfunction (Nishida *et al.*, 1998). Furthermore, vascular smooth muscle cells from salt-sensitive Dahl rats are characterized by a mutation of the coding region of the iNOS gene associated with decreased nitrite/nitrate formation (Chen *et al.*, 1998). In contrast, a study performed using various isolated smooth muscle preparations (thoracic aorta, tail artery, portal vein, anococcygeus, perfused mesenteric bed) from Dahl rats have demonstrated that the reactivity to noradrenaline, serotonin, and phenylephrine were similar in DS and DR rats (Laher and Triggle, 1984). Similar results have also been reported by other investigators who have reported absence of increased contractility in blood vessels from DS hypertensive rats on a salt diet (Abel *et al.*, 1981; Kong *et al.*, 1995). Thus, it is recognized that while endothelium-derived NO may contribute to the overall regulation of arterial blood pressure by virtue of its ability to relax vascular smooth muscle, its place in vascular dysfunction and hypertension in the Dahl model is not yet fully established.

Based on the evidence summarized above, the Dahl rat seems to be a viable model for examining the underlying pathology in salt hypertension and assess the alterations in blood vessel function in a species where the environmental factor (*i.e.*, salt) is responsible for the pathology and thus hypertension.

1.9. NATURE OF THE PROBLEM

Alterations in the behavior of blood vessels in salt-induced hypertension manifest themselves in term of changes in morphological (Lee and Triggle, 1986; Intengan and Schiffrin, 1998), mechanical (Nishida *et al.*, 1998; Tabrizchi and Duggan, 2000), and electrical (Fujii *et al.*, 1997) activity of smooth muscle cells, not necessarily in this specific order.

The role of architectural change in maintenance of high blood pressure in salt hypertension has been put forward by several investigators (Benetos *et al.*, 1995; d'Uscio *et al.*, 1997), although there is controversy over the type of changes that occur in blood vessels. While it has been suggested that blood vessels in Dahl salt-sensitive hypertensive (SSH) rats undergo increases in smooth muscle mass (Lee and Triggle, 1986), others have argued that the morphological changes in mesenteric bed of Dahl SSH rats are due to eutrophic remodeling (Intengan and Schiffrin, 1998).

There are also conflicting reports regarding alterations in functional and electrical responses in blood vessels in salt hypertension. For example, Abel *et al.* (1981) and Laher and Triggle (1984) have suggested unaltered functional properties of blood vessels, whereas Nishida *et al.* (1998) have suggested increased contractile responses to vasoconstrictors in blood vessels from Dahl SSH when compared to salt-resistant normotensive (SRN) rats.

In addition, some authors have suggested that the resting E_m was depolarized (Fujii *et al.*, 1997; Wellman *et al.*, 2001), whereas another report have shown no difference (Abel *et al.*, 1981) in resting E_m in vascular smooth muscle cells from Dahl SSH compared to SRN rats. The evidence in the literature in the context of altered behavior in blood vessels in salt-induced hypertension is inconsistent. Indeed, it seems that the inter-relationship between morphological, functional, and/or electrical change in salt-induced hypertension needs further development and investigation.

One aspect of change in blood vessel behavior from salt-induced hypertensive rat appears to be due to an altered relationship between endothelial cells, smooth muscle and Cl^-. Evidence from our laboratory has suggested that differences exist in vasoconstrictor responses to α_1-adrenoceptor agonists in blood vessels from Dahl SRN and SSH rats in Cl^--free buffer (Tabrizchi and Duggan, 2000; Bieger *et al.*, 2004). Tabrizchi and Duggan (2000) have demonstrated that the removal of Cl^- augmented cirazoline-evoked contractions in aorta of Dahl SRN rats, whereas it depressed the contraction in Dahl SSH rats. In contrast, Cl^- removal from pulmonary artery did not alter cirazoline-induced vasoconstriction in Dahl SRN rats, but it did attenuate the vasoconstriction elicited by cirazoline in SSH rats (Bieger *et al.*, 2004). This suggests that there is divergence in the behavior of conduit versus low-pressure blood vessels even within the same species. Thus it was concluded that the paradoxical effect of Cl^--free buffer on contractions produced by cirazoline in blood vessels from Dahl SRN versus SSH rat supports a close

link between Cl^- handling and endothelial cell function in these blood vessels (Tabrizchi and Duggan, 2000; Bieger *et al.*, 2004).

It is also recognized that alteration in endothelial cell function is, in part, responsible for changes in functional behavior of blood vessels in the developed state of hypertension (for review see Boulanger, 1999). Indeed, Lüscher *et al.* (1987) have demonstrated that endothelium-dependent relaxations were significantly depressed in aorta from Dahl SSH rats. In addition, Nishida *et al.* (1998) had suggested that impairment of endothelial NO production is involved in the increased contractile response to noradrenaline in Dahl SSH rats. Evidence from our laboratory has revealed that basal levels of cGMP in blood vessels of Dahl SRN are significantly higher than those of SSH rats (Tabrizchi and Duggan, 2000; Bieger *et al.*, 2004). Furthermore, it has been suggested that administration of L-arginine reduces blood pressure and restores endothelium-dependent relaxations in Dahl hypertensive rats (Chen and Sanders, 1991). Duggan and Tabrizchi (2002) have further demonstrated that relaxations elicited by Ca channel antagonists were attenuated in presence of L-NAME in aortic rings from Dahl SRN but not in SSH rats. Taken together, it seems that the signaling pathway between NO/NO synthase, Cl^- and smooth muscle cells is altered in blood vessels of SSH rats (Nishida *et al.*, 1998; Duggan and Tabrizchi, 2002; Bieger *et al.*, 2004).

Studies from our laboratory have suggested an alteration in Cl^- handling and endothelial cells in conduit and low-pressure blood vessels in Dahl SSH rats (Tabrizchi and Duggan,

2000; Bieger *et al.*, 2004). Thus the working hypothesis for this investigation is that the role of Cl^- in excitation-contraction coupling is altered in resistance blood vessels in salt-induced hypertension, perhaps due to alteration in NO/NO synthase pathways. With respect to the latter hypothesis, the questions addressed in this investigation were: (1) Is there an alteration in Cl^- handling in mesenteric blood vessels in salt hypertension?; and (2) Is the relationship between Cl^- and NO altered in mesenteric blood vessels in salt-induced hypertension?

The primary objective of the investigation was: (a) to compare the actions of the vasoconstrictor, cirazoline, on blood pressure, heart rate, mesenteric blood flow, and mesenteric vascular conductance; (b) to determine the impact of the NO synthase inhibitor, L-NAME, on basal and cirazoline-stimulated vascular conductance; (c) to examine the impact of the Cl channel antagonist, niflumic acid, on basal and cirazoline-stimulated vascular conductance; and (d) to assess any interaction between the effects of L-NAME and niflumic acid on cirazoline-mediated changes in vascular conductance in anesthetized Dahl SRN and SSH rats.

Cirazoline-induced vasoconstriction was expected to be increased (i.e., reactivity and sensitivity) as a consequence of hypertension in Dahl SSH when compared to SRN rats. The basis for the latter observation, if true, could be due to an altered NO/NO synthase cascade that occurs in Dahl SSH rats. Accordingly, L-NAME was expected not to have any effect on responses to cirazoline in blood vessels from SSH rats while potentiating

the responses in SRN rats. However, any difference in response in presence of niflumic acid was expected to be absent following treatment of animals with L-NAME.

The secondary objective was: (a) to determine the effect of Cl^- removal on α_1-adrenoceptor-mediated vasoconstriction in isolated mesenteric blood vessels; (b) to evaluate the influence of Cl^- upon addition of L-NAME on α_1-adrenoceptor-mediated vasoconstriction; and (c) to assess the response of niflumic acid in presence and absence of Cl^- on α_1-adrenoceptor-mediated vasoconstriction in isolated perfused mesenteric blood vessels and compare the differences in response between Dahl SRN and SSH rats.

Since Cl^- removal has a divergent effect in blood vessels, it was anticipated that Cl^- removal would either have an inhibitory or potentiating effect on cirazoline-mediated vasoconstriction. The role of Cl^- was suggested to be altered in salt hypertension and therefore Cl^--free buffer was expected to have a greater effect in Dahl SRN when compared to SSH rats. L-NAME was expected to have a potentiating effect on blood vessels from SRN rats and this response was expected to be altered in Cl^--free buffer. Furthermore, Cl^--free buffer was predicted to nullify the inhibitory effect of niflumic acid.

The tertiary objective of this study was: (a) to determine the influence of cirazoline on vascular smooth muscle cell resting E_m in superior mesenteric artery; (b) to determine the influence of Cl^- removal on vascular smooth muscle cell E_m in superior mesenteric artery;

and (c) to determine the influence of L-NAME on vascular smooth muscle cell E_m in superior mesenteric artery and evaluate the differences in behavior in salt hypertensive Dahl rats. In addition, the morphology of primary mesenteric blood vessels was assessed in Dahl SRN and SSH rats on salt diet for 7 weeks.

The resting E_m in superior mesenteric artery in Dahl SSH rats was expected to be either depolarized or similar to SRN rats. Cirazoline was expected to cause a membrane depolarization in vascular smooth muscle cells from Dahl SRN and SSH rats. Cl^- removal was anticipated to change the resting E_m to a greater degree in vascular smooth muscle cells from Dahl SRN when compared to SSH rats. L-NAME was expected to have a depolarizing effect in blood vessels from Dahl SRN but not in SSH rats. In addition, the blood vessels from Dahl SSH rats were likely to have undergone morphological changes.

2. METHODS

2.1.0. Preparation of animals

Female Rapp/Dahl rats (salt-resistant and salt-sensitive) were housed (two per cage) with 12 h light/dark cycles and given access to normal food (0.26% Na, 0.44% Cl; LabDiet 5P00 ProLab, PMI Nutrition International, Brentwood, MO, U.S.A.) and tap water *ad libitum*. At 6–7 weeks of age, animals were placed on a Japanese style diet containing 4% NaCl (Zeigler Brothers, Gardners, PA, U.S.A.). The animals were on 4% salt diet for 7 weeks. After 7 weeks all the Dahl SRN rats were in good health. 15% of the Dahl SSH rats fed the above diet died or exhibited significant weight loss after being placed on the diet for 7 weeks. These were excluded from the study and the remaining 85% of the healthy Dahl SSH rats were sampled.

2.1.1. Blood pressure and heart rate determination

Following 7 weeks on a 4% salt diet, each rat was weighed. Then the rat was anesthetized with halothane (5% in 100% oxygen for induction and 1.25% in 100% oxygen for maintenance). Body temperature was maintained at 36 ± 1°C using a heating lamp. A catheter (polyethylene tubing ID 0.58 mm, OD 0.965 mm) was inserted into the left femoral artery for direct measurement of arterial blood pressure. The catheters were filled with heparinized saline (25 IU ml^{-1} in 0.9% NaCl). Arterial blood pressure was recorded with a pressure transducer (Model TNF-R, Ohmeda, WI, U.S.A.) connected to a polygraph (Model 7P1F, Grass Instruments Co., MA, U.S.A.). Heart rate was derived

from the upstroke of the arterial pulse pressure by a tachograph (Model 7P44D, Grass Instruments Co., MA, U.S.A.). Arterial blood pressure and heart rate were recorded for 15 min. Thereafter, blood samples were taken randomly from some animals for plasma NO_2^-/NO_3^- and blood volume measurements.

2.1.2. Plasma NO_2^-/NO_3^- measurements

After recording blood pressure and heart rate, plasma samples for NO_2^-/NO_3^- measurements was taken (n = 10). NO_2^-/NO_3^- was only determined in rats where plasma and blood volume were not measured. The plasma concentration of NO_2^-/NO_3^- was measured using a method similar to that described by Schmidt *et al.* (1992). Samples of 180 µl of blood and 20 µl of sodium citrate (stock concentration-10% sodium citrate in 0.9% NaCl; final concentration-1% sodium citrate in blood sample) were obtained and centrifuged at 10,000 rpm for 5 min to separate the cells from plasma. The plasma layer was separated and stored. The NO_3^- in plasma was enzymatically converted to NO_2^-. Essentially, NO_3^- was reduced by incubation for 15 min at 37°C in the presence of nitrate reductase (1 IU ml^{-1}), NADPH (500 µM) and FAD (50 µM). The unused NADPH, which interferes with the assay, was oxidized by lactate dehydrogenase (100 IU ml^{-1}) and sodium pyruvate (100 mM) by incubation for 5 min at 37°C. The total NO_2^-/NO_3^- in the plasma was then assayed by addition of Griess reagent to each sample and the optical density (OD) at 540 nm (A_{540}) was measured. The total NO_2^-/NO_3^- concentration was calculated by comparison with the A_{540} of a standard solution of $NaNO_2$ also converted to NO_2^-.

2.1.3. Plasma and blood volume determination

Blood samples for plasma and blood volumes measurement were taken randomly from different groups of rats (n = 22). Plasma volume and total blood volume were determined according to Migita *et al.* (1997). A sample of 100 µl of blood was collected in two heparinized capillary tubes before and after the administration of Evans blue dye (EBD) (5 mg kg^{-1}). The samples were centrifuged for 3 min to obtain hematocrit (Hct) values. A 50 µl sample of plasma was collected from the hematocrit tubes and diluted 1:20 in distilled water. The absorbance was recorded using 4050 UV/Visible spectrophotometer (LKB Biochrom, Cambridge, England) at 620 nm (A_{620}) and corrected for the presence of hemoglobin at A_{620} (blank), and for turbidity at A_{740}. Plasma volume (PLV) was determined as follows:

$$PLV = (C_i \times V_i) / C_p$$

Where C_i and V_i are the concentration and volume, respectively, of EBD that was injected and C_p is the plasma concentration of EBD. A plasma trapping factor (t_p) of 0.96 and a total body-to-venous hematocrit ratio (F_{cells}) of 0.74 were employed (Migita *et al.* 1997) to estimate total blood volume (TBV) using the equation:

$$TBV = PLV / 1 - (Hct \times t_p \times F_{cells})$$

2.2. Study 1: Blood flow measurements

2.2.1. Surgical preparation

Additional catheters (polyethylene tubing ID 0.58 mm, OD 0.965 mm) were inserted into the left and right femoral veins for administration of drugs. After recording arterial blood pressure and heart rate, the anesthetic was switched to thiobutabarbital and titrated up to 50 mg kg^{-1} intravenously. The anesthetized animals were tracheotomized using a 14-gauge × 1/2 inch needle. The abdominal cavity was opened through a ventral midline incision and the superior mesenteric artery was exposed and carefully dissected free of connective tissue. A transonic flow probe (Model 1RB630, Transonic Systems, NY, U.S.A.) was placed on the mesenteric artery and blood flow was measured using a flowmeter (Model T106, Transonic Systems, NY, U.S.A.) and displayed on a Grass polygraph (Model 7P1F, Grass Instruments Co., MA, U.S.A.) (Tabrizchi and Pugsley, 2000). Arterial blood pressure was recorded with a pressure transducer (Model TNF-R, Ohmeda, WI, U.S.A.) connected to a polygraph (Model 7P1F, Grass Instruments Co., MA, U.S.A.). Heart rate was derived from the upstroke of the arterial pulse pressure using a tachograph (Model 7P44D, Grass Instruments Co., MA, U.S.A.). Body temperature was maintained at 36 ± 1°C using a heating lamp and monitored rectally by a mercury thermometer. After completion of surgery, each animal was allowed to stabilize for a period of 60 min while blood pressure, mesenteric blood flow and heart rate were continuously monitored prior to administration of vehicle and/or drugs. At the end of the experiment each animal was sacrificed and the heart and kidneys were removed. The

weights of the left and right kidneys, right ventricle and left ventricle + septum were taken.

2.2.2. Experimental protocol

Animals were randomly assigned to four groups (n = 6). Following a stabilization period of 60 min, each animal received a cumulative continuous infusion of cirazoline (0.4, 0.8, 1.6 and 3.2 $\mu g\ kg^{-1}\ min^{-1}$), with each dose infused for a period of 5-7 min. After completion of the first cirazoline dose-response curve, each animal was allowed to recover for 60 min. This period was sufficient to allow blood pressure, heart rate and mesenteric blood flow to return to baseline (He and Tabrizchi, 1997; Parai and Tabrizchi, 2002). Animals in each group then received a single bolus injection of saline (0.9% NaCl) and vehicle (0.4 M $NaHCO_3$ in 5% glucose); saline (0.9% NaCl) and niflumic acid ($10\ mg\ kg^{-1}$); L-NAME ($0.3\ mg\ kg^{-1}$) and vehicle (0.4 M $NaHCO_3$ in 5% glucose) and L-NAME ($0.3\ mg\ kg^{-1}$) and niflumic acid ($10\ mg\ kg^{-1}$). A period of 15 min was allowed to elapse after the bolus injection of vehicle or drugs before the second cumulative cirazoline dose-response curve was constructed.

2.3. Study 2: Perfused isolated mesenteric artery preparation

2.3.1. Tissue isolation technique

The blood pressure and heart rate were recorded for 15 min before the mesenteric blood vessels were isolated. Under halothane anesthesia, the abdominal cavity was opened and

the pancreatico-duodenal, ileo-colic and colic branches of the superior mesenteric artery were tied (McGregor, 1965). The superior mesenteric artery was then carefully separated from surrounding tissues and cannulated through an incision at the confluence with the abdominal aorta. The intestine was severed from the mesentery by cutting close to the intestinal border of the mesentery (McGregor, 1965). The mesenteric artery and its branches were flushed with physiological salt solution, transferred to a warmed organ chamber and perfused and superfused 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 120, KCl 4.6, glucose 11, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 12.5. The pH of the buffer, following saturation with a 95% O_2 : 5% CO_2 gas mixture, was 7.4. All experiments were performed in both normal Krebs and/or chloride-free buffer of the following composition (in mM): sodium propionate (C_2H_2COONa) 120, potassium propionate (C_2H_2COOK) 4.6, glucose 11, $MgSO_4$ 1.2, calcium gluconate ($Ca(C_6H_{11}O_7)_2$) 2.5, KH_2PO_4 1.2, $NaHCO_3$ 12.5. The mesenteric blood vessels were perfused at a constant rate of 5 ml min^{-1} using a Vera Varistaltic Pump Plus (Model 72315000, IL, USA). The perfusion pressure was measured by means of a pressure transducer (PD23ID, Gould Statham) and displayed on a Grass polygraph (Model 79D, Grass Instruments Co., MA, USA). The perfused blood vessels were allowed to stabilize for an hour before the start of each experiment. After the removal of the mesenteric bed, the heart and kidneys were also removed. The weights of the left and right kidneys, right ventricle and left ventricle + septum were taken.

2.3.2. Experimental protocols

2.3.2.1. Series I

Following stabilization for an hour in normal Krebs buffer, the tissues were initially exposed to a submaximal dose of cirazoline (10 nmoles) and then allowed to equilibrate for an additional hour. A control cirazoline dose-response curve (0.1 – 30 nmoles) was constructed in absence of vehicle or drugs. Perfusion pressure was allowed to return to baseline before the bolus injection of each dose of agonist was made. In three separate groups (n = 6) the effects of vehicle (0.4 M $NaHCO_3$ in 5% glucose; 10 µl 100 ml^{-1}), L-NAME (10 µM), or niflumic acid (10 µM) were examined. Blood vessels were allowed to stabilize for 30 min after the control dose-response curve and then perfused with buffer containing vehicle, L-NAME, or niflumic acid for 20 min prior to the construction of the second cirazoline dose-response curve.

2.3.2.2. Series II

In three separate groups (n = 6), the effects of vehicle, L-NAME, or niflumic acid were examined in Cl^--free buffer. A control cirazoline dose-response curve was obtained from mesenteric arteries perfused with normal Krebs as previously described. The tissues were then allowed to stabilize for 20 min while perfused with normal Krebs solution. The solution was then changed to Cl^--free buffer and after 10 min either vehicle (0.4 M $NaHCO_3$ in 5% glucose; 10 µl 100 ml^{-1}), L-NAME (10 µM), or niflumic acid (10 µM) was added to the Cl^--free buffer and 20 min was allowed to elapse before the second cirazoline dose-response curve was constructed.

2.4. Study 3: Electrophysiological Measurements

Blood vessels from three different groups (n = 5 – 6) of rats were used for membrane potential measurements. After recording the blood pressure and heart rate under halothane anesthesia, the superior mesenteric artery was isolated and removed. Arterial rings were held in place in a 5 ml sylgard lined tissue chamber superfused with physiological salt solution pregassed with a mixture of 95% O_2 : 5% CO_2 delivered at a rate of 2-3 ml per minute and warmed to 35-36°C. Blood vessels were allowed to equilibrate for 60 minutes, and the membrane potential was recorded with borosilicate capillary microelectrodes filled with KCl (3.0 M) with a tip resistance of 10-15 MΩ. The Ag/AgCl reference half-cell containing KCl (3.0 M) was connected to the bath via an agar salt bridge containing 150 mM NaCl. Impalements were made by means of a Narishige x-y-z micropositioner, typically from the endothelial side at depths > 30 μm below the surface. Criteria for impalement and measurement of smooth muscle cell membrane potential were an abrupt drop in voltage upon penetration of the cell membrane, a stable membrane potential for at least 1 minute, and a sharp return of the membrane potential to zero upon withdrawal of the electrode from the cell. Voltage signals were recorded by means of Axoclamp–2A (Axon Instruments Inc., CA, USA), the output of which was fed into a DigiData 1200 Series Interface (Axon Instruments Inc., CA, USA). Data were acquired and displayed on AxoScope (Version 1.1) and stored on a microcomputer (Bieger *et al.*, 2004).

