Modulation of Renal Autoregulation by Nitric Oxide – Cellular Mechanism

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ABSTRACT

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Normal renal function requires stable renal blood flow. It is achieved by autoregulation. Nitric oxide contributes to the regulation of multiple pathways that are relevant to myogenic autoregulation. We examined the signal transduction mechanism of nitric oxide on myogenic autoregulation in the present thesis.

Because some paracrine factors contribute to the renal autoregulation, we first isolate the effects of nitric oxide from other paracrine factors. The pressor and renal vasoconstrictor responses to nitric oxide synthase inhibition are independent of angiotensin II. There is an interaction between nitric oxide and endothelin, mediated by endothelin B receptors, that affects renal blood flow and renal autoregulation.

Y-27632, Rho-kinase inhibitor reverses completely the enhanced myogenic autoregulation by L-NAME, and L-NAME does not affect the myogenic autoregulation pre-treated by Y27632.

In conclusion, the effect of Rho-kinase with consequent inhibition of myosin light chain phosphatase may be an essential component of the modulation of myogenic autoregulation by nitric oxide. Therefore, the signal transudation of nitric oxide on renal autoregulation passes through Rho-kinase pathway.

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LIST OF ABBREVIATIONS:

ANG II	angiotensin II
BW	blood pressure
BW	body weight
CTL	control
Expt	experiment
ET	endothelin
ETA	endothelin A
ETB	endothelin B
eNOS	endothelial NOS
FC	fractional compensation
G	vascular conductance
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inibitor
GEF	guanine nucleotide exchange factor
GFR	glomerular filtration rate
iNOS	cytokine inducible NOS
L-NAME	L–nitro arginine methyl ester
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
NO	nitric oxide
NOS	nitric oxide synthase

nNOS	neuronal NOS
RAS	renin-angiotensin system
RBF	renal blood flow
RhoGDI	guanine nucleotide dissociation inhibitor.
TGF	tubuloglomerular feedback

INTRODUCTION:

A living body is composed of the various organ systems structurally and functionally linked together as an internal environment that is separate from the external environment. Cells can live and function only when the internal environment is stable. This is referred to as the term homeostasis. Many organs contribute to homeostasis. The kidney is one of the most important organs in the body because you cannot have a brain without a kidney. It is the kidney that controls extracellular fluid volume and composition. Constancy of extracellular fluid composition is necessary if membrane potential is to store and transmit information.

Basic renal functions include the regulation of extracellular fluid volume and the concentration of solutes, regulation of body pH and blood pressure (BP), as well as the excretion of end products of metabolism. In addition, the kidney secretes erythropoietin, a hormone that stimulates red blood cell production, and renin, an enzymatic hormone that triggers a chain reaction important in the process of salt conservation by the kidney, and it converts vitamin D into its active form.

The kidney is made up of repeated "nephro-vascular" units. A rat kidney contains about 30,000 such units and a human kidney contains about 1,500,000 units, all operating in parallel. The vascular component consists of the afferent arteriole, glomerulus, efferent arteriole, and peritubular capillaries. The afferent arteriole delivers blood to the glomerulus, a knot of capillaries that are specialized as filters. In an adult human about 180 L are filtered each day. Blood proceeds from the glomerulus via the efferent arteriole to the peritubular capillaries that take up the 95-99 % of filtrate that is reabsorbed from the nephron. The nephron consists of the urinary space, which surrounds

the glomerulus, the proximal tubule, Henle's loop, distal tubule, and collecting duct. The early distal tubule of every nephron passes over the vascular pole of its own glomerulus. Both the tubular and vascular regions at this point are specialized to form the juxtaglomerular apparatus. Several control mechanisms that are important to regulation of extracellular fluid volume and of renal blood flow (RBF) converge at the juxtaglomerular apparatus.

Blood pressure is the major force responsible for inducing glomerular filtration. The most common factor resulting in an alteration in the glomerular filtration rate (GFR) is alteration of glomerular capillary pressure. The GFR is controlled via three pathways, all of which are directed at adjusting glomerular blood flow by regulating the calibre and thus the resistance of the afferent arteriole. The first pathway is extrinsic to the kidney and involves a complex interaction of sensory mechanisms with the sympathetic nervous system. The second pathway, renal autoregulation, is intrinsic to the kidney and depends on changes in afferent arteriolar tone in response to the renal perfusion pressure itself. The third one is a hormonal regulatory system that responds to changes in BP and body salt content.

The extrinsic control of GFR is mediated by the sympathetic nervous system input to the afferent arterioles. It is a long-term regulation and is aimed at the defence of arterial BP. Sympathetic nervous control of RBF is largely related to arousal and physical activity (Grady and Bullivant 1992). The parasympathetic nervous system does not exert any influence on the kidneys. If BP is reduced, baroreceptors detect the reduction. This initiates neural reflexes to increase BP toward normal pressure. The sympathetic vasoconstrictor activity to the arterioles, including the renal afferent arterioles, is reflexly

raised, inducing afferent arteriolar vasoconstriction. As less blood flow enters the glomeruli through the contracted afferent arterioles, glomerular capillary blood pressure decreases, reducing the GFR. Sympathetic nerves have three effects in the kidney: modification of glomerular pressure and GFR by changes in diameter of the afferent and/or efferent arterioles; nerve stimulation causes release of renin. It is released from the juxtaglomerular apparatus of the kidney to control salt and water absorption and thus regulates BP. Nerve stimulation also causes direct stimulation of tubular salt and water reabsorption.

A hormone system regulation in the kidney is the renin-angiotensin-aldosterone system. It stimulates salt reabsorption in the distal and collecting tubules. The kidney secretes renin in response to a reduction in extracellular fluid volume or BP. Renin cleaves angiotensinogen, a plasma protein produced by the liver, into angiotensin I. Angiotensin I is converted into angiotensin II (ANG II) by angiotensin-converting enzyme which is found almost ubiquitously on endothelial cells and circulating in plasma. ANG II stimulates the adrenal cortex to secrete the hormone aldosterone that stimulates salt reabsorption by the kidneys. Together the conserved salt and water help to correct the original stimuli that activated the renin-angiotensin-aldosterone system. Other effects of ANG II in kidneys are discussed below in "interaction of ANG II".

Autoregulation

Autoregulation is defined as the kidney's ability to alter its own vascular resistance in response to changes in BP to maintain essentially constant RBF and GFR. Arterial BP is the force that drives blood flow (flow = pressure x conductance). The pressure – flow

relation is a closed one, each variable affecting the other. It is considered that pressure is the input variable and flow is the output variable.

In addition to activity-induced changes, BP fluctuates spontaneously and continuously. According to this relationship of BP and RBF, every time BP changes, blood flow should follow accordingly. But the normal renal function needs stable RBF, because unintentional shift in RBF or GFR could lead to dangerous imbalances of fluid, electrolytes, and wastes. Renal autoregulation is aimed at preventing inappropriate changes in blood flow and maintains essentially constant RBF when BP varies between 80 mmHg and 180 mmHg (Navar 1997). Autoregulation only occurs between certain pressure limits - if the pressure drops too low or soars too high, autoregulation fails, and kidney perfusion is compromised - at low pressures, perfusion drops, and at high pressures, excessive flow occurs. Within its effective range, the adjustments of afferent arteriolar resistance can compensate for changes in BP, thus preventing inappropriate fluctuations in GFR. Autoregulation dominates control of RBF in conscious and anesthetized rats, rabbits, and dogs, and with the kidney innervated or denervated (Malpas et al. 1998; Abu-Amarah et al. 1998; Just et al. 1998; Lessard et al. 1999).

Two mechanisms have been identified as contributing to autoregulation: tubuloglomerular feedback (TGF) and a myogenic system. There may be a third mechanism; Just et al. (2001) reported that in conscious, resting dog when BP was stepped from 50 mmHg to the spontaneous level, ~100 mmHg, myogenic response and TGF contribute each about 1/3 to the total response, while the remaining 1/3 seems to be brought by an unidentified additional mechanism. This putative third mechanism has not been characterized and is very little known.

The mechanism of TGF is very complicated. TGF senses changes in the concentration of NaCl in the tubular flow at the *macula densa* of the distal tubule. When such a change is detected, vasoactive chemicals are released from the *macula densa* and diffuse across to the neighboring juxtaglomerular cells of the afferent arteriole. The juxtaglomerular cells cause afferent arterioles to constrict. However, how the signal transduction occurs between the macula densa and afferent arteriole is not very clear. Schnermann showed that the renin-angiotensin system (RAS) is a necessary component for the signal transduction (Traynor et al. 1999). And the adenosine type 1 and ANG II type 1 receptors interact to induce macula densa-dependent regulation of the renal afferent arteriolar tone (Traynor et al. 1998).

The myogenic mechanism is a common property of vascular smooth muscle (Bayliss 1902). Renal myogenic autoregulation that is similar to that existing in many other vascular beds senses a variable, perhaps wall tension, which is closely related to transmural pressure. It is an active response of an artery (either constriction or dilatation) to a rapid change in transmural pressure. The blood vessel behaviour is an integrative process that depends on the endothelium, vascular smooth muscle, and extracellular matrix (Gandley et al. 1997; Osol et al. 1995; Robin et al. 2001; Sun et al. 1992). It is not only shown in intact kidney but also in those that do not have intact TGF mechanism or in hydronephrotic kidney with no remaining tubules (Hayashi et al. 1989; Loutzenhiser et al. 1987; Steinhausen et al. 1989). When the transmural pressure rises, the membrane potential of vascular smooth muscle cells depolarizes (Harder, 1984; Lombard et al. 1986; Smeda and Daniel 1988). This activates voltage-dependent calcium channels and allows rapid Ca^{2+} entry. The voltage-dependent Ca^{2+}, selective channel subtypes, includes

T- and L-type channels (McDonald et al. 1994). The smooth muscle cells of renal pre-glomerular arterial vessels are enriched in L-type channels (Goligorsky et al. 1995). Plasma membrane depolarization promotes L-type channel activation (MacDonald et al. 1994). Voltage-independent Ca^{2+} channels can be activated in response to specific receptor activation (Benham et al. 1987). Entry of Ca^{2+} through L-type channels is absolutely required for myogenic autoregulation (Davis and Hill 1999). In addition, phospholipase C activation leads to increased formation of inositol trisphosphate and release of Ca^{2+} from intracellular calcium stores (Narayanan et al. 1994). Elevated $[Ca^{2+}]i$ leads to vascular constriction by activating myosin light chain kinase (MLCK).

The common pathway of the TGF and myogenic autoregulation is involved in vascular smooth muscle constriction/dilatation machinery. Cytosolic Ca^{2+} binds with calmodulin, and the Ca^{2+}-calmodulin complex binds to and activates MLCK. The kinase in turn phosphorylates and activates myosin light chain (MLC). The phosphorylated MLC then binds with actin so that cross-bridge cycling can begin. Actin slides relative to the stationary myosin to accomplish contraction. The myosin is able to interact with actin only when the MLC is phosphorylated. The pathway is inhibited by myosin light chain phosphatase (MLCP). It causes MLC dephosphorylation and inhibits the interaction between MLC and actin, and then dilates the vascular smooth muscle.

Thus these two enzymes, MLCK and MLCP, are keys to the constriction/dilatation machinery. MLCK regulation constriction while MLCP regulates dilation. Later, the regulation pathway of MLC phosphorylation will be discussed.

Paracrine regulation

Paracrine signals are local chemical messengers whose effects are exerted only on neighbouring cells in the immediate environment of their sites of secretion. Paracrine signals originating from endothelial and epithelial cells exert profound influences on the basal tone and reactivity of the pre- and post-glomerular arterioles. The paracrine agents in kidney include nitric oxide (NO), ANG II, endothelin-1 (ET-1). They interact with each other and form a complicated network.

Nitric oxide and nitric oxide synthase

Over the last 2 decades NO has proven to be the endothelial-derived relaxing factor which accounts for smooth muscle relaxation by vasodilators such as acetylcholine and bradykinin (Furchgott and Zawadski 1980) and also for shear induced vasodilatation (Furchgott and Vanhoutte 1989).

Nitric oxide that is generated by vascular endothelium dilates vessels, inhibits platelet adhesion and leukocyte adhesion. It is not only involved in the regulation of BP but also has been characterized as a neurotransmitter and a ubiquitous messenger of cellular signals (Miyamoto et al. 1998; Palmer et al. 1987; Quyyumi et al., 1995; Zeiher et al. 1991). Its chemical nature makes it an excellent candidate for short-term and short-range signalling (Cohen et al. 1997; Nathan et al. 1994). Because NO is sufficiently small and not polar, it can diffuse freely and rapidly through the hydrophobic environment of cell membranes just like O_2 and N_2 (Balbatun A et al. 2003).

Nitric oxide is synthesized from L-arginine and oxygen by the enzyme, nitric oxide synthase (NOS) (Moncada and Higgs 1993). It is produced when NOS catalyses the

oxidation of L-arginine to L-citrulline and NO, via the intermediate N-hydroxy-arginine, in a NADPH-dependent reaction. In mammals, three distinct genes encode NOS isozymes: neuronal (nNOS), cytokine inducible (iNOS) and endothelial (eNOS) (Knowles 1994).

Neuronal NOS is expressed in the central and peripheral nervous systems and in skeletal muscles (Christopherson et al. 1997; Huang and Lo. 1998; Bolanos et al. 1999). In the brain, NO acts as a neuro-modulator influencing functions such as behavior and memory formation. In the peripheral nervous system, NO acts as a neurotransmitter participating in functions such as smooth muscle control, gastrointestinal motility, and neuroendocrine function. It also functions in skeletal muscles as a signal transducer to regulate both metabolism and muscle contractility.

The earliest medical applications of NO relate to the function of eNOS in the cardiovascular system. Cells that express eNOS include vascular endothelial cells, cardiomyocytes and others (Hemmens et al. 1998; Taylor et al. 1998). In blood vessels, NO produced by the eNOS of endothelial cells functions as a vasodilator thereby regulating blood flow and pressure (Huang and Lo. 1998; Moncada et al. 1999). Mutant eNOS knockout mice have BP that is 15% higher than wild-type littermates (van Vliet et al. 2003). Within cardiomyocytes, eNOS affects Ca^{2+} currents and contractility (Drexler et al. 1999). Expression of eNOS is usually reported to be constitutive though modest degrees of regulation occur in response to factors such as shear stress, exercise training, chronic hypoxia, and heart failure (Drexler et al. 1999).

Expression of iNOS has now been reported in a large number of cell types, and in most circumstances, the enzyme is inducible (Taylor et al. 1998; Titheradge 1999). The

iNOS gene is under transcriptional control, but the enzyme's activity is also influenced by a variety of other control mechanisms that affect mRNA stability, translation and degradation of the protein, and availability of substrate and cofactors (Stuehr et al. 1999; Hemmens et al. 1998; Taylor et al. 1998). This enzyme is found in the cytoplasm.

The development of specific inhibitors of the L-arginine NO pathway has provided powerful tools to define the role of NO in renal physiology and pathophysiology. The arginine analogue L–nitro arginine methyl ester (L-NAME) is a competitive inhibitor of iNOS, but an inactivator and essentially irreversible inhibitor of nNOS and eNOS (Dwyer et al. 1991; Griffith et al. 1996; Iadecola et al. 2000). Certainly, the effect of L-NAME on RBF is not reversible, even by massive doses of L-arginine (Baylis et al. 1990; Wang et al. 1999).

Role of NO in autoregulation

The glomeruli are situated midway between pre- and post-glomerular resistance and the autoregulatory adaptation occurs predominantly in the pre-glomerular vessels (Ulfendahl 2001; Casellas 1993). The major fraction of the changes in vascular resistance in response to changes in BP occurs at the level of the afferent arterioles (Casellas and Moore 1990; 1993 Sanchez-Ferrer 1989; Navar 1998). Efferent arterioles do not appear to have a myogenic mechanism, since they do not respond to changes in perfusion pressure even when the pre-glomerular arterioles are dilated to allow transmission of pressure to the post-glomerular network.

As in other vascular beds, NO exerts a strong vasodilator effect (Ohishi et al. 1988) and is tonically active in the renal circulation (Baylis et al. 1990). It therefore is an

important determinant for the mean level of RBF. Inhibition of NOS by L-NAME causes an important and long-lasting rise in BP that is accompanied by profound renal vasoconstriction (Baylis et al. 1990; Beierwaltes et al. 1992, Wang et al. 1999). Irrespective of this tonic influence, early studies consistently showed that despite the shift of the mean level of RBF, the autoregulatory function was not affected by the presence of NO (Baumann et al.1992; Beierwaltes et al. 1992; Majid et al. 1993). However, subsequent studies have shown that both the myogenic response (Hayashi et al. 1995; Juncos et al. 1995; Pohl et al. 1991) as well as TGF (Braam et al.1995; Ito et al. 1993; Thorup et al. 1994; Turkstra et al. 1998; Vallon et al. 1995; Wilcox et al. 1992) are attenuated by NO, whereas neither mechanism seems to be mediated by NO.

