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MECHANISMS BEHIND THE INCREASED ADRENERGIC REACTIVITY OF MESENTERIC VEINS COMPARED TO ARTERIES IN A MURINE MODEL OF HYPERTENSION

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By

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ABSTRACT

MECHANISMS BEHIND THE INCREASED ADRENERGIC REACTIVITY OF MURINE MESENTERIC VEINS COMPARED TO ARTERIES IN A MURINE MODEL OF HYPERTENSION

By

Alex A. Pérez-Rivera

One of the features in hypertension is the altered vascular reactivity that occurs to adrenergic agonists and other vasoconstrictor substances. The aim of my dissertation was to compare adrenergic reactivity of murine mesenteric arteries and veins from normotensive and hypertensive mice and to look at potential mechanisms behind reactivity differences, if any. Initial experiments showed that:

1. There were differences in the acute reactivity and time-dependent desensitization to α-AR agonists between small arteries and veins of DOCA-salt and SHAM control mice. Veins were more sensitive to the contractile effects of adrenoceptor agonists and were more resistant to desensitization when continuously stimulated. Irreversible alkylation of the alpha adrenoceptors (α-AR) with phenoxybenzamine (PBZ) rendered veins susceptible to desensitization by adrenergic agonists, just like arteries, suggesting that veins could have an increased α-AR reserve compared to arteries. This provided functional evidence that a potential reason behind the increased reactivity of veins compared to arteries could be a difference in α-AR number.

2. Subsequent experiments with receptor subtype-specific antagonists revealed that there is a differential contribution of individual α_1-AR subtypes in vasoconstractile responses of arteries and veins. The α_{1A}-AR subtype mediated

contractile responses in arteries whereas the α_{1D}-AR subtype is the main contractile isoform in mesenteric veins. The α_{1B}-AR subtype played just a minor role in contractile responses in these vessels. All three α_1-AR subtypes were expressed in arteries and veins despite the fact that only one subtype was mainly responsible for contractile responses. Protein expression for the α_{1A}- but not the α_{1B}- or α_{1D}-AR subtype was affected by deoxycorticosterone acetate-salt (DOCA-salt) hypertension as evidenced by downregulation. However, there were no differences in α_1-AR protein expression between arteries and veins.

3. Pharmacological analysis with selective α_2-AR agonists and antagonists revealed the existence of a postjunctional α_2-AR population in veins but not arteries. This differential regulation of α_2-AR in veins as opposed to arteries could be a mechanism explaining the increased reactivity seen in murine mesenteric veins. Alterations in α_2-AR mediated contractile responses was not evidenced in blood vessels taken from DOCA-salt mice.

4. Examination of neurogenic responses revealed that there were differences in the contractile responses. The α_{1B}-AR subtype, which did not mediate responses to exogenously applied catecholamines, was the main subtype mediating neurogenic contractile responses. Again, there were no differences in neurogenic neurotransmission in DOCA-salt hypertension.

Therefore, the enhanced reactivity of veins could be explained by an increased in the α_1-AR reserve of veins, by the α_1-AR subtype-selective regulation of contractile responses in mesenteric vessels and/or the selective involvement of α_2-AR in mesenteric constrictions to adrenergic agonists.

To my parents, Felix Pérez and Ana L. Rivera, for being such a great example of how a person should conduct himself and for teaching me to appreciate the value of the "little things" in life.

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LIST OF ABBREVIATIONS

α-AR	alpha adrenergic receptors
α_1-AR	alpha-1 adrenergic receptor
α_{1A}-AR, α_{1B}-AR, α_{1D}-AR	alpha-1A, alpha-1B, alpha-1D adrenergic receptors
α_2-AR	alpha-2 adrenergic receptors
α_{2A}-AR, α_{2B}-AR, α_{2c}-AR	alpha-2A, alpha-2B, alpha-2C adrenergic receptors
β-AR	beta adrenergic receptors
β_1-AR, β_2-AR β_3-AR	beta-1, beta-2, beta-3 adrenergic receptors
1K1C	one-kidney, one clip
2K1C	two-kidney, one clip
5-MU	5-methylurapidil
ANS	autonomic nervous system
ATP	adenosine 5'-trisphosphate
Ca^{++}	calcium
CEC	chloroethylclonidine
CNS	central nervous system
CO	cardiac output
COMT	catechol-o-methyl transferase
CVD	cardiovascular diseases
DAG	diacylglycerol
DOCA	deoxycorticosterone acetate

Epi	epinephrine
HEK	human embryonic kidney
IP_3	inositol 1,4,5,- trisphosphate
JNC VII	Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure
K^+	potassium
K/O	knockout
MAO	monoamine oxidase
MCFP	mean circulatory filling pressure
mRNA	messenger RNA
Na^+	sodium
NE	norepinephrine
NLA	*N*-nitro-L-arginine
NT	neurotransmitter
PBZ	phenoxybenzamine
PE	phenylephrine
PIP_2	phophatidylinositol-4,5-bisphosphate
PLC	phospholipase C
SHR	spontaneously hypertensive rat
SHR-SP	spontaneously hypertensive rat - stroke prone
SNS	sympathetic nervous system
TPR	total peripheral resistance

CHAPTER 1

Introduction

Autonomic nervous system

The autonomic nervous system (ANS) is an efferent peripheral nervous system responsible for innervating the heart, blood vessels, most visceral organs, glands and smooth muscle. Because of its importance in innervating various organs systems, the ANS is widely distributed throughout the body to regulate organ functions in a manner that is generally beyond conscious control (Ruffolo, 1994). Thus, functions like respiration, circulation, digestion, body temperature and blood pressure regulation, among others are regulated by the ANS making it of vital importance for the well-being and survival of the organism.

Divisions of the autonomic nervous system

The ANS consists of three major divisions: enteric, sympathetic and parasympathetic. The enteric division is a highly specialized neuronal system innervating the gut (Katzung, 2001). It controls motor functions of the gastrointestinal tract.

The remaining two divisions, sympathetic and parasympathetic, originate in nuclei within the central nervous system (CNS) and give rise to preganglionic efferent nerve fibers that exit from the brain stem or spinal cord and end up in ganglia located in the periphery. One of the criteria used to differentiate between these two divisions is the relative location of their preganglionic cell bodies within the CNS (Seeley et al., 1998). Sympathetic preganglionic fibers leave the CNS at the thoracic and lumbar portions of the spinal cord (thoracolumbar system) while parasympathetic preganglionic fibers leave the CNS at the cranial and sacral

portions of the spinal cord (craniosacral system). From the ganglia, postganglionic fibers run to the tissues innervated (Katzung, 2001).

The sympathetic and parasympathetic divisions are involved with visceral functions. Therefore, most organs are usually innervated by both, sympathetic and parasympathetic nerve fibers (Seeley et al., 1998). In such cases, typically the two divisions produce opposite effects. Therefore, if activation of one of the divisions increases activity, activation of its counterpart will generally produce an antagonistic effect. However, dual innervation of all organs in the body by sympathetic and parasympathetic neurons is not an universal phenomenon. For example, blood vessels, which are the focus of these research studies, are almost exclusively innervated by the sympathetic division with little or no influence from parasympathetic nerves (Seeley et al., 1998).

Neurotransmission in the autonomic nervous system

As we have seen, nerve impulses elicit responses in their effector organs. How are these processes coupled to the actual response by the effector organ? Who is the mediator of the response? Currently, the concept of chemical neurotransmission is widely accepted. The main premise being that nerves release a chemical substance or mediator, referred to as a neurotransmitter (NT), which is responsible of evoking a defined response in the effector organ.

Overview

Due to the work of pioneering pharmacologists and physiologists it is now firmly established that transmission of information between neurons in both, the sympathetic and parasympathetic divisions of the ANS involves chemical transmission in the form of a neurotransmitter. Pharmacologically, this process is of paramount importance as a number of drugs are used therapeutically that could inhibit a specific step in the neurotransmission process.

Electrical stimulation originating in the CNS results in local depolarization of the neuronal membrane. Upon reaching a particular threshold potential, an action potential or nerve impulse is initiated. This is due to the rapid influx of sodium (Na^+) ions into the cell through voltage-gated Na^+ channels further depolarizing the neuronal membrane. Inactivation of this depolarizing Na^+ current follows almost instantaneously as a result of the selective movement of potassium (K^+) ions out of the cell to terminate depolarization. As a result of these local changes in membrane potential, adjacent resting voltage-gated channels are activated (Lefkowitz et al., 1996) resulting in the propagation of the action potential along the length of the neuron.

Arrival of the action potential at the preganglionic nerve terminal results in transmission of the stimulus along the synapse. Transmission occurs in the form of quantal release of NT being stored in vesicles. Critical to NT release after the arrival of an action potential is calcium (Ca^{++}) influx into the membrane. Ca^{++} is the signal that will allow vesicles to fuse with the membrane, open to the extracellular space and release its contents (Ruffolo, 1994).

The NT will diffuse across the synapse and interact with specific receptors on the cell body of the postganglionic neuron. Activation of these, will lead to changes in ionic permeability resulting in the generation and propagation of an action potential along the length of the postganglionic neuron. As with the preganglionic nerve fiber, after arrival of the action potential at the postganglionic nerve terminal, NT release occurs through a Ca^{++}-dependent process. In this case, NT diffusion across the neuroeffector junction results in its interaction with specific receptors on the effector organ leading to a biological response (Ruffolo, 1994).

As previously stated, there are anatomical and functional distinctions between the sympathetic and parasympathetic divisions of the ANS. In addition to those, the NT being released by neurons in each of these divisions could differ. These two divisions of the ANS are similar in that the NT being released by preganglionic neurons is acetylcholine (Ruffolo, 1994). On the other hand, the NT released by postganglionic sympathetic and parasympathetic neurons differs. Norepinephrine (NE) is liberated by postganglionic sympathetic nerve terminals whereas the NT in postganglionic parasympathetic nerves is acetylcholine (Ruffolo, 1994).

Sympathetic transmission

Synthesis and storage. Neurotransmission involves release of the catecholamines NE or epinephrine (Epi) from postganglionic sympathetic nerve terminals. The biosynthetic pathways involved in the synthesis of catecholamines

are widely understood. The amino acid tyrosine is the precursor for the synthesis of the catecholamines. Tyrosine is hydroxylated by the enzyme tyrosine hydroxylase to form the catechol derivative DOPA. This hydroxylation step that takes place in the cytoplasm of postganglionic sympathetic neurons is the rate-limiting step in the biosynthesis of all catecholamines. DOPA is then decarboxylated by L-aromatic amino acid decarboxylase to form dopamine, which is taken by storage vesicles in the sympathetic nerve terminals. These vesicles contain the enzyme dopamine β-hydroxylase which catalyzes its conversion to NE. After being synthesized, NE is stored in vesicles until released after arrival of an action potential at the sympathetic nerve terminal. In the adrenal medulla, where Epi accounts for approximately 80% of the catecholamines being stored and released (Lefkowitz et al., 1996, Ruffolo, 1994), there is an additional biosynthetic step. NE is converted to Epi by the enzyme phenylethanolamine-N-methyltransferase. Epi is then stores in granules in chromaffin cells of the adrenal medulla ready to be released upon stimulation.

Release. Critical to NT release is the arrival of an action potential at the nerve terminal producing a localized depolarization that triggers Ca^{++} influx into the membrane. Ca^{++} is the signal that will allow vesicles to fuse with the membrane, open to the extracellular space and release its contents. The NT will diffuse across the synapse and interact with specifics receptors on the effector organ leading to a biological response (Ruffolo, 1994).

In fact, NE is not the only NT being released by sympathetic nerves when stimulated. Evidence have suggested that nerves in both, the central and

peripheral nervous system contain more than one substance with activity at postjunctional sites. This was proposed as early as 1976 (Burnstock, 1976) and provided a new idea of looking at neurotransmission in a way that challenged the so-called Dale's Principle - the idea that nerves utilize one and only one NT (Burnstock, 2004).

In particular, the evidence is abundant with respect to adenosine 5'-triphosphate (ATP) and its role as a cotransmitter with NE in sympathetic terminals. In the guinea pig vas deferens there is a biphasic contraction in response to nerve stimulation. The first phase phase occurs rapidly and was mimicked by exogenous ATP while the more tonic phase was mimicked by exogenous NE (Sneddon and Westfall, 1984). Pharmacological antagonism with a selective ATP-receptor and a selective alpha-adrenergic receptor selectively blocked the first and second phases, respectively. Similar results have been obtained by other investigators (von Kugelgen and Starke, 1991; Todorov et al., 1996) supporting the fact that the response to nerve-released ATP is faster while the response to NE is more gradual.

Cotransmission has also been studied in the vasculature. In rabbit mesenteric artery (von Kugelgen and Starke, 1985) as well as in canine (Bobalova and Mutafova-Yambolieva, 2001a) and guinea pig (Bobalova and Mutafova-Yambolieva, 2001b) mesenteric arteries and veins, ATP is coreleased along with NE from sympathetic nerves. However, release appears to be greater in mesenteric veins compared to arteries (Bobalova and Mutafova-Yambolieva, 2001a; Bobalova and Mutafova-Yambolieva, 2001b).

In addition to ATP, there is a growing list of other substances, particularly peptides that have been found in the adrenal medulla, nerve fibers or autonomic ganglia and that have been postulated as potential cotransmitters. These include the enkephalins, substance P, somatostatin, calcitonin gene-related peptide, vasoactive intestinal peptide and neuropeptide Y (Lefkowitz et al., 1996).

Termination of action. After being released by sympathetic nerve terminals, catecholamine actions are rapidly terminated. It is been known for quite some time that sympathetic neurons have the ability of accumulating NT (Iversen and Kravitz, 1966). These transporters or reuptake systems are localized to the neuronal synaptic membrane and serve an important role in terminating NT action (Amara and Kuhar, 1993). This is a Na^+-dependent cotransport process as NT accumulation into the nerve terminal is coupled to the inward movement of Na^+ ions down a concentration gradient (Iversen and Kravitz, 1966). Thus, this is a way in which the energy stored in transmembrane electrochemical gradients can be used to drive the NT into the sympathetic nerve terminal (Amara and Kuhar, 1993). These reuptake systems for NE and other monoamines share a common structural topology: they all have 12 hydrophobic regions presumed to be membrane spanning domains.

An additional fate for catecholamines released by sympathetic nerve stimulation is their simple diffusion away from the sympathetic nerve terminal and subsequent reuptake by extraneuronal tissues in a process commonly referred to as $uptake_2$. $Uptake_2$ is an extraneuronal transport process with low affinity for NE. This uptake system is found on many cell types: glial, hepatic, myocardial and

other cell types. This uptake system is probably of little physiological importance in the removal of catecholamines released from adrenergic nerve terminals. However, it could play some important role in the clearance of circulating catecholamines.

The life-span of catecholamines is limited not only by reuptake into neuronal or extraneuronal tissues, but also by subsequent intracellular metabolism (Trendelenburg, 1990). Once taken up by either neuronal or extraneuronal tissues, NE is metabolized by the enzymes monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). MAO and COMT are widely distributed throughout the body. However, little or no COMT is seen in adrenergic neurons (Lefkowitz et al., 1996). This is of physiological importance as these metabolizing systems are usually coupled to the uptake processes. Therefore, neuronal $uptake_1$ is usually associated with MAO whereas the extraneuronal $uptake_2$ is usually coupled to COMT (Trendelenburg, 1990).

Receptors mediating sympathetic neurotransmission

NE, the primary NT released by sympathetic nerves, interacts with different pharmacological receptors to mediate their biological effects. The existence of heterogeneity in the receptor population mediating the effects of NE was first proposed by Ahlquist (1948), who studied the actions of a series of sympathomimetic agents in several organs. He proposed the designation of alpha-adrenergic (α-ARs) and beta-adrenergic (β-ARs) receptors based on their

differential abilities to mediate either excitatory or inhibitory responses. In his original research article (Ahlquist, 1948), he stated his conclusions this way:

"The adrenotropic receptors have been considered to be of two classes, those whose action results in excitation and those whose action results in inhibition of the effector cells. Experiments described in this paper indicate that although there are two kinds of adrenotropic receptors they cannot be classified simply as excitatory or inhibitory since each kind of receptor may have either action depending upon where it is found... Tentatively the first kind of receptor has been called the alpha-adrenotropic receptor and the second kind the beta receptor... The alpha adrenotropic receptor is associated with most of the excitatory functions (vasoconstriction, and stimulation of the uterus, nictitating membrane, ureter and dilator pupillae) and one important inhibitory function (intestinal relaxation). The beta adrenotropic receptor is associated with most of the inhibitory functions (vasodilation, and inhibition of the uterine and bronchial musculature) and one excitatory function (myocardial stimulation)."

Later, data started to come out providing evidence for the existence of subtypes of β-ARs. It was discovered that certain agents could distinguish between β-mediated responses in tissues like cardiac muscle and bronchial smooth muscle (Lands et al., 1967a; Lands et al., 1967b). In one of his research papers, Lands suggested a nomenclature referring to the β-ARs mediating responses in the heart as β_1 and β_2 to those mediating physiological responses in bronchi:

"Comparison of various parameters of pharmacologic action has disclosed that, on the basis of rank order, two distinct types of receptor populations can be distinguished by exposure to structurally varied sympathomimetic amines, i.e. β-1 (cardiac acceleration-lipolysis) and β-2 (bronchodilation-vasodilation) types."

A third type of β-AR, referred to as β_3, has been discovered and characterized (Emorine et al., 1989). This particular receptor subtype has been regarded as atypical, partly because of its unusual pharmacological

characteristics: most of the typical β-AR antagonists do not effectively block its biological responses and even some behave as agonists of this particular subtype. The β_3-AR is abundantly expressed in adipose tissues (Granneman et al., 1991) and has been linked to lipolysis in humans.

Heterogeneity among α-ARs is also appreciated nowadays. Initial data pointing to that conclusion was based on the observation that NE and other sympathomimetic agents could inhibit NT release upon stimulation. On the other hand, in the presence of certain α-AR antagonists, the amount of NE released by sympathetic nerve stimulation increases dramatically. The increase in NT release elicited by these α-AR antagonists was observed in the concentration range eliciting blockade of α-ARs. This fact initially led to the conclusion that when postsynaptic α-ARs on the effector cell were occupied by the antagonist, the released NT would not be able to combine with these receptors, and thus overflow would increase without changes in release (Langer, 1974).

However, similar results were obtained in atria and heart, where the postsynaptic adrenoceptors are of the β-type (Starke et al., 1971). These results led to the hypothesis of a presynaptic negative feedback mechanism regulating NE release from sympathetic nerves. This presynaptic feedback inhibitory mechanism is mediated by an α-AR pharmacologically distinct from the postsynaptic α-AR. Clonidine and oxymetazoline at relatively low concentrations selectively activated the prejunctional α-ARs whereas phenylephrine (PE) and methoxamine did the same thing for the postjunctional receptors (Starke, 1974; Starke et al., 1975b). In the same manner, antagonists were able to differentiate

between pre- and postjunctional α-ARs. Phenoxybenzamine (PBZ) preferentially inactivated the postjunctional adrenoceptors (Dubocovich and Langer, 1974) whereas yohimbine did it for the prejunctional α-AR population (Starke, 1975a).