2.4.1. Experimental protocols

Membrane potentials from 5–6 cells were initially recorded prior to exposure of the blood vessels to drugs or Cl^--free buffer. Subsequently, tissues were exposed for 20–30 minutes to solutions containing cirazoline (3 μM) followed by normal Krebs; or Cl^--free buffer followed by Cl^--free and cirazoline (3 μM); or L-NAME (10 μM) followed by L-NAME (10 μM) and cirazoline (3 μM), and cells were then sampled for membrane potential measurements. Membrane potential recordings were carried out by sampling 5–7 cells individually after the addition of drug or Cl^--free solution. A junction potential of 4-5 mV was noted in Cl^--free buffer when pipettes were kept extracellularly during the switch from Cl^- containing to Cl^--free medium and this was corrected.

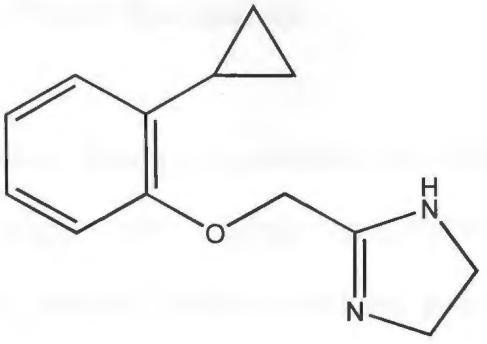
2.5. Study 4: Morphometric Measurements

Prior to removal of superior mesenteric artery, a segment of primary mesenteric artery and vein (3–4 mm in length) from Dahl SRN and SSH rats (n = 22) was isolated and placed in 4% paraformaldehyde in 0.1 M phosphate buffer solution for 24 hr before processing for routine histology. The processing of the blood vessels was done overnight where tissues were exposed to a series of solutions of buffered formalin, ethanol, xylene, and surgipath blue paraffin bath using Tissue-Tek V.I.P. vacuum infiltration process (Sakura Finietek USA, Inc., 300 E, Torrance, CA, USA). Each tissue was then sectioned at 8 μm by a microtome, and mounted on positive charged slides. The tissues were

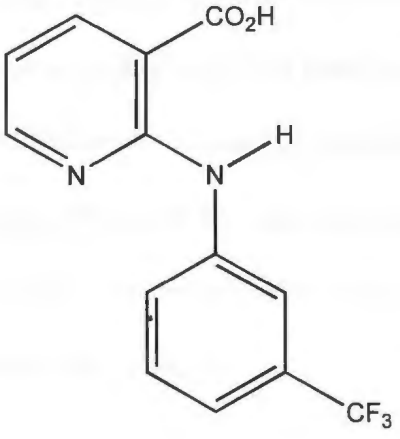
finally stained with Weigert's iron hematoxylin and van Gieson's dyes (Luna, 1968). Four to six sections of the arterial and venous rings from each rat were then examined and morphometric measurements were made of perimeter, lumen area, smooth muscle thickness, and lumen diameter using Neurolucide software (Micro Brightfield Inc., Vermount, USA) with a Microvid microscope Leitz Diaplan, (Model 020-437.035, Ernst Leitz Wetzlar GMBH, Germany) (Mong *et al.*, 2002).

2.6. Drugs and Reagents

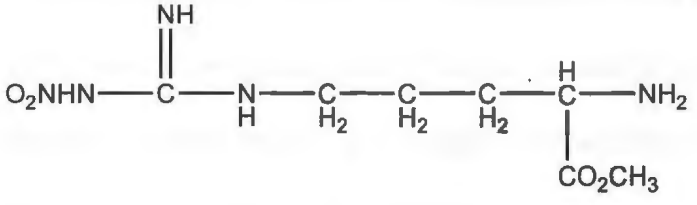
All the reagents and drugs were purchased from Sigma (Oakville, Ontario, Canada). Cirazoline hydrochloride ((2-[(2-cyclopropylphenoxy)methyl]-4,5-dihydro-1H-imidazole); $C_{13}H_{16}N_2O.HCl$), and L-NAME (N^{ω} nitro-L-arginine methyl ester; $C_7H_{15}N_5O_4.HCl$) were dissolved in saline (for *in vivo* experiments) or distilled water (for *in vitro* experiments). Niflumic acid ($C_{13}H_9F_3N_2O_2$) was dissolved in 0.4 M $NaHCO_3$ in 5% glucose solution. The structures of cirazoline, niflumic acid, and L-NAME are represented in Figure 2.



Cirazoline



Niflumic Acid



L-NAME

Figure 2. Chemical structure of cirazoline, niflumic acid, and L-NAME.

2.7. Data and Statistical Analysis

Mesenteric blood flow was corrected for body weight in each group of rats (blood flow/body weight × 100). Vascular conductance was calculated as flow divided by mean arterial blood pressure. Mean arterial blood pressure was approximated as diastolic blood pressure + 1/3 (systolic blood pressure – diastolic blood pressure)). Vascular conductance is the reciprocal of vascular resistance and is the preferred index of vascular tone where large changes in flow occur which is the usual *in vivo* situation (Lautt, 1989). Slope (m) was calculated using the linear regression equation, $y = (mlogx) + c$, in order to compare the differences in cirazoline-mediated responses between the two strains. The measured lumen was corrected using the formula $A_c = A_m \times (R_S / R_L)$ where A_c is the corrected area, A_m is the measured area, R_S is short radius of lumen, R_L is long radius of lumen (Smeda *et al.*, 1988).

All data are presented as mean ± SEM. Student's unpaired t-test was used for comparisons between hemodynamic values and analysis of variance (ANOVA) with repeated measures was used for comparison between hemodynamic, perfusion pressure, and membrane potential values. Duncan's multiple range test was used to compare between multiple means. In all cases, a probability of error less than 0.05 was selected as the criterion for statistical significance.

3. RESULTS

Both the systolic and diastolic blood pressures as well as heart rate of Dahl salt-sensitive rats were significantly ($P < 0.05$) higher than those of Dahl salt-resistant rats 7 weeks after they were placed on a 4% salt diet (Table 4). While basal mesenteric blood flow was not significantly different in Dahl SRN rats in comparison to SSH rats, basal mesenteric vascular conductance was significantly lower in the Dahl SSH when compared to SRN rats (Table 4). It was observed that the Dahl SSH rats had a slightly, but significantly, higher body weight than Dahl SRN rats 7 weeks after they were placed on the salt diet (Table 4). In addition, the ratio of right ventricle to the left ventricle plus septum was significantly smaller in Dahl SSH rats when compared to SRN rats (Table 4) indicating the presence of left ventricular hypertrophy in hypertensive animals. Moreover, the weight of the kidneys corrected for body weight was found to be greater in Dahl SSH than SRN rats on salt diet (Table 4). There were no significant differences between the plasma NO_2^-/NO_3^-, plasma volume, and total blood volume between Dahl SRN and SSH rats (Table 4).

3.1. Blood flow measurements

3.1.1. Effects of cirazoline in mesenteric bed

There were no significant differences in the baseline values of mean arterial blood pressure, heart rate, superior mesenteric blood flow and mesenteric vascular conductance

Table 4. Systolic and diastolic blood pressure (mmHg), heart rate (beats min^{-1}), mesenteric blood flow (ml min^{-1} $100g^{-1}$), mesenteric vascular conductance (ml min^{-1} $mmHg^{-1}$ $100g^{-1}$), body weight (g), ratio of right to left ventricle + septum (RV/LV+S), right kidney (g 100 g^{-1}), left kidney (g 100 g^{-1}), plasma nitrate/nitrite (μM), plasma volume (ml kg^{-1}) and total blood volume (ml kg^{-1}) of Dahl salt-resistant normotensive (SRN) and salt-sensitive hypertensive (SSH) rats after 7 weeks on a 4% salt diet.

Groups	SRN	SSH
Blood Pressure	118/80 ± 1/1	175/126 ± 1*/1*
Heart Rate	363 ± 3.0	388 ± 6.0*
Mesenteric Blood Flow[1]	2.87 ± 0.12	2.67 ± 0.12
Vascular Conductance[1]	0.023 ± 0.001	0.016 ± 0.001*
Body Weight	230 ± 1.5	255 ± 2.4*
RV/(LV + S)	0.23 ± 0.003	0.17 ± 0.003*
Right Kidney 100 g^{-1}	0.44 ± 0.006	0.53 ± 0.010*
Left Kidney 100 g^{-1}	0.43 ± 0.005	0.53 ± 0.011*
Nitrite/Nitrate[2]	0.8 ± 0.5	1.5 ± 0.5
Plasma Volume[3]	49.9 ± 1.4	48.1 ± 0.7
Total Blood Volume[3]	70.7 ± 1.7	73.7 ± 1.0

Each value represents the mean ± SEM of 76 experiments ([1]except $n = 24$; [2]except $n = 10$; [3]except $n = 22$)
*Significantly different from Dahl SRN rats; $P < 0.05$

within the groups of Dahl SRN and Dahl SSH rats (Table 5 & 6). Intravenous infusion of cirazoline (0.4, 0.8, 1.6, and 3.2 $\mu g\ kg^{-1}\ min^{-1}$) produced a dose-dependent increase in mean arterial blood pressure, and decrease in heart rate, mesenteric blood flow, and mesenteric vascular conductance in all groups. The cirazoline dose-response curve for changes in blood pressure was significantly different between Dahl SRN and SSH rats (Fig. 3A). The slope values for blood pressure vs. log[cirazoline] were not significantly different between the two strains (slope = 47.8 ± 2.6 versus 51.8 ± 4.3 respectively; mean ± SEM). There were no differencess in cirazoline-mediated decrease in mesenteric blood flow between the SRN and SSH rats (Fig. 3B). In contrast, cirazoline produced a significantly greater decrease in mesenteric vascular conductance in SRN (slope = 1.13 ± 0.07×10^3; mean ± SEM) when compared to SSH rats (slope = $8.44 \pm 0.80 \times 10^3$; mean ± SEM) (Fig. 3C). The slope values for increase in blood pressure versus decrease in heart rate from baseline at various doses of cirazoline were not different between Dahl SRN and SSH rats (0.80 ± 0.02 versus 0.88 ± 0.02 beats $min^{-1}\ mmHg^{-1}$; mean ± SEM).

In addition, administration of saline and vehicle did not significantly affect cirazoline-induced changes in mean arterial blood pressure, heart rate, mesenteric blood flow or mesenteric vascular conductance when compared to the effects of cirazoline prior to administration of saline and vehicle in Dahl SRN and SSH rats (Fig. 4, 5 & Table 6).

Table 5. Baseline values of mean arterial blood pressure (MAP; mmHg), superior mesenteric arterial blood flow (SMAF; ml min^{-1} $100g^{-1}$), and superior mesenteric arterial conductance (SMAC; ml min^{-1} $mmHg^{-1}$ $100g^{-1}$) values before and after injection of saline + vehicle, L-NAME (0.3 mg kg^{-1}) + vehicle, saline + niflumic acid (10 mg kg^{-1}), or L-NAME (0.3 mg kg^{-1}) + niflumic acid (10 mg kg^{-1}) in Dahl anesthetized Dahl salt-resistant normotensive (SRN) and salt-sensitive hypertensive (SSH) rats.

Table 5

Groups		MAP		SMAF		SMAC	
		SRN	SSH	SRN	SSH	SRN	SSH
Saline + Vehicle	Pre	129±4	177±11[a]	2.86±0.25	2.60±0.21	0.022±0.002	0.015±0.002[a]
	Post	122±4	164±12[a]	2.88±0.32	2.37±0.24	0.024±0.003	0.015±0.002[a]
L-NAME + Vehicle	Pre	124±4	168±12[a]	3.00±0.19	2.58±0.24	0.024±0.002	0.016±0.002[a]
	Post	136±5[b]	177±16[a]	2.48±0.28[b]	2.20±0.29	0.019±0.003[b]	0.013±0.002[a]
Saline + NFA	Pre	120±4	166±7[a]	2.69±0.19	2.65±0.26	0.026±0.004	0.016±0.002[a]
	Post	122±4	164±8[a]	2.89±0.39	2.26±0.23[ab]	0.024±0.003	0.014±0.001[a]
L-NAME + NFA	Pre	129±4	167±11[a]	2.59±0.13	2.88±0.31	0.020±0.001	0.018±0.002[a]
	Post	141±4[b]	175±10[a]	2.18±0.23[b]	1.99±0.16[ab]	0.016±0.002[b]	0.012±0.001[ab]

Each value represents the mean of six experiments ± SEM
[a]Significantly different from the salt-resistant rats; $P < 0.05$
[b]Significantly different from the predrug value; $P < 0.05$

Table 6. Heart rate (beats min^{-1}) values in different groups of animals before (Pre) and after (Post) treatment with saline (0.9% NaCl) + vehicle (0.4 M $NaHCO_3$ in 5% glucose), L-NAME (0.3 mg kg^{-1}) + vehicle, saline + niflumic acid (10 mg kg^{-1}), and L-NAME + niflumic acid during infusion with various doses of cirazoline (μg kg^{-1} min^{-1}) in anesthetized Dahl salt-resistant normotensive (SRN) salt-sensitive hypertensive (SSH) rats.

Table 6

Groups		Saline + Vehicle		L-NAME + Vehicle		Saline + NFA		L-NAME + NFA	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
Control	SRN	348±3	348±11	342±11	322±13	350±10	357±9	364±7	348±6
	SSH	390±12	382±13	370±16	337±9	388±14	388±24	383±10	348±12
Cirazoline 0.4	SRN	313±10[a]	323±11[a]	315±12	302±12	330±11[a]	337±5	340±7[a]	322±3[a]
	SSH	387±10	377±11	360±22	330±15	363±21	372±22	372±11	333±8
0.8	SRN	290±13[a]	297±9[ab]	288±13[ab]	297±12[a]	303±7[a]	317±7[ab]	300±7[ab]	314±2[a]
	SSH	368±16	367±14	343±23[a]	313±21	330±31	348±16	342±16[a]	322±6
1.6	SRN	290±9[a]	285±8[ab]	290±11[ab]	290±10[a]	285±7[ab]	303±7[ab]	302±3[ab]	304±2[a]
	SSH	350±19[ab]	347±17[a]	327±21[ab]	302±19[a]	327±20	325±11[a]	323±17[ab]	303±12[a]
3.2	SRN	288±10[a]	277±9[ab]	290±10[ab]	285±10[a]	280±9[ab]	293±6[abc]	304±4[ab]	286±9[ab]
	SSH	333±18[abc]	323±18[abc]	310±17[abc]	287±17[ab]	317±14[a]	312±9[a]	300±17[abc]	303±13[a]

Each value represents mean of six experiments ± SEM
[a]Significantly different from Control value; $P < 0.05$
[b]Significantly different from 0.4 μg kg^{-1} min^{-1}; $P < 0.05$
[c]Significantly different from 0.8 μg kg^{-1} min^{-1}; $P < 0.05$

Figure 3. Cirazoline dose-response curve for **(A)** mean arterial blood pressure (MAP), **(B)** superior mesenteric arterial blood flow (SMAF), and **(C)** superior mesenteric arterial conductance (SMAC) obtained from Dahl salt-resistant normotensive (SRN) (closed circles) and Dahl salt-sensitive hypertensive (SSH) (opened circles) rats. Each value represents mean ± SEM of 24 experiments. *Significantly different from corresponding value in Dahl SRN rats; $P < 0.05$.

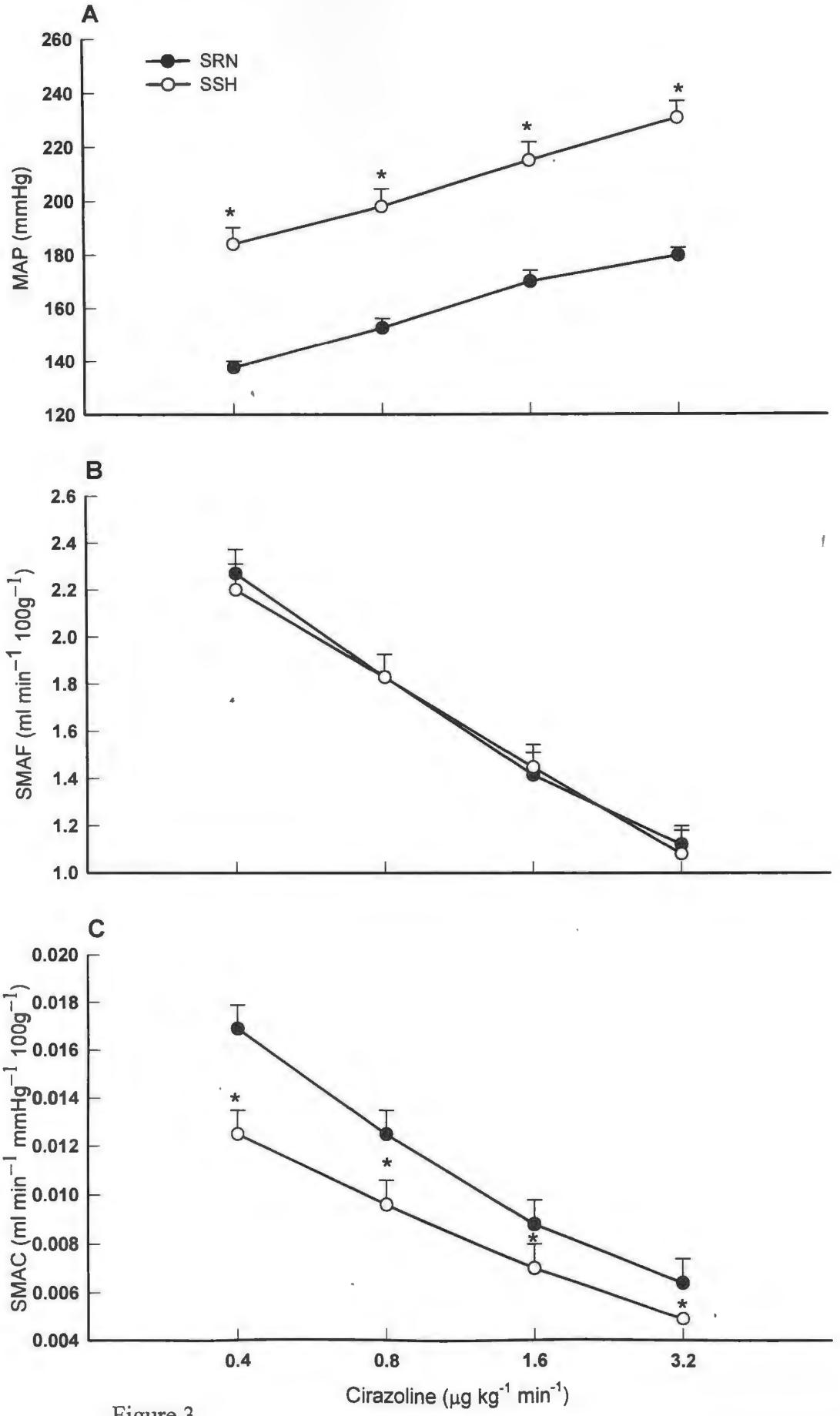

A
SRN
SSH
MAP (mmHg)
260
240
220
200
180
160
140
120
B
SMAF (ml min^{-1} 100g^{-1})
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
C
SMAC (ml min^{-1} mmHg^{-1} 100g^{-1})
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.4
0.8
1.6
3.2
Cirazoline (μg kg^{-1} min^{-1})

Figure 3

Figure 4. Cirazoline dose-response relationship in the absence (open bars) and presence of saline (0.9% NaCl) and vehicle (0.4 M $NaHCO_3$ in 5% glucose; hatched bars) on **(A)** mean arterial blood pressure (MAP), **(B)** superior mesenteric arterial blood flow (SMAF), and **(C)** superior mesenteric arterial conductance (SMAC) obtained from Dahl salt-resistant normotensive (SRN) rats. Each value represents mean ± SEM of six experiments, and is a percentage of control prior to infusion of cirazoline.