These modulating influences of NO were not found to affect the autoregulation of total RBF by different experiment in early studies. At that time, the autoregulatory function was assessed in response to stepwise artificial reductions of renal artery pressure, whereas under physiological conditions the kidney has to cope with dynamic fluctuations of BP distributed over a wide range of frequencies (Just et al. 1998). One likely reason for the negative results is that renal autoregulation is very good when assessed by the step experiment. Consequently this experiment is not especially good at detecting the effects of substances that modulate autoregulation (Cupples 1993).

Inhibition of NOS augments autoregulation of afferent arteriolar diameter in the hydronephrotic kidney (Hoffend et al. 1993; Hayashi et al. 1995) and in the isolated juxtamedullary nephron preparation (Bouriquet and Casellas 1995). Blockade of NOS also increases the magnitude of TGF responses (Navar 1996; Navar et al. 1998; Wilcox et al. 1992).

We got three physiologic responses from NOS inhibition: increased BP, profound renal vasoconstriction (≈ 60% reduction of conductance), and augmented renal myogenic autoregulation in normotensive rats in our lab (Wang et al. 1999, Wang and Cupples 2000). In these studies, time series analysis showed that inhibition of NOS causes characteristic changes in the BP-RBF transfer function in rats. The use of this analysis and parameters derived from it are discussed in the Appendix.

Pathways for NO action

This laboratory has shown that renal vascular responses to inhibition of L-NAME are constriction and augmented myogenic autoregulation. The effect on autoregulation includes both augmentation of pressure-induced constriction and augmentation of pressure-induced dilatation. One problem appears: How is the signal transduced in smooth muscle cells? The $[Ca^{2+}]i$ in smooth muscle is a major determinant of smooth muscle contractility. Channel-mediated extracellular Ca^{2+} influx can occur through Ca^{2+}-selective channels or nonselective cation channels, which can be voltage dependent or voltage independent.

The effects of NO in the renal circulation are secondary to stimulation of guanylyl cyclase and the formation of cGMP (Trottier et al. 1998). Previous findings that inhibitors of guanylyl cyclase and cGMP-dependent protein kinase attenuate the vasodilator response to NO support a primary role for cGMP in this response (Cohen et al. 1995; Loscalzo et al. 1995; Carrier et al. 1997; Olson et al. 1997). NO affects the activities of calcium and potassium channels through cGMP (Sobey and Faraci 1999; Sobey and Faraci 1997). Inhibition of NO could reduce availability of potassium

channels and thus depolarize the cell membrane or it could increase availability of L-type channels (Sobey and Faraci 1997; 1999). Each of these effects would increase $[Ca^{2+}]i$ and augment pressure-induced constriction. However, it is not clear how these effects could augment pressure-induced dilatation.

Although an increase in intracellular $[Ca^{2+}]$ plays an important role in regulating vasomotor tone, the cytosolic $[Ca^{2+}]$ is not always proportional to the extent of MLC phosphorylation and contraction. This has led to the concept of Ca^{2+} sensitization (Bradley et al. 1987; Hisaoka et al. 2001; Horowitz et al. 1996; Somlyo and Somlyo 1994; 2000). It typically refers to a process whereby Ca^{2+}-dependent contractions occur but at a Ca^{2+} concentration lower than would be expected for that mediated directly via MLCK. Ca^{2+} sensitization has also been explained by inhibition of myosin phosphatase through Rho/Rho-kinase and/or protein kinase C-based mechanisms (Kitazawa et al. 2000; Somlyo and Somlyo 1994). Many agonists cause contraction under conditions in which $[Ca^{2+}]i$ cannot be changed and are said to sensitize the vascular smooth muscle cell to $[Ca^{2+}]i$. L-NAME could increase the Ca^{2+} sensitization and augment pressure-induced dilatation through cGMP. Evidence has shown that endogenous NO-mediated vasodilatation occurs through the inhibition of Rho-kinase constrictor activity in the intact rat aorta (Chitaley et al. 2002).

In the Ca^{2+} sensitization pathway, the receptors activated by agonists are coupled to G-protein. A Gα-GAP (GTPase-activating protein), Rho-GEF (guanine nucleotide exchange factor), interacts with Gα and is the upstream 'convertor' between this trimeric G-protein and RhoA (Kozasa et al. 1978; Hart et al. 1998). Several trimeric G-proteins, Gαq, $G\alpha_{12,13,}$ and Gα1-2 can activate RhoA, depending on the receptors and cell types

involved (Katoh et al. 1998; Croxton et al. 1998; Klages et al. 1999; Hirshman and Emala 1998).

RhoA is a monomeric GTPase. It is active when bound to GTP and inactive when bound to GDP. In the resting smooth muscle, the cytosolic, inactive form of RhoA is complexed with RhoGDI (guanine nucleotide dissociation inhibitor). RhoA·RhoGDI is activated by Rho-GEFS that GTP replaces GDP, and RhoA·GTP dissociates from the complex. Then, the activated RhoA translocates to the plasma membrane (Gong et al. 1997; Somlyo et al. 2000). The Rho·GTP activates Rho-kinase, a serine/threonine-kinase. In vascular smooth muscle the major target of Rho kinase is MLCP. The activated Rho-kinase phosphorylates the regulatory subunit of MLCP and inhibits MLCP activity (Kimura et al. 1996). Thus it increases MLC phosphorylation and causes contraction. (Somlyo and Somlyo 2003).

The Ca^{2+}-sensitizing Rho/Rho-kinase pathway can be inhibited by Y-27632 a highly selective, cell permeant Rho-kinase inhibitor. In the present study, it is used to test whether blockade of Rho/Rho-kinase pathway leads a change of the effect of NO on the renal myogenic autoregulation. Because other paracrine factors, notably ET-1 and ANG II also affect Ca^{2+} sensitivity and autoregulation, we have to predict the extent of interaction between NO and ET-1/ANG II in order to isolate the effect of NOS inhibition.

Interaction between NO and ET-1

Vascular endothelium produces a number of factors that can affect vascular function. One of these substances is a contracting factor called endothelin. There are at least three isoforms of ET: ET-1, ET-2, and ET-3 (Yanagisawa et al. 1988). The most common is

ET-1, which is one of the most potent endogenous vasoconstrictors. An endothelin precursor is cleaved to ET-1 by endothelin converting enzyme found on the endothelial cell membrane (Rubanyi and Polokoff 1994). ET receptors are widely distributed not only in vascular but also in nonvascular tissues. They are classified into two subtypes termed ETA (Arai et al. 1990; Lin et al. 1991; Hosoda et al. 1991) and ETB (Elshourbagy et al. 1992) by their distinctions in affinity for the endothelins. ETA receptor has high affinity for ET-1 and ET-2 and low affinity for ET-3, and the ETB receptor has equally high affinity for all endothelins. They belong to the G protein coupled receptor family.

Endothelin A receptors are found predominantly in vascular smooth muscle and mediate vasoconstriction. In the kidney, ETB receptors are present on both endothelial and vascular smooth muscle cells, and mediate both vasodilatation and vasoconstriction. The ETB receptors can mediate both vasodilatation by release of relaxing factors from vascular endothelial cells and vasoconstriction through receptors located on smooth muscle cells (Panek et al. 1992; Takayanagi et al. 1991). Evidence has shown that ETA and ETB receptors are distributed equally in the kidney cortex; and ETB receptor is more abundant than ETA in outer medulla and papilla in the rat. Exogenous ET-1 and Sarafotoxin 6C (ETB agonist) elicit similar dose-dependent decreases in RBF which are unaffected by a selective ETA receptor antagonist (Gellai et al. 1994). ET-1 had a greater effect on the afferent than efferent arteriole in the hydronephrotic rat kidney (Loutzenhiser and Epstein 1990).

The signalling mechanisms vary between ETA and ETB receptors. ETA receptor is coupled to phospholipase C and Ca^{2+} mobilization from intracellular stores (Takuwa et al. 1990), while ETB receptor controls sodium/hydrogen exchange independently of

phospholipase C and inhibits agonist induced cAMP (Aramori and Nakanishi 1992). Both Ca^{2+} entry and Ca^{2+} sensitization mechanisms are operative under physiologically relevant conditions.

The ET-mediated increase in cGMP formation and NOS activity are not affected by ETA receptor antagonist and are blocked by ETB receptor antagonists (Mathison and Israel 2002). Therefore, ETB receptor is functionally coupled with an activated constitutive eNOS and stimulates the release of NO (Noris and Remuzzi 1999).

The efficiency of RBF autoregulation is enhanced during an acute inhibition of NO synthesis in anesthetized rats. ET may intervene in the setting of vasomotor tone through modulation of different Ca^{2+} transport systems in vascular smooth muscle cells, including voltage-dependent Ca^{2+} channel (Zhang et al. 1999). During acute blockade of NO in the rat, endothelin alters the efficiency of steady state RBF autoregulation by a preferential activation of ETB receptor (Kramp et al. 2001). In contrast, NO can displace ET from its receptor located on vascular smooth muscle (Goligorsky 1994). NO not only opposed the vasoconstrictor effects of ET but also facilitated the termination of the action of the peptide (Hercule et al. 2000).

Interaction between NO and ANG II

We have reviewed the effect of the renin-angiotensin system (RAS) and the function of its key mediator, ANG II. Traditionally, the RAS is regarded as an endocrine system. But ANG II can be found in several tissues: kidney, brain, adrenals, and the reproductive organs, suggesting paracrine/autocrine functions (Zimmerman and Dunham 1997; De Gasparo et al. 2000).

Effects of ANG II are mediated by the interaction of this peptide with two major types of receptor, AT1 and AT2. Activation of the AT1 receptor has been associated with essentially all of the cardiovascular and renal effects of ANG II (Cachofeiro et al. 1996) whereas AT2 receptor activation is involved in cell differentiation and inhibition of cell proliferation, particularly in fetal development (De Gasparo et al. 2000).

Because we investigate the interaction between NO and ANG II on myogenic autoregulation, we are interested in the AT1 receptor and signal transduction. AT1 receptor belongs to the G protein coupled receptor family (Kai et al. 1996). ANG II activates phospholipase C via G protein in vascular smooth muscle cell (Griendling et al. 1997).

ANG II is a potent renal vasoconstrictor that predominantly affects the efferent arteriole (Hall et al. 1977). In addition, it may indirectly cause constriction of the afferent arteriole through potentiation of the TGF mechanism (Schnermann 1998). Evidence has shown that both the regulation of RBF and the resetting of autoregulation are mediated by the RAS (Cupples 1993; Holm et al. 1990) and ANG II augments myogenic autoregulation in the afferent arteriole of the hydronephrotic kidney (Kirton and Loutzenhiser 1998). Ohishi (1992) reported that ANG II does not stimulate NO synthesis in kidney. It has been suggested that the function of NO is to impose a constraint on an afferent arteriolar ANG II tone (Ohishi et al.1992; Sigmon et al.1992; Ito et al. 1993; Juncos et al.1996; Qiu and Baylis 1999), and to modulate the TGF response (Wilcox et al. 1992; Thorup and Persson 1996). Whether there is an interaction between ANG II and NO on myogenic autoregulation is unknown.

Summary

Overall, renal autoregulation is essential for keeping stabilized RBF. Many paracrine factors modulate autoregulation. NO plays an important role on renal myogenic autoregulation. Inhibition of NOS enhances myogenic autoregulation by augmenting of pressure-induced constriction and pressure-induced dilatation. There are multiple pathways to regulate myogenic autoregulation by NO. These induce control of membrane potential, Ca^{2+} entry into vascular smooth muscle cells, and relaxation of vascular smooth muscle cells. Channel activation/inhibition can explain the pressure-induced constriction but not the pressure-induced dilatation. The Ca^{2+} sensitization may contribute to the pressure-induced dilatation. The present study focuses on whether regulation by phosphorylation of MLCP contributes to augmentation of autoregulation by NOS inhibitor. We investigated the effect of Rho-kinase blockade on the enhanced myogenic autoregulation by NO blockade to assess the mechanism by which NO modulates renal autoregulation.

In order to achieve this aim, we divided the project into two parts. Since paracrine factors interact with each other and form a complicated network, we first show how much they interact with NO on RBF by blocking these paracrine factors pharmacologically (ET-1 and ANG II). Then we address the central problem: whether L-NAME enhances myogenic autoregulation through Rho-kinase pathway.

MATERIALS AND METHODS:

All experiments were performed in male Wistar rats from Charles River, Canada. The study was approved by the SMBD-JGH Animal Care Committee and the McGill University Veterinarian and conducted under the guidelines of the Canadian Council on Animal Care. Rats had free access to water and food at all times prior to experiments.

Surgical procedures:

Twenty minutes prior to anesthesia, each rat received the narcotic analgesic buprenorphine (Temgesic®, 0.01 mg/kg ip, Reckitt and Colman Pharmaceuticals Inc., Wayne, N.J.). Anesthesia was induced by 5 % isoflurane in inspired gas (30 % O_2 70 % air). After induction the anesthetic concentration was reduced to ≈ 2.5 %. The animal was transferred to a servo-controlled heated table to maintain body temperature at 37°C, intubated, and ventilated by a volume-controlled animal respirator (Harvard ventilation). During the equilibration period after surgery, the inspired isoflurane concentration was reduced to the minimum concentration, about 1-1.4 %, that precluded a BP response when the tail was pinched. The tail was pinched periodically through the experiment to assure adequate anesthesia.

Cannulas were put into the right femoral artery (PE-90 with narrowed tip) and the right femoral vein (PE-50); the venous line contained a Silastic insert to allow drug infusion. A constant infusion delivered 1 % of body weight per hour. The infusion was continued throughout the whole experiment and contained 2 % charcoal washed bovine serum albumin in sterile saline (0.9 %). After the arterial cannula was placed, BP was monitored. A multiple-catheter line (Parekh 1995) for infusion of different drugs in the

renal artery was put into the left femoral artery. Then the left kidney was exposed by a subcostal flank incision and fixed in a plastic cup. It was covered with a plastic sheet to limit drying. The left renal artery and aorta were isolated and the multiple-catheter line was advanced into the left renal artery. A transit time ultrasound flow probe was placed around the renal artery to measure renal blood flow after stripping the renal artery from hilus to aorta.

A motorized clamp was placed on the aorta between the right and left renal arteries and was used to increase the amplitude of pressure fluctuations, that is, to "force" BP. The motor was driven by a program implemented in DT-VEE (Data Translation, Inc). Briefly, the program operates as a negative-feedback controller to maintain downstream pressure 15-20% below the spontaneous level of BP. It iterates at about 2 Hz, and at each iteration the target pressure is randomly changed within a predefined range, ± 5% (Wang et al 2000). In study 3, 4, and 5 forcing was used to increase BP fluctuation and then we assessed the effects of the drugs upon RBF dynamics. In each period, the target downstream pressure was adjusted so that drug actions could be compared at the same mean BP.

To achieve a homogeneous distribution of drugs within the kidney, a multiple-catheter line was used in all the studies (except study 1). The device used for intrarenal infusion consisted of a Teflon cannula inserted into the renal artery, which received several separate lines for drug infusion and one line connected to a magnetic motor that was driven at ~1.2 Hz. This provided alternating negative and positive pressure in the Teflon infusion cannula and served to mix the drugs with renal arterial blood (Parekh 1995). Placing the cannula tip into the renal artery or operating the mixing pump had no

discernible effect on BP or RBF.

Drugs and methods of administration

Because we studied the interaction of two drugs, it was necessary to determine the order of administration of the drugs. The drugs used in these studies are not reversible or are poorly reversible. Thus each experiment was performed in both directions: (1) the control (CTL) → drug 1 → drug 1 + drug2, (2) CTL → drug 2 → drug 2 + drug 1.

Table 1 lists all drugs used in this study. In addition it provides information concerning drug actions and specificities, doses and routes of administration, and sources. Captopril, L-NAME, PD 145054, BQ-123, and Y-27632 were dissolved in 0.9 % sterile saline. Endothelin-1 was dissolved in 0.9 % sterile saline and 1 % BSA; BQ-788 was prepared in 0.9 % sterile saline and 0.1 M sodium bicarbonate. Captopril and L-NAME were stored at 4°C, and other drugs were stored at -20°C.

L-NAME, captopril, and ET-1 were infused for 20 min (for positive control group only), and the other drugs were administrated for 50 min after which data were acquired.