Based on all this evidence, Langer (1974) proposed that α-ARs mediating the responses of effector organs postsynaptically be called α_1 whereas the presynaptic α-AR involved in the negative feedback regulation of neurotransmitter release be referred to as α_2:

"These results are compatible with the view that the postsynaptic alpha-receptor that mediates the response of the effector organ should be referred to as α_1, while the presynaptic alpha-receptor that regulates transmitter release should be called α_2."

In addition to their role as presynaptic receptors controlling NE release via a negative feedback mechanism, a special subpopulation of α_2-ARs is also located postjunctionally where they mediate constriction just like α_1-ARs (Daly et al., 1988; Fowler et al., 1984; Itoh et al., 1987; Polonia et al., 1986).

Alpha-1 adrenergic receptors

α_1-ARs are the membrane proteins that mediate the actions of the catecholamines NE and Epi. They are members of the G-protein coupled superfamily of receptor proteins, proposed to contain seven membrane-spanning domains that regulate effector actions through the mediation of a group of GTP-binding proteins (Ross, 1996).

α_1-AR heterogeneity

Subsequent to the original classification of adrenoceptors as α_1 or α_2, evidence began to appear suggesting that, indeed, α_1-ARs could be further subdivided into distinct subtypes. This notion was based on the different dose-response relations elicited by α_1-AR agonists, the differential sensitivity of α_1-AR mediated responses to antagonism by α_1-AR antagonists and different requirements of α_1-AR mediated responses for extracellular Ca^{++} (Nichols and Ruffolo, 1991).

Initially, Bevan (1981) found a biphasic dose-response curve for a series of sympathomimetic agonists. Additional evidence supporting this notion of heterogeneity came with Babich et al. (1987). They reported that the dose-response curve for metaraminol was not parallel to that of Epi, NE or PE. Inactivation of a portion of the α_1-AR population with PBZ showed a biphasic occupancy versus response relation for mataraminol but not to the other agonists supporting the idea that metaraminol could be acting at a different set of receptors compared to the other test agonists.

Additionally, certain antagonists were capable of producing a biphasic displacement of [^{3}H]-prazosin binding in rat brain membranes. Specifically, Morrow et al. (1985) demonstrated that dihydroergocryptine and indoramine were able to compete in a steep, monophasic manner whereas WB4101 and phentolamine exhibited shallow competition curves. Subsequent analysis of the competition curves revealed that both WB4101 and phentolamine discriminated two distinct components each one representing about 50% of the total binding

leading the investigators to suggest that in the rat cerebral cortex there were present two distinct adrenoceptor subtypes. A subsequent study by Morrow and Creese (1986) confirmed and validated the previous findings. They designated the α_1-AR population interacting with high affinity with WB4101 as α_{1A} whereas the remaining receptor population with low affinity for the same compound was named α_{1B}.

In other series of studies, chloroethylclonidine (CEC) was able to reduce the α_1-AR population in rat brain slices by about 50% (Johnson and Minneman, 1987) whereas other alkylating agents were capable of inactivating the whole α_1-AR population. These led the authors to conclude that CEC could discriminate between α_1-AR subtypes.

The ability of Ca^{++} channel blockers to selectively inhibit pressor responses to SGD 101/75 but not to other α_1-AR agonists (Timmermans et al., 1983) provided more evidence for the existence of a heterogeneous population of α_1-ARs. Furthermore, this led some people to suggest that these particular receptor subtypes could be differentiated by their Ca^{++} requirements: one particular subtype activates the release of intracellular Ca^{++} while the other is coupled to extracellular Ca^{++} influx.

However, this notion is not as crystal clear as it was intended to be. It is now known that all three α_1-AR subtypes couple via a pertussis toxin-insensitive G protein of the $G_{q/11}$ family to Ca^{++} release from intracellular stores (Guimaraes and Moura, 2001; Piascik et al., 1996; Piascik and Perez, 2001). In addition, all subtypes, to varying degrees, could activate a variety of other second messenger

pathways. They can activate Ca^{++} influx via voltage-gated Ca^{++} channels (Minneman, 1988, Perez et al., 1993) as well as phospholipase A_2 (Perez et al., 1993).

The idea of an additional subtype that can not be classified as either α_{1A} or α_{1B} was supported by receptor cloning studies. Schwinn et al. (1990), cloned a novel α_1-AR subtype from a bovine brain cDNA library. When expressed in COS7 cells, it showed a high affinity for the antagonist WB4101, a pharmacological profile very similar to the one already described for α_{1A}-AR (Morrow and Creese, 1986). However, the fact that this clone was sensitive to inhibition by the alkylating agent CEC and the lack of expression in the rat vas deferens and hippocampus, tissues where the α_{1A}-AR was previously described, led the authors to suggest that the cloned bovine brain α_1-AR cannot be classified as either α_{1A}- or α_{1B}-AR and, therefore, represented a novel α_1-AR subtype.

These findings were supported by Perez et al. (1991) who reported the cloning of a novel α_1-AR subtype with essentially the same aminoacid sequence to the one described by Lomasney et al. (1991) and thought to be the α_{1A}-AR. However, this clone isolated by Perez et al. had ligand-binding properties very different from those of the pharmacological α_{1A}-AR but it was sensitive to CEC inactivation leading the investigators to conclude this was indeed a novel adrenoceptor and named it the α_{1D}-AR.

In an effort to standardize and unify the nomenclature on α_1-AR, the International Union of Pharmacology suggested this newly cloned α_1-AR subtype

be named $\alpha_{1A/D}$ (Bylund et al., 1994). A year later, the same nomenclature committee suggested that the original designation suggested by Perez et al. (1991) be adopted (Hieble et al., 1995). Now it is recognized the existence of three α_1-AR subtypes: α_{1A} , α_{1B} and α_{1D} that could differ in their molecular biology, biochemistry and pharmacology.

Signal transduction mechanisms

The α_1-ARs utilize a variety of signaling pathways to modulate cellular function. The main signaling pathway utilized by α_1-ARs has been well-characterized. α_1-ARs are coupled to pertussis toxin insensitive G proteins of the q/11 type that upon agonist activation dissociate and activate phospholipase C (PLC). Increases in PLC activity results in the hydrolysis of phophatidylinositol-4,5-bisphosphate (PIP_2; Berridge, 1983), producing inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG are the second messengers responsible for transduction of the α_1-AR signal (Berridge and Irvine, 1984). IP_3 binds to IP_3 receptors that when activated promote Ca^{++} release from nonmitochondrial intracellular Ca^{++} stores (Streb et al., 1983). DAG activates protein kinase C, which can phosphorylate a series of intracellular substrates involved in Ca^{++} handling, such as the Ca^{++} channel itself (Piascik et al., 1996).

All three α_1-AR subtypes couple to G-proteins and the subsequent intracellular Ca^{++} mobilization that occurs. However, there are differences in the ability of the respective subtypes to activate IP_3 formation and increase

intracellular Ca^{++}. The α_{1A}-AR is the most efficiently coupled whereas α_{1D}-AR couple in a poor fashion (Theroux et al., 1996).

It is now appreciated that α_1-ARs can couple to a variety of other signaling pathways as well. In addition to translocating intracellular Ca^{++}, these receptors can activate Ca^{++} influx via voltage-gated calcium channels (Minneman, 1988, Perez et al., 1993) as well as phospholipase A_2 (Perez et al., 1993). These receptors could also signal through pertussis toxin-sensitive G proteins (Perez et al., 1993).

A common feature of G-protein coupled signaling is their potential for desensitization; α_1-ARs are no exception. Agonist occupation and stimulation of its respective receptor promotes its phosphorylation by a series of G-protein receptor kinases. A phosphorylated receptor exhibits high affinity for the arrestins; a group of proteins that will bind to the receptor, further preventing the interaction between receptor and G-proteins. In this way, arrestins promote receptor internalization contributing to the desensitization seen after prolonged exposure of a G-protein coupled receptor to an agonist (Ferguson et al., 1996; Zhang et al., 1997).

α_1-AR subtypes differ in their ability to desensitize upon agonist stimulation. Following exposure to the selective α_1-AR agonist PE, green fluorescence protein-tagged α_1-AR transfected in human embryonic kidney (HEK) cells showed differential sensitivity to desensitization. The α_{1B}-AR underwent rapid internalization, the α_{1A}-AR desensitized as well but at a slower rate compared to the α_{1B}-AR. In contrast, PE treatment did not affect cellular

location of the α_{1D}-AR (Chalotorn et al., 2002). To determine that internalization was a direct effect of arrestins, experiments were also done in cells expressing a dominant negative form of arrestin-1. In these cells, cotransfection of the dominant negative form of arrestin-1 prevented agonist-mediated internalization indicating the important role played by these proteins in agonist-mediated internalization of α_1-AR.

Cellular localization

α_1-ARs are members of the G-protein coupled superfamily of membrane receptors. It has been regarded that members of this receptor family are typically membrane-bound receptors accessible to water-soluble ligands. However, data collected have suggested that localization of α_1-ARs is not as straightforward as expected; there are major differences in cellular localization among α_1-AR subtypes.

Receptor immunoreactivity was detected on the cell margin in rat fibroblasts stably transfected with the human α_{1B}-AR cDNA (McCune et al., 2000) indicating that this particular receptor subtype was localized to the plasma membrane. In contrast, significant immunoreactivity was detected in a perinuclear orientation in fibroblasts expressing the α_{1D}-AR subtype. Experiments done in HEK cells transfected with cDNA encoding α_1-ARs essentially showed a similar expression pattern (Chalotorn et al., 2002). Membrane fluorescence was predominant in HEK cells expressing the α_{1B}-AR subtype. The α_{1A}-AR was

detected in both, the membrane and intracellularly whereas a predominant intracellular fluorescence was seen in α_{1D}-AR-expressing HEK cells.

The physiological significance of such differences in localization remains to be understood and explained. It looks that cell surface expression of the α_{1D}-AR, who has been found to be located in intracellular compartments, is controlled by heterodimerization. As shown by previous studies, α_{1A}- and α_{1B}-ARs were primarily localized to the membrane of HEK cells whereas α_{1D}-AR showed an almost complete intracellular localization (Hague et al., 2004b). Coexpression of α_{1B}- and α_{1D}-AR resulted in complete translocation of α_{1D}-AR from the intracellular sites to the plasma membrane. In contrast, coexpression of α_{1A}- and α_{1D}-AR did not result in translocation of α_{1D}-AR to the membrane. Hague et al. (2004b) showed that this effect of the α_{1B}-AR on α_{1D}-AR expression appears to only involve the α_{1B} hydrophobic core as N- and C-terminal truncation mutants were as effective as full-length receptors in translocating the α_{1D}-AR to the membrane. It could be that somehow the α_{1B}-AR specifically inhibits or obscures the N-terminal domain of the α_{1D}-AR subtype as it is known that this particular domain prevents cell surface expression (Hague et al., 2004a).

Smooth muscle contractile regulation

It is well-known that α_1-ARs play a very important role in vascular tone regulation. In addition, activation of α_1-ARs in some vascular beds results in vasoconstriction (Guimaraes and Moura, 2001; Piascik et al., 1996; Piascik and Perez, 2001). The relative contribution that each of the α_1-AR subtypes plays in

the regulation of vascular smooth muscle contraction is an important area of pharmacological research where we are starting to answer some of the questions.

Several studies from different laboratories have demonstrated in a consistent manner that in a particular blood vessel a particular α_1-AR subtype plays the dominant role in controlling vessel tone and that the dominant contractile α_1-AR is different in different vascular beds. The α_{1A}-AR has been implicated in the contractile responses of rat renal (Hrometz et al., 1999) and caudal arteries (Piascik et al., 1997). The α_{1A}-AR has also been implicated in constrictions of the murine tail and mesenteric artery (Daly et al., 2002). The α_{1D}-AR mediates contractile responses in rat femoral (Hrometz et al., 1999; Piascik et al., 1997), iliac (Piascik et al., 1997) superior mesenteric artery (Piascik et al., 1997), and aorta (Piascik et al., 1997). Contractile responses in murine aorta are predominantly α_{1D}-mediated (Chalotorn et al., 2003; Daly et al., 2002). The α_{1B}-AR appears not to be involved in smooth muscle contraction (Chalotorn et al., 2003). However, a few studies have suggested a role for this adrenoceptor subtype in rat mesenteric artery (Piascik et al., 1997) with just a minor involvement in murine vessels (Daly et al., 2002).

It looks that despite ubiquitous expression of these receptor proteins throughout the peripheral vasculature (Hrometz et al., 1999; Piascik et al., 1997), the adrenoceptor responsible for mediating contraction will differ in different vascular beds. There is diversity in terms of the specific contribution each α_1-AR

has on vascular tone, relative contributions being dependent on what vascular bed we are looking at.

Blood pressure regulation

Given their important regulatory role in vascular smooth muscle contraction, it is expected that α_1-ARs will play critical roles in the regulation of total peripheral resistance and, therefore, blood pressure. Experiments with genetically-modified mice have provided the greatest amount of scientific evidence. From these studies it can be concluded that all three α_1-AR subtypes play important roles in the pressor responses to α_1-AR agonists.

When measured by both, tail cuff and arterial catheter implantation, α_{1A}-AR knockout (K/O) mice were hypotensive under resting conditions compared to wild type controls (Rokosh and Simpson, 2002). A61603, a selective α_{1A}-AR agonist, stimulated a pressor response in control but not K/O mice while responses to PE were decreased in K/O mice compared to wild type controls. This provided the experimental evidence necessary to link this particular adrenoceptor subtype to blood pressure regulation in vivo.

α_{1D}-ARs are also important key regulators of blood pressure. Mice genetically modified to lack this particular α_1-AR subtype showed a significantly lower basal systolic and mean arterial blood pressure (Tanoue et al., 2002b). In addition, contractile responses of the aorta and pressor responses of the perfused mesenteric arterial bed were decreased.

Even though there is not a lot of evidence linking α_{1B}-ARs to vascular smooth muscle tone, experimental data taken from α_{1B}-AR K/O mice have strongly suggested that α_{1B}-AR are also mediators of the pressor responses to various α_1-AR agonists. Pressor responses to PE were decreased in these K/O animals (Cavalli et al., 1997) whereas responses to angiotensin-II and vasopressin were not altered. In addition, PE-induced constrictions of aortic rings were also decreased compared to wild type controls but contractility to serotonin was not changed.

All these summarized experimental data provide evidence of the important role that all α_1-ARs, in one way or the other have in blood pressure regulation.

Alpha-2 adrenergic receptors

α_2-ARs were initially characterized as a subset of presynaptic adrenoceptors regulating transmitter release (Langer, 1974). Now it is known that, indeed, there is a population of presynaptic α_2-ARs that mediate the negative feedback inhibition of NT release. In addition, it is now accepted that a subpopulation of postjunctional α_2-ARs is present and regulates vascular tone in conjunction with α_1-ARs in a variety of vascular beds (Daly et al., 1988; Fowler et al., 1984; Itoh et al., 1987; Polonia et al., 1986).

α_2-AR heterogeneity

There are three main α_2-AR subtypes: α_{2A}, α_{2B} and α_{2C}. One of the first to propose a classification of α_2-ARs into subtypes was Bylund (1985) who

suggested a classification of α_2-ARs based on pharmacological criteria. He noticed that prazosin competition for [^{3}H]-yohimbine binding sites uncovered in human platelet and rat lungs different α_2-ARs with marked differences in their affinities for prazosin. He suggested that the human platelet receptor showing a low affinity for prazosin be classified α_{2A} whereas the neonatal rat lung receptor having a relatively high affinity be classified α_{2B}. Later, a third α_2-AR subtype, the α_{2C}, was identified in the opossum kidney-derived cell line (Murphy and Bylund, 1988). Like the α_{2B}-, this new α_2-AR subtype has a relatively high affinity for prazosin. However, it could be distinguished from the α_{2B}-AR in that it has higher affinity for the α_2-AR antagonist rauwolscine (Blaxall et al., 1991).

Presynaptic α_2-ARs and modulation of NT release

It looks that of all α_2-AR subtypes, the α_{2A}- and α_{2C}-AR subtypes are critical for normal presynaptic control of transmitter release from sympathetic nerves as confirmed by studies in knockout mice (Hein et al., 1999). Maximal inhibition of electrically-evoked contractions was reduced by about 50% in mice lacking the α_{2A}-AR whereas no changes in prejunctional inhibition were seen in α_{2C}-AR K/O mice (Guimaraes and Moura, 2001).

Not only they were the mediators of the prejunctional negative feedback mechanism of NE release from sympathetic terminals, but Hein et al. (1999) demonstrated that these α_2-AR may regulate different aspects of NT release, the α_{2A}-AR inhibiting NT release at high frequencies of stimulation whereas the α_{2C}-AR modulated neurotransmission at low levels of nerve activity. This could

explain why, in vivo, α_{2A}- and α_{2C}-AR modulate NE release from sympathetic nerves and epinephrine release from the adrenal medulla, respectively (Brede et al., 2003).

Postsynaptic α_2-ARs and blood pressure regulation

The typical hemodynamic response when α_2-AR agonists are administered by rapid infusion consists of an initial pressor response followed by hypotension and bradycardia (Kallio et al., 1989). Three factors are responsible for the hemodynamic response due to α_2-AR stimulation (Guimaraes and Moura, 2001). First, activation of postsynaptic α_2-ARs in vascular smooth muscle is responsible for the brief initial pressor response. The hypotensive effect is due to centrally located α_2-ARs, whose activation leads to a reduction in sympathetic tone. Additionally, activation of presynaptic α_2-ARs in peripheral sympathetic neurons innervating vascular smooth muscle leading to inhibition of NT release also contributes to the hypotensive effect of selective α_2-AR agonists.

Experiments in genetically-modified mice have given insights into the role that each α_2-AR plays in blood pressure responses. Hemodynamic responses in mice with a point mutation into the α_{2A}-AR subtype showed that the hypotensive response due to infusion of α_2-AR agonists was practically absent while the initial hypertensive response was not changed (MacMillan et al., 1996). This provided functional evidence to the fact that the α_{2A}-AR mediates the hypotensive response to α_2-AR stimulation. Subsequently, results obtained in α_{2A}-AR K/O

showed complete agreement with those already obtained in the α_2-AR point mutation mice (Altman et al., 1999).