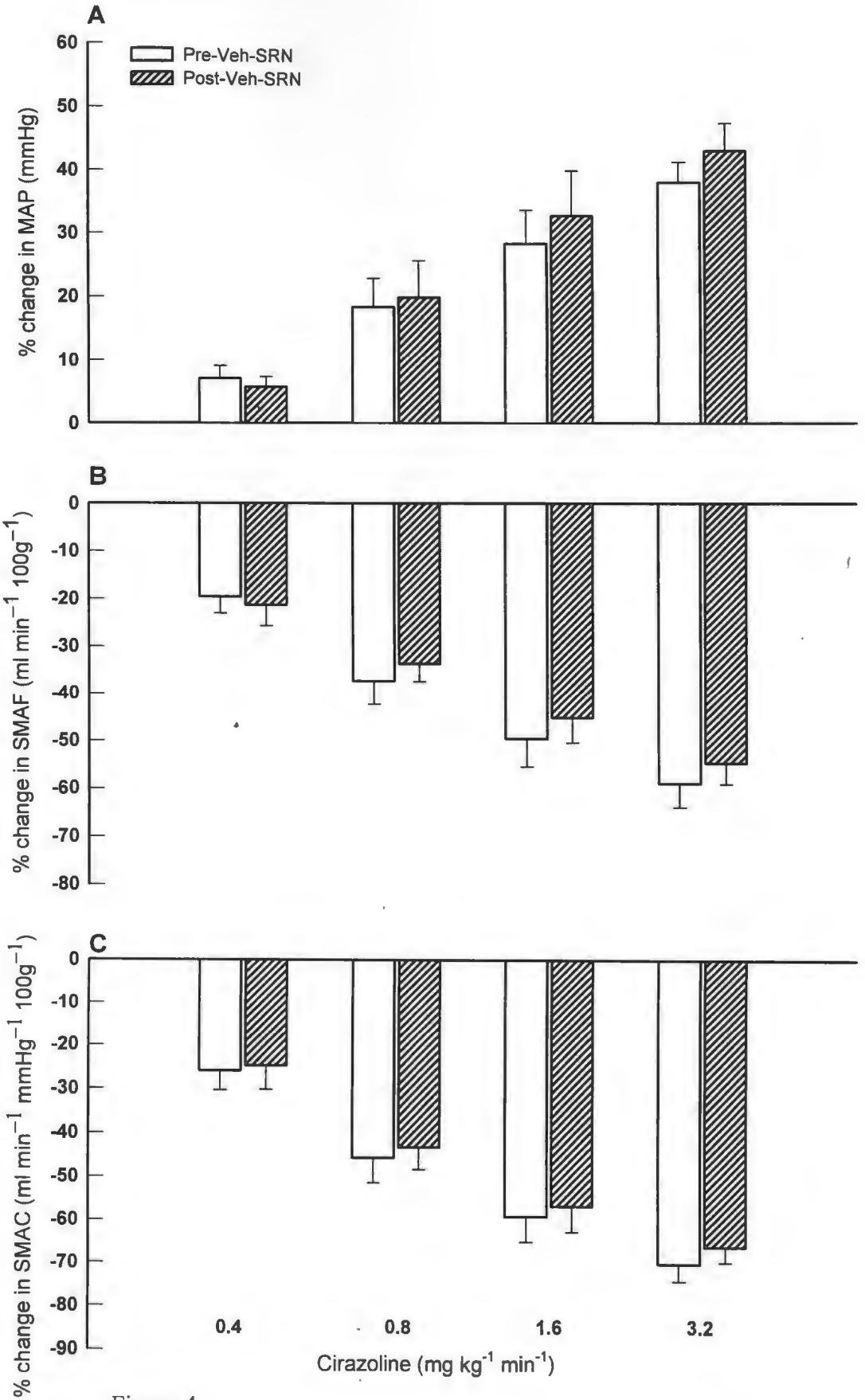

A
Pre-Veh-SRN
Post-Veh-SRN
% change in MAP (mmHg)
60
50
40
30
20
10
0
B
% change in SMAF (ml min^{-1} 100g^{-1})
0
-10
-20
-30
-40
-50
-60
-70
-80
C
% change in SMAC (ml min^{-1} mmHg^{-1} 100g^{-1})
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
0.4
0.8
1.6
3.2
Cirazoline (mg kg^{-1} min^{-1})

Figure 4

Figure 5. Cirazoline dose-response relationship in the absence (open bars) and presence of saline (0.9% NaCl) and vehicle (0.4 M $NaHCO_3$ in 5% glucose; hatched bars) on **(A)** mean arterial blood pressure (MAP), **(B)** superior mesenteric arterial blood flow (SMAF), and **(C)** superior mesenteric arterial conductance (SMAC) obtained from Dahl salt-sensitive hypertensive (SSH) rats. Each value represents mean ± SEM of six experiments, and is a percentage of control prior to infusion of cirazoline.

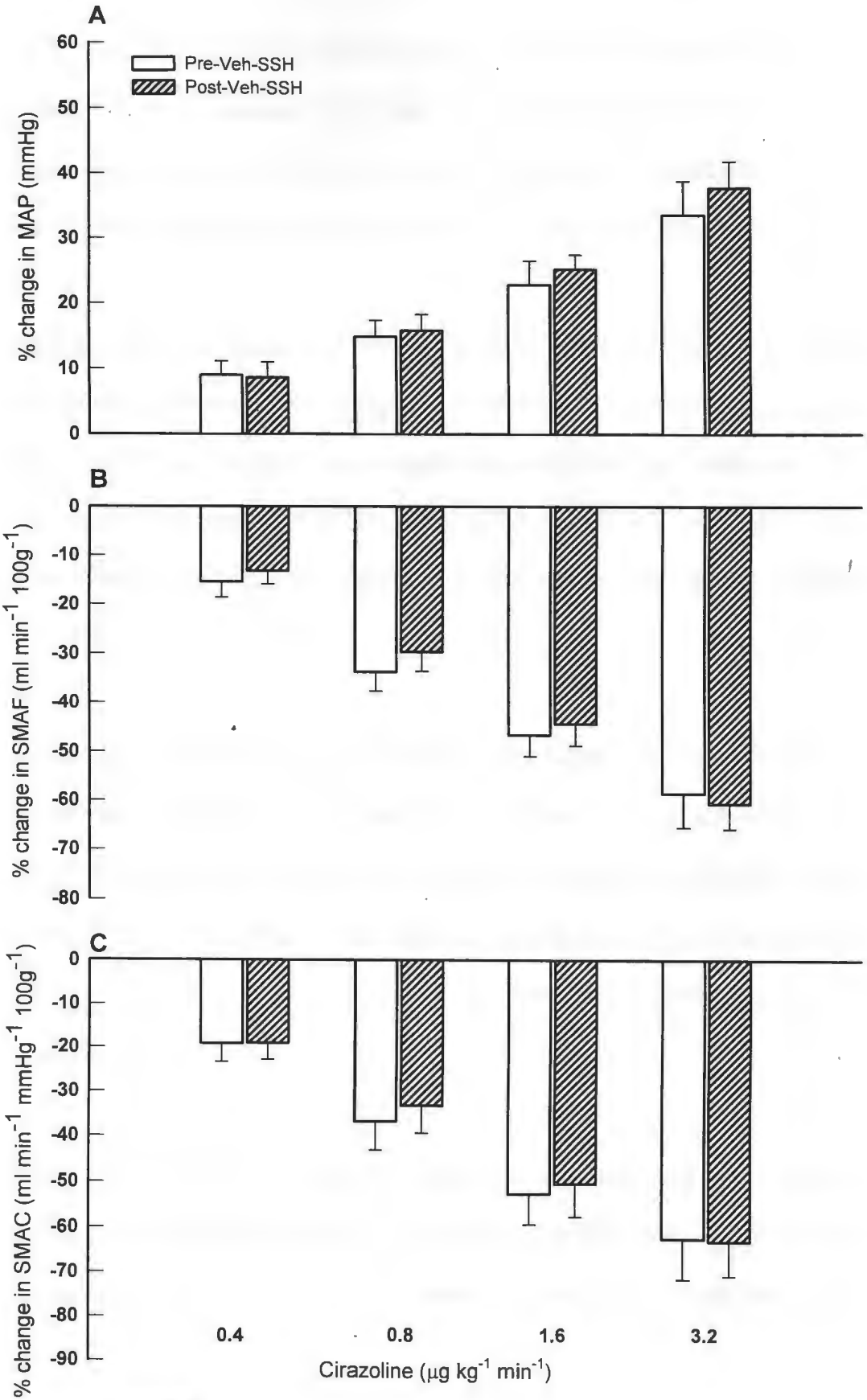

A
Pre-Veh-SSH
Post-Veh-SSH
% change in MAP (mmHg)
60
50
40
30
20
10
0
B
% change in SMAF (ml min-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
C
% change in SMAC (ml min-1 mmHg-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
0.4
0.8
1.6
3.2
Cirazoline (μg kg-1 min-1)

Figure 5

3.1.2. Effects of L-NAME on vascular effects of cirazoline in mesenteric bed

Bolus intravenous injection of L-NAME (0.3 mg kg^{-1}) significantly increased the baseline value of mean arterial blood pressure and decreased mesenteric blood flow and mesenteric vascular conductance in Dahl SRN rats but not in Dahl SSH rats (Table 5).

Administration of L-NAME did not affect cirazoline-mediated increases in blood pressure or decreases in heart rate when compared to control in either strains (Fig. 6A, 7A & Table 6). L-NAME was found to blunt cirazoline-mediated reduction in mesenteric blood flow and vascular conductance in SRN rats (Fig. 6BC). In SSH rats, L-NAME blunted cirazoline-mediated reduction in mesenteric blood flow but not vascular conductance (Fig. 7BC).

3.1.3. Effects of niflumic acid on vascular effects of cirazoline in mesenteric bed

Bolus intravenous injection of niflumic acid (10 mg kg^{-1}) did not affect the baseline values of mean arterial blood pressure, mesenteric blood flow, mesenteric vascular conductance and heart rate in Dahl SRN rats, while in the SSH rats, mesenteric blood flow was reduced but not mesenteric vascular conductance or blood pressure or heart rate (Table 5, 6).

Administration of niflumic acid did not affect cirazoline-mediated increases in mean arterial blood pressure or decreases in heart rate when compared to control in either strains of rat (Fig. 8A, 9A & Table 6). However, cirazoline-mediated reduction in

Figure 6. Cirazoline dose-response relationship in the absence (open bars) and presence of L-NAME (0.3 mg kg^{-1}; hatched bars) and vehicle (0.4 M $NaHCO_3$ in 5% glucose) on **(A)** mean arterial blood pressure (MAP), **(B)** superior mesenteric arterial blood flow (SMAF), and **(C)** superior mesenteric arterial conductance (SMAC) obtained from Dahl salt-resistant normotensive (SRN) rats. Each value represents mean ± SEM of six experiments, and is a percentage of control prior to infusion of cirazoline. *Significantly different from respective pre-drug value; $P < 0.05$.

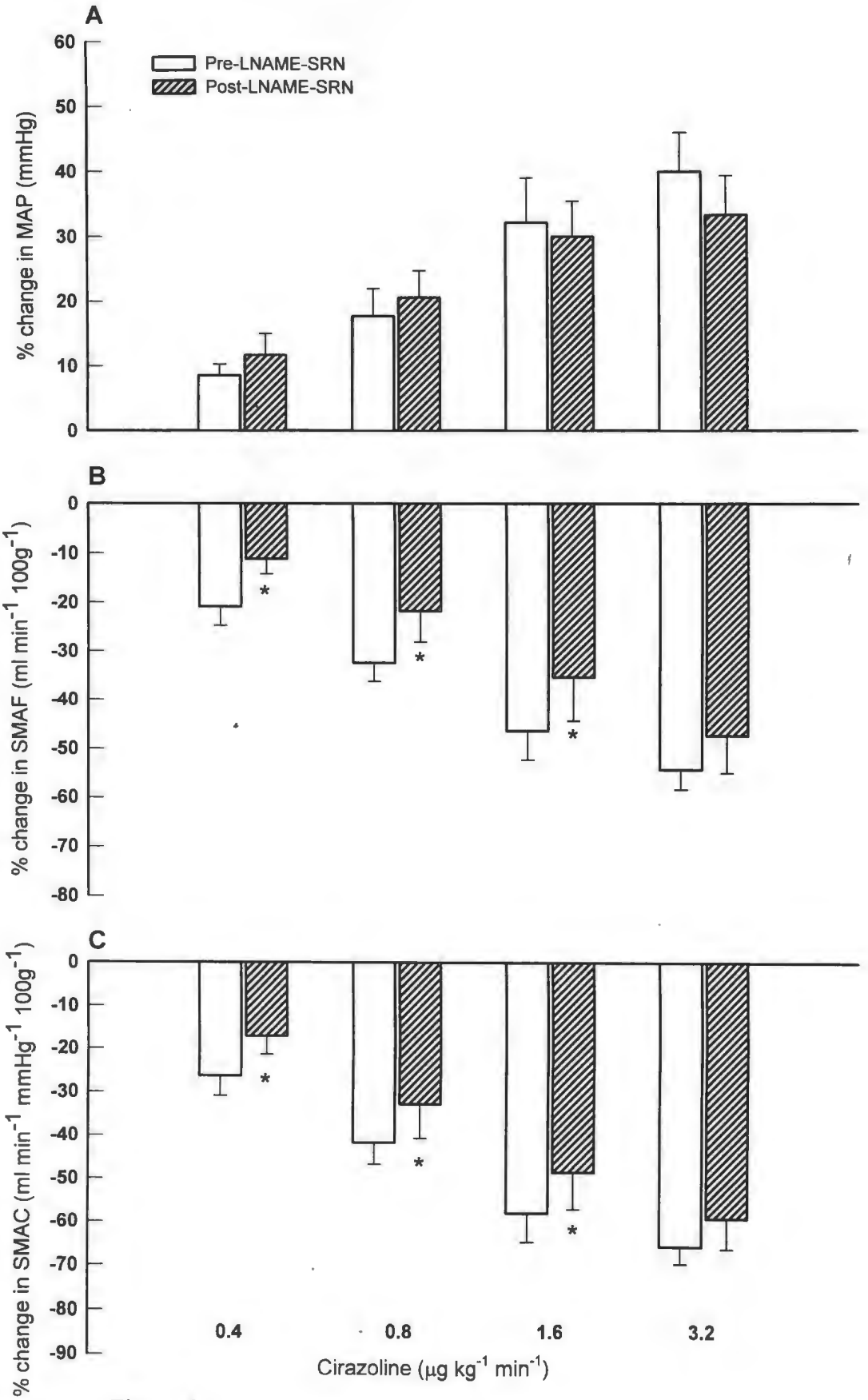

A
Pre-LNAME-SRN
Post-LNAME-SRN
% change in MAP (mmHg)
60
50
40
30
20
10
0
B
% change in SMAF (ml min-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
C
% change in SMAC (ml min-1 mmHg-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
*
0.4
0.8
1.6
3.2
Cirazoline (μg kg-1 min-1)

Figure 6

Figure 7. Cirazoline dose-response relationship in the absence (open bars) and presence of L-NAME (0.3 mg kg^{-1}; hatched bars) and vehicle (0.4 M $NaHCO_3$ in 5% glucose) on (**A**) mean arterial blood pressure (MAP), (**B**) superior mesenteric arterial blood flow (SMAF), and (**C**) superior mesenteric arterial conductance (SMAC) obtained from Dahl salt-sensitive hypertensive (SSH) rats. Each value represents mean ± SEM of six experiments, and is a percentage of control prior to infusion of cirazoline. *Significantly different from respective pre-drug value; $P < 0.05$.

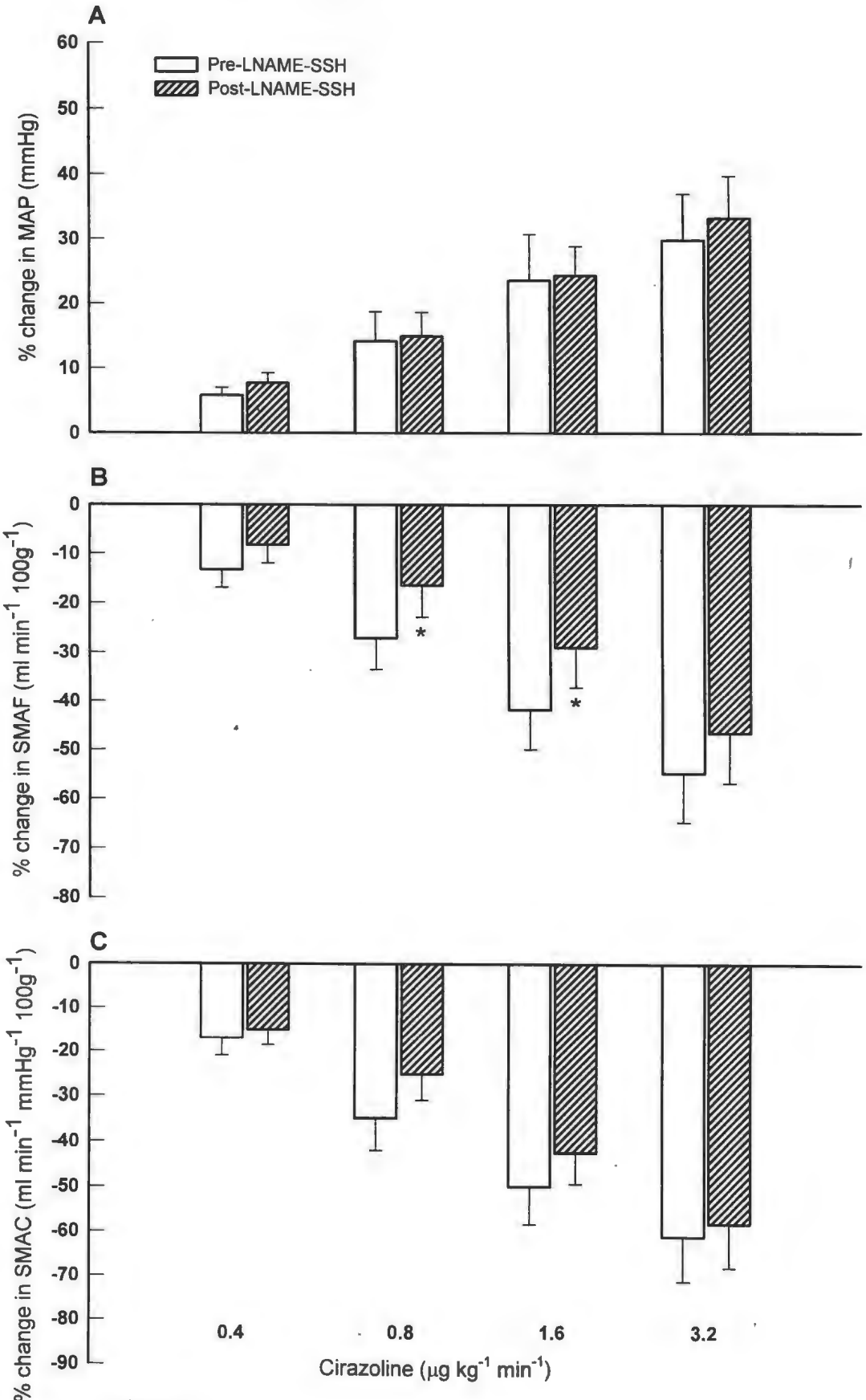

A
Pre-LNAME-SSH
Post-LNAME-SSH
% change in MAP (mmHg)
60
50
40
30
20
10
0
B
% change in SMAF (ml min-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
*
*
C
% change in SMAC (ml min-1 mmHg-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
0.4
0.8
1.6
3.2
Cirazoline (μg kg-1 min-1)

Figure 7

Figure 8. Cirazoline dose-response relationship in the absence (open bars) and presence of saline (0.9% NaCl) and niflumic acid (10 mg kg^{-1}; hatched bars) on **(A)** mean arterial blood pressure (MAP), **(B)** superior mesenteric arterial blood flow (SMAF), and **(C)** superior mesenteric arterial conductance (SMAC) obtained from Dahl salt-resistant normotensive (SRN) rats. Each value represents mean ± SEM of six experiments, and is a percentage of control prior to infusion of cirazoline. *Significantly different from respective pre-drug value; $P < 0.05$.

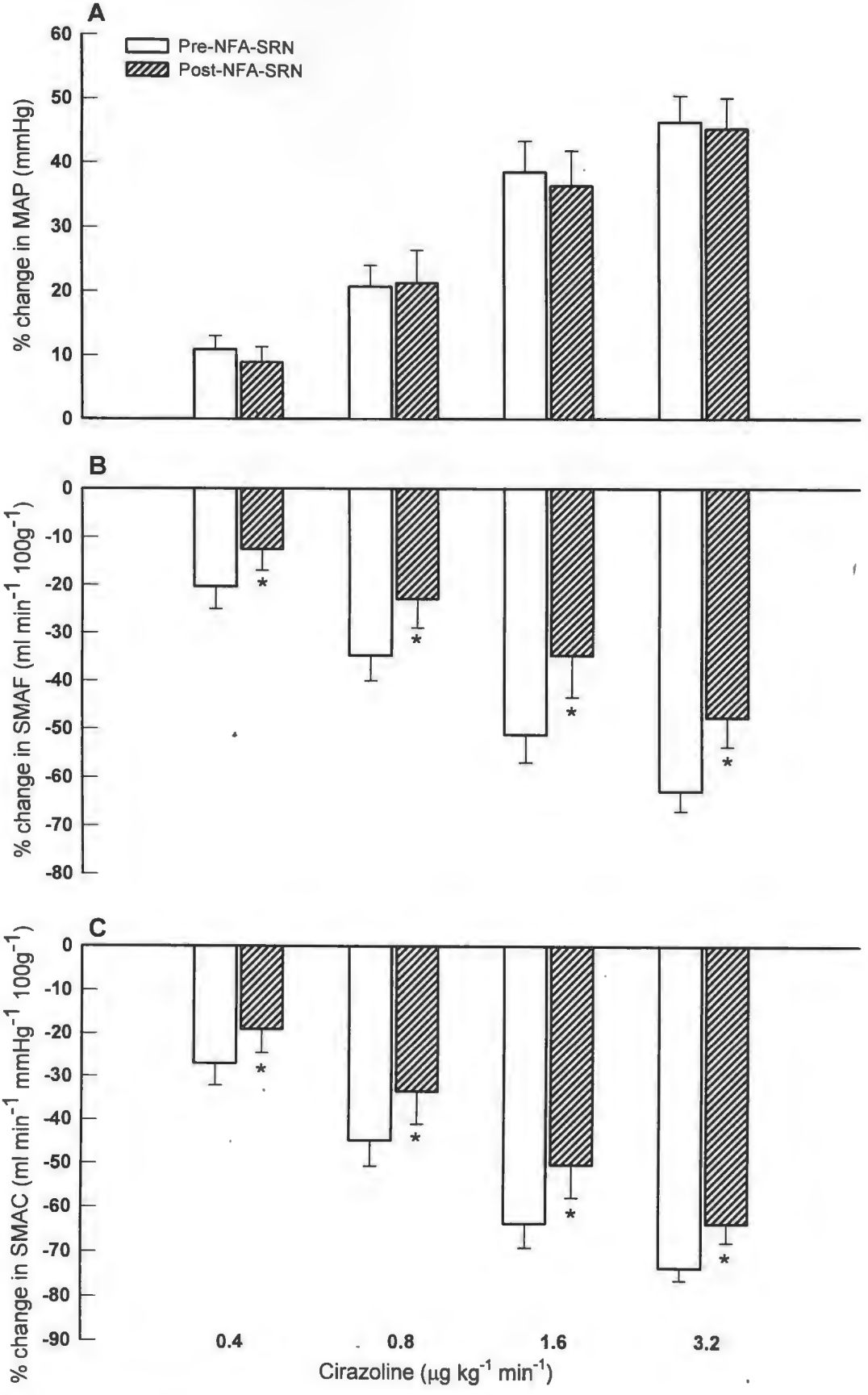

A
Pre-NFA-SRN
Post-NFA-SRN
% change in MAP (mmHg)
60
50
40
30
20
10
0
B
% change in SMAF (ml min-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
C
% change in SMAC (ml min-1 mmHg-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
0.4
0.8
1.6
3.2
Cirazoline (μg kg-1 min-1)

Figure 8

Figure 9. Cirazoline dose-response relationship in the absence (open bars) and presence of saline (0.9% NaCl) and niflumic acid (10 mg kg^{-1}; hatched bars) on **(A)** mean arterial blood pressure (MAP), **(B)** superior mesenteric arterial blood flow (SMAF), and **(C)** superior mesenteric arterial conductance (SMAC) obtained from Dahl salt-sensitive hypertensive (SSH) rats. Each value represents mean ± SEM of six experiments, and is a percentage of control prior to infusion of cirazoline. *Significantly different from respective pre-drug value; $P < 0.05$.