Drugs	Action mechanism	Stock doses	Working doses	Source and reference
L-NAME	Non-selective NOS inhibitor	10 mg/ml	(1) 10 mg/kg iv. (2) 10 μg/min x 20 min, and then 3 μg/min intrarenal artery infusion	Sigma
Captopril	Non-selective angiotensin converting enzyme inhibitor	3 mg/ml	3 mg/kg/hr iv.	Sigma
PD 145065	General non-selective ET receptor antagonist.	0.13 mM	75 nM intrarenal artery infusion	Sigma Doherty et al. 1993
BQ-123	Antagonist that selectively (1000 folds) inhibits the ETA receptor	0.79 mM	1 μM intrarenal artery infusion	ALEXIS Biochemicals Inc. Ihara et al. 1992; Mino et al. 1992
BQ-788	Antagonist that selectively inhibits (1000 fold) the ETB receptor.	0.75 mM	1 μM intrarenal artery infusion	ALEXIS Biochemicals Inc. Karaki et al. 1994; Ishikawa et al. 1994; Victorino et al. 2000
Y-27632	Highly-selective Rho-kinase inhibitor	0.03 M	10 μM intrarenal artery infusion	Welfide Corp, Japan Uehata et al. 1997.
ET-1		50 μg/ml	5 ng/kg/min	Sigma Wilkes and Boarder 1991; Pecci et al. 1993.

Table 1. All drugs used in the study. In addition it provides information concerning drug actions and specificities, doses and routes of administration, and sources.

Protocol

Study 1

Study 1 was designed to test the extent of interaction between NO and ANG II on the BP and RBF. In this study only, drugs were given systemically. A single dose of L-NAME (10 mg/kg) was given over ~ 2 min by intravenous injection. This dose has a saturating effect on BP (Beierwaltes et al. 1992) and causes profound renal vasoconstriction (Wang et al 1999, Wang and Cupples 2000). To inhibit the generation of ANG II, the converting enzyme inhibitor captopril (Sigma) was infused intravenously at 3 mg/kg/hr, a standard blocking dose (Hansell et al. 1988). The study was divided into two experiments. In one experiment (n = 6), body weight (BW) was 339 ± 25 g. L-NAME was first given after a control period, and then captopril was given. In another experiment (n = 5, BW = 312 ± 30 g), the order was reversed.

Study 2

In this and all subsequent experiments, drugs were given through the left renal artery to minimize systemic effects.

Study 2 had two objectives: one was to verify the adequate dose of PD 145065 for blockade, as well as the doses required for BQ-123 and BQ-788 for adequate blockade for study 3 and 4. The other objective was to study how much interaction there is between NO and ET-1 on RBF and BP.

In the first experiment, there were four groups. In the first group (n = 3, BW = 340 ± 52 g), ET-1 was given as a positive control. In the second group (n =6, BW = 300 ± 60 g), PD 145065 was given before L-NAME was added to the infusion. In the third group

(n = 5, BW = 318 ± 31g), the drug administration order was PD 145065 → PD 145065 + ET-1 (10 min) → PD 145065 + L-NAME, in order to test whether PD 145065 completely blocked ET-1. In the fourth group (n = 3, BW = 306 ± 20 g), BQ-123 and BQ-788 were used together to replace PD 145065 to test whether their doses were enough to block ET-1.

In the second experiment, there were two groups. In the first group, there were three subgroups. In the first subgroup, PD 145065 was given before L-NAME was added to the infusion. The drug administration of the second and third group are the same: PD 145065 → PD 145065 + ET-1 (20 min) → PD 145065 + L-NAME. In the second subgroup, the dose of PD 145065 was 75 nM (the first two subgroups were described above in the first objective). In the third subgroup (n = 4, BW = 359 ± 18 g), the dose of PD 145065 was three times that of the second subgroup to test the response of L-NAME after the infusion of a high concentration of PD 145065. In the second group (n = 8, BW = 313 ± 43 g), L-NAME was given before PD 145065 was added to the infusion.

This study showed that complete blockade of ET receptors blunted the renal vasoconstrictor response to L-NAME. The next two studies assessed the involvement of signalling through ETA and ETB receptors.

Study 3

Study 3 tested the effect of the inhibition of ETB receptor on renal autoregulation and assessed whether NO interacted with ET-1 through ETB receptor. In the first experiment (n = 6, BW = 297 ± 33 g), L-NAME was given before BQ-788 was added to the infusion. In the second experiment (n = 6, BW = 305 ± 40 g), BQ-788 was given first then L-

NAME was added to the infusion.

Study 4

Study 4 was designed to test the effect of the inhibition of ETA receptor on renal autoregulation and see whether NO interacts with ET-1 through ETA receptor. In the first experiment (n = 10, BW = 313 ± 33 g), L-NAME was given before BQ-123 was added to the infusion. In the second experiment (n = 6, BW = 310 ± 21 g), BQ-123 was given first then L-NAME was added to the infusion.

Study 5

Study 5 was a central experiment. It was designed to test the effect of MLC phosphatase on renal autoregulation and find out whether the signal transduction pathway of modulation of renal myogenic autoregulation by NO is through Ca^{2+} sensitization, via the Rho-kinase pathway. It was divided into two experiments. In the first experiment (n = 6, BW = 304 ± 35 g), L-NAME was given before Y-27632 was added to the infusion. In the second experiment (n = 7, BW = 312 ± 30 g), Y-27632 was given first then L-NAME was added to the infusion.

In the second experiment, we first infused Y-27632 at 20 μM (n = 2), but the resulting fall of BP precluded assessment of RBF dynamics. Infusing Y-27632 at 5 μM (n = 2) resulted in incomplete blockade of the renal vascular actions of L-NAME and still provoked reduction of BP. So we confirmed that the optimal concentration of Y-27632 was 10 μM.

Data acquisition and analysis

Femoral arterial pressure was measured by a pressure transducer (HP 1290C) driven by a Kent amplifier. Blood flow was measured by a Transonic Systems Inc. T420 transit time ultrasound flowmeter (probe model: 1PRB2815) or a T106 transit time ultrasound flowmeter (R1 probe). Pressure and flow were digitized on-line using 12-bit analog-to-digital conversion. The input (BP) and output (RBF) signal were low-pass filtered at 40 Hz and sampled at 100 Hz.

Power spectra, transfer function, and coherences based on a fast Fourier transform (FFT) were computed with the use of standard algorithms as shown previously (Abu-Amarah et al. 1998; Cupples et al. 1996; Wang et al. 1999; Wang et al. 2000). Data segments of 1311 sec were band-pass filtered (0.003 and 1 Hz) by using FFT-inverse FFT filter (Mos et al. 1987) with a rectangular window and subsampled to 3.125 Hz. Spectra, coherences, and transfer functions (admittance magnitude and phase) employed 1024 point segments and the Hann window; spectra were calculated with 50% overlap and transfer functions with 69% overlap.

To assess the contribution of the myogenic system to stabilization of RBF, we determined fractional compensation (FC) in the interval between the operation frequencies of the two systems. The interval from 0.05 to 0.08 Hz was used to minimize corruption by TGF (<0.05 Hz) and by myogenic transients (> 0.08 Hz). FC is calculated from gain: $FC = 1-[10^{(gain/20)}]$, which linearizes the logarithmic scale of gain. FC = 1 implies complete autoregulation, and FC = 0 implies total absence of autoregulation. To calculate slope, we examined the gain to identify linear range, fitted that range to log (frequency), and calculated the average slope. To estimate the phase peak, we calculated

the average phase in the frequency band from 0.076 to 0.137 Hz.

Statistical analysis

Results are presented as means ± SEM. Differences within experiments were assessed by one-way ANOVA using Statistica, v 5.5 (Statsoft Inc, Tulsa OK, USA). If significant effects or interactions were observed, CTL vs Drug1, CTL vs drug 2, and drug1 vs drug 2 were compared with Post Hoc Multiple Comparisons. Significance between studies was assessed by T-test. If the effects of a drug on RBF were compared in two different experiments, then these experiments must be treated by the same drugs but the drug administration order was different. The changes of RBF before and after drug in one experiment were compared with those in other experiment. $P < 0.05$ was considered to be significant.

RESULTS:

Study 1: Interaction between NO and ANG II

This study was divided into two experiments: in the first experiment, L-NAME was given first, and then captopril was added. In the second experiment, captopril was infused before L-NAME was given. All drugs were given by intravenous infusion. Chart records of BP and RBF are presented in Figure 1. Averages of BP and RBF are reported in Table 2.

Figure 1A shows that BP started to rise while RBF started to decrease after L-NAME was given for a few minutes. Both BP and RBF stabilized 10 min later. L-NAME increased BP by 31 ± 6% while RBF and conductance decreased by 49 ± 1% and 65 ± 1%, respectively. There was a significant difference (Table 2). These are typical responses to L-NAME. After the infusion of captopril, captopril could not reverse the effect by L-NAME and no significant changes were observed (Table 2). In the second experiment (Figure 1B), captopril was given before L-NAME and although changes in BP and RBF were more noticeable, these changes were again not significant (Table 2). Upon injection of L-NAME, changes of BP and RBF were the same as in Figure 1A. The response to L-NAME did not change when captopril was infused first.

Inhibition of NO caused vasoconstriction while inhibition of ANG II caused vasodilatation. Comparison of the effects of L-NAME with and without captopril showed no significant difference. Captopril did not alter the effect of L-NAME while a comparison of the effects of captopril with and without L-NAME showed that the vasodilator response to captopril was severely blunted by L-NAME. It indicated captopril did not alter the pressor or constrictor responses to L-NAME while L-NAME blocked the

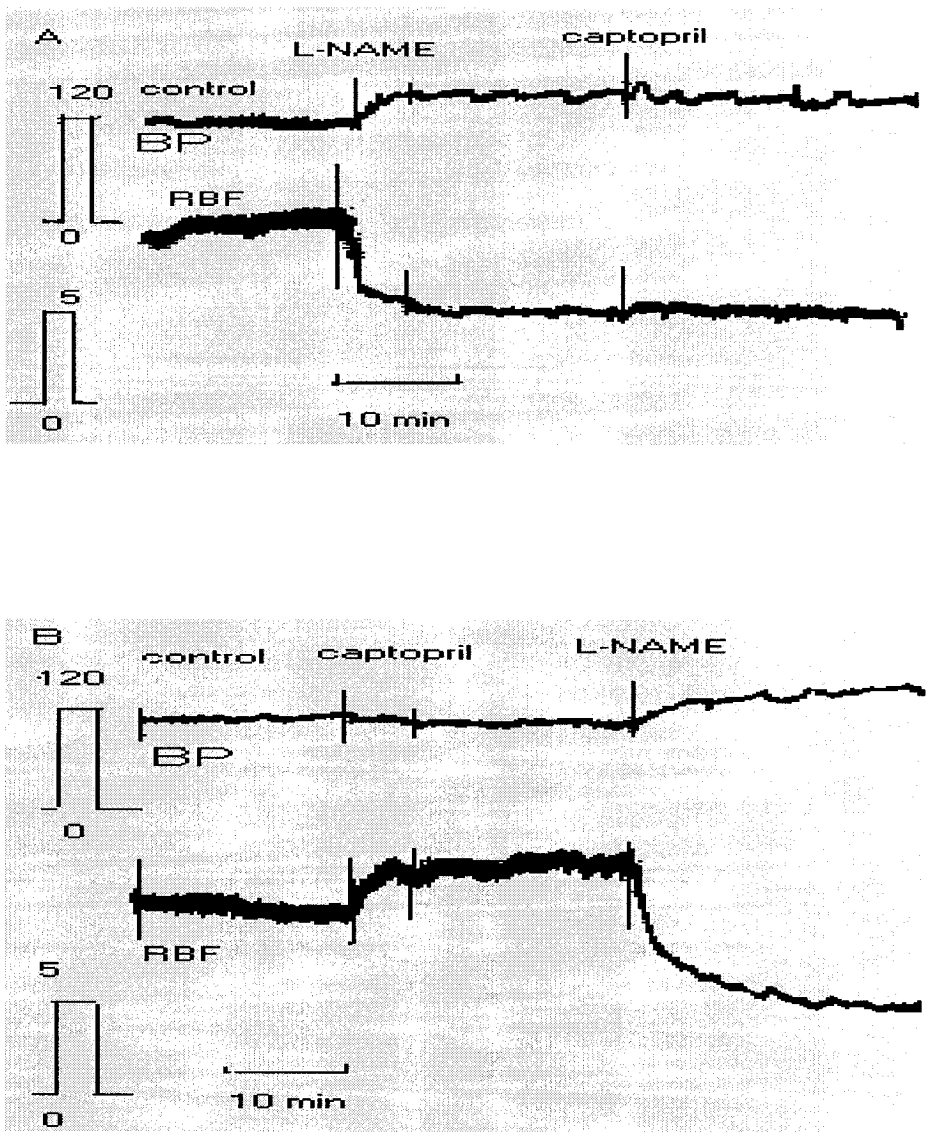

Figure 1. Chart record showing responses of blood pressure (BP) and renal blood flow (RBF) to captopril and L-NAME. Clearly responses to L-NAME are not affected by captopril, whereas the vasodilator response to captopril is severely blunted by L-NAME.

Expt		BP (mmHg)	ΔBP (%)	RBF (ml/min)	ΔRBF (%)	G (10^{-2})	ΔG (%)
1. n = 6	CONTROL	110 ± 5		8.0 ± 1.3		5.7±1.3	
	L-NAME	139 ± 3*	+31± 6	4.0 ± 0.6*	-49 ± 1	2.3±0.5*	-65 ± 1
	CAPTOPRIL	134 ± 3*	-5 ± 1	4.2 ± 0.6*	+4 ± 2	2.6±0.6*	+13 ± 3
2. n = 5	CONTROL	104 ± 4.		7.0 ± 0.9		6.9±1.5	
	CAPTOPRIL	92 ± 4	-13 ± 5	8.1 ± 1.0	+17 ± 6	8.9±1.5	+32± 9
	L-NAME	128 ± 7*ᵠ	+36 ± 3	4.0 ± 0.8*ᵠ	-53 ± 3	3.1±0.5*ᵠ	-65± 1

Table 2. The action of L-NAME and captopril on blood pressure (BP) and renal blood flow (RBF) in rats. Values are means ± SE; n = number of rats studied. BP, mean arterial pressure; RBF, renal blood flow. Statistical significance within a group (1-way ANOVA for repeated measurements and a multiple comparison): *P < 0.05 each drug given vs. control; ᵠP < 0.05 the second drug given vs. the first drug.

dilatation induced by captopril. The response to L-NAME is independent of ANGII. And the action between NO and ANG II was not important in the signal transduction pathway of NO.

Study 2: Interaction of NO and ET-1

There were two experiments in this study. One was designed to determine the adequate doses of PD 145065, BQ-123 and BQ-788 required for the blockade of ET-1. PD 145065 is a non-selective ETA/B receptor antagonist. BQ-123 is a selective ETA receptor antagonist, and BQ-788 is a selective ETB receptor antagonist. The second experiment was designed to assess how much interaction there is between NO and ET-1. Changes in RBF in response to each drug are shown in Figure 2 while BP and RBF averages are reported in Table 3. Since intrarenal artery infusion was used for these experiments, BP remained stable, making it easier to study the effects of each drug based upon RBF or conductance changes only.

The first experiment was divided into four groups. The first group was a positive control. The second group was to study effects of PD 145065 and the effects of L-NAME when pre-treated with PD 145065. The third group was designed to test whether PD 145065 could completely block ET-1. The fourth group was to test whether BQ-123 and BQ-788 together could completely block ET-1 receptors.

In the first group, ET-1 reduced RBF and conductance significantly (Table 3). The effect of renal vascular constriction by ET-1 was as a positive control to study the effect of PD 145065.

In the second group, PD 145065 was given before L-NAME was added to the

infusion. PD 145065 is a non-selective ETA/ETB receptor blocker and it had no effect on RBF and conductance. When L-NAME was infused, RBF and conductance reduced significantly. The effect of L-NAME on RBF was still significant after pre-treatment with PD 145065.

In the third group, PD 145065 was challenged with ET-1 to check whether the dose of PD 145065 was sufficient to block ET-1. We observed the response to ET-1 in the first group. If the concentration of PD 145065 was enough to block ET-1 receptors, we should not see any effect of ET-1 when PD 145065 was given first. Again, PD 145065 had no significant effect on RBF and conductance, as previously shown in the second group, and when ET-1 was infused, RBF and conductance remained the same. It indicated that PD 145065 completely blocked the response to ET-1. Finally, L-NAME was given and the typical drop in RBF and conductance following L-NAME administration was observed. Hence, the concentration of PD 145065 was adequate in blocking ET-1 receptors and significant response to L-NAME was still observed when L-NAME was given.