In α_{2B}-AR K/O mice, the initial pressor response to α_2-AR agonists was absent. The hypotensive phase occurred immediately after infusion of the α_2-AR agonist and was significantly greater than that observed in wild type mice (Link et al., 1996). This led the authors to conclude that the α_{2B}-AR mediates the initial hypertensive phase to α_2-AR activation and that this constrictor activity of α_{2B}-AR counteracts the hypotensive effect of α_2-AR agonists providing evidence for the clinical efficacy of more subtype-selective α_2-AR drugs, perhaps a selective α_{2A}- but not α_{2B}-AR agonist will be more effective as an antihypertensive drug.

It appears that α_{2C}-AR are not involved in these hemodynamic responses as hypertensive, hypotensive and bradycardic responses in α_{2C}- K/O mice were not different from wild-type mice when infused with α_2-AR agonists (Link et al., 1996).

Signal transduction mechanisms

Presynaptic α_2-ARs are primarily coupled to pertussis toxin sensitive G-proteins of the G_i/G_o family that are capable of inhibiting adenylate cyclase activity resulting in an attenuation of cAMP production in target cells (Guimaraes and Moura, 2001; Piascik et al., 1996). This results in inhibition of voltage-dependent Ca^{++} currents and activation of inwardly rectifying K^+ channels. These electrical events attenuate secretion from neuroendocrine and neuronal cells.

In vascular smooth cells, where a postjunctional population of α_2-ARs mediate contractile responses along with α_1-ARs, the α_2-AR may be linked to a Ca^{++} channel that allows translocation of extracellular Ca^{++} as it is known that in the rat tail artery and canine saphenous vein, α_2-AR mediated constriction is reduced by Ca^{++} channel blockers and almost abolished in the absence of extracellular Ca^{++} (Cooke et al., 1985; Medgett and Rajanayagam, 1984).

As seen for α_1-ARs, as G-protein coupled receptors, α_2-AR signaling is also susceptible to desensitization. Experimental finding have suggested that, like α_1-ARs, there are subtype-specific differences in susceptibility of the different α_2-ARs to desensitization (Eason and Liggett, 1992; Kurose and Lefkowitz, 1994). α_{2A}-and α_{2B}-ARs downregulate whereas α_{2C}-AR do not appear to downregulate following exposure to agonists.

Role of vascular α_1- and α_2-AR in hypertension

The sympathetic division of the autonomic nervous system is an important regulator of overall systemic blood flow and blood pressure regulation in health and disease. The observation that the increased sympathetic nervous system activity can be correlated to the pathogenesis of hypertension (de Champlain, 1990) suggest that alterations in α-AR mechanisms could be behind the increased pressures seen in hypertensive subjects. Historically, blockade of α_1-ARs as well as agonism of presynaptic α_2-ARs has been one of the most common approaches for the treatment of hypertension (Piascik et al., 1996).

According to several studies, α_1-ARs may be involved in the pathogenesis/maintenance of high blood pressure. It has been shown that there is an increased density of α_1-AR binding sites in mesenteric arteries from deoxycorticosterone acetate-salt hypertensive (DOCA-salt) rats compared to salt and water control arteries (Meggs et al., 1988). At first, this was an unexpected finding as the already known increased sympathetic tone seen in hypertensive experimental models would lead to hypothesize that instead of an upregulation there would have been a downregulation of vascular α_1-ARs.

Experiments with genetically-modified mice have provided the greatest amount of scientific evidence to the fact that all three α_1-ARs subtypes play important roles in blood pressure control (Rokosh and Simpson, 2002; Tanoue et al., 2002b; Cavalli et al., 1997). However, little is known about the pathophysiological role of each α_1-AR subtype.

An elegant study by Tanoue et al. (2002a) provided evidence that the α_{1D}-AR plays a very important role in the development of high blood pressure in response to high dietary Na^+ and subtotal nephrectomy. In these series of studies, α_1-AR K/O mice had an attenuated increase in blood pressure compared to their wild type counterparts. In addition, α_1-AR gene K/O had a favorable effect on cumulative survival due to subtotal nephrectomy and 1% salt loading. Altogether, the data presented by Tanoue et al. (2002a) supported the idea that α_{1D}-ARs could play a role in the development of salt-sensitive hypertension.

There is also evidence suggesting that salt loading causes hypertension via a mechanism involving α_2-ARs as well. To explore whether α_2-ARs are

involved in the blood pressure increases seen after salt loading, and if so, what particular subtype is responsible for the responses, α_{2B}-, and α_{2C}-K/O mice were studied along with wild type controls (Makaritsis et al., 1999). The mice were submitted to subtotal nephrectomy and given 1% saline as drinking water for up to 35 days. Only the α_{2B}-AR K/O mouse have no significant increase in blood pressure. Both, the wild type and the α_{2C}-AR K/O mouse had considerable blood pressure increases. These data suggested that a full complement of α_{2B}-AR genes is necessary to raise blood pressure in response to dietary salt loading.

As listed above, definitely vascular α_1- and α_2-ARs are important not only in the normal physiological control of blood flow and systemic arterial pressure but also in the pathophysiology behind abnormally high elevations in systemic blood pressure. In particular, it looks that α_{1D}- and α_{2B}-ARs are essential in the pathophysiology of hypertension but further studies will prove to be essential to determine the role played by the other α_1- and α_2-AR subtypes.

Hypertension

Epidemiology and statistics

In an era of incredible advances in biomedical research that has led to development of therapies for the treatment of various diseases, cardiovascular diseases (CVD) still pose a threat to our society. In the United States, CVD are one of the leading causes of death in both, men and women (American Heart Association, 2002). CVD claimed 39.4% of all deaths in the United States in 2000. This is roughly 1 of every 2.5 deaths. According to the American Heart

Association (2002), nearly 2,600 Americans die of CVD each day, about a death every 33 seconds. Not only CVD have a profound effect in our society in terms of the thousands of lives lost each year, it has also had a huge economical impact with an estimated cost of $351.8 billion dollars (American Heart Association, 2002).

Several risk factors are involved in the development of CVD. Tobacco smoke, high blood cholesterol, physical inactivity, obesity and diabetes mellitus are among the factors known to increase the risk of developing CVD (American Heart Association, 2002). Hypertension or high blood pressure, is also an important risk factor contributing to the development of CVD. Observational studies have indicated that death from both ischemic heart disease and stroke increases progressively in a linear manner with increased values of blood pressure (Chobanian et al., 2003). However, it is documented that antihypertensive therapy has reduced in a range varying from 20 to more than 50% the incidence of stroke, myocardial infarction and heart failure (Neal et al., 2000). In this regard, the need for basic cardiovascular research in an effort to develop new and more effective therapies for the treatment of hypertension continues.

Common forms of hypertension

According to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VII; Chobanian et al., 2003), hypertension is defined as a sustained systolic

blood pressure greater than 140 mmHg and/or a sustained diastolic blood pressure greater than 90 mmHg. HTN poses a significant public health problem in the United States. One in five Americans (an estimated 50 million people) is hypertensive (American Heart Association, 2002) with estimated treatment costs rounding $50.3 billion dollars. Of those with HTN, 31.6% are unaware they have it.

There are two diagnostic categories for hypertension. Around 5-10% of hypertensives have an identifiable cause such as chronic kidney disease, aorta coarctation, pheochromocytoma, renovascular hypertension, sleep apnea, thyroid or parathyroid disease, Cushing syndrome and/or other glucocorticoid or mineralocorticoid excess states (Chobanian et al., 2003). In this type, known as secondary HTN, identification and correction of the cause will most often cure this form of the disease.

On the other hand, the cause of 90-95% of the hypertension cases is not known (American Heart Association, 2002). This is the most common form of hypertension and is referred to as primary or essential. Treatment primarily involves lifestyle changes (diet, physical activity, weight loss). If lifestyle modifications are not able to control the elevated blood pressures, pharmacological treatment is then implemented. It is important to note that these approaches are introduced in an effort to control but not cure blood pressure elevations. The fact that essential hypertension has a strong familial aggregation and that some racial groups are more likely to develop it suggests that a genetic component is involved in its etiology (Garcia et al., 2003; Naber and Siffert, 2004;

O'Byrne and Caulfield, 1998). However, it is also known that environmental influences are modifiers of genetic factors that when activated could initiate the pathophysiological process (Holtz et al., 1985).

Pathophysiology of essential hypertension

Hemodynamically, mean arterial blood pressure is the product of cardiac output (CO) and total peripheral resistance (TPR). TPR, or the opposition to blood flow is mainly determined by the small arteries or arterioles. CO is the blood volume pumped by the heart per minute. The venous side of the circulation, because of its predominant role as capacitance vessels is the main determinant of CO. Given this relationship, we must expect that any increase in TPR, CO or both parameters simultaneously will elevate blood pressure levels.

In the initial stages of experimental hypertension (Ferrario et al., 1970; Smith et al., 1979) as well as in early hypertensive subjects (Schobel et al., 1993) an increase in CO is seen while TPR is within normal levels. However, in established HTN, the contribution made by CO is minimized while the effects of TPR on blood pressure are more prominent (Ferriss, 1978). Current antihypertensive approaches will lower blood pressure by either adjusting CO or TPR in an effort to correct any deregulation in the overall balance between CO and TPR necessary to maintain a normal blood pressure.

Animal models of experimental hypertension

As stated above, hypertension can be classified as either primary or secondary. In secondary hypertension, correcting the factor responsible for the increased pressure usually cures the disease. However, in primary or essential hypertension, it is usually unknown what the cause for increased pressures is suggesting that a genetic and an environmental component contribute to this type. Given the heterogeneous type of this condition, a wide range of animal models have been developed to help us in our understanding of both, secondary and essential forms of the disease.

A list of the most commonly used animal models for hypertension research is included below with a description of the major characteristics of each one. A special emphasis is placed on the DOCA-salt model as it is the model used for the studies described later. For a more detailed description, see Watson and DiPette (2003).

Genetic models of experimental hypertension

The most common genetic models currently available are the spontaneously hypertensive rat (SHR), the SHR stroke-prone (SHR-SP) strain and the Dahl salt-sensitive and salt-resistant rats. The aforementioned models share the spontaneous development of an elevated blood pressure.

The SHR was generated by selectively inbreeding the Wistar-Kyoto normotensive rat strain. Both, the SHR and SHR-SP models spontaneously develop hypertension in a manner that appears to be Na^+-independent. In

contrast, the Dahl salt-sensitive strain requires administration of increased dietary Na^+ for the development of HTN. The salt-resistant strain will only develop small elevations in blood pressure compared to the considerable elevations seen in the salt-sensitive strains.

Renal models of experimental hypertension

Kidneys play such an important role in water balance and, therefore, in blood pressure regulation (Haddy and Pamnani, 1985). Two classical models for the study of hypertension have been developed by constricting one or both renal arteries. These models are referred to as the two-kidney, one-clip (2K1C) and the one-kidney, one-clip (1K1C) hypertension models. An additional renal model worth mentioning is the renal mass reduction salt-sensitive model.

A constricting clip is placed on one renal artery but both kidneys are left intact in the 2K1C model. In 1K1C, unilateral nephrectomy is accompanied by a constricting renal arterial clip in the remaining kidney. These hypertension models are renin-dependent, in which blood pressure elevations are dependent on activation of the renin-angiotensin-aldosterone system.

The renal mass reduction model is accomplished by unilaterally nephrectomizing a kidney followed by surgical removal of about two-thirds of the remaining kidney. Considerable increases in blood pressure are obtained after placing these animals in excess salt diet. This model, along with the 2K1C and the 1K1C models represents a formidable tool for the study of mechanisms in hypertension secondary to renal etiology.

Neural models of experimental hypertension

The brain and other higher centers within the CNS play an important function in blood pressure regulation. There is evidence linking central neural control abnormalities to the pathogenesis of essential hypertension (Ferrario and Averill, 1991). Typical models in this group involve surgical manipulation of specific areas in the brain known to be involved in the regulation of blood pressure, such as the periventricular (AV3V) region. Peripheral sinoaortic deafferentation is another widely used model that, in this case, examines the role of the peripheral sympathetic nervous system (SNS) in the pathogenesis of essential hypertension.

Adrenal models of experimental hypertension

The most widely studied adrenal model is the DOCA-salt model. In this model, hypertension results by surgical uninephrectomization followed by administration of a mineralocorticoid (usually DOCA, hence the name) and excess salt. Blood pressure will significantly rise in a few weeks and if left untreated, the animal will lose weight and develop end-organ damage. This is a sodium-dependent, low renin hypertension model. Several mechanisms have been suggested to contribute to the pathogenesis of DOCA-salt hypertension. They will be reviewed in greater details in the next section.

DOCA-salt hypertension

Mechanism of action and etiology

As stated above, DOCA-salt is a model of experimental hypertension that closely resembles states of excess glucocortocoid/mineralocorticoid production. Mineralocorticoids, like DOCA, act at mineralocorticoid receptors present intracellularly. These receptors are present in high numbers in a number of mineralocorticoid-responsive organs, like the kidneys.

Mineralocorticoids are lipid-soluble hormones that will diffuse through the cell's plasma membrane and gain entrance to the cytoplasm. Once there, they will bind their specific receptors in the cytoplasm. This hormone-receptor complex translocates to the nucleus where it binds to specific response elements in DNA to activate the synthesis of messenger RNA (mRNA) that codes for specific proteins. mRNA will then move to the cytoplasm and binds to ribosomes, directing synthesis of specific proteins (Seeley et al., 1998).

In this way, mineralocorticoids directly affect ion transport by kidney epithelial cells. Their main role is to conserve Na^+ (Piano and Huether, 1998; Seeley et al., 1998). In particular, mineralocoricoids up-regulate expression of a Na^+/proton (H^+) exchanger and a Na^+ pump in the mucosal and serosal surface of the kidney epithelial cells, respectively . Activation of the Na^+/H^+ exchanger is through a non-genomic mechanism, though (Wehling et al., 1992). In this way, mineralocorticoids increase the rate of Na^+ reabsorption by the kidneys. An increased reabsorption of Na^+ will increase water reabsorption bringing an increased blood volume (Seeley et al., 1998).

Given these effects of mineralocorticoid treatment, it should be expected that Na^+ retention and the volume expansion that follows are likely the factors contributing to the increased blood pressures seen in DOCA-salt hypertension. However, this is a too simplistic model as it is known that in DOCA-salt hypertension, blood volumes are not necessarily different compared to control animals (Fink et al., 2000) and in studies where there have been detected differences in fluid volumes; it has been shown that their increases are not necessary for DOCA to maintain hypertension (Tajima et al., 1983). There are other factors implicated in the pathogenesis of DOCA-salt hypertension, these will be discussed in the next section.

Cardiovascular hemodynamics in DOCA-salt hypertension

There are contradictory views regarding the relative roles that CO and TPR play in the development of high pressure in DOCA-salt hypertension. Some studies in DOCA-salt animals showed that CO is significantly elevated without increases in TPR (Ferrario et al., 1988; Ueno et al., 1988) suggesting that DOCA-salt hypertension is due to an augmented cardiac pumping action. In addition, the increased CO is likely due to an expanded blood volume and an augmented venous return (Schenk and McNeill, 1992). Other studies have linked the elevation in blood pressure to an increase in both parameters, CO and TPR (Yamamoto et al., 1984). Differences could be attributed to the fact that the experiments by Ferrario et al. (1988) and Ueno et al. (1988), animals were not given salt; they were just supplemented with DOCA.

Effects of DOCA-salt hypertension on sympathetic nerve activity

The sympathetic nervous system (SNS) is important for the development of DOCA-salt hypertension. Evidence supporting this statement came from experiments showing that destruction of adrenergic neurons with 6-hydroxydopamine prevents or reverses development of DOCA-salt hypertension (Lamprecht et al., 1977). Direct evidence linking the sympathetic nervous system to DOCA-salt hypertension comes from studies showing that sympathetic nerve activity is elevated in DOCA-salt rats (de Champlain, 1990; Oparil, 1986). Abnormal catecholamine levels also result from DOCA-salt treatment. Particularly, there is a tendency for an increase in plasma catecholamines as evidenced by Bouvier and de Champlain (1986) and de Champlain et al. (1987). There was a correlation between blood pressure and catecholamine levels leading the authors to suggest that blood pressure elevations, in fact, could be linked to sympathetic nerve activity (de Champlain et al., 1987).

DOCA-salt hypertension and cardiovascular morphological changes

It is well established that DOCA-salt treatment cause significant changes in the cardiovascular system that contribute to the overall pathology of this model. The heart hypertrophies as an adjustment to the high pressures seen after DOCA-salt treatment (Tomanek and Barlow, 1990). It is generally believed that the heart enlarges as a compensatory mechanism to continue working as an

effective pump in face of the high pressure environment. Another common finding seen in DOCA-salt hypertensive animals, particularly rats, is the induction of structural changes in the vasculature, arteries in particular (Vial et al., 1982; Walker and Boyd, 1983). Vascular structural changes lead to lumen narrowing in these vessels. As a result, a decreased in the internal radius leads to an increased in total peripheral resistance, further contributing to the increased blood pressures.

Vascular reactivity in DOCA-salt hypertension

One of the features of DOCA-salt hypertension is the altered vascular reactivity that occurs to adrenergic agonists and other vasoconstrictors as well. Mesenteric arteries from DOCA-salt hypertensive rats (Suzuki et al., 1994) showed an enhanced adrenergic reactivity compared with arteries taken from normotensive rats. Similarly, other studies have shown an enhanced reactivity of DOCA-salt arteries compared to controls (Ekas and Lokhandwala, 1980; Longhurst et al., 1988; Meggs et al., 1988; Perry and Webb, 1988). The enhanced responsiveness seen in these studies was manifested as either an increase in potency and/or an increase in the maximal contraction elicited by adrenergic agonists. This phenomenon is of physiological relevance as it has been postulated that the enhanced arterial reactivity seen in DOCA-salt hypertension is at least, partly responsible for the increased total peripheral resistance observed in hypertension. Increases in arterial resistance could directly be responsible for the maintenance of elevated blood pressures.

However, it looks that the direction of the change (increased, decreased, no change) in vascular adrenergic reactivity to vasoconstrictor substances will vary depending on the experimental conditions or vascular bed studied as others have shown that sensitivity to adrenergic agonists is normal in caudal (Hermsmeyer et al., 1982) and mesenteric arteries (Luo et al., 2003) of DOCA-salt rats.

What is even more striking is that very little is known about vascular reactivity to NE of small capacitance veins despite the fact that changes in venoconstrictor tone could also have effects on circulatory hemodynamics. It has been demonstrated that an increased venous tone will result in blood pressure changes by virtue of increases in venous return and, therefore, CO. The elevated blood pressures seen in DOCA-salt hypertension could be a combination of both, abnormal arterial as well as venous reactivity to adrenergic stimulation.