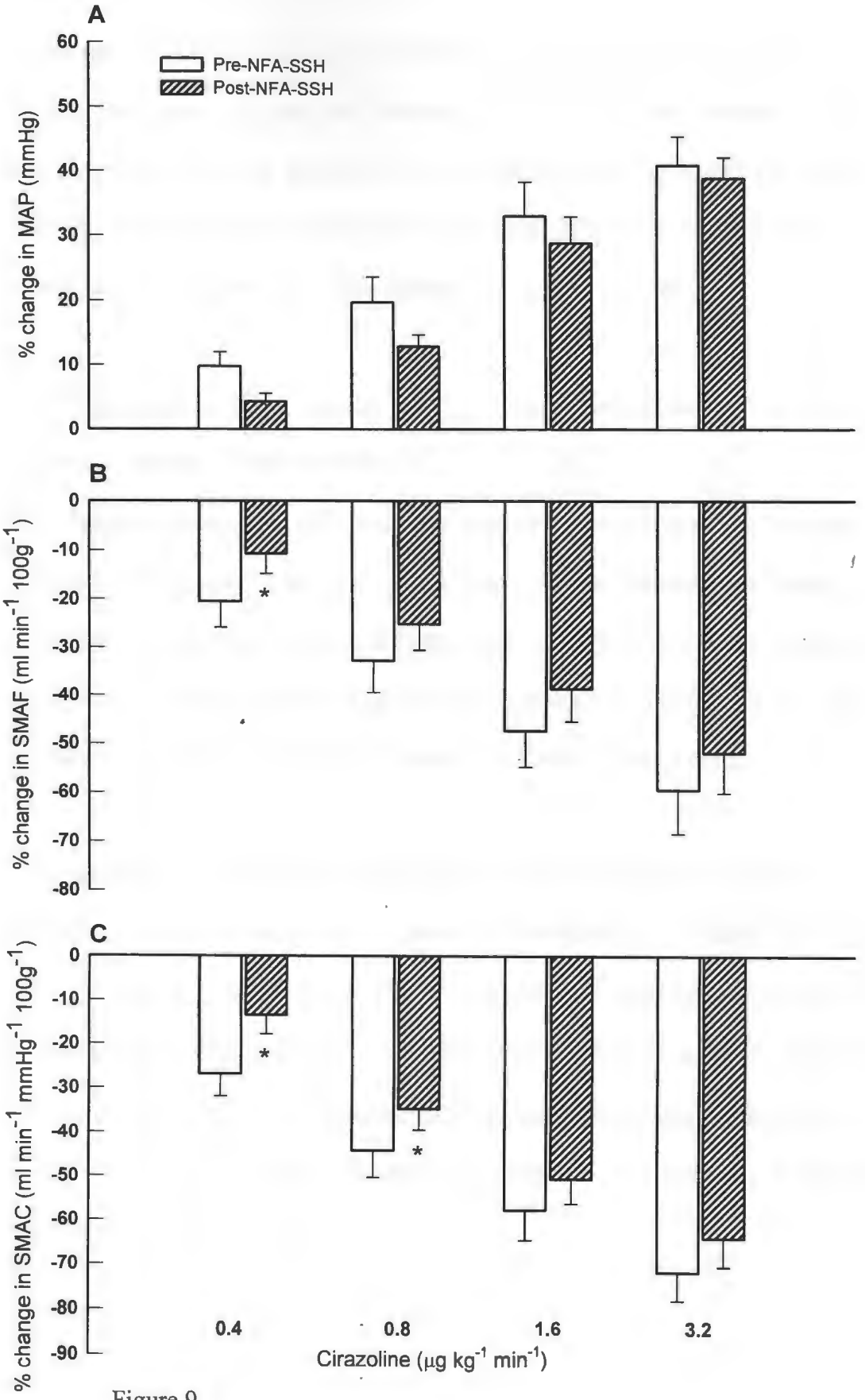

A
Pre-NFA-SSH
Post-NFA-SSH
% change in MAP (mmHg)
60
50
40
30
20
10
0
B
% change in SMAF (ml min-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
*
C
% change in SMAC (ml min-1 mmHg-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
*
*
0.4
0.8
1.6
3.2
Cirazoline (μg kg-1 min-1)

Figure 9

mesenteric blood flow and mesenteric vascular conductance at all the doses of α_1-adrenoceptor agonist in Dahl SRN rats was significantly attenuated following niflumic acid treatment (Fig. 8BC). In the SSH rats, treatment with niflumic acid was only able to attenuate cirazoline-mediated reduction in mesenteric blood flow and mesenteric vascular conductance at the lower dose of α_1-adrenoceptor agonist (Fig. 9BC).

3.1.4. Effect of concomitant administration of L-NAME and niflumic acid on vascular effects of cirazoline in mesenteric bed

Bolus intravenous injection of L-NAME (0.3 mg kg^{-1}) and niflumic acid (10 mg kg^{-1}) significantly increased the baseline value of blood pressure and attenuated mesenteric blood flow and vascular conductance in SRN rats. In the SSH rats, the combination of L-NAME and niflumic acid reduced the baseline values of mesenteric blood flow and vascular conductance but not blood pressure or heart rate (Table 5 & 6).

Administration of L-NAME and niflumic acid combined significantly attenuated cirazoline-mediated increase in blood pressure in the SRN but not in SSH rats (Fig. 10A, 11A). In contrast to the actions of niflumic acid alone, the concomitant administration of L-NAME and niflumic acid significantly attenuated α_1-adrenoceptor mediated reduction in mesenteric blood flow and vascular conductance in a similar manner in both strains (Fig. 10BC, 11BC). The inhibitory effect was significant at all four doses of cirazoline in Dahl SRN as well as SSH rats.

Figure 10. Cirazoline dose-response relationship in the absence (open bars) and presence of L-NAME (0.3 mg kg^{-1}) and niflumic acid (10 mg kg^{-1}; hatched bars) on **(A)** mean arterial blood pressure (MAP), **(B)** superior mesenteric arterial blood flow (SMAF), and **(C)** superior mesenteric arterial conductance (SMAC) obtained from Dahl salt-resistant normotensive (SRN) rats. Each value represents mean ± SEM of six experiments, and is a percentage of control prior to infusion of cirazoline. *Significantly different from respective pre-drug value; $P < 0.05$.

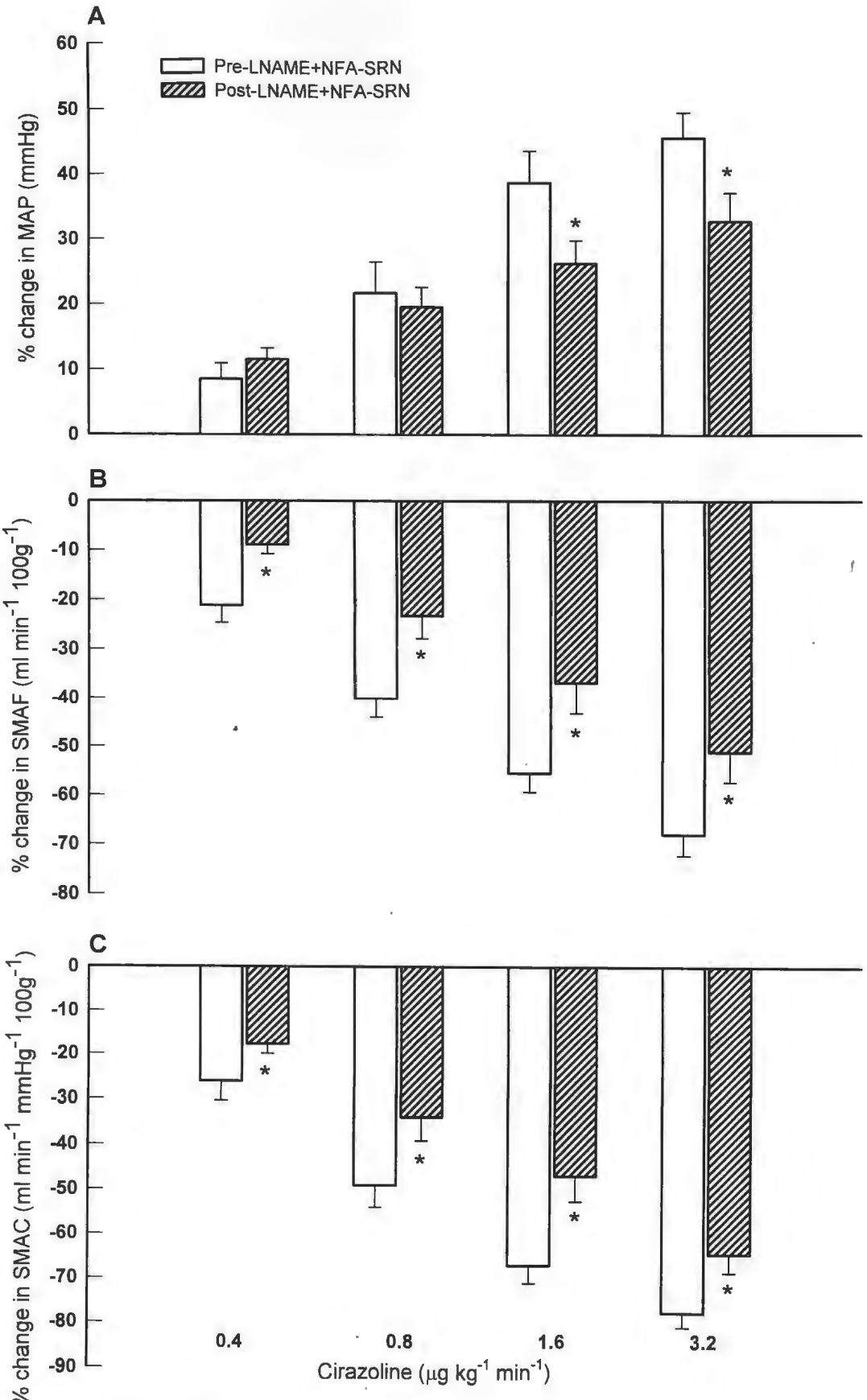

A
Pre-LNAME+NFA-SRN
Post-LNAME+NFA-SRN
% change in MAP (mmHg)
60
50
40
30
20
10
0
B
% change in SMAF (ml min-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
C
% change in SMAC (ml min-1 mmHg-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
0.4
0.8
1.6
3.2
Cirazoline (μg kg-1 min-1)

Figure 10

Figure 11. Cirazoline dose-response relationship in the absence (open bars) and presence of L-NAME (0.3 mg kg^{-1}) and niflumic acid (10 mg kg^{-1}; hatched bars) on **(A)** mean arterial blood pressure (MAP), **(B)** superior mesenteric arterial blood flow (SMAF), and **(C)** superior mesenteric arterial conductance (SMAC) obtained from Dahl salt-sensitive hypertensive (SSH) rats. Each value represents mean ± SEM of six experiments, and is a percentage of control prior to infusion of cirazoline. *Significantly different from respective pre-drug value; $P < 0.05$.

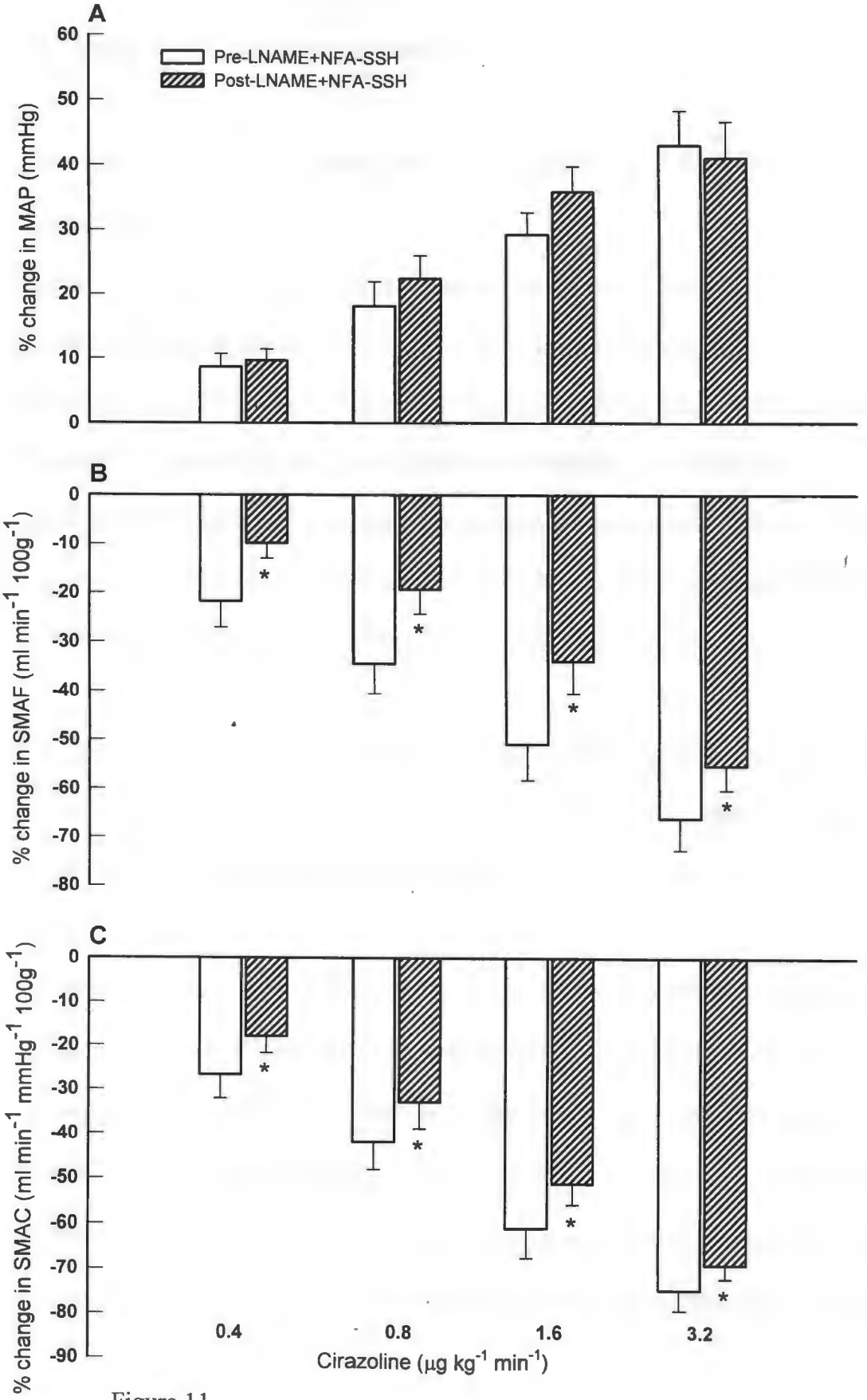

A
Pre-LNAME+NFA-SSH
Post-LNAME+NFA-SSH
% change in MAP (mmHg)
60
50
40
30
20
10
0
B
% change in SMAF (ml min-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
C
% change in SMAC (ml min-1 mmHg-1 100g-1)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
0.4
0.8
1.6
3.2
Cirazoline (μg kg-1 min-1)

Figure 11

3.2. Studies in isolated perfused mesenteric bed preparation

3.2.1. Effect of cirazoline in isolated mesenteric bed perfused with normal Krebs and Cl^--free buffer

The basal perfusion pressures in isolated mesenteric arteries of Dahl SRN and SSH rats, perfused with normal Krebs, were 22.6 ± 1.2 and 21.0 ± 1.2 mmHg (mean ± SEM; n = 36), respectively. There was no significant difference between basal perfusion pressures of Dahl SRN versus SSH rats. Bolus injections of cirazoline (0.1-30 nmoles) evoked dose-dependent constrictor responses in the isolated mesenteric arteries from Dahl SRN and SSH rats (Fig 12-17). Cirazoline-evoked increases in perfusion pressure were found not to be significantly different between the Dahl SRN and SSH rats (Fig 12-17).

Addition of vehicle in normal Krebs buffer did not significantly affect cirazoline-evoked vasoconstriction when compared to the effects of cirazoline prior to administration of vehicle in Dahl SRN and SSH rats (Fig 12AB).

A switch of perfusion medium from normal Krebs to Cl^--free buffer produced a transient increase in perfusion pressure which returned to basal levels within 10 min. This transient increase in perfusion pressure in Cl^--free buffer was significantly smaller in Dahl SRN when compared to SSH rats (26.4 ± 3 and 44.1 ± 7 mmHg, respectively; mean ± SEM; n = 12). In Cl^--free buffer when compared to normal Krebs, cirazoline-induced vasoconstriction in blood vessels from Dahl SRN rats was significantly attenuated at the

Figure 12. Cirazoline dose-response relationship in rat isolated mesenteric arterial beds obtained from Dahl salt-resistant normotensive (SRN) **(A)** and salt-sensitive hypertensive (SSH) **(B)** rats, perfused with normal Krebs in the absence (open columns) and the presence of vehicle (0.4 M $NaHCO_3$; hatched columns). Each value represents the mean of six experiments ± SEM.

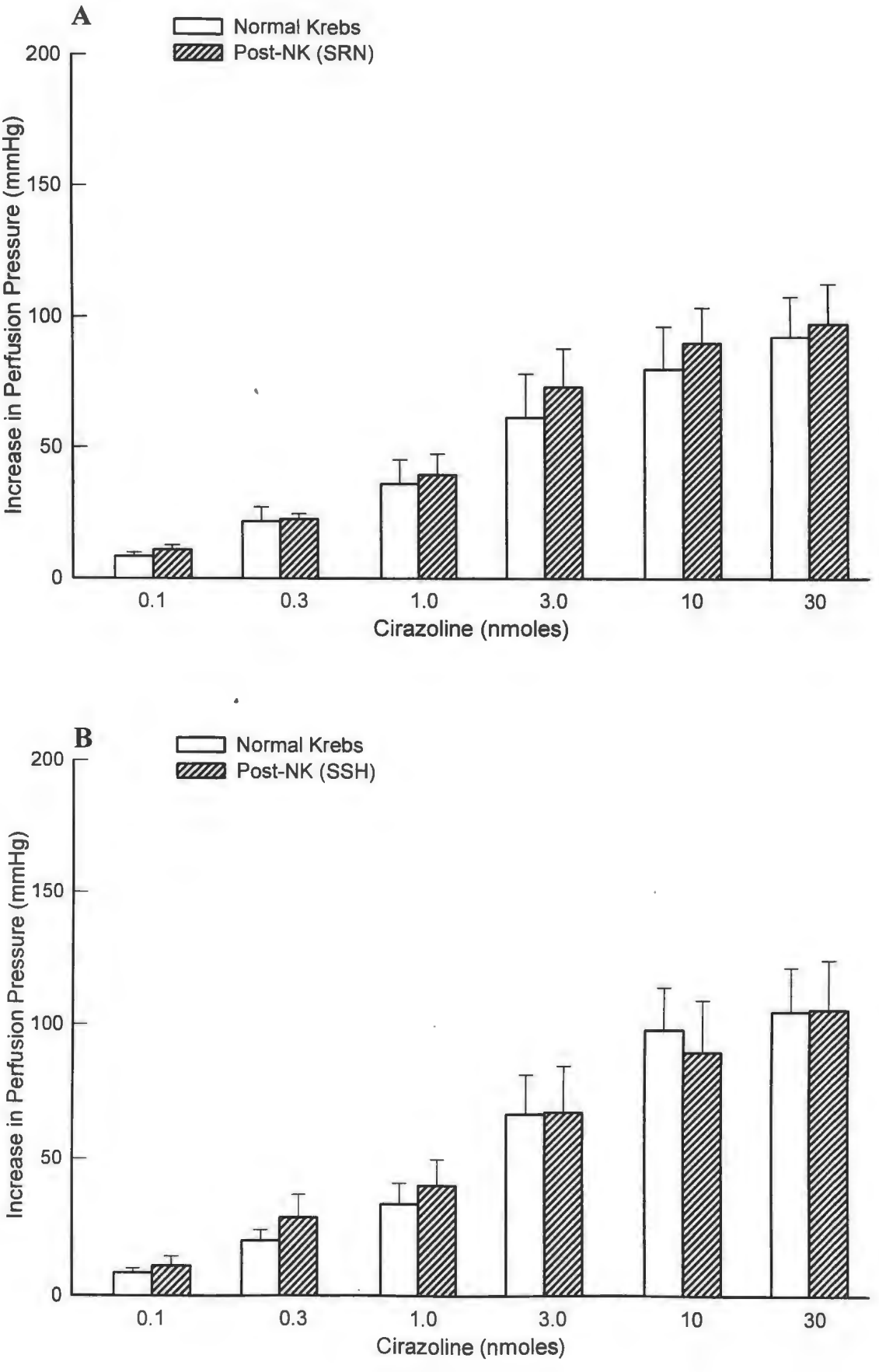

A
Normal Krebs
Post-NK (SRN)
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)
B
Normal Krebs
Post-NK (SSH)
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)

Figure 12

Figure 13. Cirazoline dose-response relationship in rat isolated mesenteric arterial beds obtained from Dahl salt-resistant normotensive (SRN) **(A)** and salt-sensitive hypertensive (SSH) **(B)** rats, perfused with normal Krebs (open columns) and with Cl^--free buffer (hatched columns). Each value represents the mean of six experiments ± SEM.