In the fourth group, BQ-123 and BQ-788 were infused together to test whether they completely blocked ET-1 receptors. The protocol was the same as in the third group except that PD 145065 was substituted with BQ-123 and BQ-788. BQ-123 and BQ-788 did not alter RBF and conductance. ET-1 did not decrease RBF and conductance significantly while L-NAME reduced RBF and conductance significantly. In comparison with the third group, the same effects were observed using BQ-123 and BQ-788.

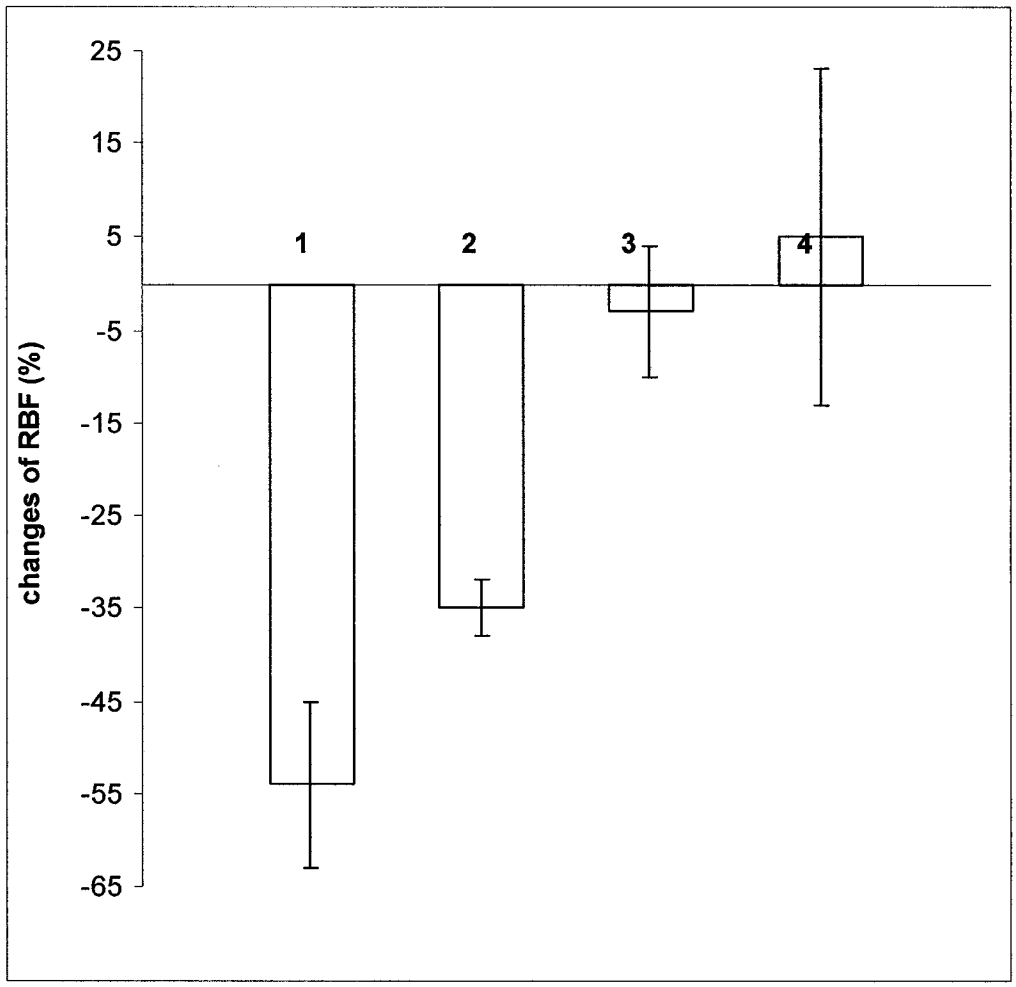

Figure 2. The effect of L-NAME with or without PD 145065 and PD 145065 with or without L-NAME on renal blood flow (RBF). 1- L-NAME only; 2- L-NAME pre-treated with PD 145065; 3-PD 145065 only; 4- PD145065 pre-treated with L-NAME.

Expt	Group		BP (mmHg)	RBF (ml/min)	ΔRBF (%)	G (10^{-2})	ΔG (%)
1.	1. n = 3	CONTROL	122 ± 11	7.8 ± 0.2		6.6±0.8	
		ET-1	129 ± 13	4.4 ± 0.1*	-44 ± 1	3.5±0.4*	-47 ± 0.3
	2. n = 6	CONTROL	106 ± 3	7.2 ± 0.8		6.2±0.8	
		PD 145065	112 ± 4	6.9 ± 0.7	-3 ± 3	6.1±1.3	-3 ± 7
		L-NAME	115 ± 3	4.3 ± 0.5*ϕ	-38 ± 1	3.6±0.9*ϕ	-42 ± 2
	3. n = 5	CONTROL	109 ± 6	7.7 ± 1.0		7.3±1.3	
		PD 145065	117 ± 6	7.6± 0.6	+3 ± 9	6.6±0.7	-4 ± 9
		ET-1	117 ± 5	7.4 ± 0.5	-2 ± 5	6.4±0.5	-2 ± 5
		L-NAME	117± 5	4.9 ± 0.3 *γ	-34 ± 3	4.2±0.4*γ	-35 ± 3
	4. n = 3	CONTROL	118 ± 6	7.7 ± 0.4		6.6±0.4	
		BQ-123 & BQ-788	115 ± 5	7.6 ± 0.6	-3 ± 2	6.7±0.4	+1±3
		ET-1	116 ± 5	8.2 ± 0.3	+9± 10	7.2±0.5	+8±10
		L-NAME	113 ± 7	5.8 ± 0.5*γ	-29 ± 4	5.2±0.6*γ	-28±5
2.	1. 1. n= 6	CONTROL	106 ± 8	7.2 ± 2.0		6.2±0.8	
		PD 145065	112 ± 9	6.9 ± 1.7	-3 ± 3	6.1±1.3	-3 ± 7
		L-NAME	114 ± 8	4.3 ± 1.2*ϕ	-38 ± 1	3.6±0.9*ϕ	-42 ± 2
	2. n= 5	CONTROL	110 ± 5	7.7 ± 10		7.3±1.3	
		PD 145065	117 ± 6	7.6 ± 0.6	+3 ± 9	6.6±0.7	-4 ± 9
		ET-1	117 ± 7	7.4 ± 0.5	-2 ± 5	6.4±0.5	-2 ± 5
		L-NAME	118± 5	4.9 ± 0.3 *γ	-34 ± 3	4.2±0.4*γ	-35 ± 3
	3. n= 4	CONTROL	109 ± 6	10.5 ± 0.8		9.7±0.7	
		PD 145065 x 3	108 ± 3	9.8 ± 0.7	-7 ± 3	9.1±0.4	-6 ± 8
		L-NAME	99 ± 2	5.7 ± 0.1*ϕ	-34 ± 1	5.8±0.2*ϕ	-31 ± 1
	2. n = 8	CONTROL	121 ± 4	8.4 ± 0.7		7.0±0.5	
		L-NAME	135 ± 3	3.8 ± 0.4*	-55± 3	2.9±0.3*	-59±4
		PD 145065	134 ± 2	4.0 ± 0.4*	+6 ± 7	3.0±0.3*	6±6

Table 3. Actions of L-NAME, ET-1 and ET-1 blockers on blood pressure (BP) and renal blood flow (RBF) in rat. Values are means ± SE; N = number of rats studied. Statistical significance within a group (1-way ANOVA for repeated measurements and a t-test): *P < 0.05 each drug given vs. control; ϕP < 0.05 the second drug vs the first drug; γP< 0.05 the third drug vs the second drug.

In the first and the fourth groups, the numbers of experiment performed were three. In order to avoid variation, we normalized the data. ΔRBF_{ET-1} was -44 ± 1 % and ΔG_{ET-1} was -47 ± 0.3 % in the first group while ΔRBF_{L-NAME} was -29 ± 4 % and ΔG_{ET-1} was -28 ± 5 % for the fourth group. There was a significant difference (Table 3).

The second experiment of this study was divided into two groups. In the first group, the effects of L-NAME were tested after a treatment with PD 145065. In the second group, the effects of PD 145065 were studied following a treatment with L-NAME.

In the first group, there were three subgroups. The first two subgroups were the same as group 2 and 3 from the first experiment, where the adequate concentrations of the drugs have been determined. In the first subgroup, PD 145065 was given before L-NAME was added to the infusion and no changes in RBF and conductance were observed. When L-NAME was infused, RBF and conductance were reduced significantly (ΔRBF_{L-NAME} = -38 ± 1% and ΔG_{L-NAME} = -42 ± 2%, $P < 0.05$). In the second subgroup, ET-1 was used to challenge PD 145065. As previously, PD 145065 alone did not alter RBF and conductance. When ET-1 was infused, ET-1 did not alter RBF and conductance. This indicated that PD 145065 efficiently blocked the response to ET-1. Finally, when L-NAME was given, RBF and conductance dropped significantly. In the third subgroup, the sequence of drugs infused was the same as the second subgroup but the concentration of PD 145065 was tripled. Again, PD 145065 did not alter RBF and conductance; ET-1 did not alter them either. When L-NAME was given, RBF and conductance again decreased significantly (Table 3).

In brief, the comparison of the effect of PD 145065 in RBF and conductance at different doses showed no significant difference in the first group (n = 15) while L-

NAME significantly reduced RBF and conductance (-36 ± 1 % and -35 ± 1%, respectively) following a treatment with PD 145065.

In the second group, L-NAME was given before PD 145065 was added to the infusion. First, we observed the characteristic drop in RBF and conductance after L-NAME. When PD 145065 was given, RBF and conductance remained stable. PD 145065 did not alter the response to L-NAME.

In summary, this study demonstrated that when we challenged PD 145065 by adding ET-1, RBF and conductance were not altered. It indicated that the doses of PD 145065, and BQ-123 with BQ-788 together were adequate in blocking ET-1 receptors. A comparison of the effect of L-NAME with and without PD 145065, or L-NAME pre-treated with PD 145065 showed that L-NAME still reduced RBF and conductance significantly. When the dose of PD 145065 was increased to three times its original concentration, the same result was again observed. ET-1 blockade attenuated, but did not block the vasoconstriction response to L-NAME while the effects of PD 145065 alone on RBF and conductance were not significantly different when it is pre-treated with L-NAME.

PD 145065 blunts the constrictor response to subsequent L-NAME, but does not reverse the response to prior L-NAME. Thus failure to reverse the constriction induced by L-NAME indicates that endothelin should not be considered as a mediator of this response. Instead, this pattern indicates the presence of an interaction between NO and ET-1.

Study 3: Interaction between NO and ETB receptor

This study was divided into two experiments. In the first experiment, L-NAME was

given before and while BQ-788 was added to infusion. In the second group, it was reversed and BQ-788 was administered before and while L-NAME was given.

In the first experiment, we saw a typical response of RBF and conductance to L-NAME. Averages of BP, RBF, conductance, FC, slope of gain reduction by the myogenic mechanism and phase peak are shown in Table 4. L-NAME alone reduced RBF by 55 ± 4% and reduced renal conductance by 56 ± 4%, $P < 0.05$. BP power and RBF dynamics from forcing periods are shown in Figure 3. Because all forcing started at about the same level of BP, the spectral power in BP after drugs were the same as the BP power in the control forcing period (Figure 3A). Figure 3B shows the coherence between BP and RBF. Under control condition, coherence was high above 0.1 Hz and declined at low frequencies. L-NAME decreased coherence below 0.1 Hz. During the control period, gain was positive in the pressure passive region of the spectrum (> 0.2 Hz) consistent with vascular compliance permitting amplification of BP fluctuation into RBF (Figure 3B). Spontaneous operation of myogenic system created a resonance peak at about 0.2 Hz and reduced gain to < 0 at lower frequencies. L-NAME increased gain above 0.2 Hz. The slope of gain reduction below the corner frequency was increased after L-NAME, $p < 0.05$ (Figure 3C); FC also increased, $P < 0.05$. In the control, two phase peaks were apparently centred at 0.13 and 0.035 Hz. The peak at 0.13 Hz was significantly increased by L-NAME (Figure 3D). Hence, L-NAME enhanced myogenic autoregulation.

When BQ-788 was infused, it further decreased RBF and conductance in a significant fashion. The BP power did not change (Figure 3A) and the coherence was the same as L-NAME (Figure 3B). The slope of gain, phase peak and FC were also unchanged (Figure 3C). The lack of difference between L-NAME and BQ-788 transfer

functions demonstrated that BQ-788 had no effect on myogenic autoregulation when added to L-NAME.

In the second experiment, BQ-788 was given first and then L-NAME was added. After the infusion of BQ-788, a significant decrease in RBF and conductance was observed (Table 4). The BP power was the same and coherence decreased to below 0.25 Hz (Figure 4A and 4B). The slope of gain, FC, and phase peak all increased significantly (Table 4, Figure 4 C-D). Hence, BQ-788 enhanced myogenic autoregulation.

Notice that the admittance phase decreased below 0 at 0.65 Hz and it was at its lowest at 0.8 Hz. This 'signature' – a strong peak in gain and an abrupt phase transition from positive to negative is characteristic of an autonomous oscillation (Cupples WA personal communication). It is clear that this event originated in the kidney (no signal in BP power) and it was reliably induced by BQ-788 although we know that on occasion it can also be induced by L-NAME. Its function, however, is unknown.

Upon the addition of L-NAME, L-NAME reduced RBF and conductance significantly. Coherence increased slightly after L-NAME, although it was still lower than the control one (Figure 4B). The slope of gain below the corner frequency increased close to the control level (Figure 4C); FC and the phase peak reduced to a level between that of the control and BQ-788 (Figure 4D). It seemed that L-NAME reversed the enhancement of myogenic autoregulation by BQ-788. Hence, L-NAME reduced the effect of BQ-788 on myogenic autoregulation, suggesting that there was some interaction between NO and ET-1 through the ETB receptor.

Expt		BP (mmHg)	RBF (ml/min)	ΔBRF (%)	ΔG (%)	FC	SLOPE (dB)	PEAK (rad)
1 n = 6	CONTROL	98 ± 4	6.5 ± 0.7			0.32 ± 0.05	29.9 ± 2.4	1.26 ± 0.06
	L-NAME	101 ± 4	2.9 ± 0.5*	-55 ± 4	-56 ± 4%	0.59±0.06*	49.4 ± 4.8*	1.78±0.11*
	BQ-788	102 ± 4	2.1 ± 0.3*	-29 ± 5	-29±6%	0.49±0.04*	45.9 ± 6.4	1.74±0.13*
2 n = 6	CONTROL	102 ± 5	7.8 ± 0.6			0.34±0.09	30.1 ± 3.8	1.04 ± 0.12
	BQ-788	108 ± 3	4.0 ± 0.7*	-50 ± 4	-52±5%	0.57±0.04*	41.8 ± 4.9*	1.47±0.05*
	L-NAME	110 ± 3	2.1 ± 0.4*$^{\Phi}$	-48 ± 4	-49 ±4%	0.38±0.04$^{\Phi}$	25.8 ± 2.3$^{\Phi}$	1.25 ± 0.06

Table 4. The interaction between L-NAME and BQ-788 on blood pressure (BP) and renal blood flow (RBF) dynamics. Values are means ± SE; n = number of rats studied. BP, mean arterial pressure; RBF, renal blood flow. Statistical significance within a group (1-way ANOVA for repeated measurements and a t-test): *P < 0.05 each drug given vs. control; $^{\Phi}$P < 0.05 the second drug given vs the first drug given.