It is important to remember, however, that vascular beds could vary in their specific reactivity to different contractile agonists, including catecholamines. A variety of factors could determine reactivity of vascular smooth muscle cells. Among these, mechanical factors acting on the vascular wall could influence reactivity in a variety of ways (Johansson, 1981). It is generally believed that tension production in muscle tissue is affected by muscle length. Therefore, active tension is primarily a function of the extent of overlap of the contractile apparatus (Seow, 2000). In other words, at given length, the degree of overlap between contractile fibers is optimal resulting in maximal contraction.

The physiological correlates and implications of these length-tension relationships on vascular reactivity of smooth muscle cells examined in vitro are

beyond the scope of this dissertation. However, it is known that with the variation in blood pressure, smooth muscle cells lining the arterial wall are constantly subjected to length changes. This could theoretically lead to changes in vessel reactivity. Therefore, reactivity changes are not always a receptor-dependent phenomenon.

The mouse in hypertension research: genetic advances

Reserachers have always been interested in creating tools that allow for control of a particular gene in order to study and understand its function. Lately, there has been a considerable progress in the development of techniques that permit the creation of genetically-modified animals. These latest developments have boosted biomedical research, including cardiovascular, in the direction of identifying the specific functions played by particular genes and to determine what processes may be regulated by them. In addition, these new developments have allowed scientists to study how a certain gene contributes to a determined pathophysiological state. For technical reasons, mice have generally been the most widely used animals for the development of these transgenic and/or knockout models. For detailed reviews, see Pray (2002), Smith (2000) and Zambrowicz and Sands (2003).

Transgenic technology

This experimental approach involves the injection of "foreign" genetic material into the nuclei of fertilized eggs. This genetic material will then

incorporate into the genome of the cell. These transformed eggs willl be inserted back into pregnant females and brought to term. Transgenic animals have foreign DNA introduced into their own genome. In this way, an animal is produced that expresses a particular gene of interest. A major disadvantage, though, is that it could not be predicted or control where in the genome the foreign genetic material was inserted. As the pattern of expression of a given gene could be determined by its location, this could result in mouse lines with varying phenotypes even though they carry the same transgene.

Knockout technology

Conventional knockouts

As the name suggests, a knockout mouse is one in which a specific gene has been replaced or "knocked out" with an inactive or mutated allele. By "knocking out" the expression of a gene, researchers are able to remove a particular gene of interest in order to define what effect the gene has in an organism and its probable role in disease. In cardiovascular research, this is a powerful tool in studying the particular role played by a gene in the development or maintenance of hypertension.

Even though conventional knockout technology has exciting applications in biomedical research as it allows researchers to better understand how a particular gene contributes to a certain disease or pathophysiological process, it also contains a number of limitations. First, because of developmental effects some knockout mice die even before the researcher has a chance to use them.

In addition, it can not be assumed that a particular gene will exhibit identical functions in both, mice and humans. Therefore, results obtained with knockout animals are only suggestive of particular phenomena that could happen in humans. Last, in conventional knockout technology both gene alleles are deleted from all cells. Sometimes, this is not desired

Conditional knockouts

Newer technologies have been developed that allowed for the refinement of conventional approaches to knockout animals. In conditional knockout mice the gene of interest is deleted from a particular organ, cell type or stage of development. This allows researchers to use this technique to knock out certain portions of genes at specific times when the gene of interest would be particularly important.

The most widely used method in the development of conditional knockouts is the so called Cre-loxP recombinase system. In general terms, Cre recombinase is the enzyme that will recognize two target sequences called loxP. This enzyme will cut out a gene that is in between these two target sequences. The beauty of this technique is that this enzyme is only expressed in certain cell types. Therefore, the targeted gene will only be knocked out out of those cells and only when the researcher wants them to be.

The mouse in hypertension research: challenges for the future

However, in order to take advantage of these genetically-altered animals, baseline cardiovascular data have to be developed in mice that will allow for comparisons with data taken from transgenic/knockout animals. In this respect, we could not assume that data already recollected in other species, like rats, will apply to the mouse as it is been shown that vascular reactivity to some agonists could differ between the two species (Douglas et al., 2000; Russell and Watts, 2000). Nevertheless, determination of the mouse cardiovascular phenotype is not easy due to its small size. This has required the adaptation, for use in the mouse, of surgical and technical methodologies used in the classical experimental animal models.

In 1996, Johns et al. reported the successful development of the 2K1C and DOCA-salt model in mice. Mice that underwent either the renal artery clip or DOCA-salt treatment, exhibited blood pressures around 140 mmHg, significantly higher than the pressures recorded in control mice. They recorded blood pressures indirectly (tail-cuff) as well as directly (intra-arterial catheter) and were able to obtain a close correlation between both sets of results. The contralateral kidney in the 2K1C mice and the remaining kidney of DOCA-salt mice were significantly larger in size than those of their respective controls providing additional evidence regarding the effectiveness of these treatments.

Not only mice are relatively resistant to the development of really high blood pressures, but additional cardiovascular parameters are different. It appears that plasma NE concentrations are 3 to 10 times higher in mice than is

in rats or humans (Janssen and Smits, 2002). Whether these differences are due to technical artifacts or due to real species differences is still not known. This is particularly a striking finding as basal renal sympathetic nerve activity is reduced in the mouse compared to the rat (Ling et al., 1998). It is known that renal nerves in the mouse are thinner than in the rat. Therefore, whether this reduced firing frequency in mice is a real phenomenon or due to the lower number of axons in a given preparation is not known (Janssen and Smits, 2002).

As we have seen, cardiovascular parameters in mice could be somehow different from those already known in rats. Although some considerable progress has been made already, still some more work have to be performed in order to fully characterize cardiovascular physiological parameters in mice that will enable for accurate hemodynamic predictions.

CHAPTER 2

Hypothesis and Specific Aims

Overall hypothesis

Blood pressure regulation is dependent on TPR and CO (Beevers et al., 2001). Historically, many studies have looked at the role of small arteries in blood pressure regulation as these are the main site of vascular resistance. However, it should be known that an increased CO also can contribute to increases in blood pressure. Capacitance function largely resides in systemic veins and venules. A reduction in capacitance of systemic veins will shift blood from peripheral vascular beds towards the thoracic cavity (Ricksten et al., 1981). In this way, augmented venous return leads to higher stroke volume and CO and contributes to blood pressure regulation as well. Because of their predominant role as resistance vessels, it has been well characterized the impact that changes in arterial adrenergic vascular reactivity has on blood pressure regulation. However, very little is known about vascular reactivity in veins. A few studies have compared sensitivity to adrenergic stimulation in arteries and veins and have found that veins are more sensitive to the contractile effects of adrenergic agonists (Luo et al., 2003). Similar studies have concluded that veins are more sensitive not only to exogenous but also to nerve-released catecholamines (Kreulen, 1986; Hottenstein and Kreulen, 1987; Luo et al., 2003).

I tested the hypothesis that...

"In murine mesenteric vessels, veins will have an enhanced reactivity to NE compared to mesenteric arteries. This enhanced reactivity of veins is due to differences in the α_1-AR reserve of veins, in the α_1-AR subtype-selective regulation of contractile responses in mesenteric vessels and/or the selective involvement of α_2–ARs in mesenteric constrictions to adrenergic agonists."

The specific aims were:

Specific aim 1: Are there differences in the acute reactivity and time-dependent desensitization to α-AR agonists between small arteries and veins of DOCA-salt and SHAM control mice? If so, is α-AR reserve a factor behind these differences?

Specific aim 2: Do the relative contributions of individual α_1-AR subtypes in mediating the vasoconstriction of mesenteric arteries and veins from SHAM control and DOCA-salt mice differ? Is a particular α_1-AR subtype involved in contractile responses of murine mesenteric arteries as opposed to veins and vice versa? Are there differences in protein expression for these α_1-AR subtypes? Do expression changes in DOCA-salt hypertension?

Specific aim 3: Do postjunctional α_2-ARs play a role in contractile responses to adrenergic agonists that could explain the differential reactivity seen in murine mesenteric arteries and veins? Does α_2-AR reactivity changes in DOCA-salt hypertension?

Specific aim 4: Do the same α_1-ARs mediate contractile responses to exogenous and endogenous catecholamines in mesenteric arteries? Are there changes in neurogenic mechanisms in DOCA-salt hypertension?

CHAPTER 3

Increased Reactivity of Murine Mesenteric Veins to Adrenergic Agonists: Functional Evidence Supporting Increased Alpha-1 Adrenoceptor Reserve in Veins Compared to Arteries

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INTRODUCTION

The SNS is an important contributor to hypertension and other CVD (de Champlain, 1990). The main effector of the SNS which plays an important role in the regulation of vascular tone is the catecholamine NE, and to a lesser extent Epi (McCulloch and McGrath, 1998). These vasoactive agents modulate vascular tone by directly acting upon specific receptor proteins present on vascular smooth muscle cells. α-ARs play a fundamental role in regulation of systemic arterial blood pressure and blood flow (Piascik and Pérez, 2001).

Blood pressure regulation is dependent on TPR and CO (Beevers et al., 2001). Hypertension can result from an increase in either CO or TPR. In established hypertension the usual hemodynamic abnormality is increased TPR (Schobel et al., 1993; Smith et al., 1979; Ferrario et al., 1970). Since small arteries are the main site of vascular resistance, many studies have compared the ability of NE to contract arteries from normotensive and hypertensive individuals. Data derived from these studies is conflicting. Some have shown an enhanced reactivity of arteries from DOCA-salt rats to adrenergic agonists compared to control. (Ekas et al., 1980; Longhurst et al., 1988; Meggs et al., 1988; Perry and Webb, 1988; Suzuki et al., 1994). Other studies showed that sensitivity to adrenergic agonists is normal in caudal (Hermsmeyer et al., 1982) and mesenteric arteries (Luo et al., 2003) of DOCA-salt rats.

Increased CO also can contribute to increases in blood pressure. The splanchnic bed is a major blood reservoir containing up to 30% of total blood

volume (Greenway, 1983). This capacitance function largely resides in systemic veins and venules. A reduction in capacitance of systemic veins will shift blood from peripheral vascular beds towards the thoracic cavity (Ricksten et al., 1981). In this way, augmented venous return leads to higher stroke volume and CO. Data from animal and human studies support a role for decreased venous capacitance in the development of hypertension as increased CO often occurs in the initial stages of experimental hypertension (Ferrario et al., 1970; Smith et al., 1979) as well as in the early stages of human hypertension (Schobel et al., 1993).

Mean circulatory filling pressure (MCFP) is the effective driving force for venous return to the heart. MCFP is elevated in renal hypertensive dogs, 2-kidney, 1-clip hypertensive rats, SHR and in the DOCA-salt hypertensive rats (Ferrario et al., 1970; Edmunds et al., 1989; Martin et al., 1998; Fink et al., 2000). MCFP is dependent on venoconstrictor tone and blood volume. Most studies done in animals and hypertensive humans have revealed that blood volume does not increase in hypertension (Schobel at al., 1993; Ferrario et al., 1970). Therefore, increased MCFP in hypertension development is primarily due to venoconstriction. Multiple factors determine venomotor tone but sympathetic-mediated vasoconstriction is the most important (Pang, 2001). However, little is known about venous reactivity to NE in hypertension.

We sought to test the hypothesis that increased venoconstriction in hypertension is due to enhanced reactivity to NE. We studied vascular reactivity in a murine model of DOCA-salt hypertension. In this salt-sensitive, low renin

experimental model, SNS activity has been found to play an important part (de Champlain, 1990) and venous capacitance has been shown to be decreased by the SNS as determined by changes in MCFP (Fink et al., 2000), making this hypertension model relevant for the studies performed here. Furthermore, α_1-AR antagonists are effective antihypertensive agents in DOCA-salt hypertension (Nabata et al., 1985).

We compared acute reactivity and time-dependent desensitization to α-AR agonists in small arteries and veins of DOCA-salt and SHAM control mice. We did these studies because if altered vascular responses to NE contribute to hypertension (a chronic condition), those responses should be either larger, or more sustained in vessels from hypertensive versus normotensive rats. We also examined potential mechanisms behind differences in vascular reactivity of arteries and veins.

MATERIALS AND METHODS

Animals: C57/BL male mice (25-30g) used in these experiments were obtained from Charles River Labs (Portage, MI). Upon arrival at the animal care facility, mice were maintained according to the standards approved by the Michigan State University All-University Committee on Animal Use and Care. Mice were individually housed in clear plastic cages with free access to standard pelleted chow (Harlan/Teklad 8640 Rodent Diet) and tap water. Mice were housed in temperature and humidity-controlled rooms with a 12 hours on/12 hours off light cycle. Animals were allowed a period of 2-3 days of acclimatization prior to entry into any experimental protocol.

DOCA-salt surgery: Mice were unilaterally nephrectomized under anesthesia provided by intraperitoneal injection of approximately 70 – 80 μL of a solution containing ketamine (100 mg/mL) and xylazine (20 mg/mL) in a 9:1 ratio, respectively. The skin over the left flank was shaved and a 1.5 cm incision was made through the skin and underlying muscle caudal to the rib cage. The left kidney was exteriorized and removed after ligation of the renal artery and vein with 4-0 silk sutures (Ethicon, Inc, Somerville, NJ). The muscle and skin layers were then closed separately with 4-0 silk sutures. A small area between the shoulder blades of the back was shaved and a 1 cm incision was made through which DOCA-salt pellets were implanted subcutaneously resulting in a dose of 150 mg/kg DOCA. All DOCA mice were given salt water containing 1% NaCl and 0.2% KCl. Normotensive SHAM mice were also unilaterally nephrectomized, received no DOCA pellet implantation, and were given tap water. Both groups

were placed on standard pelleted rodent chow. After recovery, the mice were housed under standard conditions for 4 weeks after which systolic BP was determined by the tail-cuff method.

In-vitro preparation of mesenteric vessels: Mice were anesthetized and the small intestine with its associated mesenteric vessels was removed and placed in oxygenated (95% oxygen, 5% carbon dioxide) Krebs' physiological saline solution of the following composition (mmol): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 11. A segment of the intestine with associated vessels was removed and pinned flat in a silicone elastomer-lined (Sylgard, Dow Corning) petri dish. A section of mesentery containing vessels close to the mesenteric border was cut out using fine scissors and forceps. The preparation was transferred to a smaller silicone elastomer-lined recording bath and pinned flat. Second or third-order mesenteric veins or arteries (100-200 μm diameter) were isolated for study by carefully clearing away the surrounding fat tissue. The recording bath containing the preparation was mounted on the stage of an inverted microscope (Olympus CK-2) and superfused with warm (37 °C) Krebs' solution at a flow rate of 7 mL/min. All preparations were allowed a 20 minute equilibration period during which the vessels relaxed to a stable resting diameter.

Kidney and cardiac ventricle weight: Kidneys and cardiac ventricles from SHAM control and DOCA-salt mice were excised, blotted dry and weighed. Tissue weight was normalized to body weight.

Video monitoring of vessel diameter: The output of a black and white video camera (Hitachi model KP-111) attached to the microscope was fed to a

PC Vision Plus frame-grabber board (Imaging Technology Inc, Woburn, MA) mounted in a personal computer. The video images were analyzed using computer software (Diamtrak, Adelaide, Australia). The digitized signal was converted to an analog output (DAC-02 board; Keithley Megabyte, Tauton, MA) and fed to a chart recorder (EZ Graph; Gould, Inc, Cleveland, OH) for a record of vessel diameter. Changes in vessel diameter as small as 1.8 μm could be resolved.

Concentration-response studies: All drugs were added in known concentrations to the superfusing Krebs' solution. Concentration-response curves were obtained after application of the adrenergic agonists NE (Sigma, St. Louis, MO) and PE (Sigma, St. Louis, MO). Each agonist concentration was applied for 3 minutes and there was a 20 minute interval between successive applications. A single concentration-response curve was obtained from each preparation.

Desensitization studies: Mesenteric vessels were taken from SHAM and DOCA-salt mice, isolated and prepared as described in the sections above. In this series of experiments, vasoconstriction of arteries and veins was examined using NE (veins: 10^{-6} M; arteries: 10^{-5} M) and PE (veins/arteries: 10^{-5} M) concentrations which elicited maximal constrictions in these vessels. The adrenergic agonist was continuously applied to the superfusing Krebs' solution and blood vessels were exposed to the adrenergic agonist for 30 minutes. The vasoconstrictor state of arteries and veins at different time points was examined.

Effect of PBZ on NE- or PE-elicited initial constriction and the vasoconstrictor reactivity of mesenteric arteries and veins upon a 30 minute incubation period with adrenergic agonists: The PBZ-pretreatment protocol was done according to previously published protocols (Watts et al., 1996). After the initial 20 minute equilibration period, tissues were incubated for 10 minutes with one concentration of PBZ (0.3, 3, 10, 30 nM) followed by a 30 minute incubation in 100 μM sodium thiosulfate ($Na_2S_2O_3$). Preparations were then washed for an additional 30 minutes with Krebs' physiological saline solution after which they were challenged with maximal concentrations of the adrenergic agonists NE (veins: 10^{-6} M; arteries: 10^{-5} M) and PE (veins/arteries: 10^{-5} M). The initial constriction and vasoconstrictor reactivity of arteries and veins throughout a 30 minute period was examined.

Data analysis: Constrictions of blood vessels to the different treatments are expressed as percentage constriction (percentage reduction from the resting diameter). Half maximal effective agonist concentration (EC_{50}) and maximum response (E_{max}) were calculated from a least-squares fit of individual agonist concentration response curves using the following logistic function from Origin 5.0 (Microcal Software, Inc, Northampton, MA):

$$Y = \{(E_{min} - E_{max})/[1 + (x/EC_{50})^n]\} + E_{max}$$

where E_{min} is the minimum response and was constrained to zero, n is the slope factor. All data is expressed as mean ± SEM. Statistical differences between groups was assessed by Student's two-tailed unpaired t-test. When more than two groups were compared, an analysis of variance (ANOVA) was used with

Student-Newman-Keuls multiple comparison as a post test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad InStat for Windows 95 (GraphPad Software, San Diego, CA).

RESULTS

General. Four weeks after the start of DOCA-salt treatment, systolic blood pressure in the DOCA-salt treated mice (n = 56) was significantly higher than systolic blood pressure in the SHAM (n = 47) control mice (123 ± 1 mmHg –vs- 101 ± 1 mmHg, $P<0.05$). In agreement with other studies documenting hypertrophy of kidneys and cardiac ventricles of DOCA-salt rats (Young et al.,1994) and mice (Peng et al., 2001), kidney and ventricular weight when normalized for body weight, were higher in the DOCA-salt treated group (10.0 ± 0.2 mg/g body weight –vs- 8.1 ± 0.1 mg/g body weight and 4.7 ± 0.1 mg/g body weight –vs- 4.0 ± 0.09 mg/g body weight, respectively, $P<0.05$). The inner diameter of mesenteric arteries from SHAM and DOCA-salt mice was 154 ± 8.1 μm and 166.2 ± 6.9 μm, respectively ($P>0.05$). The diameter of mesenteric veins from SHAM and DOCA-salt mice was 193.7 ± 3.2 μm and 169.8 ± 5.1 μm, respectively ($P<0.05$).