[a]significantly different from value in normal Krebs; $P < 0.05$.

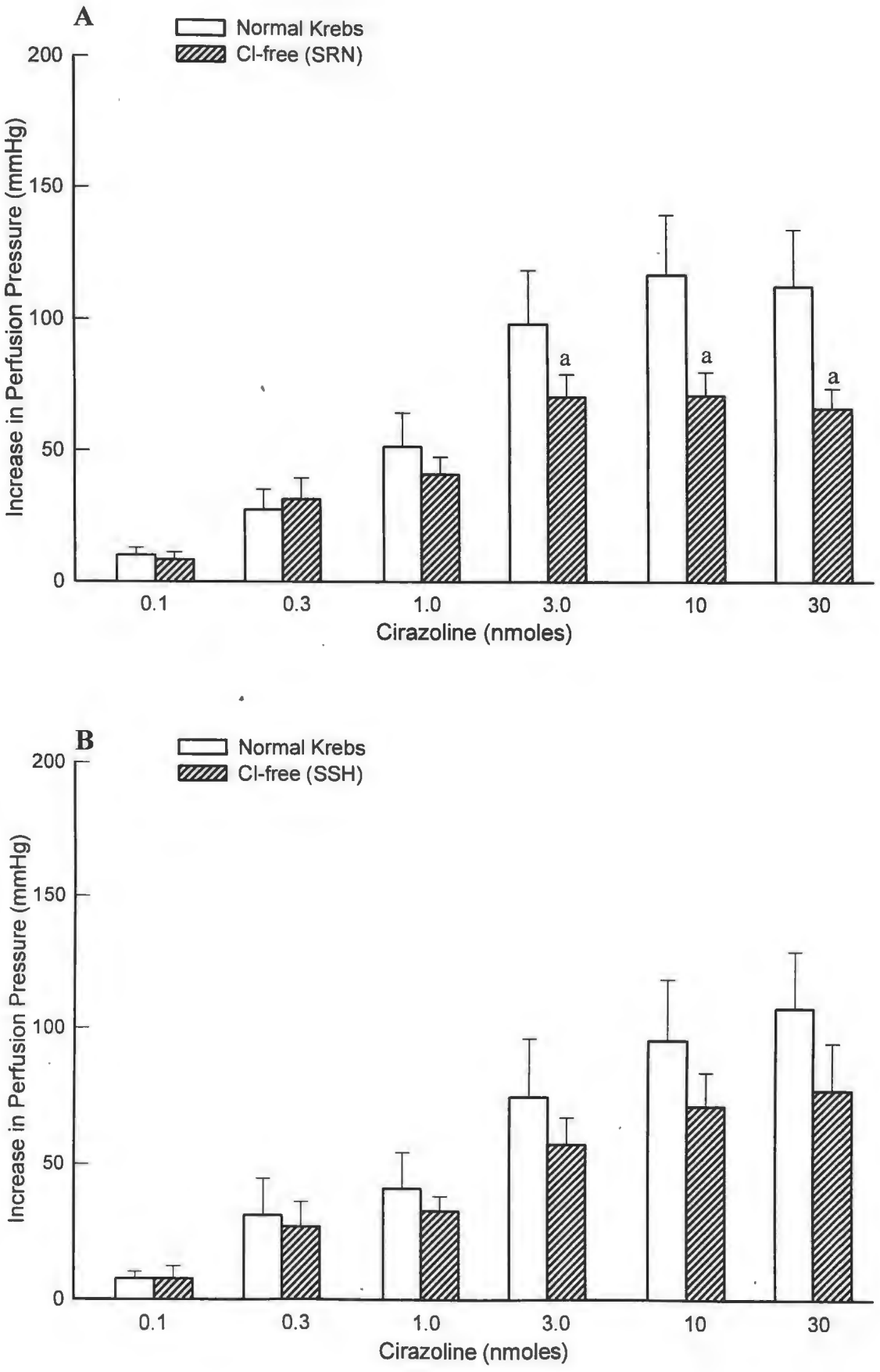
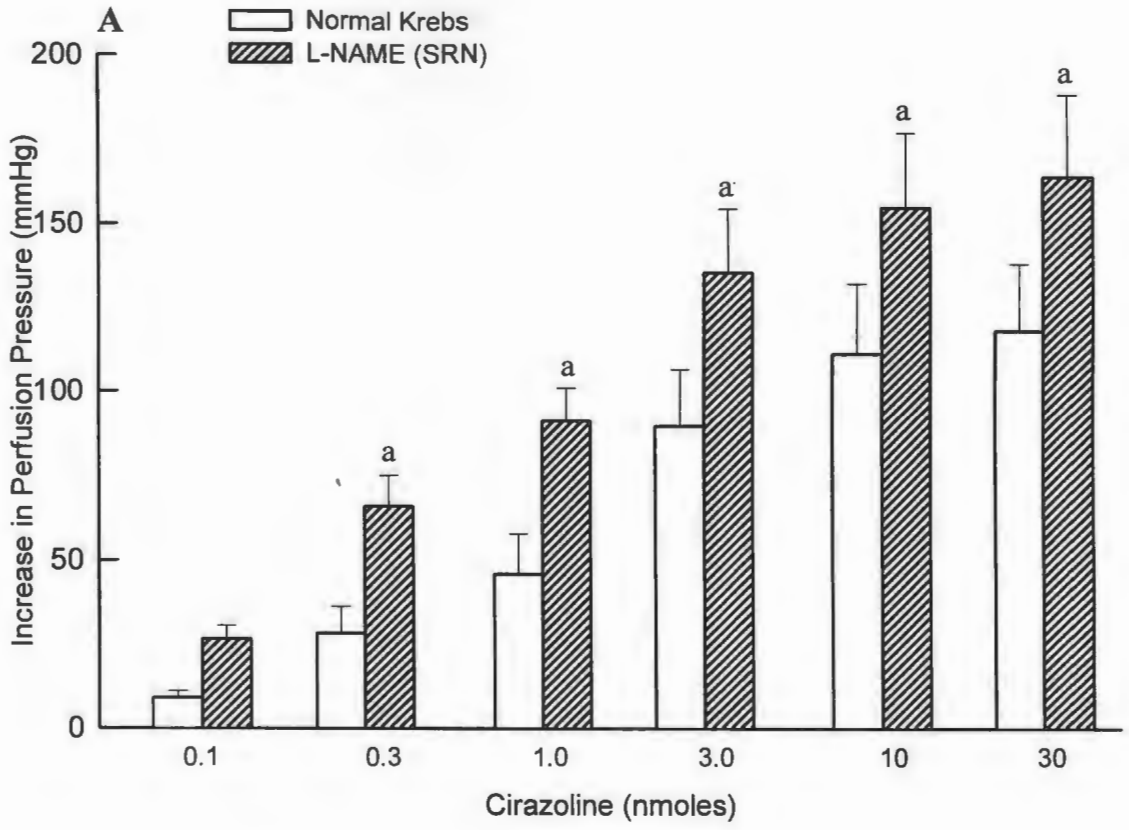

A
Normal Krebs
Cl-free (SRN)
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
a
a
a
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)
B
Normal Krebs
Cl-free (SSH)
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)

Figure 13

Figure 14. Cirazoline dose-response relationship in rat isolated mesenteric arterial beds obtained from Dahl salt-resistant normotensive (SRN) **(A)** and salt-sensitive hypertensive (SSH) **(B)** rats, perfused with normal Krebs in the absence (open columns) and the presence of L-NAME (10 μM; hatched columns). Each value represents the mean of six experiments ± SEM. [a]significantly different from value in normal Krebs. [b]significantly different from corresponding value in Dahl SRN rats; $P < 0.05$.


A
Normal Krebs
L-NAME (SRN)
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
a
a
a
a
a
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)

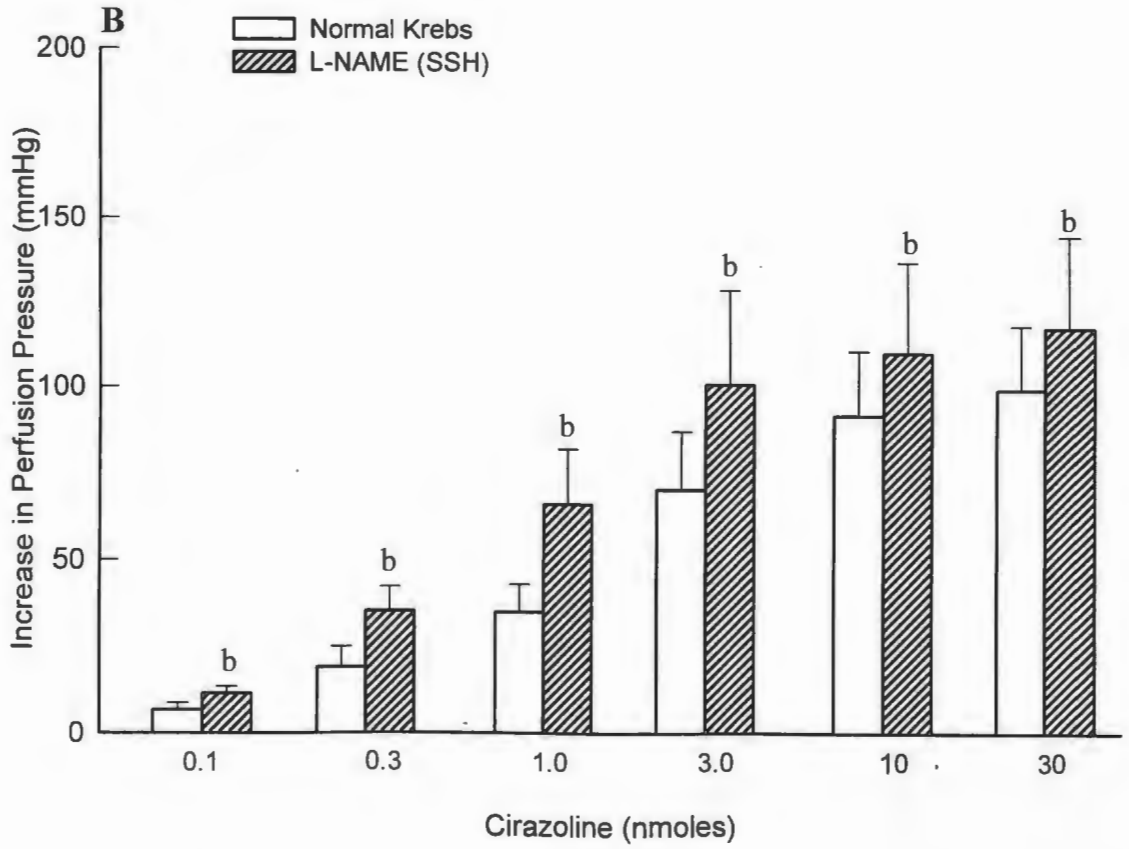

B
Normal Krebs
L-NAME (SSH)
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
b
b
b
b
b
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)

Figure 14

Figure 15. Cirazoline dose-response relationship in rat isolated mesenteric arterial beds obtained from Dahl salt-resistant normotensive (SRN) **(A)** and salt-sensitive hypertensive (SSH) **(B)** rats, perfused with normal Krebs buffer (open columns) and Cl^--free buffer in the presence of L-NAME (10 μM; hatched columns). Each value represents the mean of six experiments ± SEM. [a]significantly different from value in normal Krebs; $P < 0.05$.

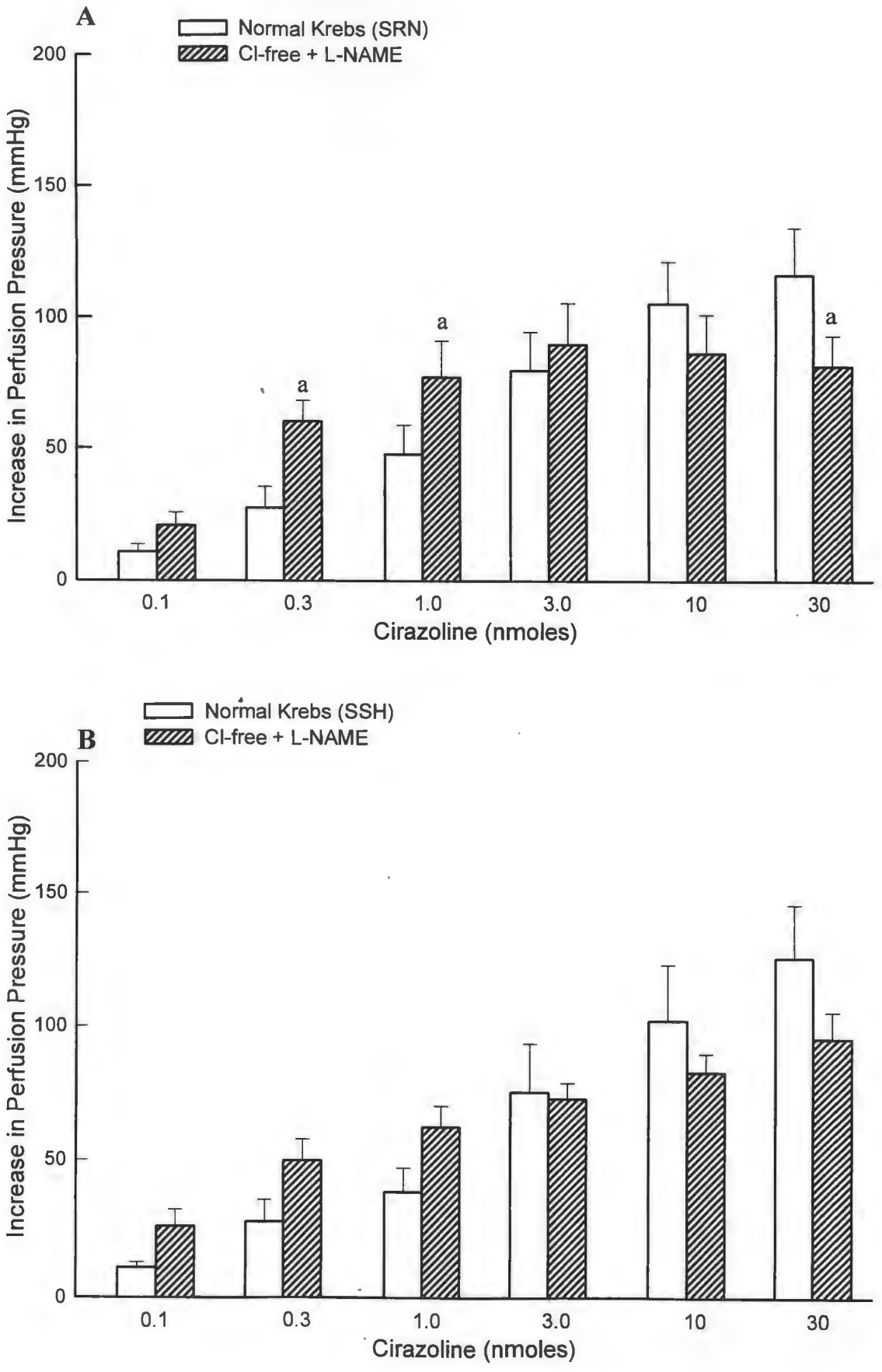

A
Normal Krebs (SRN)
Cl-free + L-NAME
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
a
a
a
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)
B
Normal Krebs (SSH)
Cl-free + L-NAME
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)

Figure 15

Figure 16. Cirazoline dose-response relationship in rat isolated mesenteric arterial beds obtained from Dahl salt-resistant normotensive (SRN) **(A)** and salt-sensitive hypertensive (SSH) **(B)** rats, perfused with normal Krebs in the absence (open columns) and the presence of niflumic acid (10 μM; hatched columns). Each value represents the mean of six experiments ± SEM. [a]significantly different from value in normal Krebs. [b]significantly different from corresponding value in Dahl SRN rats; $P < 0.05$.

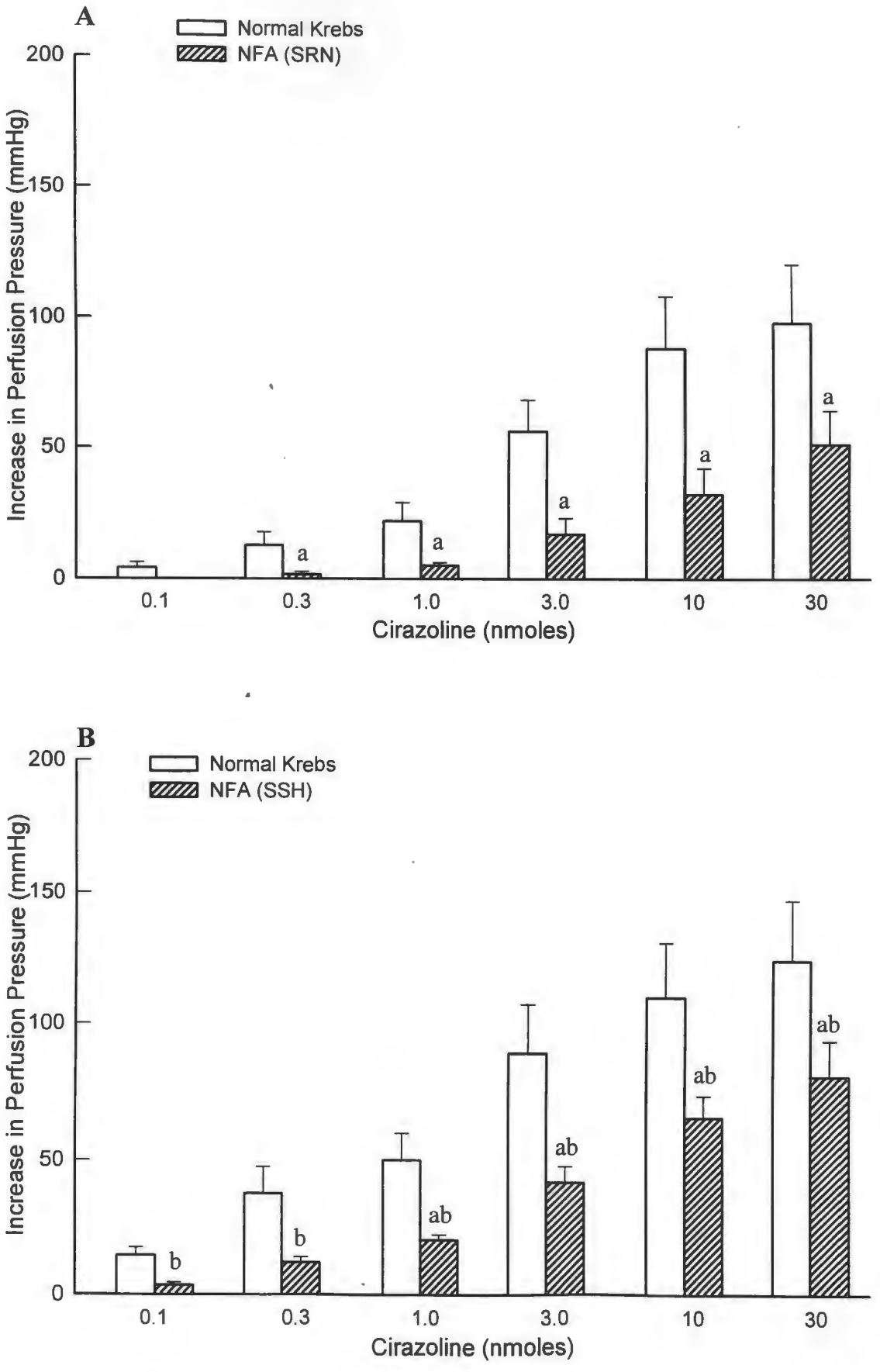
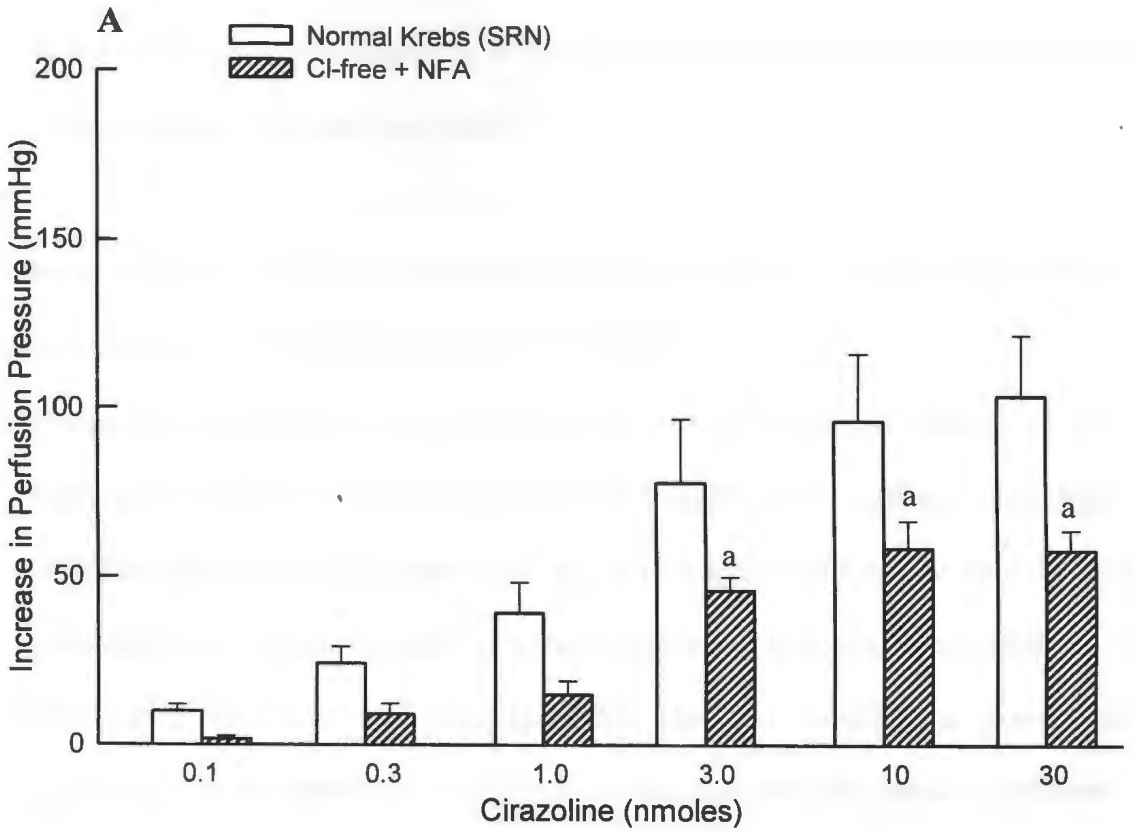

A
Normal Krebs
NFA (SRN)
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
a
a
a
a
a
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)
B
Normal Krebs
NFA (SSH)
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
b
b
ab
ab
ab
ab
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)

Figure 16

Figure 17. Cirazoline dose-response relationship in rat isolated mesenteric arterial beds obtained from Dahl salt-resistant normotensive (SRN) **(A)** and salt-sensitive hypertensive (SSH) **(B)** rats, perfused with normal Krebs buffer (open columns) and Cl^--free buffer in the presence of niflumic acid (10 μM; hatched columns). Each value represents the mean of six experiments ± SEM. [a]significantly different from value in normal Krebs; $P < 0.05$.