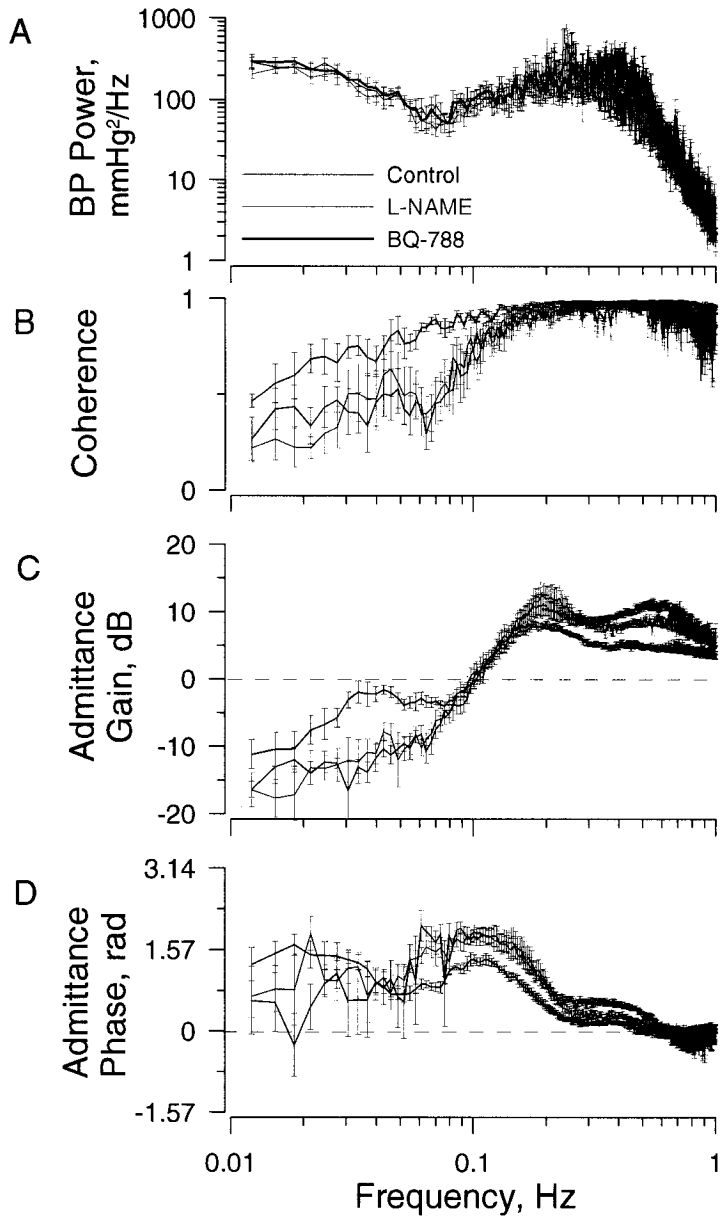

Figure 3. Blood pressure power spectra and renal blood flow (RBF) dynamics when L-NAME was given before BQ-788. A. Similar blood pressure (BP) (input) spectra were achieved in all conditions. B. Coherence was high during control and somewhat reduced at frequencies < 0.1 Hz by L-NAME and BQ-788. C. Gain reduction by the myogenic system was enhanced by L-NAME and not further altered by BQ-788. D. The phase peak of the myogenic system was significantly enhanced by L-NAME and not further altered by BQ-788.

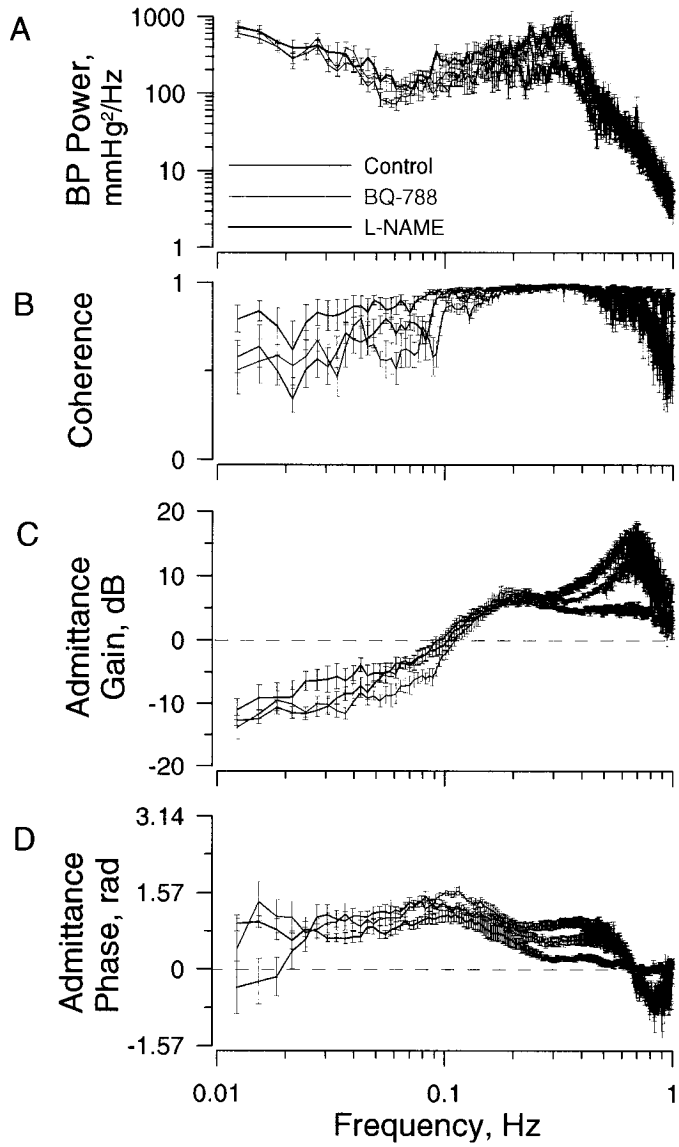

Figure 4. Blood pressure power spectra and renal blood pressure (RBF) dynamics when BQ-788 was given before L-NAME. A. Similar blood pressure (BP) spectra were achieved in all conditions. B. Coherence was high during control and somewhat reduced at frequencies < 0.1 Hz by BQ-788 and L-NAME. C. Gain reduction by the myogenic system was enhanced by BQ-788 and this was reversed by L-NAME. D. The phase peak of the myogenic system was significantly enhanced by L-NAME and not further altered by BQ-788. BQ-788 reliably induced a gain peak and phase transition from negative to positive at ~0.65 Hz. This pattern is the signature of an autonomous oscillator.

In summary, ETB activation dilates renal vasculature via eNOS activation, but removal of ETB does not inactivate all NOS – there are alternate activation pathways. For myogenic autoregulation, both L-NAME and BQ-788 augment myogenic autoregulation. Their effects are, at least, mutually occlusive. Therefore they are series linked in a common pathway and no alternate pathway exists for either to affect the myogenic mechanism. We see little or no evidence for an action of either upon tubuglomerular feedback.

Study 4: Interaction between NO and ETA receptor

This study was designed to investigate the level of interaction between L-NAME and BQ-123. BQ-123 is selective ETA receptor blocker. This study was the same as study 3 except that BQ-123 was infused instead of BQ-788. This study involved two experiments. In the first experiment, L-NAME was given before and during BQ-123. In the second experiment, the administration order was reversed: BQ-123 was given before and during L-NAME.

Averages of BP, RBF, conductance, slope of gain and phase peak are shown in Table 5. In the first experiment, ten rats were used, but the results were not reliable and only one data seemed to make sense. The reason will be discussed in the Discussion. Results for this data are presented in the Table 5 and Figure 5. When L-NAME was given, the typical response to L-NAME was observed: RBF and conductance decreased considerably. Under all similar BP power conditions (Figure 5A), coherence decreased below 0.1 Hz (Figure 5B). The slope of gain, FC, and the phase peak all increased (Figure 5C - 5D).

When BQ-123 was infused, there was no change in BP, RBF and conductance. BP power and coherence did not change much either (Figure 5A - 5B). The slope of gain decreased slightly (Figure 5C) while the phase peak and FC were unchanged. This suggested that BQ-123 could not affect the enhanced myogenic autoregulation by L-NAME.

In the second experiment (Figure 6), BQ-123 was given before and while L-NAME was added to infusion. When only BQ-123 was infused, RBF and conductance slightly increased (Table 5). BP power and coherence were the same as the control (Figure 6A - 6B). The slope of gain, FC, and the phase peak remained unchanged. BQ-123 did not have any effect on myogenic autoregulation.

L-NAME was then infused, decreasing RBF and conductance significantly. The BP power was similar to the control (Figure 6A) and coherence was reduced at 0.1 Hz (Figure 6B). The slope of gain, FC, and the phase peak increased significantly, again showing a typical effect of L-NAME on myogenic autoregulation. Although BQ-123 was added before and during L-NAME, L-NAME's effects were unaltered. It indicated that BQ-123 had no effect upon the action of L-NAME.

Constriction induced by NOS inhibition is independent of activation of ETA receptors and myogenic autoregulation enhanced by NOS inhibition is also independent of activation of ETB receptors.

Expt		BP (mmHg)	RBF (ml/min)	ΔRBF (%)	ΔG (%)	FC	SLOPE (dB)	PEAK (rad)
1 n = 1	CONTROL	92	4.8			0.26	31.3	1.2
	L-NAME	101	2.6	-41	-50	0.70	49.2	1.8
	BQ-123	94	2.8	+1	+15	0.60	46.3	1.7
2 n = 6	CONTROL	100 ± 3	6.0 ± 0.7			0.08±0.09	17.3 ± 3.7	0.7 ± 0.2
	BQ-123	93 ± 2	6.7 ± 0.8	+12 ± 5	+21 ± 6	-0.07±0.10	16.5 ± 4.5	0.6 ± 0.2
	L-NAME	95 ± 3	3.5 ± 0.6$^{\Psi}$	-47 ± 8	-48 ± 8	0.11±0.12	26.3 ± 6.2	1.0 ± 0.2

Table 5. The interaction of L-NAME and BQ-123 on blood pressure (BP) and renal blood flow (RBF) dynamics. Values are means ± SE; In group 1, only 1 result was considered to be valid, In group 2, n = number of rats studied. BP, mean arterial pressure; RBF, renal blood flow. Statistical significance within a group (1-way ANOVA for repeated measurements and a t-test): *P < 0.05 each drug given vs. control; $^{\Psi}$P < 0.05 the second drug given vs the first drug given.

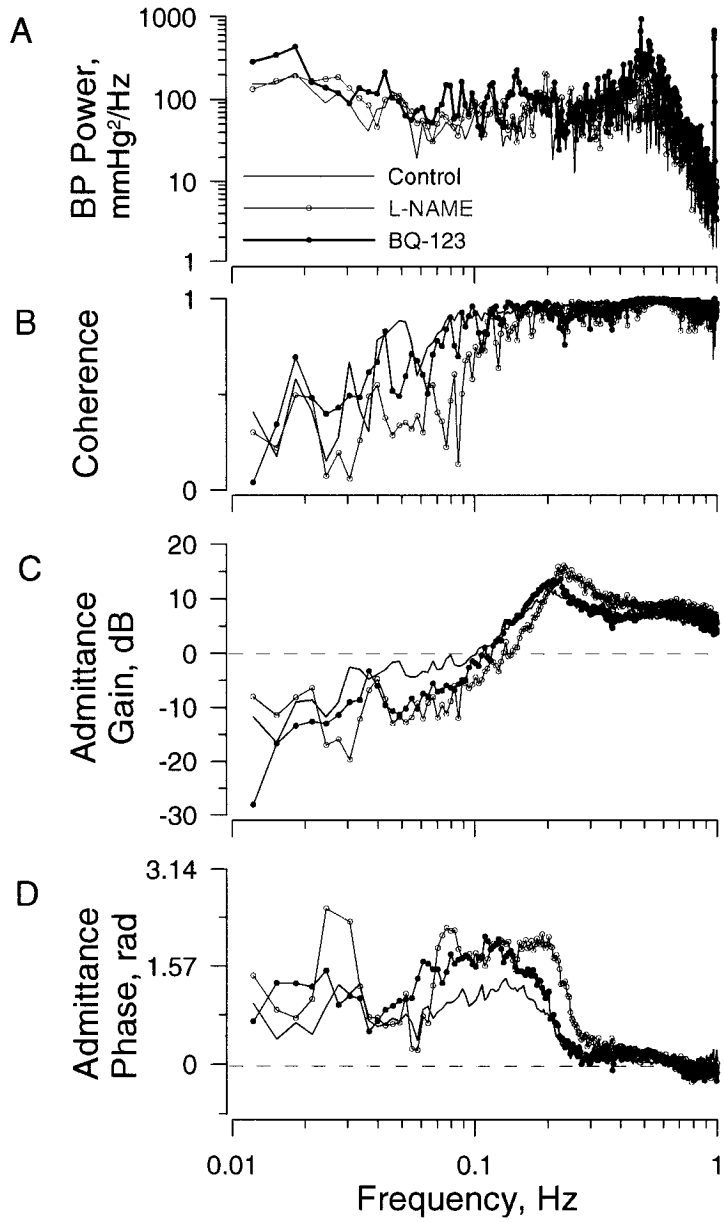

Figure 5. Blood pressure power spectra and renal blood flow (RBF) dynamics when L-NAME was given before BQ-123. A. Similar blood pressure (BP) (input) spectra were achieved in all conditions. B. Coherence was high at frequencies >0.1 Hz and somewhat reduced at frequencies < 0.1 Hz. C. Gain reduction by the myogenic system was enhanced by L-NAME and not further altered by BQ-123. D. The phase peak of the myogenic system was significantly enhanced by L-NAME and not further altered by BQ-123.

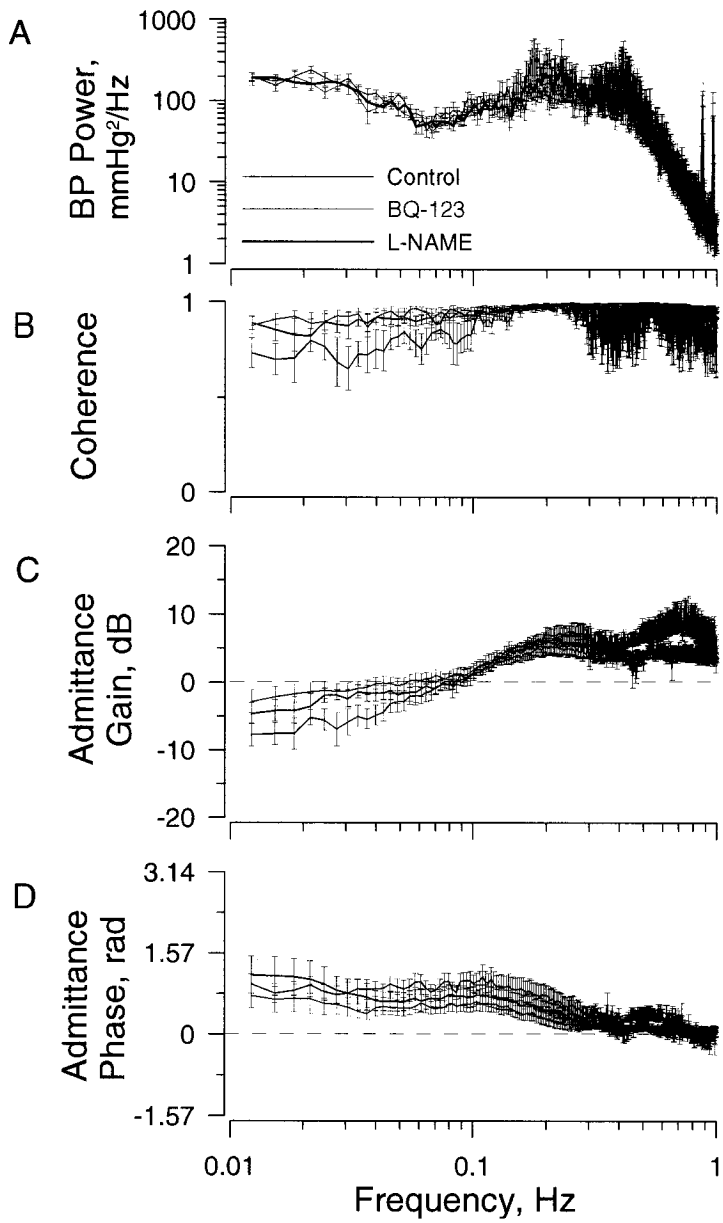

Figure 6. Blood pressure power spectra and renal blood flow (RBF) dynamics when BQ-123 was given before L-NAME. A. Similar blood pressure (BP) spectra were achieved in all conditions. B. Coherence was high under all conditions. C. Gain reduction by the myogenic system was not affected by BQ-123 and was significantly enhanced by L-NAME. D. The phase peak of the myogenic system was not altered by BQ-123 and was significantly enhanced by L-NAME.

Study 5: Interaction of NO and Rho-kinase

This study is the central section that will lead to the end of the story. It was designed to test whether the signal transduction pathway of the modulation of renal myogenic autoregulation is through Ca^{2+} sensitization, NO/Rho-kinase pathway. Y-27632 is a highly selective Rho-kinase inhibitor (Uehata et al. 1997) and it is a strong vascular dilator, IC_{50} = 0.8 μM. This study was divided into two experiments. In the first experiment, L-NAME was given before and during Y-27632. In the second one, Y-27632 was given before and during L-NAME.

In this study, the initial concentration of Y-27632 used was 20 μM. When Y- 27632 was infused, BP decreased to 60- 70 mmHg, which was below the autoregulation range (data not shown). Although the concentration of 10 μM still decreased BP, it maintained BP within autoregulation range and could achieve complete blockade of autoregulation as shown by the transfer function. The concentration was further decreased to 5 μM. Although it still caused BP to fall, that concentration led to incomplete blockade of myogenic responses to L-NAME (Figure 7). Hence, the concentration of Y-27632 used in this study was 10 μM.