α_1-ARs mediate constrictions of arteries and veins. The adrenergic agonist NE produced a concentration-dependent constriction of mesenteric veins (10^{-10} – $3x10^{-6}$ M) and arteries (10^{-7} - $3x10^{-5}$ M) from DOCA-salt and SHAM control mice (Fig. 1A). Similarly, the selective α_1-AR agonist PE produced a concentration-dependent constriction of mesenteric veins (10^{-10} – $3x10^{-5}$ M) and arteries (10^{-7} – $3x10^{-5}$ M) in both treatment groups (Fig. 1B). NE and PE were both more potent in constricting mesenteric veins from SHAM and DOCA-salt mice as there was a leftward shift in the concentration-response curve obtained

in veins when compared to arteries (Fig.1A, Fig. 1B, Table 1). However, the magnitudes of responses of veins and arteries to various doses of NE and PE were similar between DOCA-salt and SHAM groups (Fig. 1A, Fig. 1B, Table 1).

The role of α_2-ARs in mediating vasoconstriction of arteries and veins from DOCA-salt and SHAM control mice was assessed. The α_2-AR clonidine (10^{-7} M – 10^{-5} M) and UK 14,304 (10^{-7} M – 10^{-5} M) did not elicit constrictions in mesenteric arteries or veins from SHAM and DOCA-salt mice.

Differential desensitization in arteries and veins. Our concentration-response studies showed that murine mesenteric veins were more sensitive than arteries to the constrictor effects of NE and PE. Mesenteric arteries exhibited similar maximal responses but higher concentrations were needed to achieve them. Given those differences, we decided to further examine the potential mechanisms behind the marked differences in reactivity between mesenteric arteries and veins to adrenergic stimulation. This next series of experiments explored whether arteries and veins desensitize in a similar way when exposed to maximum concentrations of NE and PE. The concentrations used in this series of experiments were those responsible for inducing a maximal response in arteries and veins according to our concentration-response studies (Fig. 1). NE produced an initial peak constriction in both arteries (10^{-5} M) and veins (10^{-6} M) from DOCA-salt and SHAM control mice (Fig. 2A, Fig. 2B). However, arteries exhibited a time-dependent desensitization as their diameter returned to the initial resting diameter during the 30 minute agonist application (Fig. 2B). This effect was more prominent in SHAM arteries compared to DOCA-salt arteries.

After 30 minutes of continuous agonist exposure, the diameter of SHAM arteries was about 10% of the initial peak constriction caused by NE while in DOCA-salt arteries the response declined to about 50% of the initial peak constriction (Fig. 3A). However, mesenteric veins maintained a tonic constriction despite continuous exposure to NE (Fig. 2A). After 30 minutes exposure, DOCA-salt and SHAM vein diameter was about 80% of the initial peak constriction elicited by NE (Fig. 3A). Continuous exposure of arteries and veins to the selective α_1-AR agonist PE (10^{-5} M) revealed a marked difference between mesenteric arteries and veins. Incubation with PE elicited a constriction in arteries and veins (Fig. 2C, Fig. 2D). PE responses in DOCA-salt and SHAM arteries (Fig. 3B) completely desensitized upon continuous exposure to PE. However, after 30 minute exposure to PE the diameter of DOCA-salt and SHAM veins was between 80-90% of the initial peak constriction (Fig. 3B).

α-AR alkylation studies with PBZ: effects on agonist-induced initial constriction. To further determine whether the increased reactivity to adrenergic agonists seen in veins was due to differences in adrenergic receptor concentrations, we incubated the vessels with the α-AR alkylating agent PBZ (0.3, 3, 10 and 30 nM) and compared the effects on the NE- or PE-elicited initial peak constriction. Incubation of SHAM veins with PBZ (0.3 nM) did not affect their initial constriction in response to NE (Fig. 4A). However, higher PBZ concentrations (3, 10 and 30 nM) produced a significant concentration-dependent reduction in the NE-elicited peak constriction (Fig. 4A). DOCA-salt mesenteric veins were more resistant to PBZ alkylating effects as the peak

contractile response to NE was significantly inhibited only at the highest (30 nM) PBZ concentration (Fig. 4A).

Preincubation of SHAM veins with PBZ (0.3 nM) did not affect the peak constriction elicited by PE compared to control responses (Fig. 4B). However, incubation with higher concentrations (3, 10 and 30 nM) of PBZ significantly inhibited peak constriction (Fig. 4B). A similar inhibition was seen in DOCA-salt veins (Fig. 4B) as PBZ (0.3 nM) did not affect the peak contractile response seen after PE application compared to veins not exposed to PBZ. However, incubation at the higher doses (3, 10 and 30 nM) significantly inhibited the peak contractile response (Fig. 4B).

Incubation of SHAM control and DOCA-salt mesenteric arteries with all PBZ concentrations completely inhibited their contractile response to NE (Fig. 4A). All PBZ concentrations blocked PE-induced constrictions of SHAM and DOCA-salt arteries (Fig. 4B).

Effects of α-AR alkylation with PBZ on desensitization. The ability of 30 minutes exposure to NE to desensitize mesenteric veins preincubated with different concentrations of the alkylating agent PBZ was assessed. As preincubation with any PBZ concentration completely inhibited contractile responses in arteries, these studies were not performed in arteries. PBZ (0.3 nM) pretreatment did not change reactivity of SHAM veins to NE applied for 30 minutes, as NE caused a sustained constriction (Fig. 5A). In contrast, veins pre-incubated with PBZ (3nM) were not able to maintain a contractile response throughout the 30 minute period when compared to non PBZ-treated veins (Fig.

5A). As 30nM PBZ markedly reduced the peak NE-induced constriction in SHAM veins (Fig. 4A), we could not assess desensitization in these tissues. DOCA-salt veins were more resistant to the PBZ inhibitory effect since only veins incubated with the highest PBZ concentration (30nM) failed to maintain contractility to NE applied for 30 minutes (Fig. 5B). As PE was much less efficacious than NE in stimulating constriction in the blood vessels studied, an analysis examining the effects of a 30 minute PE incubation time period on vasoconstriction could not be performed.

DISCUSSION

α_1-ARs mediate direct vasoconstriction of mesenteric arteries and veins. α_1-ARs mediate vasoconstriction as PE mimicked the constricting effects of NE. Furthermore, the α_2-AR agonists clonidine and UK 14,304, did not constrict any artery or vein. However, others have proposed a vasoconstrictive role for α_2-ARs in blood vessels (reviewed by Civantos Calzada and Aleixandre de Artinano, 2001). McCafferty et al. (1999) showed that in the pithed mouse, α_{2B}-ARs mediate pressor responses to α_1 and α_2-AR agonists. It is possible that pressor responses caused by α_{2B}-AR activation are not mediated by vasoconstriction in murine mesenteric vasculature. Alternatively, α_2-AR constriction mechanisms may be active in vivo but not in vitro.

Adrenergic vascular reactivity is not altered in DOCA-salt mice. Vascular reactivity of arteries and veins to α_1-AR stimulation is not altered in DOCA-salt compared to SHAM. Despite the difference in resting venous diameter between SHAM control and DOCA-salt veins, vascular reactivity was not altered. In agreement with our data, NE responses of subcutaneous veins taken from hypertensive patients were unchanged compared to control subjects (Lind et al., 1997). However, studies done in DOCA-salt rats showed that mesenteric arterial adrenergic reactivity is enhanced compared to SHAM rats (Suzuki et al., 1994; Longhurst et al., 1988; Perry and Webb, 1988; Ekas and Lokhandwala, 1980). This discrepancy could be due to the differences in size of the vessels studied or the different methods used to assess vascular reactivity. Suzuki et al. (1994), Longhurst et al. (1988) and Ekas and Lokhandwala (1980)

measured perfusion pressure changes of the main branches of the superior mesenteric artery. Perry and Webb (1988) measured isometric force development of large mesenteric arterial strips. We assessed vascular reactivity by measuring diameter changes in unpressurized small mesenteric arteries (< 200 μm diameter). In addition, there may be different physiological processes regulating adrenergic constriction in mice and rats as vascular mechanisms can differ between the two species (Douglas et al., 2000). Our studies agree with those in caudal arteries (Hermsmeyer et al., 1982) and mesenteric arteries (Luo et al., 2003) which show that the reactivity to adrenergic agonists does not change in DOCA-salt rats.

Veins are more sensitive to the vasoconstrictive effects of NE and PE. We showed that veins are more sensitive than arteries to adrenergic stimulation. It could be argued that increased venous reactivity is due the fact that these experiments were carried out in unpressurized vessels and arteries and veins have different flow-pressure characteristics. However, previous studies have demonstrated that the increased sensitivity of mesenteric veins compared to arteries to either adrenergic agonists (Naito et al., 1998) or to sympathetic nerve stimulation (Hottenstein and Kreulen, 1987) is maintained when arteries and veins were pressurized to physiological levels. Therefore, increased venous adrenergic reactivity compared to arteries is not a function of vessel pressure.

Given this increased sensitivity of veins to adrenergic agonists, we tested the hypothesis that the increased adrenergic reactivity of veins is due to a larger α_1-AR concentration. The α-AR alkylating agent PBZ was used to assess

receptor reserve in arteries and veins. The initial NE-elicited constriction was reduced by low concentrations of PBZ in SHAM veins but only by the highest PBZ concentration in DOCA-salt veins. All PBZ doses completely inhibited NE responses in arteries. These data suggest that there is a larger α_1-AR reserve in DOCA-salt compared to SHAM veins. These data also suggest that murine mesenteric veins express more α_1-ARs than arteries.

Veins are resistant to desensitization. An increased α-AR population in veins led us to predict that veins would be more resistant to desensitization than arteries. Arteries exhibited a time-dependent desensitization by NE that was more prominent in vessels taken from SHAM mice. In response to continuous exposure to PE, arteries from SHAM and DOCA-salt mice desensitized completely. Desensitization in arteries was more prominent when the vessels were exposed to PE than when exposed to NE suggesting that α_2-mediated constriction elicited by NE could offset desensitization of α_1-ARs. However, α_2-ARs do not play a direct vasoconstrictive role in the small arteries and veins studied here (see above). Upregulation of α_1-ARs in DOCA-salt mesenteric arteries could explain why there was not a complete desensitization of these vessels in response to continuous exposure to NE. Upregulation of α_1-ARs occurs in mesenteric arteries of DOCA-salt rats (Meggs et al., 1988). Given this, DOCA-salt arteries should be more resistant to α_1-AR desensitization than SHAM arteries upon exposure to PE. That was not found as both groups of arteries completely desensitized.

Increased post-receptor activation events in DOCA-salt arteries could account for the relative resistance to desensitization seen in those vessels. Phosphatidylinositol activity was found to be greater in mesenteric (Takata et al., 1989) and femoral arteries of DOCA-salt rats with no apparent change in receptor number or binding affinity (Eid and de Champlain, 1988). On the other hand, mesenteric veins maintained a tonic constriction upon continuous exposure to both NE and PE suggesting that mesenteric veins have an increased α_1-AR reserve compared to arteries.

α_1-AR subtypes have different susceptibilities to desensitization induced by sustained NE stimulation. In HEK cells stimulated continuously with NE, Zhang et al. (1997) showed that the α_{1A} subtype easily desensitized. Desensitization of the α_{1D} subtype was delayed with α_{1B} desensitization being intermediate. Other studies (Chalotorn et al., 2002) have shown that continuous exposure to PE in transiently transfected HEK 293 cells increased internalization of α_{1A} and α_{1B} but not α_{1D}-ARs. Internalization was faster for the α_{1B} subtype. As there are differences in desensitization and internalization properties of α_1-ARs, it will be important to identify the subtype expression in murine mesenteric vessels and to determine if expression changes in DOCA-salt hypertension.

PBZ-pretreated veins are susceptible to desensitization. Our studies suggest that there is an increased α_1-AR concentration in veins compared to arteries. The increased receptor concentration could account for the relative resistance of veins to desensitization. We hypothesized that decreasing the α_1-AR reserve in veins would render them more susceptible to desensitization by

adrenergic agonists. PBZ-treated veins showed a partial desensitization to NE exposure similar to that seen in arteries. PBZ-treated SHAM veins were more susceptible to desensitization compared to DOCA-salt veins, which only desensitized after treatment with the highest PBZ concentration. These results suggest that veins have an increased α-AR population compared to arteries and that there is an upregulation in DOCA-salt veins compared to SHAM veins.

Responses to PE in PBZ-treated vessels were also inhibited in SHAM and DOCA-salt veins. However, the inhibition seen in these vessels was greater than that seen in PBZ-treated veins subsequently challenged with NE. Inhibition of PE responses between SHAM and DOCA-salt veins upon PBZ pretreatment did not differ. However, responses to PE were completely abolished in mesenteric arteries previously treated with any PBZ concentration. PE may be less efficacious than NE in stimulating constrictions in mesenteric vessels and this could explain the greater sensitivity to PBZ in veins challenged with PE.

α_1-ARs activate a variety of second messenger pathways (Pérez et al., 1993). There could be a larger α_1-AR reserve for one signaling pathway over the other and there could be preferential activation of one of these pathways in veins as opposed to arteries. This concept of a larger receptor reserve in one signaling pathway over the other has been shown for the 5-HT_{2A} receptor (Kurrasch-Orbaugh et al., 2003)

It could also be that different α_1-AR subtypes are involved in mediating constriction in arteries and veins and they could differ in their sensitivity to PBZ. Studies done in α_{1B}-adrenoceptor knockout mice concluded that α_{1A} as well as

α_{1D} adrenoceptors are involved in vasoconstriction with a minor role for α_{1B} adrenoceptors (Daly et al., 2002). Yamamoto and Koike (2001) also concluded that α_{1D}-like receptors are present in the mouse mesenteric artery. Whether these receptors play a predominant role in constrictions of murine mesenteric veins is not yet known.

Conclusion. Murine mesenteric veins are more sensitive than arteries to the constricting effects of NE and PE and reactivity is not altered in DOCA-salt hypertension. Studies with PBZ indicate that murine mesenteric veins express more α_1-ARs than arteries. This would account for the greater venous reactivity to NE and resistance to desensitization compared to mesenteric arteries. Our data also indicate that there is an up-regulation of α_1-ARs in DOCA-salt veins. These results support the importance of adrenergic regulation of venomotor tone in the long-term control of arterial blood pressure.

Table 1. Response of mesenteric arteries and veins from SHAM control and DOCA-salt mice to the adrenergic agonists PE and NE. Data are expressed as mean ± SEM. Numbers in parentheses refer to the number of animals from which the data were obtained. E_{max} is the maximum constriction based on data fitted to a logistic equation. EC_{50} is the negative logarithm of the molar concentration of agonist producing half maximal constriction. [a] Significantly different compared to respective artery EC_{50}.

	E_{max} (%)		EC_{50} (-log M)	
	VEIN	**ARTERY**	**VEIN**	**ARTERY**
NE				
SHAM	40.6±7.5 (4)	39.3±11.7 (5)	7.2±0.2[a] (4)	5.7±0.1 (5)
DOCA-salt	48.8±6.0 (5)	31.9±3.7 (4)	6.8±0.2[a] (5)	5.5±0.01 (4)
PE				
SHAM	30.5±2.8 (4)	32.9±5.0 (5)	7.1±0.2[a] (4)	5.3±0.1(5)
DOCA-salt	31.0±3.7 (4)	34.2±3.7 (5)	6.8±0.3[a] (4)	5.2±0.05 (5)

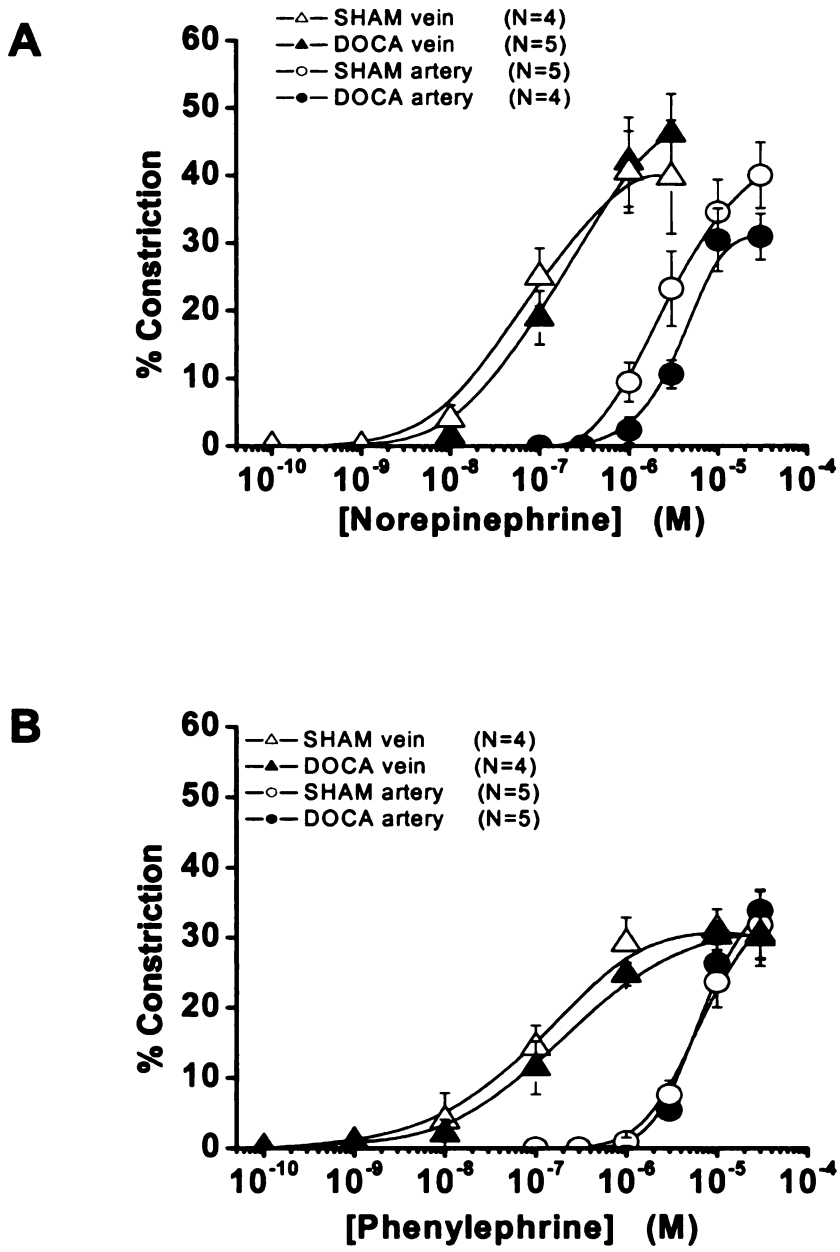

Figure 1. Concentration-response curves for the adrenergic agonists (A) norepinephrine and (B) phenylephrine obtained in mesenteric arteries and veins from SHAM control and DOCA-salt mice. Veins were more sensitive to the contractile effects of the agonists. Vascular reactivity was not altered in DOCA-salt vessels compared to their SHAM controls. Data are mean ± SEM. N indicates the number of animals from which preparations were obtained.