A
Normal Krebs (SRN)
Cl-free + NFA
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
a
a
a
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)

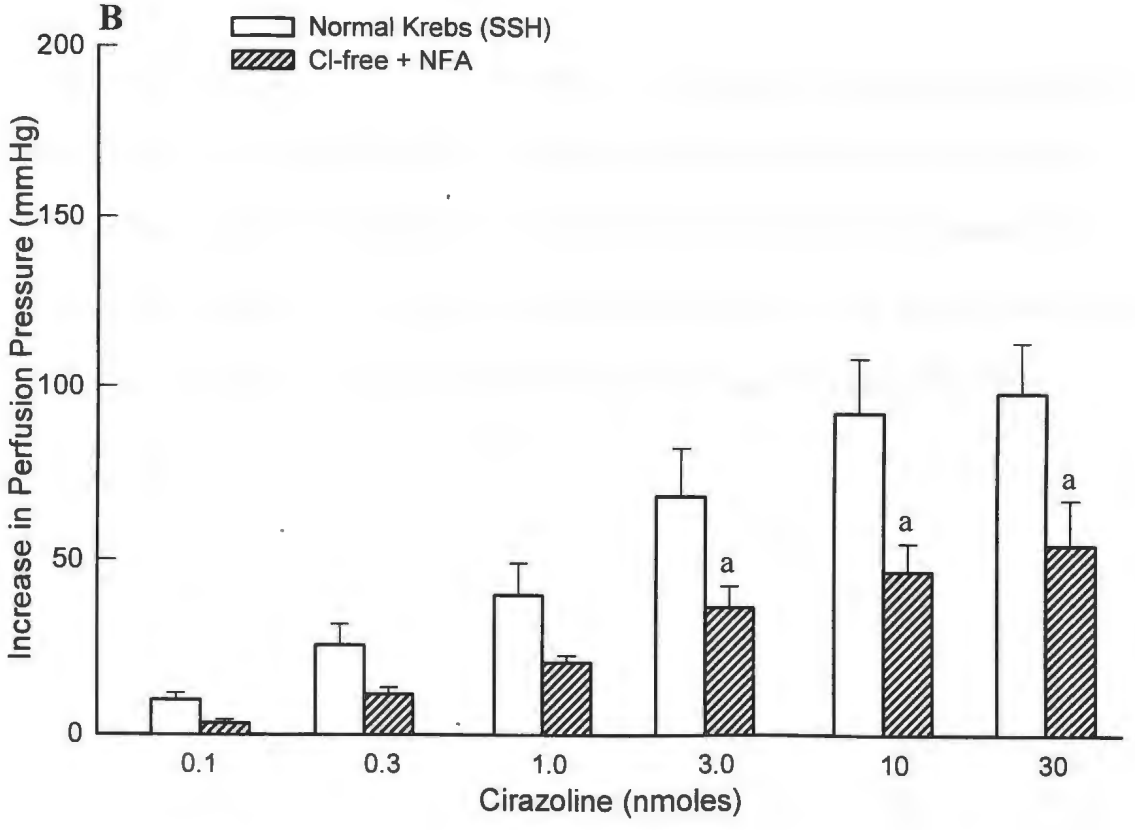

B
Normal Krebs (SSH)
Cl-free + NFA
Increase in Perfusion Pressure (mmHg)
200
150
100
50
0
a
a
a
0.1
0.3
1.0
3.0
10
30
Cirazoline (nmoles)

Figure 17

higher administered three doses (3-30 nmoles), but in contrast, there were no significant changes in Dahl SSH rats (Fig 13AB).

3.2.2. Effect of L-NAME on cirazoline-induced vasoconstriction in isolated mesenteric bed perfused with normal Krebs and Cl^--free buffer

The addition of L-NAME (10 μM) to the perfusate either in normal Krebs or Cl^--free buffer did not result in a change in the basal perfusion pressure in blood vessels from Dahl SRN and SSH rats. The presence of L-NAME in normal Krebs buffer enhanced the vasoconstrictor actions of cirazoline in Dahl SRN rats (0.3-30 nmoles), while it had no significant effect in Dahl SSH rats (Fig 14AB). Moreover, the cirazoline dose-response relationship in the presence of L-NAME in normal Krebs was significantly different between the two strains (Fig 14AB).

In Cl^--free buffer, the addition of L-NAME caused a significant potentiating effects at the lower doses of cirazoline (0.3 and 1.0 nmoles) while inhibiting cirazoline-mediated effects at the higher dose (30 nmoles) in Dahl SRN rats (Fig 15A). In contrast to the latter, the presence of L-NAME in Cl^--free buffer did not have any significant effect on cirazoline-mediated responses in blood vessels from Dahl SSH rats (Fig 15B).

3.2.3. Effect of niflumic acid on cirazoline-induced vasoconstriction in isolated mesenteric bed perfused with normal Krebs and Cl^--free buffer

The addition of niflumic acid (10 μM) to the perfusate either in normal Krebs or Cl^--free buffer did not result in a change in the basal perfusion pressure in blood vessels from Dahl SRN and SSH rats. The presence of niflumic acid in the normal Krebs significantly attenuated the vasoconstrictor actions of cirazoline in Dahl SRN (0.3-30 nmoles) and SSH rats (1-10 nmoles) (Fig 16AB). The inhibitory action of niflumic acid was significantly
more pronounced in blood vessels from Dahl SRN when compared to SSH rats (Fig 16AB).

The presence of niflumic acid in Cl^--free buffer did not produce any additional inhibition of cirazoline-mediated vasoconstriction in blood vessels of either Dahl SRN or SSH rats when compared to the action of niflumic acid in normal Krebs (Fig 17AB).

3.3. Electrophysiological investigation in superior mesenteric artery

The resting membrane potential of smooth muscle cells in normal Krebs buffer from Dahl SRN rats and SSH rats was –67.8 ± 0.4 mV (mean ± SEM; n = 16) and –67.3 ± 0.5 mV (mean ± SEM; n = 16), respectively. There was no significant difference between the resting membrane potential of smooth muscle cells between the two strains (Table 7). Perfusion with cirazoline (3 μM) in normal Krebs did not produce any depolarization of

Table 7. Resting membrane potential (mV) recorded from superior mesenteric artery from Dahl salt-resistant normotensive (SRN) and salt-sensitive hypertensive (SSH) rats on a 4% salt diet for 7 weeks

Groups	NK	Cirazoline	Post-Cirazoline
SRN	–67.7 ± 0.6 (30)(5)	–67.0 ± 0.8 (35)(5)	–68.2 ± 0.8 (20)(5)
SSH	–67.4 ± 1.2 (27)(5)	–67.1 ± 1.2 (38)(5)	–68.9 ± 0.9 (27)(5)
	NK	Cl^--free	Cl^--free + Cirazoline
SRN	–68.3 ± 0.8 (32)(6)	–60.8 ± 1.2[a] (35)(6)	–62.1 ± 1.0[a] (35)(6)
SSH	–67.7 ± 0.8 (31)(5)	–57.7 ± 0.8[ab] (38)(5)	–56.0 ± 1.2[ab] (37)(5)
	NK	NK + L-NAME	NK + L-NAME + Cirazoline
SRN	–67.4 ± 1.2 (26)(5)	–61.8 ± 1.9[a] (36)(5)	–61.6 ± 1.2[a] (34)(5)
SSH	–67.0 ± 1.0 (30)(6)	–67.3 ± 0.4[b] (34)(6)	–68.7 ± 0.9[b] (31)(6)

Each value represents mean of 5-6 experiments ± SEM. Number in parenthesis represents number of cells and tissues, respectively.
[a]Significantly different from normal Krebs (NK); $P < 0.05$
[b]Significantly different from SRN rats; $P < 0.05$

vascular smooth muscle cells of either Dahl SRN or SSH rats (Table 7). However, exposure of blood vessels to Cl^--free buffer caused a significant depolarization in smooth muscle cells from Dahl SRN and SSH rats 10 min after switching the perfusion medium from normal Krebs to Cl^--free buffer (Table 7). The latter depolarization was significantly larger in Dahl SSH rats when compared to SRN rats. The addition of cirazoline in Cl^--free buffer did not produce any additional depolarization of smooth muscle cells in either SRN or SSH rats. While perfusion with L-NAME (10 μM) resulted in a significant depolarization of vascular smooth muscle cells in Dahl SRN, it did not do so in SSH rats (Table 7). Addition of cirazoline in combination with L-NAME did not cause any additional depolarization of smooth muscle cells from SRN or any change in the resting membrane potential of vascular smooth muscle cells in SSH rats (Table 7).

3.4. Morphometric assessment in primary mesenteric blood vessels

Morphological examination of sections of the primary mesenteric arteries revealed significantly larger perimeter, lumen area, smooth muscle wall thickness, and lumen diameter in blood vessels from Dahl SSH rats when compared to SRN rats (Table 8). Interestingly, while smooth muscle thickness and lumen diameter were found to be 1.8 and 1.4 fold greater in SSH compared to SRN rats, the ratio of smooth muscle thickness to lumen diameter (0.35 ± 0.03 versus 0.41 ± 0.03) was found not to be significantly different between the two strains (Table 8). In contrast, the primary mesenteric veins from Dahl SRN and SSH rats did not reveal any difference in perimeter, lumen area,

Table 8. Morphometric measurements from primary mesenteric arteries from Dahl salt-resistant normotensive (SRN) and salt-sensitive hypertensive (SSH) rats on a 4% salt diet for 7 weeks.

Group	Perimeter (μm)	Lumen area (μm^2)	Smooth muscle wall thickness (μm)	Lumen diameter (μm)	Smooth muscle thickness/lumen diameter
SRN	319 ± 18	6927 ± 909	28.0 ± 1.0	90 ± 6	0.35 ± 0.03
SSH	487 ± 24*	13516 ± 1407*	50.4 ± 2.8*	124 ± 7*	0.41 ± 0.03

Each value represents mean of 22 experiments ± SEM
*Significantly different from corresponding value in SRN rats; $P < 0.05$

smooth muscle wall thickness, lumen diameter, or ratio of smooth muscle thickness to lumen diameter (0.06 ± 0.01 versus 0.07 ± 0.01) (Table 9). There were significant differences noted between the morphology of primary mesenteric arteries compared to veins in Dahl SRN and SSH rats (Table 8 & 9).

Table 9. Morphometric measurements from primary mesenteric veins from Dahl salt-resistant normotensive (SRN) and salt-sensitive hypertensive (SSH) rats on a 4% salt diet for 7 weeks.

Group	Perimeter (μm)	Lumen area (μm^2)	Smooth muscle wall thickness (μm)	Lumen diameter (μm)	Smooth muscle thickness/lumen diameter
SRN	$619 \pm 68^*$	$21871 \pm 3442^*$	$7.84 \pm 0.8^*$	$161 \pm 14^*$	$0.06 \pm 0.01^*$
SSH	$648 \pm 35^*$	$20842 \pm 3690^*$	$11.07 \pm 0.8^*$	$157 \pm 14^*$	$0.07 \pm 0.01^*$

Each value represents mean of 10 experiments ± SEM
*Significantly different from corresponding value in mesenteric arteries; $P < 0.05$

4. DISCUSSION

There is evidence to suggest that a high-salt diet is a factor responsible in the development of hypertension in susceptible human and experimental models (Tobain, 1991; Simchon *et al.*, 1991). The Dahl salt-sensitive rats develop high blood pressure when placed on a high-salt diet, whereas the salt-resistant rats do not (for review see Rapp, 1982). The evidence in the current literature seems to suggest that the development of high blood pressure in the Dahl salt-sensitive rat is graded and primarily depends on the amount (Pfeffer *et al.*, 1984) and the duration of the salt diet (Simchon *et al.*, 1991). Ganguli *et al.* (1979) have reported when Dahl salt-resistant rats are placed on an 8% salt diet for 3 days, cardiac output increased and peripheral resistance decreased by 18% and 14% respectively, while the average blood pressure remained the same (110 versus 112 mmHg). In contrast, with a similar salt diet duration in Dahl salt-sensitive rats, the cardiac output and peripheral resistance increased by 10% each, and the arterial pressure became elevated by approximately 20% when compared to rats on a low-salt diet (133 versus 111 mmHg) (Ganguli *et al.*, 1979). Based on the assumption that blood pressure is the product of cardiac output and total peripheral vascular resistance it can be suggested that the early elevation in blood pressure appears to be due to increases in both cardiac output and total peripheral resistance. However, the latter theory is not supported by evidence presented by Simchon *et al.* (1991). They have reported that at 4 weeks the hemodynamic basis for hypertension in salt-sensitive rats on an 8% salt was primarily due to increased cardiac output, without any change in the total peripheral resistance. However, at 8 weeks the situation was reversed and cardiac output was reduced while

total peripheral resistance increased (Simchon *et al.*, 1991). In light of these findings, it seems that in the Dahl salt-sensitive rat, cardiac output is elevated only in the early stages of high salt consumption, and that the continued development of high arterial pressure in this model subsequently is due to a progressive rise in total peripheral resistance (Boegehold *et al.*, 1991; Simchon *et al.*, 1991). Perhaps not surprisingly, similar data has also been presented in humans. It has been demonstrated that normotensive subjects on high-salt diet produced forearm vasodilation and failed to increase arterial pressure significantly, whereas the hypertensive subjects augmented forearm vasoconstrictor responses and had elevated blood pressure, perhaps suggesting the hemodynamic basis of elevated blood pressure in the subpopulation was due to increased peripheral resistance (Mark *et al.*, 1975).

Associated with these hemodynamic changes within the circulatory system during the development of salt-induced hypertension there appears to be significant alteration in the behavior of blood vessels in these animals (Lüscher *et al.*, 1987; Tabrizchi and Duggan, 2000). These alterations in behavior manifest themselves in term of change in morphological (Lee and Triggle, 1986; Intengan and Schiffrin, 1998), mechanical (Nishida *et al.*, 1998; Tabrizchi and Duggan, 2000), and electrical (Fujii *et al.*, 1997) activity of smooth muscle cells. In addition, alteration in the interrelationship between smooth muscle and endothelial cells also seems to occur (Tabrizchi and Duggan, 2000). Collectively, these changes appear to contribute to difference in the behavior of blood vessels in salt-induced hypertension when compared to salt-resistant normotensive rats (Lüscher *et al.*, 1987; Nishida *et al.*, 1998). Against this background, the current

investigation was undertaken to assess the hemodynamic, electrical, mechanical, and morphological differences in mesenteric blood vessels from Dahl SRN and SSH rats placed on a 4% salt diet for 7 weeks.

4.1. Hemodynamic measurements

4.1.1. Blood pressure and heart rate

In the present study, it was found that the female Dahl salt-sensitive rats had significantly higher blood pressure and were hypertensive whereas the salt-resistant rats remained normotensive after 7 weeks on a 4% salt diet. The evidence in the literature also suggest that the Dahl salt-sensitive rats had a higher blood pressure when compared to salt-resistant rats on a 4% salt diet (152/109 ± 9/9 versus 120/83 ± 4/5 mmHg) (Laher and Triggle, 1984) (180/130 ± 1/1 versus 101/76 ± 1/1 mmHg) (Tabrizchi and Duggan, 2000).

In this study, a 4% salt diet was used because 8% salt treatment has been suggested to result in development of severe hypertension where mortality rate is high over a 7 week time frame. Tobain (1991) have reported a 53% mortality rate in Dahl hypertensive rats placed on an 8% salt diet for 8 weeks. This experimental design was designed to produce a moderate elevation of blood pressure with low mortality in animals placed on a high-salt diet. The mortality rate associated with the present investigation was 10%. Moreover, the average amount of salt commonly present in an adult human American diet is about 2% (Tobain, 1991) and hence using 4% salt seems more closely related to human

salt intake. Laher (1983) has reported that Dahl salt-sensitive rats develop a blood pressure of 151/109 ± 6/7 mmHg and 207/135 ± 17/21 mmHg (using indirect tail-cuff method) on 4% and 8% salt diet, respectively. Similarly, Pfeffer *et al.* (1984), using the same indirect blood pressure measurement, have reported that Dahl salt-sensitive rats developed a mean blood pressure of 148 ± 4 mmHg on a 4% salt diet and 193 ± 6 mmHg on an 8% salt diet. The blood pressure of the halothane anesthetized Dahl salt-sensitive rats used in the present study were 175/126 ± 1/1 mmHg, measured using intra-arterial catheter, after 7 weeks on a 4% salt diet. Based on the published data in the literature it would not be unreasonable to suggest that the elevated blood pressure in these rats at this stage of hypertension was due to an elevated peripheral resistance consistent with reduced conductance measured here (Ganguli *et al.*, 1979; Simchon *et al.*, 1991).

There was no particular reason for using female Dahl rats except that these have been used previously in our laboratory. In fact, it has been suggested that there is no sex difference in the response of blood pressure to high salt intake in Dahl rats (Dobešova *et al.*, 1995), although the mortality rate of the female rats was found to be lower compared to the male rats on high-salt diet (Bayorh *et al.*, 2001).

In this study, the heart rate from Dahl SSH rats was significantly higher when compared to SRN rats. However, others have reported that heart rate was not different between the Dahl SRN and SSH rats on low- and high-salt diets, although there were a tendency of higher heart rate in SSH rats (He *et al.*, 1997; Bayorh *et al.*, 1998). The sample size (n =

76 versus n = 10 and 20, respectively; $P < 0.05$) was quite large compared to others, which might be why the results reached a statistical significance level. Based on the present data it can be suggested that the increase in arterial blood pressure in Dahl SSH rats might be associated with an increase in heart rate.

4.1.2. Superior mesenteric arterial blood flow

In the present study, the basal mesenteric blood flow is slightly but not significantly higher in the Dahl SRN compared to SSH rats. According to Poiseuille's law, flow is directly proportional to the forth power of the radius of the blood vessel ($Q \propto r^4$; where Q represents rate of blood flow through a vessel and r represents radius of the blood vessel). This might represent a slight difference in radius of the blood vessels and suggests that the blood vessels from SSH rats are in a constricted state. Conductance is the reciprocal of resistance (R) and hence can be given as flow/pressure gradient ($F/\Delta P$) (Lautt, 1989). Indeed, change in conductance rather than blood flow in a blood vessel is a more appropriate index of function as flow is normalized for any change in pressure in calculation of values of conductance (Lautt, 1989). In this study, it was found that the basal mesenteric vascular conductance was significantly lower in Dahl SSH rats indicating increased peripheral resistance in mesenteric blood vessels which might be, in part, due to reduced basal NO release in the latter blood vessels (for review see Marin and Rodriguez-Martinez, 1997). In spite of a constricted artery, mesenteric blood flow was not significantly different between the two strains owing to the presence of high pressure in blood vessel of SSH rats as can be explained by Ohm's law if we assume that P is the

pressure difference and R is the peripheral resistance (*i.e.*, $F = P/R$). Furthermore, it seems that organ blood flow measured using microspheres in conscious rats indicates that the flow rates of the brain, heart, lung, liver, spleen, intestine, skeletal muscle, and skin are not different between Dahl SRN and SSH rats (He *et al.*, 1997). In contrast, the rate of blood flow to the kidney was significantly reduced in Dahl SSH when compared to SRN rats (He *et al.*, 1997). Taken together, it can be inferred that although the blood flow to the mesenteric bed is not different between the Dahl SRN and SSH rats, the conductance is significantly reduced suggesting that the increase in blood pressure is most likely due to increased resistance to flow. The observation that superior mesenteric blood flow remained constant in Dahl SSH rats despite increases in perfusion pressure (BP) can be attributed to autoregulation. The maintenance of constant flow despite changes in arterial pressure is called autoregulation.