In the first experiment, L-NAME was given first and then Y-27632 was added. When L-NAME was infused, RBF and conductance decreased significantly (Table 6). The action of L-NAME on RBF was consistent with previous results. BP power was the same as the control (Figure 8A) while coherence decreased below 0.1 Hz (Figure 8B). The admittance gain increased at 0.2 Hz. The slope of gain, FC, and the phase peak increased significantly (Table 6). This was a classical response by L-NAME as shown in previous experiments.

Expt		BP (mmHg)	RBF (ml/min)	Δ RBF (%)	ΔG (%)	FC	SLOPE (dB)	PEAK (rad)
1	CONTROL	91 ± 3	7.1 ± 0.4			-0.02±0.11	20.5 ± 4.1	0.7 ± 0.1
n = 6	L-NAME	96 ± 3	3.8 ± 0.5*	-46 ± 5	-50 ± 5	0.31±0.11*	35.5 ± 9.3*	1.2 ± 0.2*
	Y-27632	86 ± 2ᶠ	8.0 ± 0.6 ᶠ	+122 ± 20	+152 ± 25	0.05±0.07ᶠ	12.3 ± 1.3ᶠ	0.5 ± 0.1ᶠ
2	CONTROL	92 ± 1	6.8 ± 0.5			0.21±0.08	22.2 ± 2.3	0.9 ± 0.1
n = 7	Y-27632	84 ± 2*	8.8 ± 0.6*	+25 ± 4	+40 ± 5	-0.18±0.08*	6.8 ± 0.8*	0.3 ± 0.0*
	L-NAME	86 ± 2	8.5 ± 0.7	-12 ± 10	-2.1 ± 4.3	-0.06±0.06*	11.1 ± 1.9	0.5 ± 0.1*

Table 6. The interaction of L-NAME and Y-27632 on blood pressure (BP) and renal blood flow (RBF) dynamics. Values are means ± SE; n = number of rats studied. BP, mean arterial pressure; RBF, renal blood flow. Statistical significance within a group (1-way ANOVA for repeated measurements and a t-test): *$P < 0.05$ each drug given vs. control; ᶠ$P < 0.05$ the second drug given vs the first drug given.

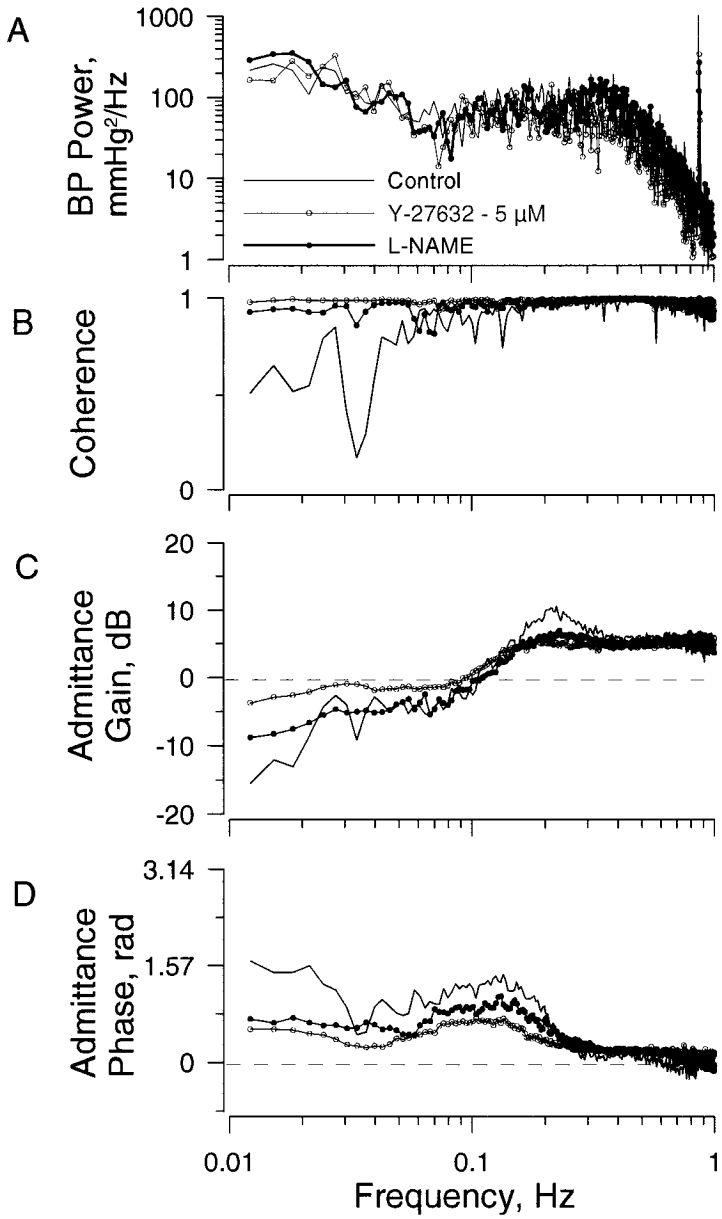

Figure 7. Results of an experiment showing incomplete blockade of the response of renal blood flow (RBF) dynamics to a low dose, 5 μM, of Y-27632.

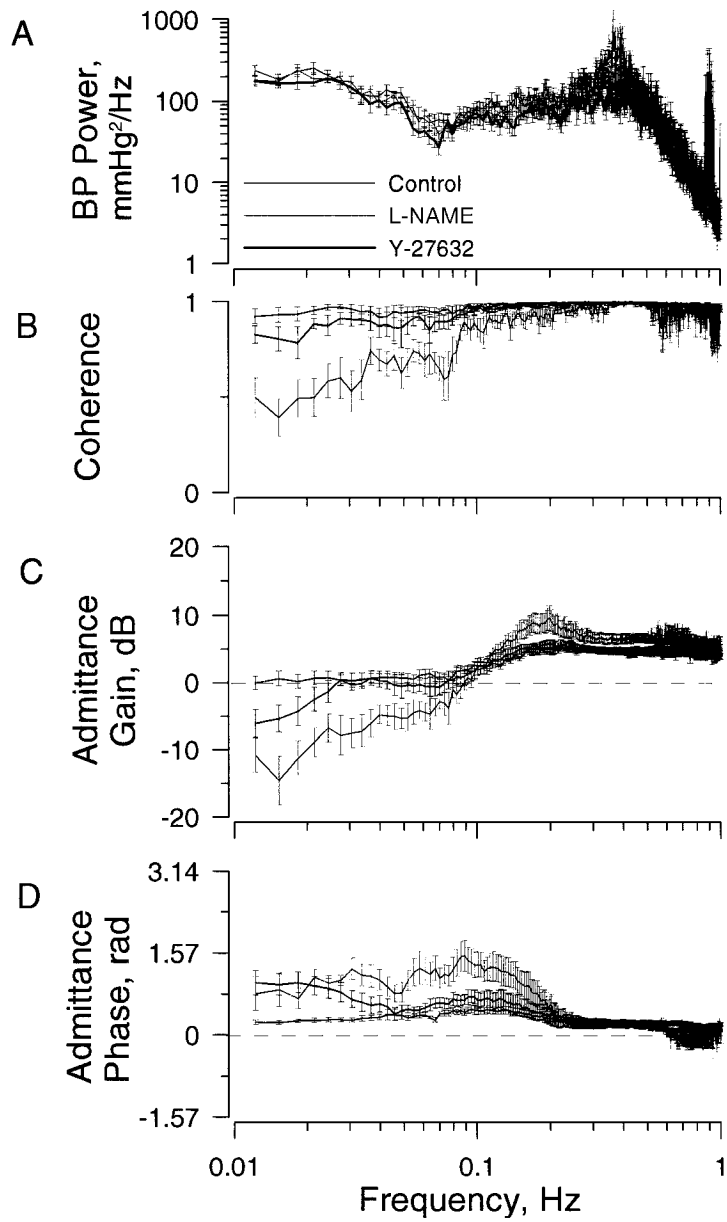

Figure 8. Blood pressure power spectra and renal blood flow (RBF) dynamics when L-NAME was given before Y-27632. A. Similar blood pressure (BP) (input) spectra were achieved in all conditions. B. Coherence was high during control, reduced at frequencies < 0.1 Hz by L-NAME and restored by Y-27632. C. Gain reduction by the myogenic system was enhanced by L-NAME and not markedly impaired by Y-27632. D. The phase peak of the myogenic system was significantly enhanced by L-NAME and markedly reduced by Y-27632.

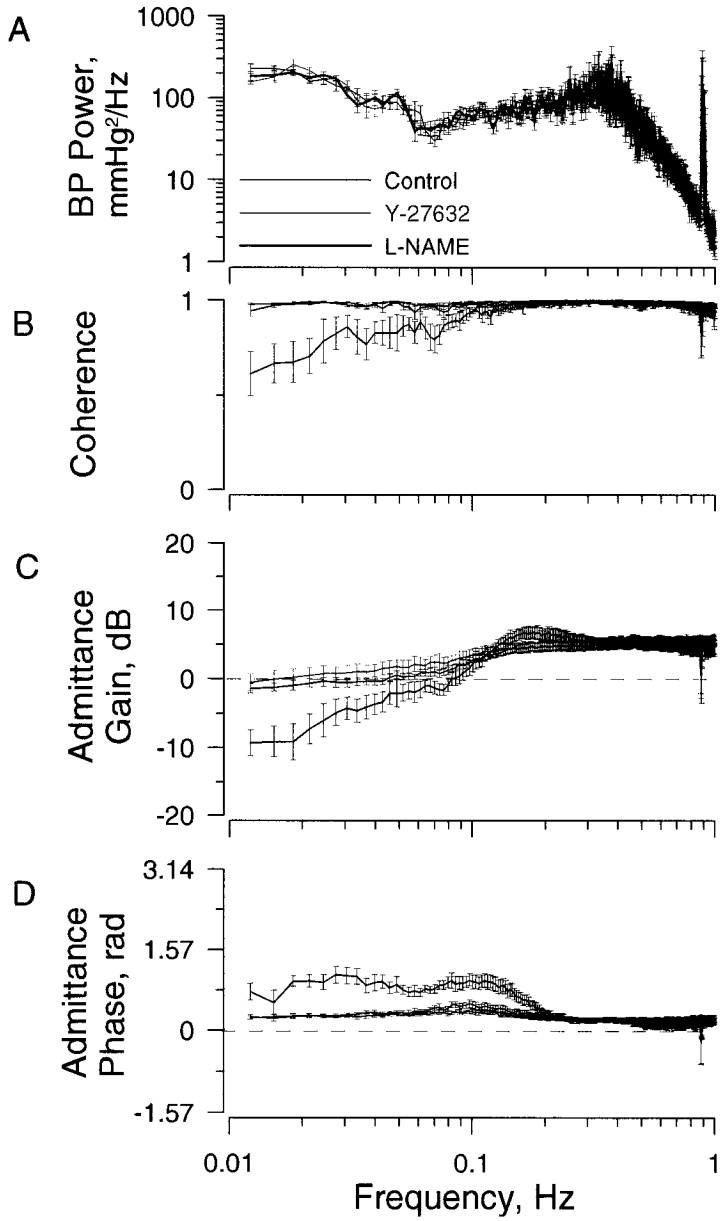

Figure 9. Blood pressure power spectra and renal blood flow (RBF) dynamics when Y-27632 was given before L-NAME. A. Similar blood pressure (BP) (input) spectra were achieved in all conditions. B. Low Frequency coherence was increased by Y-27632 and not altered by L-NAME. C. Gain reduction by the myogenic system was reduced by Y-27632 and not altered by L-NAME. D. The phase peak of the myogenic system was significantly was reduced by Y-27632 and not altered by L-NAME.

When Y-27632 was added by intrarenal infusion, BP decreased significantly (Table 6). While BP decreased, RBF and conductance increased significantly (ΔRBF = +122 ± 20 %, and ΔG = +152 ± 25 %, $P < 0.05$). BP power remained unaltered (Figure 8A) while coherence increased and surpassed the control level (Figure 8B). The gain decreased at 0.2 Hz and increased below 0.1Hz (Figure 8C). The slope of gain, FC, and the phase peak all decreased noticeably ($P < 0.05$). Values are reported in Table 6 and the changes are depicted in Figure 8. Y-27632 completely inhibited the effect of L-NAME.

In the second experiment, Y-27632 was infused first and then L-NAME was given. When Y-27632 was given, BP decreased significantly while RBF and conductance increased significantly (Table 6). BP power remained unchanged (Figure 9A) and coherence was the same as the control (Figure 9B) while the gain decreased at 0.2 Hz, but increased below 0.1 Hz, making a straight line. The slope of gain and FC decreased significantly (Figure 9C). The phase peak decreased markedly, almost forming a straight line (Figure 9D). These changes indicated an absence of autoregulation. Y-27632 strongly inhibited normal autoregulation.

Upon the administration of L-NAME, BP, RBF, conductance, phase peak, and slope of gain were slightly changed (Table 6). RBF dynamics were essentially the same as those of Y-27632. We can conclude that Y-27632 abolished normal myogenic autoregulation and L-NAME, although a potent vasoconstrictor, could not counteract the effect of Y-27632.

For vasoconstriction, constriction induced by NOS inhibition is abolished by Rho-kinase inhibitor. Dilatation induced by Y-27632 is independent of NOS inhibition. For myogenic autoregulation, Y- 27632 paralysed myogenic autoregulation and TGF. Its

effect is independent of L-NAME. Y –27632 not only reversed myogenic autoregulation, but also abolished TGF.

DISCUSSION:

The kidneys play an important role in maintaining fluid and electrolyte balance and elimination of potentially toxic metabolic wastes and foreign compounds from the body. They are regulated by nerve, hormone, and renal autoregulation.

Arterial BP is the force that drives blood flow through organs in the body. According to this relationship, blood flow should follow accordingly but renal function and preservation of renal structure both require stable RBF. Any unintentional shift in RBF or GFR could lead to dangerous imbalances of fluid, electrolytes, and wastes. Although many neural and hormonal circuits regulate renal function, autoregulation is the only mechanism that stabilizes RBF when BP fluctuates. Renal autoregulation is aimed at preventing inappropriate changes in blood flow and maintains essentially constant RBF when BP varies between ~80 mmHg and ~180 mmHg (Navar 1997). Within its effective range, the adjustments of afferent arteriolar resistance can compensate for changes in BP, thus preventing inappropriate fluctuations in GFR. Impairment of autoregulation leads to the development of progressive renal damage and eventually to renal failure (Bidani, et al. 1987). Autoregulation is a complex phenomenon, and includes myogenic and TGF mechanisms. Myogenic autoregulation is a rapid response to spontaneous blood pressure fluctuation (Halpern et al. 1984; Cupples et al. 1996), so it is very important for normal renal function.

Nitric oxide is a signal molecule produced from L-arginine by NOS. It causes vasodilatation and decreases blood pressure. Many evidence has shown that NO modulates myogenic autoregulation in several organs including the kidney (Bouriquet and Casellas 1995; Pohl and De Wit 1996; De Wit et al. 1998; Nurkiewicz and

Boegehold 1999; Turkstra et al. 2000). There are two potential sites of NO production within the kidney: arterial and arteriolar endothelium which contains eNOS, and the macula densa which contains nNOS. L-NAME was used to inhibit NOS in the present study. The compound is a competitive inhibitor of the inducible and neuronal isoforms of NOS, but an essentially irreversible inhibitor of endothelial NOS (Griffith and Kilbourn 1996). The effects of L-NAME on RBF dynamics are not reversible, even by massive doses of L-arginine (Wang et al. 1999).

Three physiologic responses to NOS inhibition were observed in our lab: increased BP, profound renal vasoconstriction (≈ 60% reduction of conductance), and augmented renal myogenic autoregulation. Our data have shown enhanced myogenic autoregulation in the kidney, but not in the gut of normotensive rats (Wang et al. 1999, Wang and Cupples 2000, Cupples et al. 2002). In these studies, time series analysis showed that inhibition of NOS causes characteristic changes in the BP-RBF transfer function in rats: increased FC, increased slope of gain reduction by the myogenic mechanism and increased amplitude of the associated phase peak.

Endothelial and epithelial cells in kidney secrete many agents including NO, ANG II, ET-1 that affect vascular function. ET-1, ANG II and NO all modulate autoregulation: the first question we addressed was how they interact with each other; the second question was what is the NO signal transduction pathway. Inhibition of Rho kinase will block Ca^{2+} sensitivity pathway and cause activation of MLCP. If NO signal transduction occurs through the same pathway, we would expect to see relaxation of vascular muscle and attenuate myogenic autoregulation.