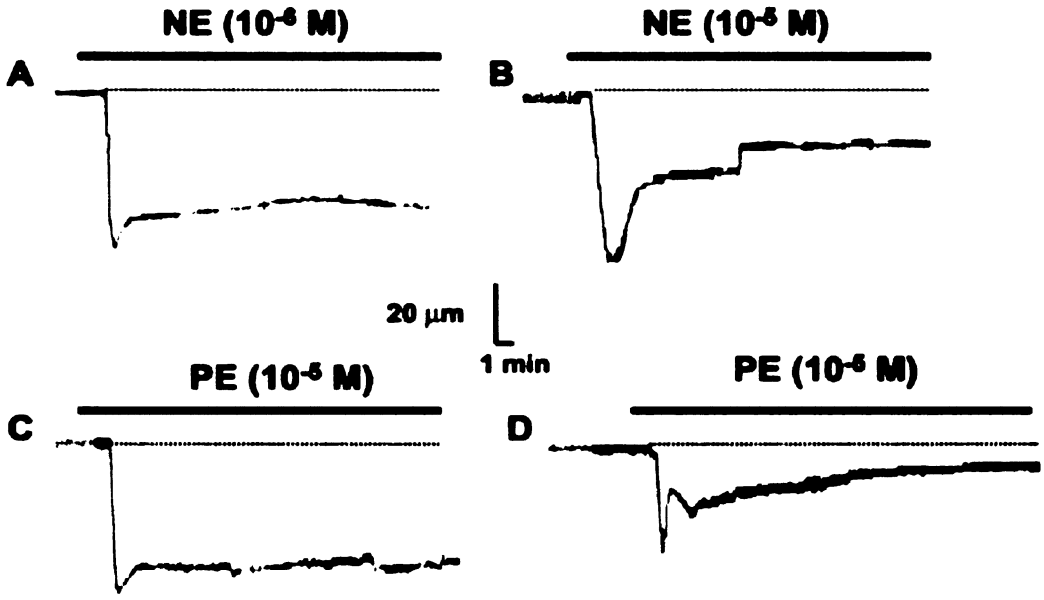

Figure 2. Representative traces showing maintained constrictions in a vein (A, C) but not an artery (B, D) when exposed to maximum concentrations of NE or PE. Agonists were applied at the indicated concentration during the period indicated by the bar above each trace. The first 15 minutes of incubation are shown.

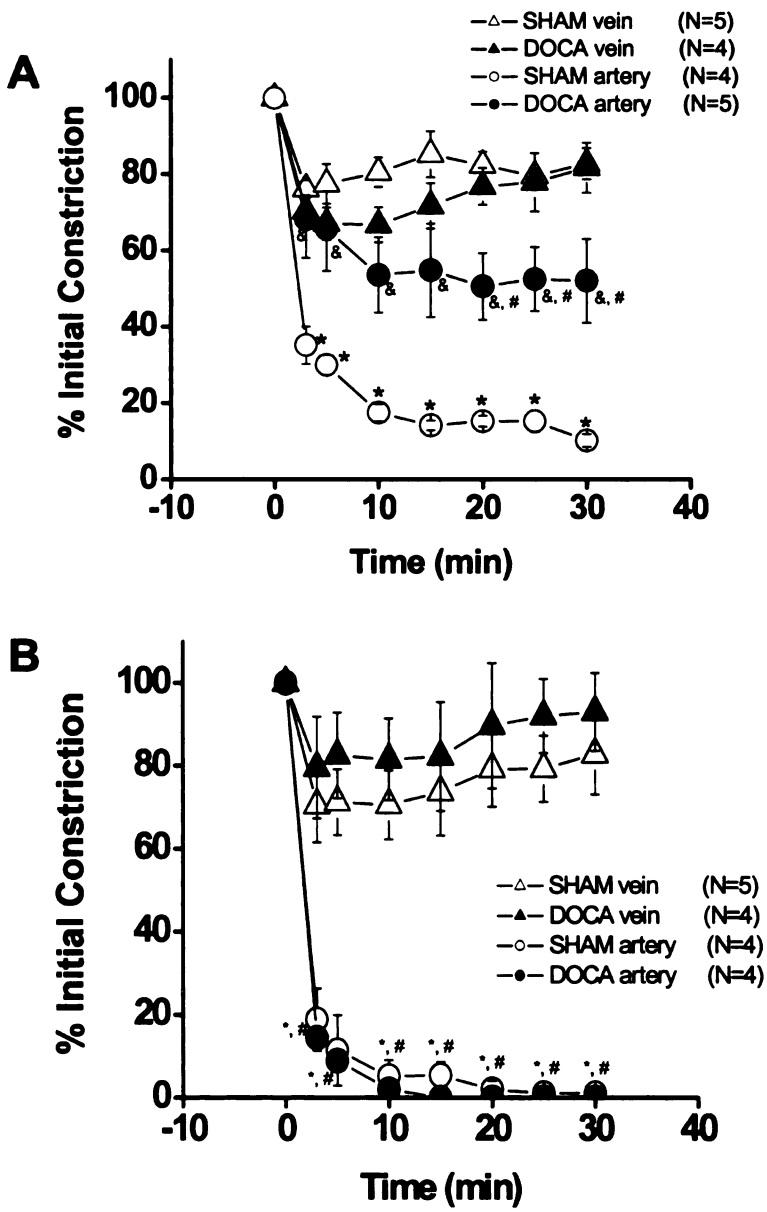

Figure 3. Mesenteric arteries but not veins desensitize during a 30 minute incubation period with the adrenergic agonists NE (A) and PE (B). Blood vessels were exposed for 30 minutes to near maximum agonist concentration. Veins maintained a tonic constriction upon challenge with NE and PE. This tonic constriction was not different between SHAM control and DOCA-salt veins. Arteries showed a time-dependent desensitization to NE that was more prominent in the SHAM arteries. PE completely desensitized SHAM and DOCA-salt arteries. Data are mean ± SEM. N indicates the number of animals from which the preparations were obtained.*: $P<0.05$ SHAM artery -vs- SHAM vein, #: $P<0.05$ DOCA artery -vs- DOCA vein, &: $P<0.05$ DOCA artery -vs- SHAM artery.

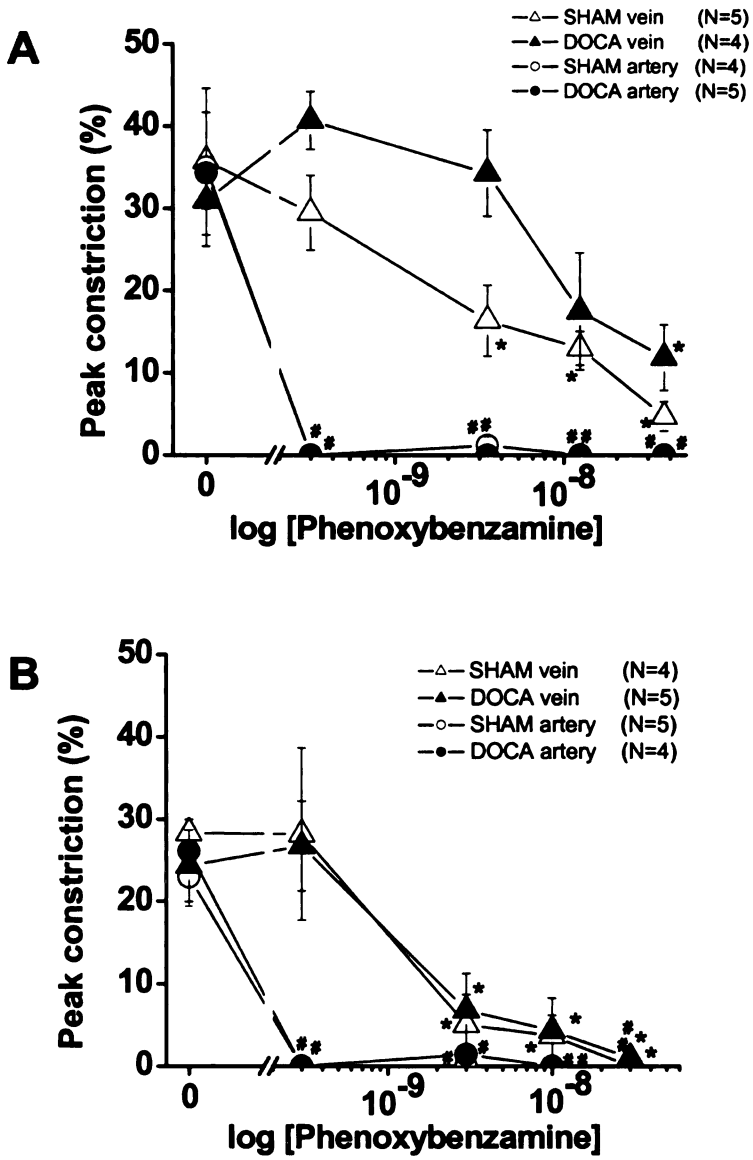

Figure 4. Effect of PBZ on NE- (A) and PE-induced (B) initial constriction in SHAM control and DOCA-salt arteries and veins. Blood vessels were incubated for 10 minutes with PBZ (0.3 – 30 nM) prior to challenge with NE or PE. PBZ (0.3 – 30 nM) pretreatment completely abolished NE- and PE-elicited constrictions of mesenteric arteries from SHAM as well as DOCA-salt mice. PBZ (3 - 30 nM) significantly reduced constrictions of SHAM veins while only PBZ (30 nM) significantly reduced the initial response in DOCA-salt veins. PBZ (3 - 30 nM) pretreatment significantly inhibited PE-induced constrictions of SHAM and DOCA-salt veins. Data are mean ± SEM from N mice. *, #: $P<0.05$ -vs- No PBZ.

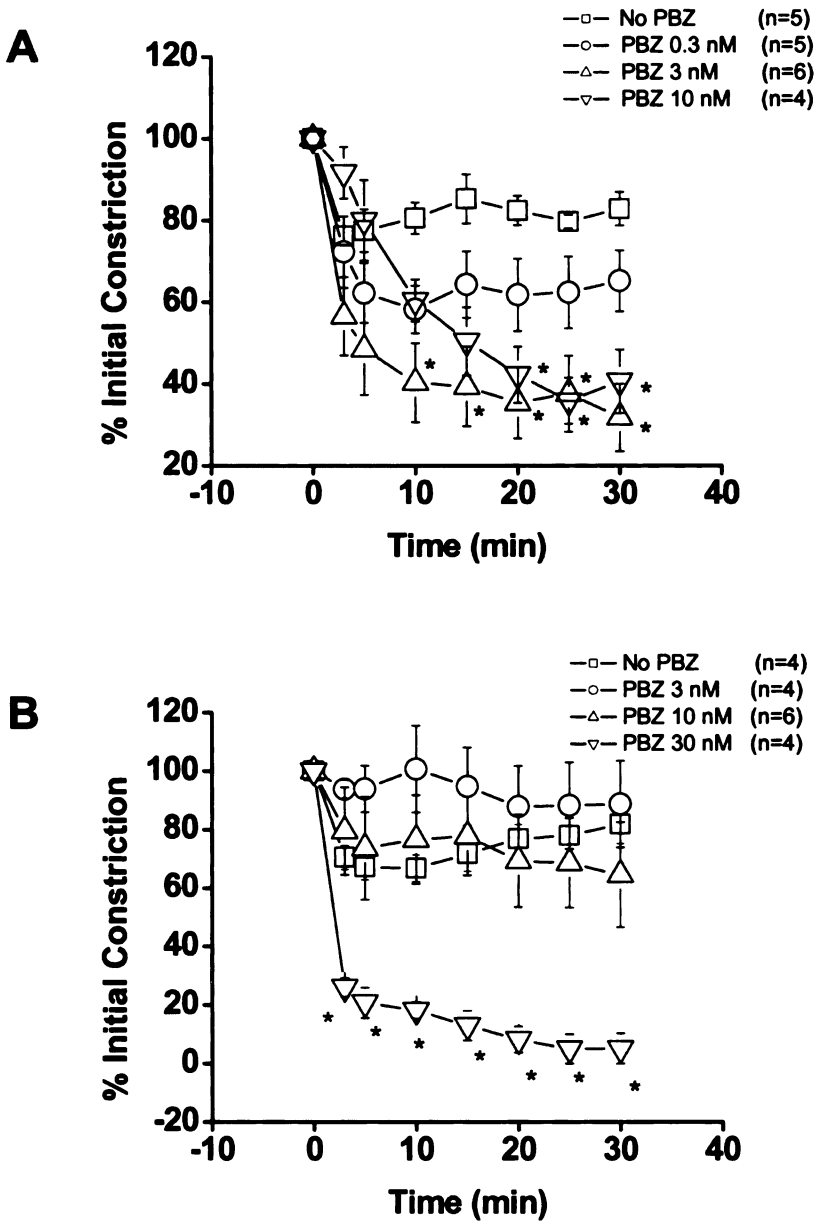

Figure 5. Effect of the alkylating agent PBZ on the time course of NE-induced desensitization of SHAM control (A) and DOCA-salt (B) veins upon a 30 minute exposure period. Blood vessels were incubated for 10 minutes with PBZ (0.3 – 30 nM) prior to challenge with NE (10^{-6} M). SHAM veins significantly desensitized when exposed for 30 minutes to NE when pretreated with PBZ (3 - 30 nM). DOCA-salt veins desensitized significantly only when pretreated with the highest PBZ (30 nM) concentration. Data are mean ± SEM from N number of mice. *: P<0.05 -vs- No PBZ.

CHAPTER 4

Alpha-1 Adrenergic Receptor Function and Protein Expression in Arteries and Veins from Normal and Hypertensive Mice

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INTRODUCTION

α_1-ARs are a subset of membrane proteins that mediate the actions of the neurotransmitters norepinephrine (NE) and epinephrine. In blood vessels, α_1-ARs are important mediators of smooth muscle contraction. Three genes encode distinct α_1-AR subtypes (Lomasney et al., 1991; Schwinn et al., 1990). Based on that evidence it was proposed that these subtypes be named α_{1A}-, α_{1B}-, and α_{1D}-ARs (Hieble et al., 1995). It is been speculated that α_1-AR subtypes could perform different functions in tissues as they differ in cellular distribution (Chalotorn et al., 2002; Hrometz et al., 1999; McCune et al., 2000), coupling to G-proteins (Theroux et al., 1996) and internalization and desensitization characteristics (Chalotorn et al., 2002).

Previous studies have shown that α_1-ARs are expressed throughout the vasculature (Hrometz et al., 1999; Piascik et al., 1997). Because these receptors are expressed ubiquitously in peripheral arteries, they all could participate in the contractile effects of catecholamines in these vessels. However, data obtained so far have provided evidence that the particular α_1-AR subtype mediating contractions varies according to the vascular bed studied. For example, the α_{1A}-AR mediates contractile responses of rat renal (Hrometz et al., 1999) and caudal arteries (Piascik et al., 1997) and murine tail and mesenteric arteries (Daly et al., 2002). The α_{1D}-AR mediates contractile responses in rat femoral (Hrometz et al., 1999; Piascik et al., 1997), iliac, superior mesenteric artery, and aorta (Piascik et al., 1997). Contractile responses in murine aorta are predominantly α_{1D}-mediated (Daly et al., 2002; Chalotorn et al., 2003). For the most part, the α_{1B}-

AR is not involved in vascular smooth muscle contraction (Chalotorn et al., 2003). However, a few studies have suggested a role for this adrenoceptor subtype in rat mesenteric artery (Piascik et al., 1997) with just a minor involvement in murine vessels (Daly et al., 2002).

In contrast to arteries, few studies have attempted to examine in a comprehensive way the role that α_1-AR subtypes play in contractile responses of veins. It is known that the α_{1D}-AR is the main adrenoceptor subtype mediating contractile responses in canine mesenteric veins (Daniel et al., 1997) whereas the α_{1B}-AR is the main functional adrenoceptor subtype in rat vena cava (Sayet et al., 1993). In the human saphenous vein, both, α_{1A}- and α_{1B}-AR subtypes are the main functional receptor subtypes (Yan et al., 2001). Although not widely appreciated, the venous side of the circulation is important for blood pressure control. Increases in arterial resistance or cardiac output (CO) can cause elevations in blood pressure. A reduction in capacitance of systemic veins will shift blood from peripheral vascular beds toward the thoracic cavity (Ricksten et al., 1981). In this way, augmented venous return leads to higher stroke volume and CO. Data from animal studies support a role for decreased venous capacitance in the development of hypertension, as increased CO often occurs in the initial stages of experimental hypertension (Ferrario et al., 1970). Therefore, identifying the α_1-AR subtype(s) mediating venoconstriction will help to understand the contribution of veins to the hemodynamic changes that occur in hypertension.

As seen, particular α_1-AR subtypes could make variable contributions to the regulation of specific vascular beds. However, whether their relative contribution changes under conditions of high blood pressure is not established. Moreover, relatively little is known about α_1-AR subtype protein expression in hypertension, particularly in veins. A few studies have detailed the expression profile of α_1-AR in pulmonary (Xiao et al., 2004), portal (Zhu et al., 2000) and genetic systemic hypertension (Jackson and Insel, 1993) but α_1-AR subtype expression changes have not been investigated in DOCA-salt hypertension. In this salt-sensitive, low-renin experimental model, sympathetic nerve activity has been found to play an important role (de Champlain, 1990). Furthermore, α_1-AR blockade can prevent hypertension in DOCA and salt-treated rats (Sanchez et al., 1989) making this model of particular relevance.

Our aim was to determine the relative contribution of individual α_1-AR subtypes in mediating agonist-induced vasoconstriction of mesenteric arteries and veins from SHAM and DOCA-salt mice. These small arteries and veins are important players in blood pressure regulation as they are the major determinants of total peripheral resistance and vascular capacitance, respectively. We also compared α_1-AR subtype protein expression and looked at potential differences between normotensive and DOCA-salt hypertensive vessels.

MATERIALS AND METHODS

Animals. C57/BL male mice (25 - 30g) were obtained from Charles River Labs (Portage, MI). Upon arrival at the animal care facility, mice were maintained according to the standards approved by the Michigan State University All-University Committee on Animal Care and Use. Mice were individually housed in clear plastic cages with free access to standard pelleted chow (Harlan/Teklad 8640 Rodent Diet) and tap water. Mice were housed in temperature and humidity-controlled rooms with a 12 hours on/12 hours off light cycle. Animals were allowed a period of 2-3 days of acclimatization prior to entry into any experimental protocol.