Cardiac output measured in anesthetized rats using electromagnetic flow probes and microspheres was 180-250 (Kawaue and Iriuchijima, 1984) and 291-343 (Tabrizchi and Pang, 1993) ml min^{-1} kg^{-1}, respectively. Furthermore, superior mesenteric blood flow measured using implanted flow probe in conscious spontaneously hypertensive (4.78 ± 1.54 ml min^{-1} $100g^{-1}$) and control (4.36 ± 0.66 ml min^{-1} $100g^{-1}$) rats were found not to be different between the two strains (Iriuchijima, 1983). Based on the values reported for cardiac output in rat, the blood flow to superior mesenteric artery seems to contribute to about 15% of the total cardiac output (Tabrizchi and Pang, 1993; Iida, 1995). Taking into account the cardiac output values obtained using electromagnetic flow probe (Kawaue

and Iriuchijima, 1984), the superior mesenteric artery seems to receive 12-14% of the total cardiac output in Dahl rats. Adding together the celiac blood flow, the gut receives a total of 20% of the cardiac output (Iida, 1995). Thus, it is not unreasonable to suggest that this bed contributes substantially to the overall peripheral resistance and therefore blood pressure of these animals.

4.1.3. Plasma volume and total blood volume

The body weight of Dahl SSH was significantly higher when compared to SRN rats on a salt diet for 7 weeks in the present study. Previous data from our laboratory also suggest an increase in body weight of Dahl SSH when compared to SRN rats on a high-salt diet (Tabrizchi and Duggan, 2000; Bieger *et al.*, 2004). In contrast, others have reported a decrease (De Simone *et al.*, 1996; Intengan and Schiffrin, 1998) or no difference (Ganguli *et al.*, 1979; Pfeffer *et al.*, 1984) in body weight in SSH when compared to SRN rats on a salt diet. Despite the higher body weight in Dahl SSH rats, there was no expansion of plasma volume and total blood volume in SSH rats in this study. Greene *et al.* (1990) measured blood volume for 3 days using ^{51}Cr-labeled erythrocytes and have reported an increase in blood volume in both Dahl SRN and SSH rats on an 8% salt diet which were not different between the two groups. Dobešova *et al.* (1995) used Evans blue to measure blood volume and has also reported that the blood volume was not different between the two strains on a salt diet. However, since blood volume of SRN and SSH rat was not significantly different it can be assumed that hypertension in Dahl salt-sensitive rat is probably not due to an abnormal increase in blood volume (Greene *et al.*, 1990; Dobešova *et al.*, 1995). In contrast, Simchon *et al.* (1991) using ^{51}Cr-labeled erythrocytes to

measure blood volume have reported that the Dahl SSH rats on an 8% salt diet had a significant expansion of blood volume at 4 and 8 weeks when compared to SRN rats. The current data seems to support the view that a transient increase in blood volume occurs initially during high salt intake after which the blood volume returns to normal levels (Greene *et al.*, 1990). The consensus seems to be that blood volume increases, but about the same in Dahl SRN and SSH rats (Greene *et al.*, 1990; Dobešova *et al.*, 1995).

4.1.4. Left ventricular hypertrophy

It is recognized that a prolonged volume expansion and/or increase in afterload (*i.e.* increase peripheral resistance) will ultimately lead to ventricular hypertrophy. There are several studies that suggest a link between salt intake and left ventricular growth in both experimental (Meggs *et al.*, 1988; De Simone *et al.*, 1996) and clinical conditions (Schmieder *et al.*, 1988). For example, it has been reported that salt ingestion is a powerful determinant of left ventricular hypertrophy in patients with essential hypertension (Schmieder *et al.*, 1988; Du Cailar *et al.*, 1989). The explanation offered is that saline overload leads to volume overload and increases left ventricular mass. Thus dietary salt restriction promotes regression of cardiac hypertrophy (for review see Coca and De la Sierra, 1997). In the experimental situation, Pfeffer *et al.* (1984) have indicated that the Dahl salt-sensitive rats develop graded ventricular hypertrophy in response to salt intake. They have demonstrated that the increase in left ventricular weight in salt-sensitive rats was related to the level of arterial pressure. Essentially, it was demonstrated that the left ventricular weights of the salt-sensitive rats were 14%, 32%, and 54% greater than those of the salt-resistant rats on their respective low (0.4%), moderate (4%), and

high (8%) salt diets (Pfeffer *et al.*, 1984). In addition, De Simone *et al.* (1996) had also indicated that left ventricular hypertrophy is more marked in Dahl salt-sensitive than in salt-resistant rats.

Two forms of left ventricular hypertrophy may be distinguished: concentric and eccentric hypertrophy (for review see Coca and De la Sierra, 1997). When the primary stimulus to hypertrophy is left ventricular pressure overload, there is a resultant rise in intracavitary pressure, which leads to a progressive thickening of the ventricular wall and enhanced wall thickness/chamber radius ratio leading to concentric hypertrophy (Grossman *et al.*, 1975). However, when the primary stimulus is left ventricular volume overload, there is left ventricular chamber enlargement, which leads to an increase in the internal ventricular diameter and a normal wall thickness/chamber radius ratio leading to eccentric hypertrophy (Grossman *et al.*, 1975). Now, if the peripheral resistance is elevated, a greater load is placed on the heart and this increase in afterload will eventually result in increased left ventricular wall mass (Grossman *et al.*, 1975). In this study, an increase in blood pressure but not in blood volume was observed in the Dahl SSH when compared to SRN rats suggesting that the left ventricular hypertrophy observed is most likely concentric and is due to increased peripheral resistance (*i.e.* impedance to flow). Although it is also possible that volume overload in response to salt loading occurred prior to 7 weeks on a salt-diet which could have contributed to cardiac hypertrophy in Dahl hypertensive rats.

A high-salt diet has also been shown to increase renal growth in Dahl salt-sensitive and salt-resistant rats, although the rate of growth differs between strains (McCormick *et al.*, 1989). It has been reported that 4% salt-diet causes renal hypertrophy associated with glomerulosclerosis in Dahl SSH rats (Takizawa *et al.*, 1997). The present evidence suggests that the SSH rats undergo significant hypertrophy of the kidneys which might be due to high-salt diet and hypertension in these animals.

4.2.1. Effects of cirazoline in mesenteric bed in vivo

It was of further interest to investigate if there were any functional changes associated with the hemodynamic alterations that had occurred in Dahl salt-sensitive rats on a high-salt diet. Functional responses to various agonists are believed to be altered in blood vessels from Dahl SSH rats (for review see Rapp, 1982). Lüscher *et al.* (1987) have demonstrated that in Dahl SSH rats, contractions in response to noradrenaline in aortic rings are increased while relaxation in response to acetylcholine is depressed. It should be noted that in a blood vessel, a given response to an agonist might be quite different *in vivo* than *in vitro*.

4.2.1.1. Cirazoline effect on blood pressure: In the present study a selective α_1-adrenoceptor agonist, cirazoline, was infused in anesthetized animals. While cirazoline-mediated pressor action in Dahl SSH rats was different from SRN rats the slope of the cirazoline dose-response curves was not significantly different. In this study, the slope of cirazoline dose-response curve can be defined as reactivity of the blood vessel. This

indicates that cirazoline did not produce a greater contractile response; only the initial starting point was different. Several investigators have also suggested that the vasoconstrictor response to an agonist (noradrenaline, serotonin) is not different in blood vessels (caudal artery, thoracic aorta, tail artery, portal vein, perfused mesenteric bed) from Dahl SSH compared to SRN rats on a high-salt diet (Abel *et al.*, 1981; Laher and Triggle, 1984; Kong *et al.*, 1995).

4.2.1.2. Cirazoline effect on vascular conductance: The cirazoline dose-response curve for vascular conductance was found to be shallower in Dahl SSH when compared to SRN rats. This may be a reflection of the difference in the initial state of the blood vessel diameter and/or be related to alteration in the architecture of the vasculature in Dahl SSH when compared to SRN rats.

4.2.1.3. Cirazoline effect on heart rate: Abnormalities in the baroreflex control of heart rate have been identified in experimental (Gordon *et al.*, 1981) and human hypertension (Eckberg, 1979). Slope values were calculated for the regression lines relating the increase in blood pressure from baseline versus decrease in heart rate from baseline value at various doses of cirazoline. The slopes were found not to be different between Dahl SRN and SSH rats (). In contrast, Gordon *et al.* (1981) have reported significant difference in slope values from conscious Dahl SRN (1.84 ± 0.12 msec $mmHg^{-1}$) and SSH rats (1.11 ± 0.06 msec $mmHg^{-1}$). They have suggested impaired baroreflex function in hypertensive Dahl salt-sensitive rats on high-salt diet (Gordon *et al.*, 1981). In this

study the animals were anesthetized and the anesthetic might have blunted the baroreflex control in both the strains.

4.2.2. Effect of cirazoline in isolated mesenteric bed perfused with normal Krebs

Cirazoline-mediated vasoconstrictor responses were further investigated in isolated mesenteric blood vessels. *In vitro* studies have shown no difference (Abel *et al.*, 1981; Laher and Triggle, 1984) or an increase (Lüscher *et al.*, 1987; Nishida *et al.*, 1998) in response to vasoconstrictors in blood vessels from Dahl SSH compared to SRN rats. In the current investigation, the basal perfusion pressure in the isolated mesenteric bed was found not to be different between the Dahl SRN and SSH rats. Indeed, the vasoconstrictor responses to the α_1-adrenoceptor agonist, cirazoline, were found not to differ in either reactivity or magnitude in the perfused mesenteric bed of Dahl SSH when compared to SRN rats in our present study. Laher and Triggle (1984) and Kong *et al.* (1995) had also indicated that there were no alterations in the response to a number of vasoconstrictors in the same tissue from Dahl SRN and SSH rats on a high salt diet. In fact, it was inferred that the sustained hypertension in Dahl rats had a negative effect on the contractility of the mesenteric arterial system (Kong *et al.*, 1995). Cirazoline-induced vasoconstriction is mediated via the activation of α_{1A}-adrenoceptor subtype in mesenteric blood vessels (Horie *et al.*, 1995; Williams and Clarke, 1995). Furthermore, radioligand binding experiments showed no differences in overall α_1-adrenoceptor population in mesenteric blood vessels in Dahl SRN and SSH rats on a 7% salt diet for 5 days or 3 weeks (Caveney *et al.*, 1997). In the present investigation a lack of increased sensitivity

and an altered magnitude in response to cirazoline may be due to receptor desensitization and/or morphological changes in blood vessel of Dahl SSH when compared to SRN rats.

4.3.1 Effect of L-NAME on vascular effects of cirazoline in mesenteric bed in vivo

It was of further interest to investigate if L-NAME had a different response in blood vessels from Dahl SRN and SSH rats. It is recognized that the basal release of NO in blood vessels is decreased in hypertension (for review see Marin and Rodriguez-Martinez, 1997). Previous reports from our laboratory had indicated that while the presence of L-NAME did not have any impact on cirazoline-evoked vasoconstriction in isolated aorta (Tabrizchi and Duggan, 2000) and pulmonary artery (Bieger *et al.*, 2004) from Dahl SSH rats, it substantially accentuated cirazoline-evoked contractions in blood vessels from Dahl SRN rats. As demonstrated in the present experiment, the basal release of NO makes a significant contribution to the maintenance of vascular tone *in vivo* in Dahl SRN rats on a high salt diet. Administration of L-NAME significantly increased the basal blood pressure (10 ± 2%; mean ± SEM), decreased mesenteric blood flow (18 ± 4%; mean ± SEM), and mesenteric vascular conductance (25 ± 4%; mean ± SEM) in SRN but not in SSH rats. This clearly suggests that the blood vessels in SRN rats are in a constant state of vasodilation due to basal release of NO. Although in the present study we do not find any difference in plasma nitrite/nitrate concentration in Dahl SRN and SSH rats, perhaps indicating that the produced NO is compartmentalized. Interestingly, in Dahl SRN rats, the treatment with L-NAME blunted cirazoline-induced vasoconstriction. This simply implies that in absence of NO, an already constricted

vessel cannot be substantially further constricted by the stimulation of α_1-adrenoceptors. The inhibition of NO synthase activity in the majority of cases will result in the potentiation of vasoconstriction in a blood vessel *in vitro* subsequent to stimulation of post-junctional α-adrenoceptors (Tabrizchi and Duggan, 2000, Bieger *et al.*, 2004). However, the behavior of blood vessels towards the NO synthase inhibition can be quite variable *in vivo*. The basis of the difference in the latter case could be related to pharmacokinetic and/or pharmacodynamic aspects of the drugs as well as regional differences, and/or due to other compensatory mechanisms that may play a role in anesthetized animals.

4.3.2. Effect of L-NAME on cirazoline-induced vasoconstriction in isolated mesenteric bed perfused with normal Krebs

The inhibition of NO synthase *in vivo* did not result in potentiation of cirazoline-induced vasoconstriction in blood vessels from Dahl SRN rats. Hence the effect of L-NAME was further examined in the isolated mesenteric bed. In this study, when isolated mesenteric blood vessels were perfused with L-NAME there was no change in basal perfusion pressure in Dahl SRN and SSH rats. In contrast, perfusion with L-NAME significantly increased α_1-adrenoceptor-mediated vasoconstriction in Dahl SRN but not in SSH rats suggesting an impaired synthesis of NO via NO synthase pathway in salt hypertension. This clearly corroborates the observation made in the whole animal that, in SSH rats, there is a reduction in NO production. Indeed, it has also been demonstrated that the

basal levels of cGMP are significantly lower in blood vessels of Dahl SSH when compared to SRN rats (Tabrizchi and Duggan, 2000; Bieger *et al.*, 2004).

The endothelial cell dysfunction observed in Dahl SSH rats cannot be explained by strain differences. There are a number of studies that have reported that placing Dahl salt-sensitive rats on a low-salt diet (0.1-0.45% NaCl) will not result in the development of hypertension in this strain. In these Dahl salt-sensitive normotensive rats, endothelial cell function is normal and NO/NO synthase pathway behaves in the similar manner as the Dahl salt-resistant normotensive rats on either high or low-salt diets (Raij *et al.*, 1988; Boegehold, 1992; Nishida *et al.*, 1998). Furthermore, responses to vasoconstrictors and vasodilators are not altered in Dahl salt-sensitive rats when compared to Dahl salt-resistant rats fed a low-salt diet (Abel *et al.*, 1981; Lüscher *et al.*, 1987).

4.4.1. Effect of niflumic acid on vascular effects of cirazoline in mesenteric bed in vivo

The effect of Cl^-, particularly Ca^{2+}-dependent Cl channels in mesenteric bed in Dahl SRN and SSH rats were investigated. It is known that among the various Cl channel antagonists, niflumic acid appears to be the most potent and selective inhibitor of Ca^{2+}-dependent Cl channels (for review see Large and Wang, 1996). Evidence in the literature appears to indicate that niflumic acid is capable of selectively inhibiting Ca^{2+}-dependent Cl channels by blocking the open state of Cl channels (Hogg *et al.*, 1994). In addition, niflumic acid has been found to produce a concentration-dependent and reversible inhibition of noradrenaline-evoked contractions in the rat aorta (Criddle *et al.*, 1996). As

well, a comparative study of putative Cl channel antagonists (niflumic acid, indanyloxyacetic acid 94 and diphenylamine-2-carboxylic acid) revealed that niflumic acid was the most effective in attenuating α_1-adrenoceptor-mediated changes in blood flow and vascular conductance in the mesenteric vascular bed *in vivo* (Parai and Tabrizchi, 2002). Collectively the current evidence would appear to support the idea that niflumic acid is capable of inhibiting receptor-mediated contraction in blood vessels by affecting Ca^{2+}-dependent Cl channels.

In this study, niflumic acid did not have any effect on the pressor responses of cirazoline. This is consistent with our previous findings in hypertensive and normotensive animals (He and Tabrizchi, 1997; Parai and Tabrizchi, 2002). However, it was reported that treatment of animals with niflumic acid (the same dose as the present investigation) significantly attenuated the constrictor actions of cirazoline on mesenteric blood flow and mesenteric vascular conductance *in vivo* (Parai and Tabrizchi, 2002). In the present investigation, it is apparent that niflumic acid inhibited the α_1-adrenoceptor-mediated decrease in mesenteric blood flow and mesenteric vascular conductance substantially more in Dahl SRN than in Dahl SSH rats. This can be taken to suggest that differences exist between blood vessel behaviors in SRN versus SSH rats with respect to the receptor-mediated signal transduction process. These differences are associated with niflumic acid-sensitive processes during α_1-adrenoceptor mediated vasoconstriction. Thus, based on current findings, it is possible that alteration to Cl channel activity is, in part,

responsible for differences in α_1-adrenoceptor mediated vasoconstriction in salt-induced hypertension.

4.4.2. Effect of niflumic acid on cirazoline-induced vasoconstriction in isolated mesenteric bed perfused with normal Krebs

The inhibitory effect of niflumic acid was further examined in isolated mesenteric blood vessels. Niflumic acid has been found to produce a concentration-dependent and reversible inhibition of noradrenaline-evoked (Criddle *et al.*, 1997), as well as, α_1-adrenoceptor-mediated (He and Tabrizchi, 1997) vasoconstriction in isolated rat mesenteric blood vessels. A further investigation of the effects of niflumic acid in isolated mesenteric bed revealed that it was able to inhibit α_1-adrenoceptor-mediated vasoconstriction substantially more in Dahl SRN than in SSH rats. The latter evidence clearly supported the findings *in vivo* which indicated that niflumic acid had a greater inhibitory action on cirazoline-mediated effects in SRN rats. Taken together, this reemphasizes the fact that differences exist between blood vessel behavior in SRN versus SSH rats with respect to Ca^{2+}-dependent Cl channels as evidenced by the inhibitory actions of niflumic acid on mesenteric blood vessels both *in vivo* and *in vitro*.

In the present study, however, niflumic acid seems to produce a more intense inhibitory effect *in vitro* compared to *in vivo* in both Dahl SRN and SSH rats. This may be attributed to pharmacokinetic and/or pharmacodynamic properties of niflumic acid and/or other compensatory mechanisms that may play a role in anesthetized animals.

4.5. Effect of L-NAME and niflumic acid on vascular effects of cirazoline in mesenteric bed in vivo

The observation that niflumic acid had a greater inhibitory response to cirazoline-mediated effects *in vivo* and *in vitro* in Dahl SSH rats raised the question whether this difference in behavior is due to endothelial dysfunction in Dahl SSH rats. To elucidate this point both L-NAME and niflumic acid were used together *in vivo*. A report by Lamb and Barna (1998b) has suggested that the endothelium may regulate vascular tone via an effect on vascular smooth muscle Cl channels, although there was no electrophysiological evidence presented in that particular study. In the latter study, noradrenaline-induced contractions of rat aorta from normotensive rats were potentiated in low Cl^- buffer depending on the presence of endothelial NO (Lamb and Barna, 1998b). In addition, a dichotomous effect of Cl^- removal on cirazoline-evoked contractions in blood vessels from Dahl SSH versus SRN rats was reported (Tabrizchi and Duggan, 2000). This study demonstrated that while Cl^- replacement with propionate ions augmented cirazoline-evoked contractions in blood vessels of Dahl SRN rats, it depressed the contraction in blood vessels of Dahl SSH rats, suggesting the role that Cl^- plays in α_1-adrenoceptor-mediated excitation-contraction-coupling processes of blood vessel is modified in salt-induced hypertension (Tabrizchi and Duggan, 2000). In the present study, the differences in the effects of niflumic acid on cirazoline-mediated vasoconstriction in the mesenteric bed in Dahl SRN versus SSH rats were abolished by pretreatment of animals with L-

NAME. This appears to suggest that the tonic release of NO has a significant effect on niflumic acid-sensitive pathways.

4.6. Effect of removal of extracellular Cl^- in mesenteric bed

Since it was not possible to remove Cl^- *in vivo*, the intestine was taken and investigated for the effect of Cl^--free medium (replaced by propionate) in isolated mesenteric blood vessels. This study provides evidence of an integral role for Cl^- in electromechanical function of mesenteric blood vessels in Dahl rats. Removal of Cl^- from the perfusion media resulted in a transient vasoconstriction in the mesenteric bed of both in the Dahl SRN and SSH rats. The vasoconstrictor response was significantly higher in Dahl SSH compared to SRN rats. These phenomena were also observed in a similar preparation in 2K1C hypertensive and sham normotensive rats (He and Tabrizchi, 1997). It has also been suggested that removal of Cl^- from the extracellular fluid results in a transient membrane depolarization in smooth muscle cells (Aickin and Brading, 1982; Aickin and Vermüe, 1983). The observed transient vasoconstriction may have occurred as a consequence of sudden shift in Cl^- equilibrium due to exposure of the preparation to Cl^--free medium. Cl^--free medium would be expected to cause efflux of Cl^- from the cytoplasm resulting in depolarization, opening of Ca channels, and subsequent contraction (for review see Large and Wang, 1996). Overall, Cl^- appears to play a role in electromechanical response in vascular smooth muscle (for review see Chipperfield and Harper, 2000).