The main finding of this study was that Y-27632 not only abolished enhanced

myogenic autoregulation by L-NAME but it also abolished TGF. Y-27632 completely abolished autoregulation in renal vascular muscle.

Experiment methods

Body weight, spontaneous arterial pressure, and initial RBF for all experiments were given. They showed that the animals used were of comparable physiological status at the beginning of the experiment.

There were two methods used in the present study: intravenous and intrarenal arterial infusion. In most labs, L-NAME and other drugs are given intravenously in vivo or by direct vascular perfusion in vitro. Intrarenal arterial infusion is rarely used.

An effective method for intrarenal arterial infusion was developed by Parekh (1995). A special set of devices is used to deliver a drug directly to the kidney. The intent is that the drug should affect only the kidney and should not affect the systemic circulation and other tissues. It also provides stable BP during the whole experiment. Thus, it is easier to isolate the renal responses under study. The device for intrarenal infusion consists of a multiple-catheter system with a cannula inserted into the renal artery, which is connected to different lines for drug infusion. This makes it easier to match drug delivery to the kidney. This system also includes a mixing (push-pull) pump which allows for proper mixing of the drug and blood.

With usual methods of intrarenal drug infusion, the drug goes only to a localized part of the kidney. The mixing pump here can resolve the problem. It mixes the drug with the blood and pumps it into the renal artery to provide an even distribution of the drug in the kidney. Systemic effects of drugs usually have secondary effects on the kidney. The use

of intrarenal artery infusion limits the drug effects to the kidney. One tenth of normal amount used can achieve comparable concentration in the kidney because RBF is about one tenth of cardiac output, so systemic toxicity is less of a problem. It also allows one to use some drugs that would otherwise be too toxic for in vivo applications.

The catheter is inserted directly into the renal artery from the aorta. In the event of a leak, the drug would affect the systemic circulation and BP would change dramatically. We would observe a significant difference in BP. However, if the insertion of the tip was done cautiously, that problem should be avoided. If leaking occurred, the result was taken out.

Results obtained were as predicted for each experiment. Sometimes, BP was not completely stable because it fluctuated slightly by itself. Another reason might be that the drug was not completely eliminated in the kidney and went into the systemic circulation through blood flow. However, the effect of the latter was so small that there was no significant difference in BP. We obtained satisfying results in most experiments except with the Rho-kinase inhibitor, Y-27632. Y-27632 is a very strong vasodilator. Even though it was infused by intrarenal infusion, BP still decreased significantly (Table 6). We repeated the experiment several times and obtained the same results. This confirmed that it was not due to a technical problem but that the drug itself was too strong to maintain stable BP. In this case, it was likely that a small amount of drug has not been eliminated in the kidney went into the system and caused reduction of the BP. We failed to find a concentration that would not affect BP and be effective in the kidney.

Although we cannot overcome the effect of Y-27632 in the systemic circulation, we do not think it affected the analysis. Renal autoregulation range lies between 80 to 180

mmHg and we were still able to obtain a perfusion pressure that was high enough to study myogenic autoregulation. The result we got showed that the responses to Y-27632 on renal autoregulation were dose-dependent. Therefore, the result can be interpreted as the effect of Y-27632. In the future, we can isolate and confirm the effects in the kidney through renal vascular perfusion in vitro.

Interaction between NO and ANG II

L-NAME was used to block the production of NO by inhibiting NOS. The observed rise of BP and profound renal vasoconstriction are classical responses to acute inhibition of NO synthase (Baylis et al. 1990; Beierwaltes et al. 1992; Majid et al. 1993). In our experiment, BP increased by 31 ± 6% and conductance declined by 65 ± 1% after treatment with L-NAME. These results agree with those reported by other investigators (Wang et al. 1999; Wang and Cupples 2001; Kramp et al. 2001). Captopril inhibits the conversion of ANG I into ANG II. ANG II is a potent vasoconstrictor. In our experiment, captopril caused BP to drop by 12 ± 5%, and increased conductance by 32 ± 9%. The renal vasodilatation by captopril is the classical response to captopril (Cupples 1993).

Traditionally, the RAS is regarded as an endocrine system, but ANG II can be generated locally in several tissues including the kidney (Zimmerman and Dunham 1997; De Gasparo et al. 2000). Thus it is also considered as a paracrine factor. ANG II is a potent renal vasoconstrictor that predominantly affects the efferent arteriole (Hall et al. 1977). In addition, it may indirectly cause constriction of the afferent arteriole through potentiation of the TGF mechanism (Schnermann 1998). It is a very important modulator of TGF (Navar 2003). Cupples (1993) reported that ANG II is necessary for

autoregulation resetting to operate at reduced arterial pressure (< 80 mmHg) and that it modulates the pathway controlling autoregulatory vasodilatation. Furthermore, a low concentration of ANG II (3 pM) does not alter afferent arteriolar diameter but augments myogenic reactivity in a protein kinase C-dependent manner (Kirton and Loutzenhiser 1998). Just et al. (2002) reported that ANG II enhances the sensitivity of the myogenic response, and that the relative contribution of the myogenic response to overall autoregulation remains unaltered in physiological range.

Our experiment showed that captopril did not alter the constriction effect of L-NAME. The effect of L-NAME with or without captopril was no significant difference. These results were in agreement with the report that inhibiting NO decreased RBF when ANG II was blocked (Gomez-Alamillo et al. 2003). On the other hand L-NAME blocked the dilatation induced by captopril. Captopril did not alter the pressor or constrictor responses to L-NAME. Therefore, the response to L-NAME is independent of ANG II. .

Interaction between NO and ET-1

This study was performed using intrarenal drug delivery. Therefore, BP did not change significantly and we only concentrated on comparing the differences in RBF or conductance. After infusing L-NAME, conductance decreased by 59 ± 4%. In comparison with the previous study, L-NAME affected vascular conductance to the same extent. From this, we observed that the effect of L-NAME by intrarenal infusion was the same as that by intravenous infusion.

Of the ET-1 receptor antagonists employed in this study, PD 145065 is a general blocker, which inhibits both ETA and ETB receptors (Cody et al. 1993; Doherty et al.

1993). BQ-123 is an ETA receptor blocker (Ihara et al. 1992), and BQ-788 is an ETB receptor inhibitor (Pang et al. 1998).

Several groups have shown that PD 145065 effectively blocks the action of ET-1 (Dobrowolski et al. 1997; Wellings et al. 1993; 1994). They tested the action of PD 145065 as follow: ET-1 was first given and then PD 145065 was infused to inhibit the effect of ET-1 (Wellings et al. 1993), or ET-1 and PD 145065 were both given together (Allcock et al 1995; Dobrowolski et al. 1997). Few experiments were first given an ETA/ETB receptor blocker and then ET-1 to test the interaction of both drugs. Our study was designed to block ET-1 within a physiological range. If exogenous ET-1 was first given, it takes a long time to completely block ET-1 receptors because the effect of ET-1 is hard to reverse. Therefore, PD 145065 was given first here.

In the first experiment, we got the effect of exogenous ET-1 as a positive control in the first group. ET-1 is a potent vasoconstrictor. It caused a significant decrease in RBF and conductance (Table 3). From this, we concluded that ET-1 worked effectively.

In the second group, the effect of PD 145065 was as a negative control. Although ET-1 is a potent constrictor, inhibition of ET-1 did not alter RBF and vascular conductance significantly. The relative abundance of ETA and ETB receptors present in the kidney varies depending on the species (Benigni et al. 1999). In the rat, the number of ETB receptors is higher than that of ETA receptors in the kidney. ETB receptor mediates constriction or dilatation depending on the dose of the ETB receptor agonist. When given exogenously, an ETB agonist causes renal vasodilatation (Gellai et al. 1994). The result can be interpreted by two ways: one possibility is that the amount of ET-1 present in the circulation is too small (the effect of the ETA blocker cannot be shown); and the other

possibility is that the amount of PD 145065 may be too small or that PD 145065 is not in an active form in the kidney. Therefore, the effect of PD 145065 cannot be shown after its infusion. L-NAME reduced conductance by 59 ± 4% alone while it reduced conductance by 42 ± 2% when PD 145065 was added. Although PD 145065 alone did not affect vascular conductance, it reduced the effect of L-NAME in RBF and conductance significantly, which, in turn, indicated that PD 145065 had an effect in the kidney but whether the dose of PD 145065 was adequate needed to be verified.

In the third group, ET-1 challenged the effect of PD 145065. The result that ET-1 didn't change RBF and conductance (Table 3) can be interpreted that PD 145065 at the concentration employed effectively inhibited binding to ET receptors of not only the endogenous ET-1 but also the exogenous ET-1. When L-NAME was added to the ET-1 infusion, the effect of L-NAME was significantly less than usual, meaning that PD 145065 blunted the effect of L-NAME.

In the fourth group, the doses of the two blockers of ETA and ETB receptor subtypes were tested. The additive effect of the two receptor blockers (BQ-123 and BQ-788, respectively) on RBF and conductance should be equal to that of the general receptor. What we got was almost what we predicted. In rats pre-treated with BQ-123 and BQ-788 together, exogenous ET-1 had no effect on conductance while L-NAME reduced conductance by 28 ± 5%. There was no significant difference between this blunting of L-NAME response and that achieved after the combined ETA/ETB receptor antagonist PD 145065. From these results, we confirmed that the doses of BQ-123 and BQ-788 were sufficient in blocking ET-1 (Table 3).

From this first experiment, PD 145065 was confirmed to block ET-1 completely at a

given concentration. It was also found that when pre-treated with PD 145065, the effect of L-NAME was significantly blunted, PD 145065 blunted the response to L-NAME while replacing PD 145065 with both BQ-123 and BQ-788 blocked ET-1 as efficiently.

In the second experiment, the interaction between NO and ET-1 was studied. There were three subgroups in the first group. The first and second subgroups were as described in the second and third groups in the first experiment. Again, when pre-treated with PD 145065, the effect of L-NAME decreased significantly. Because the constrictor response to L-NAME was bunted, but not blocked, it remained possible, though unlikely, that the ET receptor blockade was somehow inadequate. Therefore, in the third subgroup, three times the original concentration of PD 145065 was used to confirm the blunting effect to L-NAME. The result was not significantly different to the original dose. Therefore, PD 145065 showed little or no evidence for dose dependency (in blunting of L-NAME response). In the second group, L-NAME was given before and during PD 145065 infusion. After the classical response to L-NAME, PD 145065 did not alter conductance. The effect of PD 145065 was not different with or without L-NAME.

It has been reported (Banting et al 1996, Qiu and Baylis, 1999) that the pressor response to NO synthase inhibition by L-NAME is largely mediated by ET-1. Since the pressor response to L-NAME is a consequence of intense peripheral vasoconstriction, this would imply important blunting of L-NAME-induced vasoconstriction in important vascular beds such as the kidney. In the current study the same ET receptor antagonist was employed, with positive controls, and a forward and reverse experimental design. Yet even at a high dose PD 145065 could prevent only a fraction of the constrictor response to L-NAME and contributes modestly to the renal vasoconstriction induced by

L-NAME and in an interactive fashion.

Hence, it was found in these ANG II and ET interaction experiments that there was no significant interaction between ANG II and NO whereas there was significant interaction between ET and NO (at least for vasoconstriction). This is consistent with results of Kramp (2001) and leads to the next set of studies in the interaction between ET-1 with NO was explored to determine which ET receptor subtype mediates the interaction and to determine if the autoregulatory mechanism is involved.

Interaction between NO and ETB receptor

Stimulation of ETB receptors on vascular endothelial cells results in vasodilatation, probably via release of NO and prostacyclin (Luscher 1994), although renal vasoconstriction is often seen in response to ETB stimulation due to the presence of ETB receptors on renal vascular smooth muscle (Gellai et al. 1994). In the first experiment, L-NAME was infused before and during BQ-788 infusion; in the second, BQ-788 was infused before and during L-NAME infusion. It is important to remember that this is a double inhibitor study and that both drugs were given at blocking doses {Table 1 for BQ-788, Beierwaltes et al (1992) for L-NAME}. It is also important to recall that the experiments examine two related, though not necessarily identical actions – vasoconstriction and myogenic autoregulation.

Thus L-NAME causes strong vasoconstriction (Tables 2 & 3) and augments both steady-state and dynamic autoregulation (Wang et al. 1999; Turkstra et al. 2000; Kramp et al. 2001; Cupples and Wang 2001; Cupples et al. 2002). As in previous studies (eg. Gellai et al. 1994), selective ETB blockade by BQ-788 caused renal vasoconstriction

(Table 4) and augmented myogenic autoregulation (Table 4 and Figure 5) in the current study. As expected from the study of Kramp et al. (2001), there was significant interaction between ETB blockade and NOS inhibition. However, the interactive effects on vasoconstriction differed from those on myogenic autoregulation.

Prior infusion of L-NAME blunted the constrictor response to BQ-788 while prior BQ-788 did not affect the constriction in response to L-NAME. This pattern of responses suggests that part of the effect of ETB blockade is mediated via deactivation of eNOS. Certainly, Noris and Remuzzi (1999) reported that the ETB receptors are functionally coupled with eNOS and stimulate the release of NO in cultured endothelial cells. However, eNOS responsive to other stimuli (for instance, shear stress, acetylcholine, bradykinin) clearly also contributes to setting renal vascular conductance. (Furchgott and Zawadski 1980, Pohl et al. 1991). In other words, there is a good deal of redundancy in NO-dependent signalling.

A somewhat different pattern emerged with respect to myogenic autoregulation. Given alone, both BQ-788 and L-NAME augmented autoregulation, in particular the myogenic component. This is the expected response to L-NAME, but had not previously been reported for ETB blockade. Importantly, BQ-788 given after L-NAME did not affect myogenic autoregulation while L-NAME given after BQ-788 actually impaired myogenic autoregulation. Thus the presence of either drug precluded the response to the other – they are mutually occlusive. The simplest explanation of this finding is that ETB receptors and eNOS are coupled in series in the same signal pathway that modulates myogenic autoregulation. One implication of this conclusion is that only one pool of eNOS, that coupled to ETB receptors, modulates myogenic autoregulation.

Here we review the characteristic meaning of a transfer function (Figure 3) to explore the "event" centered at ~0.65 Hz. This event consists of a gain peak associated with a phase transition from negative to positive. This is the signature of an autonomous oscillator (ie an oscillation that originates in the renal vasculature). The results from Stauss and from Malpas suggest that vascular smooth muscle can not oscillate any faster (Stauss and Kregel 1996; Malpas et al. 1999). This oscillation is occasionally seen in all laboratories in normotensive rats, more often – though not routinely – seen in rats treated with L-NAME, and is reliably observed in stroke-prone, spontaneously hypertensive rats (AK Bidani, personal communication). This is the first demonstration that it can be induced reliably by a single agent (BQ-788). However the frequency ratio between this event and the myogenic mechanism (~0.65 Hz : ~0.2 Hz) is high order, ~7:2 (3.58 ± 0.11). Such a high order ratio makes sustained interaction between the two systems unlikely and contributes to overall system stability (Lessard et al. 1999).

The result is consistent with the view that endothelin resets RBF autoregulation efficiency during acute blockade of NO through ETB receptor (Kramp et al. 2001). BQ-788 and L-NAME both cause the renal vasoconstriction and enhanced renal myogenic autoregulation, but BQ-788 did not affect the effect of L-NAME while L-NAME blunted enhanced myogenic autoregulation by BQ-788. First, with respect to vasoconstriction, ETB blockade requires an intact NO system for its full effect though there is residual effect in the absence of NO, whereas L-NAME does not require ETB function for its full effect: we conclude that NO and ETB are not the same pathway but there is a little the common pathway. ETB stimulation is only one of several pathways leading to NOS

activation.

Secondly, for myogenic autoregulation ETB and NO are in the same pathway and the interaction is different from that of vasoconstriction. In this case, ETB blockade has some effects on myogenic autoregulation in inhibiting NOS, or L-NAME has some effect beyond inhibiting eNOS. We know that nNOS and iNOS are present in the kidney. Unpublished results from this laboratory indicate that blockade of iNOS has very little effect on RBF or on myogenic autoregulation and that blockade of nNOS certainly does not impair myogenic autoregulation. Thus, in the case of myogenic autoregulation the interaction between ETB receptors and eNOS is obligatory, suggesting that NO generated in response to ETB activation is the only pool of NO that can modulate the myogenic mechanism.

Here we can see that autoregulation does not follow directly from constriction. Similar results were reported before. In our lab, a strong constrictor phenylephrine does not have much effect on myogenic autoregulation (unpublished data).