DOCA-salt surgery. Mice were unilaterally nephrectomized under anesthesia using a solution containing ketamine (500 mg/ml) and xylazine (20 mg/ml) in a 9:1 ratio, respectively. Animals (25–30g) received about an 80 μL volume of the anesthetic solution. The skin over the left flank was shaved and a 1.5 cm incision was made through the skin and underlying muscle caudal to the rib cage. The left kidney was exteriorized and removed after ligation of the renal artery and vein with 4-0 silk sutures (Ethicon, Inc; Somerville, NJ). The muscle and skin layers were then closed separately with 4-0 silk sutures. A small area between the shoulder blades was shaved and a 1 cm incision was made through which DOCA-salt pellets were implanted s.c. to provide a dose of 150 mg/kg DOCA. DOCA mice were given tap water containing 1.5% NaCl and 0.2% KCl. SHAM mice were also unilaterally nephrectomized, received no DOCA pellet implantation and were given tap water. Both groups of mice were placed on

standard pelleted rodent chow. After recovery, the mice were housed under standard conditions for 4 weeks after which systolic BP was determined by the tail-cuff method.

In-vitro preparation of mesenteric vessels. Mice were euthanized with a lethal dose of pentobarbital (50 mg/kg i.p.). The small intestine with its associated mesenteric vessels was removed and placed in oxygenated (95% O_2, 5% CO_2) Krebs' physiological saline solution of the following composition (mmol): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 11. A segment of the intestine with the associated vessels was removed and pinned flat in a silicone elastomer-lined (Dow Corning; Midland, MI) Petri dish. A section of mesentery containing vessels close to the mesenteric border was cut out using fine scissors and forceps. The preparation was transferred to a smaller silicone elastomer-lined recording bath and pinned flat. Second or third-order mesenteric arteries or veins were isolated for study by carefully clearing away the surrounding fat tissue. The recording bath containing the preparation was mounted on the stage of an inverted microscope (Olympus CK-2) and superfused with warm (37°C) Krebs' solution at a flow rate of 7 ml/min. All preparations were allowed a 20 min equilibration period during which the vessels relaxed to a stable resting diameter.

Video monitoring of vessel diameter. The output of a black and white video camera (Hitachi model KP-111) attached to the microscope was fed to a PC Vision Plus frame-grabber board (Imaging Technology Inc; Woburn, MA)

mounted in a personal computer. The video images were analyzed using Diamtrak software version 3.5 (http://www.diamtrak.com; Adelaide, Australia).

Concentration-response studies. All drugs were added in known concentrations to the superfusing Krebs' solution. Control concentration-response curves were obtained in arteries (0.1 – 30 μM) and veins (0.001 – 30 μM) after application of the selective α_1-AR agonist phenylephrine (PE; Sigma-Aldrich; St. Louis, MO). Each agonist concentration was applied for 3 min and there was a 20-minute interval between successive applications. The contribution of α_{1A}-, α_{1B}-, and α_{1D}-ARs to PE-induced contractile responses was studied by comparing concentration-response curves in the absence and in the presence of the selective α_{1A}-AR antagonist 5-MU (10, 100 nM, Sigma Aldrich; St. Louis, MO), the selective α_{1B}-AR antagonist L-765,314 (100 nM, 1 μM, Sigma Aldrich; St. Louis, MO), and the selective α_{1D}-AR antagonist BMY-7378 (100, 300 nM, Sigma Aldrich; St. Louis, MO).

In a second set of studies, contractile responses to PE were examined in the presence of simultaneous application of 5-MU (100 nM) and L-765,314 (1 μM) to block α_{1A} and α_{1B}-ARs in arteries and in the combined presence of L-765,314 (1 μM) and BMY-7378 (300 nM) to block α_{1B} and α_{1D}-ARs in mesenteric veins. In all experimental protocols, preparations were preincubated with the antagonists for 20 minutes prior to application of the agonist and were continuously exposed to the antagonist throughout the experiment. A single concentration-response curve was obtained for each preparation either in the absence or presence of specific receptor antagonists.

Western blot analysis

Protein isolation. Mesenteric arteries and veins from SHAM and DOCA-salt mice were removed and cleaned of surrounding mesentery and fat. Tissues taken from five different mice were pooled and frozen in liquid nitrogen, pulverized in a liquid nitrogen–cooled mortar and pestle, and solubilized in lysis buffer (0.5 mol/L Tris HCl [pH 6.8], 10% SDS, and 10% glycerol) with protease inhibitors (0.5 mmol/L phenylmethylsulfonyl fluoride, 10 µg/µL aprotinin, and 10 µg/mL leupeptin). Homogenates were centrifuged (11000*g* for 10 minutes, 4°C), and supernatant total protein was measured (BCA, Sigma-Aldrich; St. Louis, MO).

Immunoblotting protocol. Supernatant (4:1 in denaturing loading buffer, boiled for 5 minutes) was loaded (35 µg protein), separated on 10% denaturing SDS-polyacrylamide gels, and transferred to Immobilon-P membranes (Millipore). Membranes were blocked for 4 to 6 hours in Tris-buffered saline plus Tween 20 (0.1%) containing 5% milk and 0.025% sodium azide. Goat anti α_{1A}-AR antibody (1:500, Santa Cruz Biotechnology; Santa Cruz, CA), goat anti α_{1B}-AR antibody (1:500, Santa Cruz Biotechnology; Santa Cruz, CA) and rabbit anti α_{1D}-AR antibody (1:500, Santa Cruz Biotechnology; Santa Cruz, CA) were incubated with blots overnight (4°C). After washes, secondary antibody (1:2000) linked to horseradish peroxidase: donkey anti-goat IgG-HRP (Sigma-Aldrich; St. Louis, MO) or anti-rabbit HRP-linked IgG (Cell Signaling; Beverly, MA) was added for 1 hour and incubated with blots at 4°C. Enhanced chemiluminescence was

performed by using standard reagents (Amersham Biosciences; Piscataway, NJ). Blots for the different α_1- AR subtypes appear at approximately 50 kDa. Each blot was washed and redeveloped by using a mouse α-smooth muscle actin primary antibody (1:1000, Oncogene Research Products; La Jolla, CA) followed by incubation for one hour with an anti-mouse IgG HRP-linked secondary antibody.

Data analysis. Constrictions of blood vessels caused by different treatments are expressed as percentage constriction (percentage reduction from the resting diameter). Half maximal effective agonist concentration (EC_{50}) and maximum response (E_{max}) were calculated from a least-squares fit of individual agonist concentration response curves using the following logistic function from Origin 7.0 (Microcal Software, Inc; Northampton, MA):

$$Y = \{(E_{min} - E_{max})/[1 + (x/EC_{50})^n]\} + E_{max}$$

where E_{min} is the minimum response (set at 0), n is the slope factor. Data are expressed as mean ± standard error of the mean (SEM). Statistical differences between groups were assessed by Student's two-tailed unpaired t-test. When more than two groups were compared, analysis of variance (ANOVA) was used with Student-Newman-Keuls multiple comparison as a post test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad InStat (GraphPad Software; San Diego, CA).

RESULTS

General. Four weeks after the start of DOCA-salt treatment, systolic blood pressure in DOCA-salt (n=59) mice was higher than in SHAM (n=53) mice (139 ± 2 mmHg –vs- 105 ± 3 mmHg, respectively; $p < 0.05$). The resting diameter of mesenteric arteries from SHAM and DOCA-salt mice was 149 ± 4 μm and 158 ± 4 μm, respectively ($p > 0.05$). The resting diameter of mesenteric veins from SHAM and DOCA-salt mice was 165 ± 4 μm and 179.8 ± 6 μm, respectively ($p > 0.05$).

α_{1A}-ARs mediate constriction of mesenteric arteries but not veins. We examined concentration-response curves in the absence and presence of 5-MU (10, 100 nM), a selective α_{1A}-AR antagonist, in an effort to assess the role of this subtype in PE-mediated constrictions of arteries and veins. Arteries exhibited a concentration-dependent constriction to PE. 5-MU did not change resting diameter of arteries or veins. In SHAM arteries, 5-MU (10, 100 nM) produced a significant rightward shift of the PE concentration-response curves (Figure 1A, Table 1). PE-induced constrictions were also antagonized by 5-MU (10, 100 nM) in DOCA-salt arteries (Figure 1B, Table 2).

PE constricted veins in a concentration-dependent manner. 5-MU (10, 100 nM) did not alter resting diameter and it did not affect PE-induced constriction of SHAM (Figure 2A, Table 1) or DOCA-salt (Figure 2B, Table 2) veins.

α_{1B}-ARs play a minor role in contractile responses of mesenteric arteries and veins. The selective α_{1B}-AR antagonist L-765,314 (100 nM, 1 μM) was used as a pharmacological tool to assess the role of the α_{1B}-AR subtype in

PE-induced contractile responses of arteries and veins. In SHAM arteries, the α_{1B}-AR antagonist (100 nM) did not change PE contractile responses (Figure 3A, Table 1). There was a tendency for a leftward shift, although not significant, in DOCA-salt arteries preincubated with L-765,314 (100 nM; Figure 3B, Table 2). Preincubation of both SHAM (Figure 3A, Table 1) and DOCA-salt (Figure 3B, Table 2) arteries with L-765,314 (1 μM) caused a rightward shift in the concentration-response curves. L-765,314 (100 nM) did not affect contractile responses to PE in SHAM (Figure 4A, Table 1) and DOCA-salt (Figure 4B, Table 2) veins. However, preincubation with L-765,314 (1 μM) caused rightward shifts in SHAM and DOCA-salt veins concentration-response curves.

α_{1D}-ARs mediate constriction of mesenteric veins but not arteries. Contractile responses to PE were obtained in the absence and presence of the selective α_{1D}-AR antagonist BMY-7378 (100, 300 nM). The α_{1D}-AR antagonist did not change resting diameter of arteries and veins by itself. PE constricted arteries from both treatment groups in a concentration-dependent manner while BMY-7378 (100, 300 nM) did not affect PE-induced constriction of SHAM (Figure 5A, Table 1) or DOCA-salt (Figure 5B, Table 2) arteries.

In contrast, PE-induced constriction in veins was competitively inhibited by the α_{1D}-AR antagonist. Both concentrations of BMY-7378 antagonized PE-induced constrictions as shown by the rightward shift in the concentration-response curve of SHAM veins (Figure 6A, Table 1). A similar inhibition was seen in DOCA-salt (Figure 6B, Table 2) veins.

Combined blockade of α_{1A} and α_{1B}-ARs in arteries produces a greater inhibition than blockade of individual receptors. The data presented so far suggested that the α_{1A}-AR is the predominant contractile isoform in mesenteric arteries with a minor contribution from the α_{1B}-AR subtype. For that reason, we decided to examine contractile responses in SHAM and DOCA-salt arteries in the presence of combined α_{1A} and α_{1B}-AR antagonism with 5-MU (100 nM) and L-765,314 (1 μM), respectively. Contractile responses in SHAM (Fig. 1A) and DOCA-salt (Fig. 1B) arteries were shifted to the right compared to curves obtained in the presence of either 5-MU or L-765,314 (Table 3).

Combined blockade of α_{1B} and α_{1D}-ARs in veins produces a greater inhibition than blockade of either receptor alone. In veins, α_{1D}-ARs are the main contractile adrenoceptor with a minor contribution from α_{1B}-ARs. Therefore, combined antagonism of α_{1B} and α_{1D}-ARs with L-765,314 (1 μM) and BMY-7378 (300 nM), respectively, resulted in significant rightward shifts of contractile responses in SHAM (Fig. 6A) and DOCA-salt (Fig. 6B) veins compared to curves obtained in the presence of either L-765,314 or BMY-7378 alone (Table 3).

α_1-AR protein expression in mesenteric arteries and veins. Western blot analysis revealed that arteries and veins express all three adrenoceptor subtypes. Western immunoblotting revealed a downregulation in the expression of α_{1A}-ARs in mesenteric arteries from DOCA-salt mice compared to SHAM mice (Figure 7). As with arteries, veins also expressed the protein for the α_{1A}-AR. However, no differences in expression were seen between SHAM and hypertensive vessels (Figure 7).

Western immunoblotting for the α_{1B}-AR revealed expression in arteries and veins from SHAM and DOCA-salt hypertensive mice (Figure 8) but DOCA-salt treatment did not alter protein expression levels for this adrenoceptor.

The α_{1D}-AR subtype was also expressed in mesenteric arteries. DOCA-salt treatment did not alter α_{1D}-AR protein expression in these vessels (Figure 9). Similarly, the protein for the α_{1D}-AR is expressed in mesenteric veins but protein expression levels for this adrenoceptor subtype were not affected by DOCA-salt treatment (Figure 9).

DISCUSSION

5-MU inhibits PE responses in arteries but not veins. 5-MU right-shifted PE concentration-response curves in arteries but not veins. Therefore, our results provide evidence for functional α_{1A}-ARs in mesenteric arteries but not veins as shown by others for murine tail and mesenteric artery (Daly et al., 2002). It appears that vascular α_1-ARs may preferentially affect resistance in small vessels via α_{1A}-ARs (Daly et al., 2002). This suggests that the main role of α_{1A}-ARs in mice may be to alter blood flow via changes in peripheral resistance.

α_{1B}-AR antagonism reveals a minor involvement of this adrenoceptor in contractile responses in arteries and veins. L-765,314, a selective α_{1B}-AR antagonist, was used to test the role of the α_{1B}-AR in contractile responses of mesenteric arteries and veins. At a low concentration, L-765-314 did not shift the PE-induced concentration-response curves in arteries or veins. However, significant antagonism was seen with L-765,314 (1 μM) in SHAM and DOCA-salt arteries. In SHAM and DOCA-salt veins, L-765,314 (1 μM) antagonized responses to PE as well but not at a concentration of 100 nM. This data argue that α_{1B}-ARs provide just a small component to contractile responses to PE in mesenteric arteries and veins. This completely agrees with previous studies showing that this adrenoceptor subtype plays a minor role in vascular contractile responses (Chalotorn et al., 2003).

α_{1D}-ARs mediate constrictions of veins but not arteries. Contractile responses of mesenteric arteries and veins were examined in the absence or presence of the α_{1D}-AR antagonist BMY-7378 as an approach to defining the role

of α_{1D}-AR in arterial and venous contractile responses. In SHAM or DOCA-salt arteries, the α_{1D}-AR antagonist did not affect PE contractile responses. In contrast to our findings, others have found (Yamamoto and Koike, 2001) that functional "α_{1D}-like" adrenoceptors are present in murine mesenteric arteries. This difference could be due to the fact that they used NE, a non-selective α_1/α_2 adrenoceptor agonist, and we used a selective α_1-AR agonist. However, our results are in agreement with those of others (Daly et al., 2002), who showed that α_{1A} but not α_{1D}-ARs have a major vasoconstrictor role in murine mesenteric arteries.

On the other hand, BMY-7378 was a competitive antagonist of PE-induced constrictions in mesenteric veins providing pharmacological evidence that the α_{1D}-AR subtype is involved in PE-induced constriction of murine mesenteric veins. This is in agreement with the finding that in canine mesenteric vein there is a subpopulation of "α_{1D}-like" ARs (Daniel et al., 1997).

Of particular importance is the fact that different receptor subtypes mediate contractile responses to PE in mesenteric arteries and veins. It is therefore, imperative to determine what is the physiological significance of such differentiation in responses between arteries and veins. For example, it is known that the α_{1D}-AR contributes to blood pressure regulation as α_{1D}-AR K/O mice have lower arterial pressures relative to controls (Tanoue et al., 2002b). In addition, pressor responses to catecholamines in these mice were decreased. The α_{1D}-AR has also been implicated in the pathogenesis of hypertension. α_{1D}-AR K/O mice submitted to subtotal nephrectomy and salt loading showed an

attenuated increase in blood pressure compared to control animals suggesting that α_{1D}-ARs play an important role in the development of salt-induced hypertension (Tanoue et al., 2002a). These data were confirmed by a recent study (Hosoda et al., 2005) showing that α_{1B}-AR K/O but not α_{1D}-AR K/O mice developed a comparable level of hypertension to wild-type mice after salt loading.

The fact that the α_{1D}-AR is involved in the pathogenesis of salt-induced hypertension and that we have shown in these studies that it mediates contractile responses to PE in mesenteric veins but not arteries could point to a contribution of veins to blood pressure regulation. It is known that the splanchnic mesenteric vascular bed contains up to 30% of blood volume (Greenway, 1983). This capacitance function largely resides in veins and venules. A reduction in capacitance of systemic veins will shift blood from peripheral vascular beds toward the thoracic cavity (Ricksten et al., 1981) leading to increases in CO; one of the determinants of systemic blood pressure. However, this is still a controversial hypothesis and more experimentation will be needed to get a more definitive conclusion.

All α_1-AR subtypes are expressed in murine mesenteric arteries and veins. We have provided functional evidence that α_{1A}-ARs mediate contractile responses in mesenteric arteries whereas α_{1D}-ARs are the main subtype responsible for constriction in veins while α_{1B}-ARs play a minor role in contractile responses. We next tested whether the differential contractile responses we

observed in mesenteric arteries and veins were due to selective expression of particular adrenoceptor subtypes.

Western immunoblotting analysis revealed that both mesenteric arteries and veins express the α_{1A}-AR protein but there were differences in α_{1A}-AR expression between SHAM control and DOCA-salt hypertensive arteries. There is a downregulation in the expression of α_{1A}-AR in arteries from hypertensive mice. It is known that in DOCA-salt hypertension there is an increased in sympathetic nerve activity (de Champlain, 1990; Oparil, 1986). In addition, there is a tendency for an increase in plasma catecholamine concentration (Bouvier and de Champlain, 1986; de Champlain et al., 1987). A positive correlation between blood pressure and catecholamine levels suggests blood pressure elevations are linked to sympathetic nerve activity (de Champlain et al., 1987). Sympathetic overactivity can cause adrenoceptor downregulation. Whether increased sympathetic activity occurs in DOCA-salt mice is not established. However, it appears that plasma NE concentrations are generally 3 to 10 times higher in mice than in rats or humans (Janssen and Smits, 2002).

An important finding here was that despite α_{1A}-AR downregulation in DOCA-salt arteries, PE-induced constriction is not compromised. It could be argued that this is because of a greater affinity for PE of the remaining α_{1A}-AR in DOCA-salt arteries. It is also possible that an increased in postreceptor events are involved in the maintained adrenergic reactivity in DOCA-salt arteries in the face of a downregulation of functional adrenoceptors. There were no differences in α_{1A}-AR protein expression between SHAM and DOCA-salt hypertensive veins.