4.6.1. Effect of cirazoline in isolated mesenteric bed perfused with Cl^--free buffer

It was of further interest to investigate the effect of cirazoline when Cl^- is removed from the perfusion medium. Previous reports from our laboratory have suggested a difference in vasoconstrictor response in conduit systemic (high pressure) (Tabrizchi and Duggan, 2000) and pulmonary blood vessels (low pressure) (Bieger *et al.*, 2004) from Dahl SRN and SSH in Cl^--free buffer. Thus the vasoconstrictor response in mesenteric blood vessels was examined in Cl^--free buffer. It was found that α_1-adrenoceptor mediated vasoconstriction in mesenteric blood vessels from Dahl SRN rats was inhibited in Cl^--free buffer, which is expected if Cl^- is involved in smooth muscle contraction (for reviews see Large and Wang, 1996; Chipperfield and Harper, 2000). The inhibitory effect of Cl^--free medium was absent in Dahl SSH rats. This may be taken to suggest that Cl^- plays a greater role in blood vessels from SRN rats. As mentioned previously, Cl^- replacement was found to augment cirazoline-evoked contractions in aorta from Dahl SRN rats, whereas it depressed the contractions in SSH rats (Tabrizchi and Duggan, 2000). In contrast, Cl^- removal from pulmonary artery did not alter cirazoline-induced vasoconstriction in Dahl SRN rats, but it did attenuate the vasoconstriction elicited by cirazoline in SSH rats (Bieger *et al.*, 2004). Based on the data from our laboratory it seems clear that the impact of the removal of Cl^- from extracellular space and its replacement with propionate ions on excitation-contraction coupling is not uniform among various blood vessels in the same species, and that Cl^- seems to play a variable

role on functional responses of blood vessels, this being dependent of on the type of blood vessel involved, as well as, pathology.

4.6.2. Effect of L-NAME on cirazoline-induced vasoconstriction in isolated mesenteric bed perfused with Cl^--free buffer

The influence of L-NAME in blood vessels from Dahl SRN rat were further examined in Cl^--free buffer. It is evident that the removal of Cl^- from extracellular space had no effects on the potentiating effect of L-NAME at the lower doses of cirazoline, while the responses at the higher doses of the agonist were blocked in Dahl SRN rats. This evidence may be taken to suggest that the role that Cl^- may play during the process of excitation-contraction coupling is dependent on the concentration of agonist. Such may not be the case in every blood vessel. For example, in conduit blood vessels, Cl^--free medium did not have a biphasic effect on the action of L-NAME on cirazoline-mediated contraction in SRN rats (Tabrizchi and Duggan, 2000). In contrast, in pulmonary artery, the potentiating effects of L-NAME of the concentration-response curve to cirazoline were abolished in Cl^--free medium (Bieger *et al.*, 2004). The effect of L-NAME in Cl^--free medium further reinforces the view of divergence of effects of Cl^- on the process of excitation-contraction coupling in different blood vessels even within the same species.

4.6.3. Effect of niflumic acid on cirazoline-induced vasoconstriction in isolated mesenteric bed perfused with Cl^--free buffer

It was of further interest to examine if the inhibitory effect of niflumic acid is altered in Cl^--free medium. Niflumic acid is capable of attenuating cirazoline-mediated vasoconstriction in mesenteric blood vessels by inhibiting Ca^{2+}-dependent Cl channels. There was no additional inhibitory effect of niflumic acid on the actions of cirazoline in Cl^--free medium. This would be expected as Cl^- would not be making any contribution to the process of excitation-contraction coupling in a Cl^--free medium, thus niflumic acid should not be capable of producing any further inhibitory effect which was the case. Although a greater inhibitory response to niflumic acid in Dahl SRN rats compared to SSH rats was observed in normal Krebs buffer, this difference in inhibitory effect was absent in Cl^--free medium. Therefore, the *in vivo* and *in vitro* findings suggest that alterations in Cl^- handling and Ca^{2+}-dependent Cl channels are, in part, responsible for altered responses in blood vessels from Dahl SSH rats.

4.7. E_m measurements

The absence of increased reactivity to cirazoline in blood vessel from Dahl SSH rats directed the study towards assessment of E_m of these blood vessels. There is conflicting evidence in the literature regarding alterations in the resting membrane potential of vascular smooth muscle cells of arteries from Dahl SRN when compared to SSH rats. In this study, the resting E_m in smooth muscle cells from the superior mesenteric artery was

not different in Dahl SRN when compared to SSH rats. Abel *et al.* (1981) have reported no significant difference in resting membrane potential of caudal arteries in Dahl SSH (–50.2 ± 1.1 mV) and SRN (–51.4 ± 0.9 mV) rats fed an 8% salt diet. Measurements of K^+, Na^+, and Cl^- content by electron-probe X-ray analysis showed no significant difference in intracellular K^+, Na^+, or Cl^- for Dahl SRN and SSH rats on either low- or high-salt diets (Abel *et al.*, 1981). In contrast, Fujii *et al.* (1997) have reported that the resting E_m was more positive in the superior mesenteric arteries of Dahl SSH (–41.4 ± 0.5 mV) when compared to SRN rats (–47.0 ± 0.7 mV). Wellman *et al.* (2001) had also reported that the membrane potential of cerebral artery myocytes from Dahl SSH rats (–36.8 ± 0.8 mV) was depolarized compared with similar cells from SRN animals (–49.7 ± 1.2 mV). In contrast, to these observations, it was previously reported that the resting E_m of pulmonary artery from Dahl SSH (–52.1 ± 1.04 mV) rats is more hyperpolarized when compared to SRN rats (–46.4 ± 1.58 mV) (Bieger *et al.* 2004). This divergence was further interpreted to be due to humoral/hormonal factors acting to alter vascular smooth muscle membrane properties in the low-pressure bed (pulmonary circulation) since the pulmonary artery does not experience high blood pressure in Dahl rats, thus promoting hyperpolarization (Bieger *et al.* 2004). Certainly, the lack of consistency between the measured E_m in various blood vessels in Dahl SSH and SRN rat may also point to the different nature of E_m in blood vessel in different region of the body.

E_m measurements performed in other types of hypertensive rats also seems to differ depending on the strains used. For example, Hermsmeyer (1976) have reported that the

resting E_m of arterial vascular smooth muscle cells from spontaneously hypertensive rats are less negative than those from matched Wistar-Kyoto normotensive rats. In contrast, it has been reported that the resting E_m was found not different between DOCA-salt hypertensive and control rats (Hermsmeyer *et al.*, 1982). However, Bratz *et al.* (2002) have demonstrated that vascular smooth muscle cells from L-NNA-treated hypertensive rats are depolarized (–36.8 ± 0.8 mV) compared to control rats (–44.5 ± 1.0 mV). Although, apparently, there is no difference in the resting E_m in smooth muscle cells from Dahl SRN and SSH rats in the present investigation, there might be alteration in transmembrane signaling processes related to E_m changes.

4.7.1. Effect of cirazoline on E_m

The resting E_m in vascular smooth muscle cells and the contractile response to cirazoline in isolated mesenteric bed was not different between the two strains. Thus further studies were carried out to investigate if cirazoline produces a change in E_m in these blood vessels. The issue of agonist-induced changes in the membrane potential of vascular smooth muscle cells remains controversial. Su *et al.* (1964) have shown in the rabbit pulmonary artery that there was no change in E_m during contraction by sympathetic nerve stimulation and noradrenaline. Experiments performed by Casteels *et al.* (1977) confirmed the previous observations that, in the main pulmonary artery, a mechanical response can be elicited without depolarization of the membrane at low concentrations of noradrenaline. Large (1984) have also provided further evidence that noradrenaline can initiate contraction by a mechanism which is not dependent on a change in membrane

potential in rat anococcygeous muscle and pharmacomechanical responses appear to be responsible for contraction independent of changes in E_m of vascular smooth muscle cells. Also 5-hydroxytryptamine did not produce a change in the membrane potential of pig coronary artery (Frieden and Beny, 1995). In contrast, others have shown a significant change in membrane potential associated with contraction (Haeusler, 1978; Haeusler and De Peyer, 1989). They have reported that noradrenaline produced 15-17 mV depolarizations in vascular smooth muscle cells from rabbit main pulmonary artery (Haeusler, 1978). In rabbit aorta, methoxamine depolarized the smooth muscle cells from –54.6 mV to –40 mV (Haeusler and De Peyer, 1989). Mulvany *et al.* (1982) have also shown that noradrenaline stimulation depolarized the smooth muscle cells from a resting membrane potential of –59.2 mV to –40 mV (a difference of ~ 19 mV) in rat mesenteric arteries. Interestingly, in the present investigation, addition of cirazoline did not depolarize the smooth muscle cells from Dahl SRN and SSH rats. The present observation may suggest that the vascular smooth muscle is quiescent in nature and/or the pharmacomechanical coupling might be playing a major role in contraction of smooth muscle.

It has been suggested that niflumic acid is capable of inhibiting α_1-adrenoceptor-mediated vasoconstriction, most probably by inhibiting the Ca^{2+}-dependent Cl channels and subsequently inhibiting depolarization (for review see Large and Wang, 1996). In the present investigation, niflumic acid attenuated cirazoline-mediated vasoconstriction. Indeed, while cirazoline does not seem to produce sustained membrane depolarization,

one cannot rule out the possibility of a transient depolarization since measurements were made after 10 min exposure. The latter could be due to the activation of Ca^{2+}-dependent Cl channels which is blocked by niflumic acid. This could explain the inhibitory action of the latter chemical in cirazoline-elicited vasoconstriction in our present study.

4.7.2. Effect of Cl^--free buffer on E_m

The replacement of extracellular Cl^- with propionate ions in isolated mesenteric bed caused inhibition of cirazoline-mediated vasoconstriction, which raised the question if Cl^- removal will have an effect on the E_m. Removal of Cl^- from the extracellular space should cause an acute redistribution of Cl^- across the cell membrane resulting in membrane depolarization. Aickin and Vermüe (1983) have reported that complete removal of extracellular Cl^- causes a decline in intracellular Cl^- activity from 51.1 ± 4.0 mM to an apparent level of 3.3 ± 0.9 mM. The decline in intracellular Cl^- concentration could be described by a single exponential process with a mean time constant of 6.7 ± 1.3 min (Aickin and Vermüe, 1983). They have reported that removal of Cl^- from extracellular fluid results in a transient membrane depolarization in guinea-pig vas deferens (Aickin and Brading, 1982) and ureter (Aickin and Vermüe, 1983). In rat anococcygeous muscle low Cl^- depolarized the membrane from –59.3 ± 0.6 mV to –50.7 ± 1.4 mV (replaced by benzenesulphonate) or –53.2 ± 1.8 mV (replaced by isethionate) (Large, 1984). On the other hand, Davis *et al.* (1997) have reported that on switching the superfusing solution from normal to Cl^--free buffer, E_m hyperpolarized from –63.0 ± 2.9 mV to –69.4 ± 2.5 mV and intracellular Cl^- concentration fell to an apparent level of 4.4

mM from 35.0 mM. Recent evidence from our laboratory indicates that replacement of Cl^- with propionate ions produces significant hyperpolarization in pulmonary artery of Dahl SRN but not in SSH rats (Bieger *et al.*, 2004). In the present investigation, Cl^--free buffer caused a significant depolarization of the membrane in Dahl SRN (7.5 ± 0.8 mV; mean ± SEM) and SSH rats (10 ± 1.0 mV; mean ± SEM). Based on the time constant for decline of intracellular Cl^- concentration in guinea-pig ureter (Aickin and Vermüe, 1983), more than 90% of the intracellular Cl^- was expected to be removed in 30 min in the present investigation. A transient vasoconstriction was observed in mesenteric blood vessels in Cl^--free buffer. Although some of the intracellular Cl^- might have been replaced by propionate, it is perhaps reasonable to assume that a net reduction in negative ion inside the cell might have been responsible for the depolarizing effect observed in the present investigation.

Surprisingly, while there is a depolarization of vascular smooth muscle in Cl^--free medium, cirazoline-evoked vasoconstriction was attenuated in this medium. This strongly implies that Cl^- are of critical importance in the process of excitation-contraction coupling involving the activation of α_1-adrenoceptors irrespective of the differences in membrane potential (for review see Chipperfield and Harper, 2000). However, it is also evident that the blood vessels from the Dahl SSH rats underwent a significantly larger depolarization compared to SRN rats in Cl^--free buffer. This may in part explain the lesser impact of Cl^--free medium on cirazoline-evoked vasoconstriction in blood vessels of Dahl SSH in comparison to SRN rats. Thus, our observation could be taken to suggest

that there is some degree of dissociation but not a complete dissociation between changes in the resting membrane potential and vasoconstriction initiated as consequence of the stimulation of α_1-adrenoceptors in mesenteric blood vessels.

4.7.3. Effect of L-NAME on E_m

The functional studies have clearly indicated that the presence of L-NAME has an effect on the blood vessels from Dahl SRN but not in SSH rats. Thus it was investigated if L-NAME had an effect on the E_m in these blood vessels. The functional studies clearly suggest an impaired NO production associated with salt hypertension (Lüscher *et al.*, 1987; Tabrizchi and Duggan, 2000; Bieger *et al.*, 2004). Addition of L-NAME was found to cause depolarization in blood vessels of SRN rats (5.6 ± 0.8 mV; mean ± SEM) but such an effect was not detected in SSH rats. This depolarization might again explain the exaggerated response to cirazoline in the presence of L-NAME in SRN rats. It seems plausible to suggest that NO may have a direct or an indirect (*i.e.*, via cGMP) effect on the resting membrane potential of mesenteric vascular smooth muscle cells, in Dahl SRN but not SSH rats. It has been suggested that NO can directly activate Ca^{2+}-dependent K channels, leading to endothelium-dependent hyperpolarization of vascular smooth muscle cells, resulting in vasodilation (Bolotina *et al.*, 1994). Furthermore, NO has also been suggested to cause membrane hyperpolarization (*i.e.*, from –39 ± 1 mV to –48 ± 1 mV) by opening voltage-gated K channels in pulmonary artery (Yuan *et al.*, 1996). Bratz *et al.* (2002) have demonstrated that addition of L-NNA caused a significant depolarization in vascular smooth muscle cells from normotensive rats, with E_m changing from –44.5 ± 1.0

mV to –39.0 ± 1.9 mV, but had no effect on smooth muscle cells from L-NNA-treated hypertensive rats. Also endothelium removal resulted in depolarization in smooth muscle cells from normotensive when compared to L-NNA-treated hypertensive rats (Bratz *et al.*, 2002). It was further demonstrated that superfusion with L-arginine hyperpolarized the smooth muscle cells in L-NNA-treated hypertensive and normotensive rats (Bratz *et al.*, 2002). Thus it can be suggested that NO seems to have a hyperpolarizing effect on resting membrane potential of smooth muscle cells from Dahl SRN but not SSH rats which might be, in part, due to opening of K channels (Bolotina *et al.*, 1994; Yuan *et al.*, 1996).

The present study demonstrates that L-NAME produces an effect on the E_m in smooth muscle cells from Dahl SRN but not SSH rats. As an extension of this, it can be suggested that perhaps the basal release of NO is playing a role in maintaining the resting E_m in Dahl SRN rats. If NO is playing a role in maintaining the resting E_m in Dahl SRN but not in SSH rats, this further raises the question of why is the resting E_m not different between the two strains. This might be explained by the fact that in smooth muscle cells of Dahl SRN rats, there is perhaps a change in ionic conductance that contributes in such a manner that the resting E_m is reset at a level which is similar to Dahl SSH rats.

Evidence from the E_m recordings demonstrates that there is no difference between the resting E_m in vascular smooth muscle cells from superior mesenteric blood vessels in Dahl SRN and SSH rats. Furthermore, addition of cirazoline does not depolarize the

smooth muscle cells from Dahl SRN and SSH rats. It is observed that removal of Cl^- results in a larger depolarization of the smooth muscle cells in Dahl SSH than SRN rats. While L-NAME treatment depolarizes the smooth muscle cells in Dahl SRN, it has no effect in the SSH rats. It is possible that the modified role of NO on membrane potential may have a direct bearing on the differences observed in Cl^- handling in blood vessels of Dahl SRN versus SSH rats.

4.8. Morphology

In addition to exploring functional and electrical properties in mesenteric blood vessels, simultaneous assessment of morphology was done in the mesenteric blood vessels from Dahl SRN and SSH rats on a high-salt diet. There are studies that have presented evidence of vascular hypertrophy in mesenteric blood vessels of Dahl SSH but not Dahl SRN rats fed a high-salt diet (Lee and Triggle, 1986; Intengan and Schiffrin, 1998). Furthermore, mesenteric blood vessels of Dahl salt-sensitive rats fed a low-salt diet do not undergo morphological changes (Lee and Triggle, 1986). In the present study, an increase in smooth muscle thickness (*i.e.*, vascular hypertrophy) in primary mesenteric arteries from Dahl SSH rats was observed. Furthermore, it has been suggested that these morphological changes play a critical role in alteration of blood vessels function (McGregor and Smirk, 1968; Intengan and Schiffrin, 2001). In fact, it has been argued that alteration in responsiveness observed in blood vessels of Dahl SSH rats is, in part, due to changes in vascular morphology (Intengan and Schiffrin, 1998).

In the present study, there were significant changes in morphometry of primary mesenteric arteries but not veins of Dahl SSH rats. Alteration in architecture of the primary mesenteric blood vessels will most likely reflect changes that occur in smaller blood vessels (e.g., tertiary) (Lee and Triggle, 1986; Intengan and Schiffrin, 1998). However, there is no potentiation in the vasoconstrictor actions of cirazoline in Dahl SSH when compared to Dahl SRN rats *in vivo* as well as *in vitro*. An absence of increased reactivity, and/or magnitude in response, in blood vessels from Dahl SSH rats might possibly be due to secondary compensatory event that occurred in blood vessels such as receptor desensitization.

Furthermore, we have applied the Laplace's law to our *in vitro* studies. As an extension of Laplace's law, it has been further proposed that the "media thickness/lumen diameter" ratio, of a blood vessel is a good correlate of the ability of the vessel to contract against intravascular pressure (for review see Zervoudaki and Toutouzas, 2003). Since the isolated mesenteric bed was perfused at a constant flow rate and thus it can be assumed that the blood vessels contracted against a constant intravascular pressure. Based on morphological examination it was revealed that the media thickness/lumen diameter ratio of these blood vessels was not different between the two strains. Therefore, this might provide the alternate explanation for the absence of increase in magnitude of response in blood vessels from Dahl SSH when compared to SRN rats in the current investigation and other studies using the same preparation (Laher and Triggle, 1984; Kong *et al.* 1995). Overall, the evidence from our present investigation seems to support the view that any

alteration in the behavior of mesenteric blood vessels to the actions of cirazoline can be attributed to changes in signal transduction rather than a difference in architecture of blood vessels.

4.9. Summary

To summarize the findings from this study, it can be inferred that, although the mean arterial blood pressure is higher in Dahl SSH rats, there is certainly lack in sensitivity and/or magnitude in the vasoconstrictor responses to α_1-adrenoceptor agonist, cirazoline, in mesenteric blood vessels of Dahl SSH when compared to SRN rats. This similarity in vasoconstrictor response can be attributed to the similar resting E_m and similar media thickness/lumen diameter ratio in blood vessels in Dahl SRN and SSH rats. The evidence also suggest an integral role for Cl^- in electromechanical function of mesenteric blood vessels in Dahl rats and this role might be altered in salt hypertensive animals. Interestingly, these observations also seem to support the concept of a partial dissociation between electrical activity and mechanical function in vascular smooth muscle cells following the stimulation of α_1-adrenoceptors. Of special interest, is the finding that NO may regulate electrical activity and mechanical function in mesenteric blood vessels from Dahl SRN but not in SSH rats. The dysfunction of the NO system and Cl^- channels could contribute to the hypertension observed in the SSH model.

This study reveals the fact that high salt consumption is responsible for hemodynamic, electrical, mechanical, and morphological changes in blood vessels from Dahl SSH rats. Similar changes may also occur in salt-sensitive humans on a moderate high-salt diet over a period of time. The high blood pressure and morphology of the blood vessels most probably would lead to left ventricular hypertrophy and heart failure. Hence, a reduction in salt intake in every individual can be suggested as a preventive measure, and in cases where this is not helpful, pharmacological intervention is the other option.

4.10. LIMITATIONS

As with all experiments, there are certain limitations associated with the present study. The anesthetic used might have an effect on the measurements of blood pressure, heart rate, and mesenteric blood flow. Also since the animals were anesthetized, the altered baroreflex mechanisms in Dahl SSH rats were not observed. It would have been optimum if cardiac output was measured simultaneously during the drug infusion. In the isolated perfused mesenteric blood vessels, the arterioles were absent which are mainly responsible for maintaining elevated peripheral vascular resistance. Furthermore, the membrane potential of vascular smooth muscle was recorded from the superior mesenteric artery. The ideal scenario would be to measure the E_m in the smaller resistance arteries along with functional changes. Furthermore, the nitrite/nitrate measurements were done in the plasma sample. The NO/NO synthase activity was not assessed in the endothelial cells. It would have been optimal if the eNOS expression were investigated in the blood vessels from these rats.

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