Interaction of NO and ETA receptor

When L-NAME was given before and during BQ-123, we could not detect any effect on conductance of ETA receptor blockade by BQ-123. When BQ-123 was given first, no effect of BQ-123 was observed either. Upon the subsequent addition of L-NAME, conductance was reduced by 48 ± 8 %. It indicates that there may be no interaction between the two drugs. We would interpret that BQ-123 has little effect on conductance by itself (presumably because there isn't much ET around to block) and equally does not do much to the vasoconstrictor response to L-NAME.

As for the transfer function, we did not get a satisfactory result although the second experiment was better than the first one. Therefore, we would discuss about the BQ-123 + L-NAME group first, and then discuss about the group where L-NAME was given before and during BQ-123. When BQ-123 was given, the transfer function did not change; the curve of the phase and gain overlapped the control one (Figure 6). With the addition of L-NAME, we observed the typical increase in myogenic autoregulation. The phase peak and slope of gain reduction were significantly increased after L-NAME. This suggested that BQ-123 did not alter renal autoregulation, and not did it affect the effect of L-NAME on myogenic autoregulation either.

L-NAME used in these experiments also served the purpose of verifying whether the rat responded in a "normal" way as previous studies have shown the responses to L-NAME. The result of the experiment that was treated with L-NAME before and during BQ-123 could not be analysed in a meaningful way since we failed to obtain the L-NAME response in most rats in this group. Only one result can be used. From those data (shown in Figure 5), we can see the typical control myogenic autoregulation and enhancement of myogenic autoregulation by L-NAME. After adding BQ-123, there was no significant change. This suggested that ETA receptor had no effects on the enhancement of myogenic autoregulation by L-NAME.

We think that the other results were unreliable because we failed to obtain either normal autoregulation during the control period or enhancement of myogenic autoregulation by L-NAME as we did in experiment 3. We discussed this issue with Dr. R.D. Loutzenhiser, another expert in renal autoregulatory mechanisms. Based on the pattern of response (there was effective constriction, but little autoregulation), we believe

that there was a small amount of endotoxin contamination in the intrarenal cannula, but not in any of the other cannulas. If a bit of endotoxin (lipopolysaccharide, LPS) got into the kidney, it could be enough to paralyse the myogenic system without affecting RBF responses to L-NAME. That was the most probable reason. These experiments are currently being repeated.

Kramp et al. (2001) reported that ET-1 modulates some of the vascular actions of acute NOS inhibition through ETB receptor and not ETA receptor. Inhibition of NO synthesis when the ETA receptor was blocked, maintained the resetting of steady-state autoregulation to a lower pressure limit, whereas activation of the ETA receptor, when the ETB receptor was blocked, markedly impaired RBF autoregulation. It suggests an implication of the ETB receptor in the autoregulatory resetting during NOS inhibition. Other evidence shows that ET-mediated increase in cGMP formation and NOS activity were not affected by ETA receptor antagonist and were blocked by ETB receptor antagonist. Thus ET signalling through NO/cGMP pathway is mediated by ETB receptor, not ETA receptor (Mathison et al. 2002).

Our result is consistent with these reports but there are some differences. The research areas are not exactly same. Kramp et al. (2001) reported that endothelin resets RBF autoregulatory efficiency; we studied the myogenic mechanism which is a part of renal autoregulation. We observed the result that L-NAME and BQ-788 both enhanced myogenic autoregulation, but BQ-788 did not affect the effect of L-NAME while L-NAME blunted the enhanced myogenic autoregulation by BQ-788. They transduce the vascular myogenic autoregulation signal pathway are same pathways but it is different for constriction. Beyond this interaction, there are other effectors. The BQ-788 – L-NAME

result is actually close to what Kramp et al. (2001) reported. They compared L-NAME alone to L-NAME after BQ-788 and they observed the significant impairment of autoregulation. It is similar that our result with BQ-788. They used BQ-610 to block ETA receptors and saw no interaction between BQ-610 and L-NAME, it is very similar to our results with BQ-123. Therefore our experiment is similar to Kramp's and the results are consistent with their findings, but our experiments research further the autoregulation mechanism.

Interaction of NO and Rho-kinase

The kidney is one of the vital organs, which contribute the homeostasis in the body. Autoregulation is a very important mechanism to stabilize renal function. Myogenic autoregulation is the faster of two components that regulate RBF and GFR. Impaired autoregulation contributes to the development of renal failure. NO is one of paracrine factors which impair myogenic autoregulation. Inhibition of NO could enhance myogenic autoregulation.

We concentrated on how the signal of NO is transduced in cellular mechanism. Our experiment confirms that L-NAME augments myogenic autoregulation when BP fluctuates around a constant mean value. This means that both pressure-induced constriction and pressure-induced dilatation are enhanced. It is very easy to explain how L-NAME enhances pressure-induced constriction by one or more of the pathways that are targets of cGMP (K^+ channels, Ca^{2+} channels, etc). Thus reduced open probability of K^+ channels will tend to depolarize the membrane leading to increased $[Ca^{2+}]_i$. Similarly, increased open probability of Ca^{2+} channels would elevate $[Ca^{2+}]_i$ and both would

enhance constriction.

It is not at all clear how this would enhance pressure-enhanced dilatation because they affect only the myosin light chain kinase pathway that activates smooth muscle. Calcium sensitization pathway was a good candidate to explain the renal vascular effects of L-NAME. The Rho-kinase pathway for adjusting Ca^{2+} sensitivity is also a target of cGMP. In brief, the Rho-kinase pathway for Ca^{2+} sensitivity begins with binding of GTP by the small GTPase Rho. GTP-bound Rho activates Rho-kinase, which in turn phosphorylates and inhibits myosin light chain phosphatase. Myosin light chain phosphatase is the enzyme that deactivates (relaxes) smooth muscle. Rho-kinase can be blocked by the highly selective inhibitor, Y-27632 (Uehata et al. 1997; Fu et al. 1998, Ishizaki et al. 2000, Somlyo and Somlyo 2003). If the NO signal is transduced through this pathway, inhibition of Rho kinase will reverse and prevent the effect of L-NAME. Uehata et al. (1997) reported that Y-27632 reduces blood pressure in hypertensive rats more than in normal rats. Recently, it has been shown that NO inhibits Rho-kinase activity in the intact rat aorta, and that endogenous NO-mediated vasodilatation occurs through the inhibition of Rho-kinase constrictor activity in the intact rat aorta (Chitaley et al. 2002; Somlyo and Somlyo 2003).

In the first experiment, after the classical response to L-NAME was observed, the transfer function changed to almost a straight line by Y-27632 (Figure 7). It not only reversed myogenic autoregulation, but also abolished TGF. The TGF signature was removed by Y-27632 as effectively as by furosemide. Furosemide is a diuretic that inhibits the transporter that is used as a sensor by TGF. In fact, the transfer function after Y-27632 is strongly similar to that reported by Cupples and Loutzenhiser (1998) in the

hydronephrotic kidney when perfusion pressure was low and there was no myogenic autoregulation. It indicated that Y-27632 could abolish the constriction caused by L-NAME and the augmentation of myogenic autoregulation by L-NAME.

In the second experiment, Y-27632 was first given, causing a significant change in the transfer function and abolishing myogenic autoregulation and TGF. When L-NAME was added to the infusion, the transfer function did not change significantly. Y-27632 is a potent vascular dilator, paralysing myogenic autoregulation and TGF. Inhibition of Rho-kinase can prevent the constrictor response to L-NAME and the augmentation of myogenic autoregulation by L-NAME.

Thus, despite the fact that L-NAME is a strong constrictor, it has no effect once Rho-kinase is inhibited and a Rho-kinase blocker can abolish any effect of L-NAME. From this, we conclude that Rho-kinase is downstream in the NO signalling transduction in smooth muscle constriction, although further research needs to be done to very this conclusion.

Conclusion

Many paracrine factors contribute to regulate renal autoregulation and to maintain stable RBF and normal renal function. These factors interact with each other and form a complicated network in the kidney. NO is one of these factors that play an important role in renal myogenic autoregulation. We found that ANG II and ETA did not affect the action of NO on myogenic autoregulation but that ET-1 modulates NO through ETB receptor. At last, Y-27632 was used to study the Rho-kinase pathway. Rho-kinase was found to be a downstream cascade element in NO signalling pathway. Because Y-27632

paralysed vascular muscle, we can conclude that Y-27632 is a very potent dilator and that it can abolish any type of autoregulation, not just myogenic autoregulation.

In conclusion, our findings suggest that L-NAME enhances myogenic autoregulation through a Ca^{2+} sensitization pathway, NO/Rho-kinase pathway but further research is required.

FUTURE DIRECTION

The next step in elucidating the signal transduction of NO on renal myogenic autoregulation will involve another potent Rho-kinase blocker, HA-1077 (fasudil). It also inhibits cyclic nucleotide (cAMP and cGMP) dependent kinases at concentration three times higher than those needed to inhibit Rho-kinase (Uehata et al. 1997). Renal vasodilatation by NO is largely or totally dependent on cGMP and largely mediated by cAMP. Therefore, I expect HA-1077 to give similar results to those obtained with Y27632 after L-NAME, and HA-1077 alone should enhance myogenic autoregulation with minimal vasoconstriction and L-NAME should have no effect on it.

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Appendix

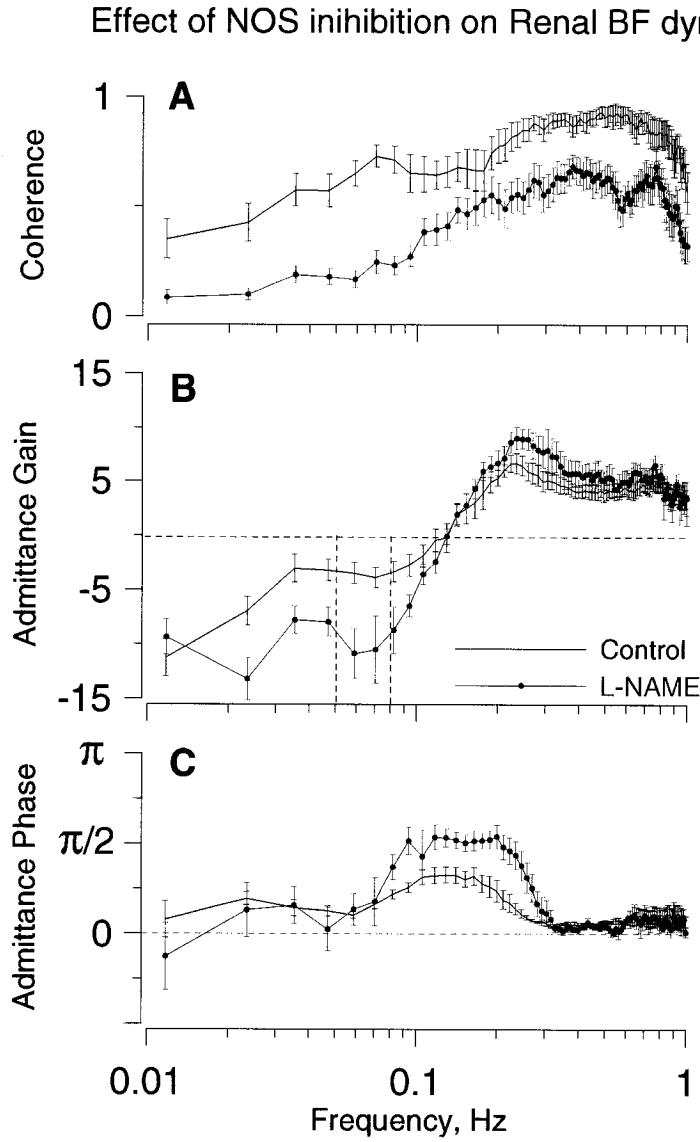

Figure A1. Effect of nitric oxide synthase inhibition on spontaneous renal blood flow dynamics. Panel A shows coherence between blood pressure (BP) and renal blood flow (RBF). Panels B and C show admittance gain, normalized and presented in deciBels, and phase, in radians. Two regions of gain reduction associated with positive phase peaks are seen below 0.04 Hz and between 0.08 and 0.2 Hz; the latter is the myogenic mechanism while the former is tubuloglomerular feedback. The effects of L-NAME to enhance gain reduction by the myogenic mechanism and increase the corresponding phase peak are apparent. The dotted vertical lines in panel B denote the region from 0.05 to 0.08 Hz in which myogenic autoregulation is essentially complete, but tubuloglomerular feedback has not begun to affect admittance.

This is the characteristic figure by NOS inhibition in the BP-RBF transfer function in rats in our laboratory (Figure A1). Here some parameters, coherence, slope of gain and phase peak, and fractional compensation (FC) are reflected the renal autoregulation in rats. Coherence is an index of the degree to which the input (BP) and output (RBF) signals are linearly related; coherence = 1 means that all variation in the RBF variable can be explained as a linear function of variation of the BP, while coherence = 0 suggests that the signals are unrelated. Reduction of coherence after L-NAME probably results from increasing complexity of the system.

The transfer function is an input – output relationship and is used to separate events in frequency that cannot be separated in space. In the current case, tubuloglomerular feedback and the myogenic mechanism operate on the same blood vessels and so can not be separated spatially. Because each controller has its own natural frequency, they can be separated in frequency. Many evidences and data show that the afferent arteriole dominates renal vascular conductance in the kidney (Carmines et al. 1990; Casellas and Moore 1993). Thus the BP-RBF transfer function predominantly reflects the function of the afferent arteriole under the conditions employed here. Gain is calculated as magnitude divided by conductance and expressed in deciBels (dB). That is, gain = 20 x log (admittance magnitude/conductance). Thus gain > 0 means that BP fluctuations are actually amplified into blood flow as expected from passive, compliant vessels; gain = 0 means that the vasculature behaves as a stiff tube; gain < 0 means that flow is actively being stabilized, for instance, by autoregulation. The temporal relationship between BP and RBF is expressed in admittance phase. Figure A1 shows two discrete regions of gain reduction at < 0.2 and < 0.04 Hz that are associated with positive phase peaks. The

combination of gain reduction with a positive phase peak indicates an autonomous, or autoregulatory, system (Holstein-Rathlou and Marsh 1994).

When the kidney is denervated, the vasculature is pressure passive at frequencies above ~0.3 Hz, resulting in slightly positive admittance phase that is independent of frequency, and positive gain resulting from vascular compliance (Holstein-Rathlou and Marsh 1994). The myogenic system generates the resonance peak in gain at ≈ 0.25 Hz and the accompanying broad phase rise and it stabilizes RBF at frequencies < 0.1 Hz (gain < 0). Tubuloglomerular feedback operates at 0.03-0.05 Hz. It produces the gain reduction below 0.05 Hz and the small phase peak at ≈ 0.03 Hz (Abu-Amarah et al. 1998; Holstein-Rathlou et al. 1994; Wang et al. 1999; Wang et al. 2000). Both the amplitude of the phase peak and the slope of gain reduction are informative concerning the internal dynamics of the system (Just et al. 1998). A phase peak ≈ $\pi/4$ radians and slope of gain reduction ≈ 20 dB/decade are characteristic of a first-order system, one that responds to the level of the input variable while a phase peak ≈ $\pi/2$ radians and slope of gain reduction ≈ 40 dB/decade are characteristic of a rate sensitive (second order) system that responds to the level of the input and to its rate of change. In the example shown, L-NAME significantly increased the slope of gain reduction from 38 ± 5 to 55 ± 6 dB/decade, and the phase peak from 1.0 ± 0.1 to 1.7 ± 0.1 rad.

To assess the contribution of the myogenic system to stabilization of RBF, we determined fractional compensation (FC) in the interval between the operating frequencies of the two systems. The interval from 0.05 to 0.08 Hz was used to minimize corruption by tubuloglomerular feedback (< 0.05 Hz) and by myogenic transients (> 0.08 Hz). Fractional compensation is calculated from gain: $FC = 1 - [10^{(gain/20)}]$, which

linearizes the logarithmic scale of gain. Thus FC = 1 implies complete autoregulation and FC = 0 implies total absence of autoregulation. In the example shown, FC increased significantly from 0.30 ± 0.07 during control to 0.55 ± 0.08 after L-NAME.

Panel A shows L-NAME reduced coherence at all frequencies, particularly below 0.1 Hz. Panel B shows that L-NAME increased gain in the region above 0.3 Hz and increased the slope of gain reduction below 0.2 Hz, Panel C shows that L-NAME enhanced the resonance peak in gain at ≈ 0.25 Hz, increased the slope of gain and enhanced the phase peak at ≈ 0.15 Hz. Thus, the myogenic autoregulation is enhanced by L-NAME (The data were provided by Dr. Cupples).