It is not clear why DOCA-salt hypertension does not affect expression of α_{1A}-AR in veins.

The α_{1B}-AR had a minor influence on PE-induced constrictions in mesenteric vessels but was expressed in arteries and veins. The function of this receptor subtype in vessels remains unclear. The α_{1D}-AR, which is the predominant adrenoceptor subtype involved in venous contraction to PE, was expressed in veins. In arteries, our functional experiments did not provide any evidence for a contractile role for α_{1D}-AR. However, the protein is clearly expressed in these resistance vessels. Protein expression for the α_{1B}-AR and the α_{1D}-AR subtypes was unaffected by DOCA-salt treatment. Therefore, at least in mice, the α_{1A}-AR is the only adrenoceptor subtype whose expression changes upon DOCA-salt treatment. Selective changes in receptor expression in DOCA-salt hypertension could point to an important physiological function of the α_{1A}-AR in blood pressure regulation. However, at this point we could not reject a role for the α_{1B}- and α_{1D}-AR as all three adrenoceptor subtypes play important roles in blood pressure homeostasis as well (Tanoue et al., 2002c). The mechanisms for receptor subtype-selective downregulation in DOCA-salt hypertension remain to be established.

Conclusions. There is a differential regulation of α_1-AR subtypes mediating contractile responses in murine arteries and veins: α_{1A}- being the predominant contractile receptor in arteries, the α_{1D}-AR mediates venous constriction to PE whereas the α_{1B}-AR has a minor involvement in both, arteries and veins. All three α_1-AR subtypes are expressed in arteries and veins of SHAM

and DOCA-salt mice but only α_{1A}-AR expression changes in DOCA-salt hypertension. These studies highlight the fact of a differential adrenergic contractile regulation in murine mesenteric arteries as opposed to veins. Future experiments will determine the physiological significance of such adrenoceptor subtype-specific responses.

Table 1. PE responses in SHAM mesenteric arteries and veins in the absence or presence of antagonists for the α_{1A}-, α_{1B}- and the α_{1D}-ARs. Data are mean ± SEM. Numbers in parentheses are the number of animals from which data were obtained. E_{max} is the maximum constriction based on data fitted to a logistic equation. EC_{50} is the negative logarithm of the molar concentration of agonist producing half maximal constriction. *: $p < 0.05$ –vs- control.

	E_{max} (%)		EC_{50} (- log M)	
	VEIN	*ARTERY*	*VEIN*	*ARTERY*
SHAM				
5-methylurapidil (α_{1A}-AR antagonist)				
PE (control)	36.5 ± 2.8 (9)	20.6 ± 1.7 (16)	6.5 ± 0.1 (9)	5.4 ± 0.06 (16)
PE/5-MU (10^{-8} M)	37.2 ± 3.4 (6)	17.4 ± 2.0 (5)	6.5 ± 0.1 (6)	4.8 ± 0.1* (5)
PE/5-MU (10^{-7} M)	40.2 ± 1.4 (8)	17.6 ± 3.2 (8)	6.2 ± 0.09 (8)	4.9 ± 0.07* (8)
L-765,314 (α_{1B}-AR antagonist)				
PE (control)	41.6 ± 3.0 (7)	19.9 ± 1.8 (10)	6.5 ± 0.1 (7)	5.5 ± 0.08 (10)
PE/L-765,314 (10^{-7} M)	34.3 ± 4.6 (5)	19.6 ± 2.6 (5)	6.4 ± 0.2 (5)	5.4 ± 0.07 (5)
PE/L-765,314 (10^{-6} M)	38.2 ± 4.1 (5)	19.1 ± 1.4 (5)	6.0 ± 0.1* (5)	4.7 ± 0.04* (5)
BMY-7378 (α_{1D}-AR antagonist)				
PE (control)	37.4 ± 2.7 (11)	23.0 ± 2.7 (4)	6.1 ± 0.1 (11)	5.5 ± 0.08 (4)
PE/BMY-7378 (10^{-7} M)	32.7 ± 3.7 (4)	25.7 ± 1.8 (4)	5.8 ± 0.2* (4)	5.4 ± 0.03 (4)
PE/BMY-7378 (3×10^{-7} M)	35.9 ± 1.4 (8)	23.6 ± 2.0 (8)	5.3 ± 0.1* (8)	5.3 ± 0.06 (8)

Table 2. PE responses in DOCA-salt hypertensive mesenteric arteries and veins in the absence or presence of antagonists for the α_{1A}-, α_{1B}- and the α_{1D}-ARs. Data are mean ± SEM. Numbers in parentheses are the number of animals from which data were obtained. E_{max} is the maximum constriction based on data fitted to a logistic equation. EC_{50} is the negative logarithm of the molar concentration of agonist producing half maximal constriction. *: $p < 0.05$ –vs-control.

	E_{max} (%)		EC_{50} (- log M)	
	VEIN	*ARTERY*	*VEIN*	*ARTERY*
DOCA-salt				
5-methylurapidil (α_{1A}-AR antagonist)				
PE (control)	34.9 ± 2.2 (6)	20.6 ± 1.0 (14)	6.6 ± 0.1 (6)	5.3 ± 0.06 (14)
PE/5-MU (10nM)	35.0 ± 4.0 (5)	21.1 ± 1.3 (17)	6.6 ± 0.2 (5)	5.0 ± 0.07* (17)
PE/5-MU (100nM)	32.3 ± 2.5 (7)	17.1 ± 3.5 (5)	6.3 ± 0.2 (7)	4.4 ± 0.2* (5)
L-765,314 (α_{1B}-AR antagonist)				
PE (control)	38.6 ± 3.5 (6)	21.0 ± 1.5 (10)	6.2 ± 0.1 (6)	5.3 ± 0.09 (10)
PE/L-765,314 (100 nM)	34.3 ± 2.6 (5)	25.8 ± 1.4 (5)	6.3 ± 0.2 (5)	5.5 ± 0.09 (5)
PE/L-765,314 (1μM)	29.7 ± 3.9 (5)	15.7 ± 4.3 (5)	5.5 ± 0.06* (5)	4.9 ± 0.1* (5)
BMY-7378 (α_{1D}-AR antagonist)				
PE (control)	33.2 ± 2.0 (17)	23.5 ± 2.4 (4)	6.3 ± 0.1 (17)	5.5 ± 0.05 (4)
PE /BMY-7378 (100nM)	36.5 ± 2.0 (5)	24.3 ± 2.6 (4)	5.7 ± 0.1* (5)	5.5 ± 0.03 (4)
PE/BMY-7378 (300nM)	29.4 ± 4.9 (4)	21.7 ± 2.0 (5)	5.4 ± 0.09* (4)	5.4 ± 0.02 (5)

Table 3. Concentration-response curves for SHAM and DOCA-salt arteries and veins in the presence of the single and combined application of α_{1A}- and α_{1B}-AR or α_{1B}- and α_{1D}-AR antagonists. Data are mean ± SEM. Numbers in parentheses are the number of animals from which the data were obtained. E_{max} is the maximum constriction based on data fitted to a logistic equation. EC_{50} is the negative logarithm of the molar concentration of agonist producing half maximal constriction. *: $p < 0.05$ –vs- control, &: $p < 0.05$ –vs- PE/BMY-7378 or PE/L-765,314, #: $p < 0.05$ –vs- PE/5-MU or PE/L-765,314.

	E_{max} (%)		EC_{50} (- log M)	
	SHAM	*DOCA*	*SHAM*	*DOCA*
Arteries				
PE (control)	20.6 ± 1.7 (16)	20.6 ± 1.0 (14)	5.4 ± 0.06 (16)	5.3 ± 0.06 (14)
PE/5-MU (100nM)	17.6 ± 3.2 (8)	17.1 ± 3.5 (5)	4.9 ± 0.07* (8)	4.4 ± 0.2* (5)
PE/L-765,314 (1µM)	19.1 ± 1.4 (5)	15.7 ± 4.3 (5)	4.7 ± 0.04* (5)	4.9 ± 0.1* (5)
PE/5-MU (100nM)/ L-765,314 (1µM)	13.3 ± 3.1 (5)	15.4 ± 3.4 (5)	4.1 ± 0.1*,# (6)	4.2 ± 0.08*,# (5)
Veins				
PE (control)	37.4 ± 2.7 (11)	33.2 ± 2.0 (17)	6.1 ± 0.1 (11)	6.3 ± 0.1 (17)
PE/BMY-7378 (300nM)	35.9 ± 1.4 (8)	29.4 ± 4.9 (4)	5.3 ± 0.1* (8)	5.4 ± 0.09* (4)
PE/L-765,314 (1µM)	38.2 ± 4.1 (5)	29.7 ± 3.9 (5)	6.0 ± 0.1 (5)	5.5 ± 0.07* (5)
PE/BMY-7378 (300nM)/ L-765,314 (1µM)	29.9 ± 3.1 (5)	27.9 ± 3.9 (5)	5.0 ± 0.01*,& (5)	4.9 ± 0.1*,& (5)

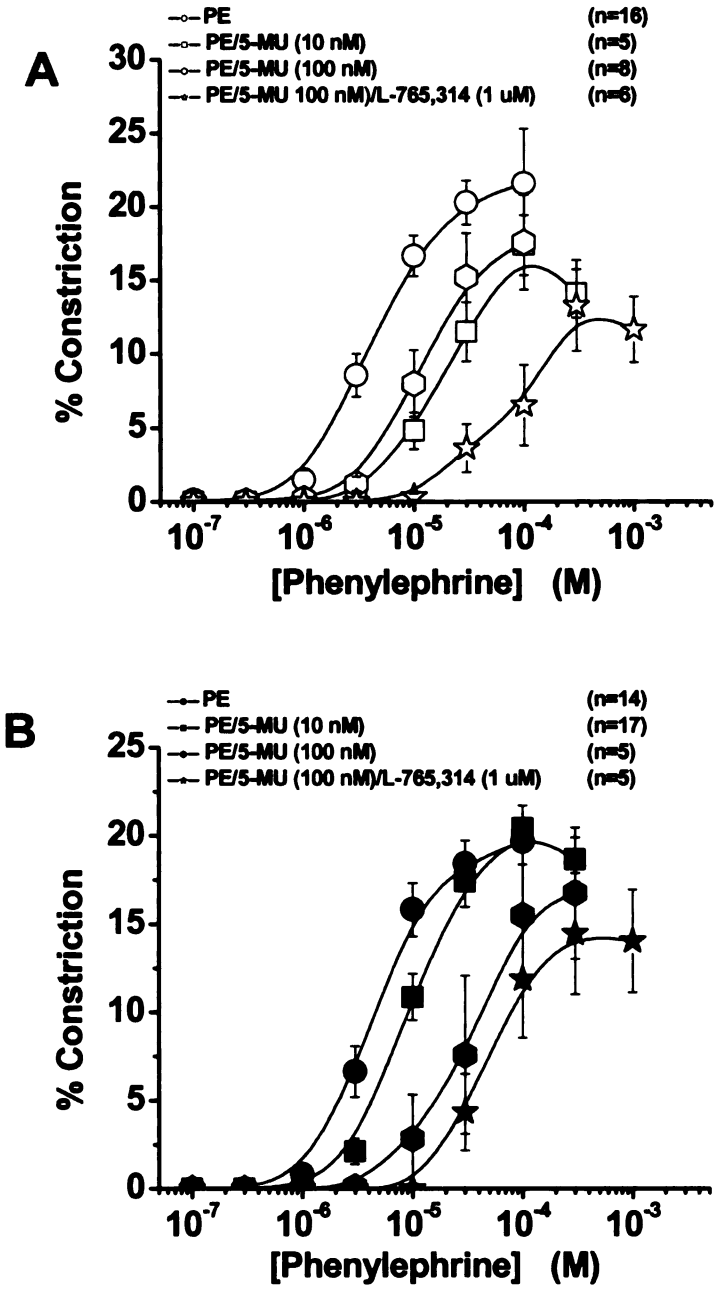

Figure 1. PE concentration-response curves from SHAM (A) and DOCA-salt (B) arteries. PE responses were obtained in the absence and presence of 5-MU, a selective α_{1A}-AR antagonist and during combined application with the selective α_{1B}-AR antagonist L-765,314. Data are mean ± SEM from "n" animals.

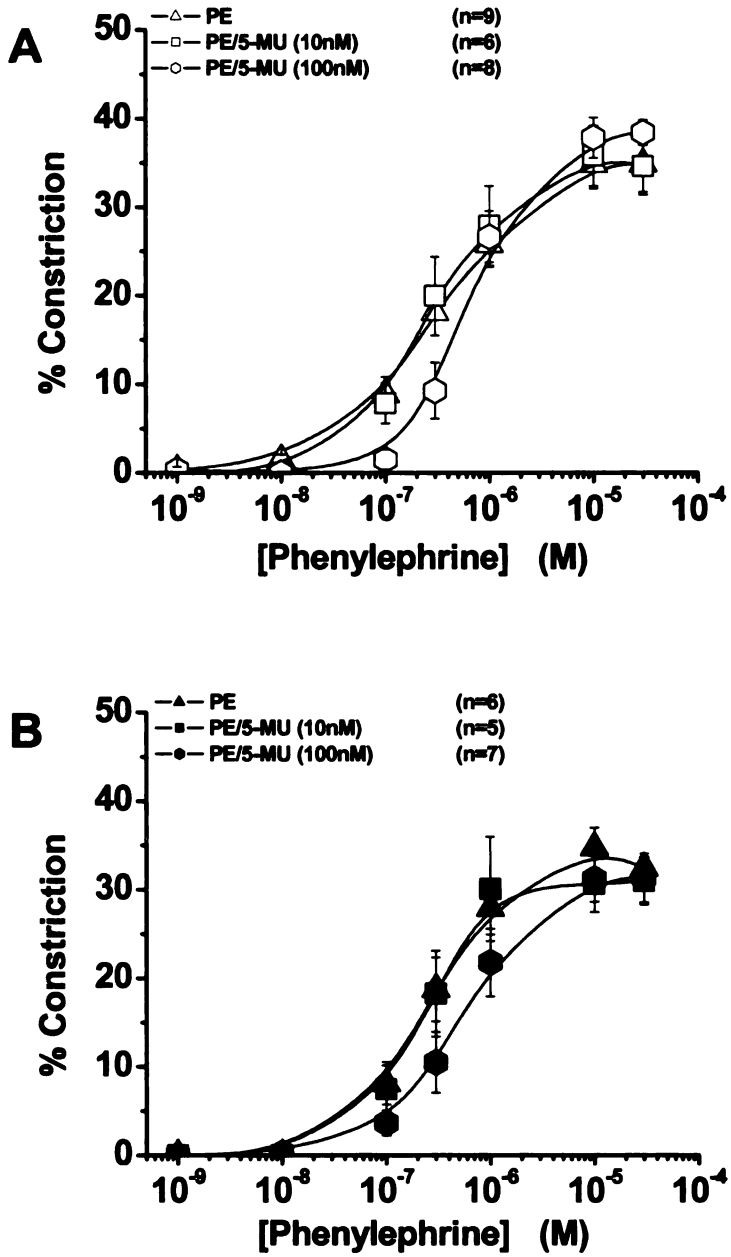

Figure 2. Concentration-response curves for the selective α_1-AR agonist PE in the absence and presence of 5-MU, a selective α_{1A}-AR antagonist. 5-MU did not affect PE-induced constrictions in SHAM (A) and DOCA-salt (B) veins. Data are mean ± SEM from "n" animals.

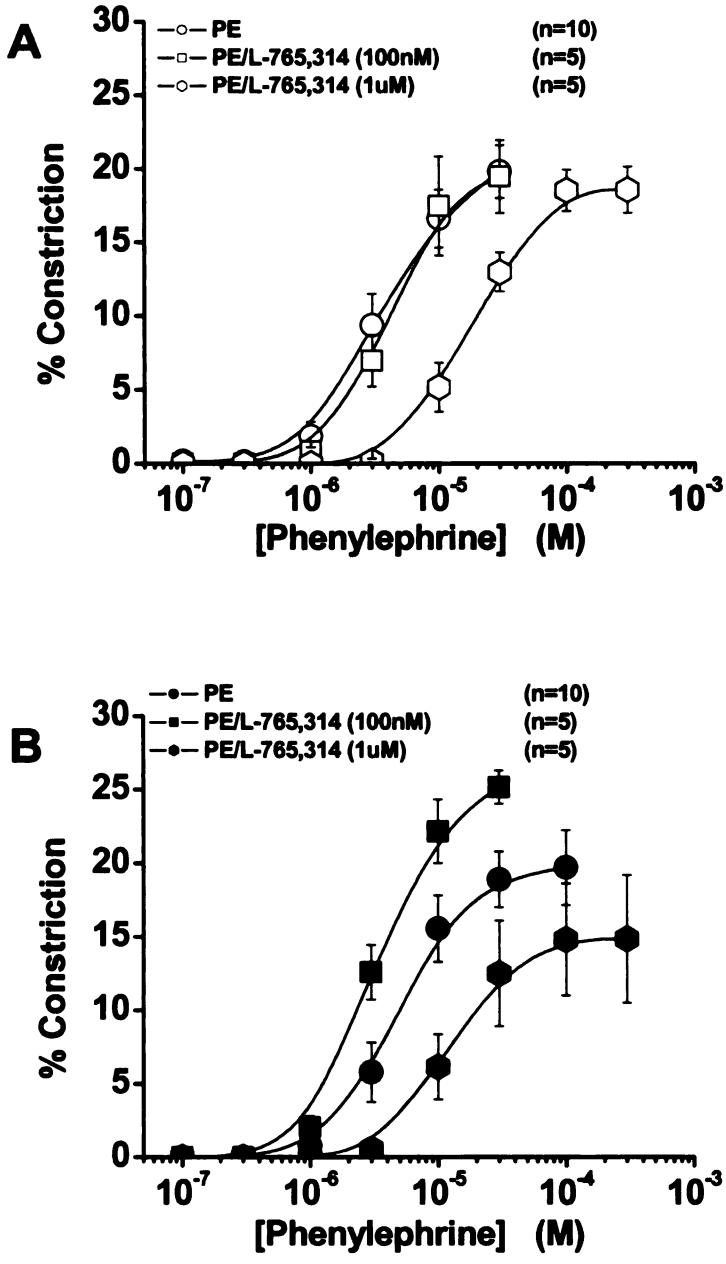

Figure 3. Effects of the selective α_{1B}-AR antagonist L-765,314 on PE-induced constrictions of SHAM (A) and DOCA-salt (B) arteries. L-765,314 (100 nM) was not effective in antagonizing responses to PE. However, L-765,314 (1 μM) competitively antagonized PE-induced constrictions in SHAM and DOCA-salt arteries. Data are mean ± SEM from "n" animals.

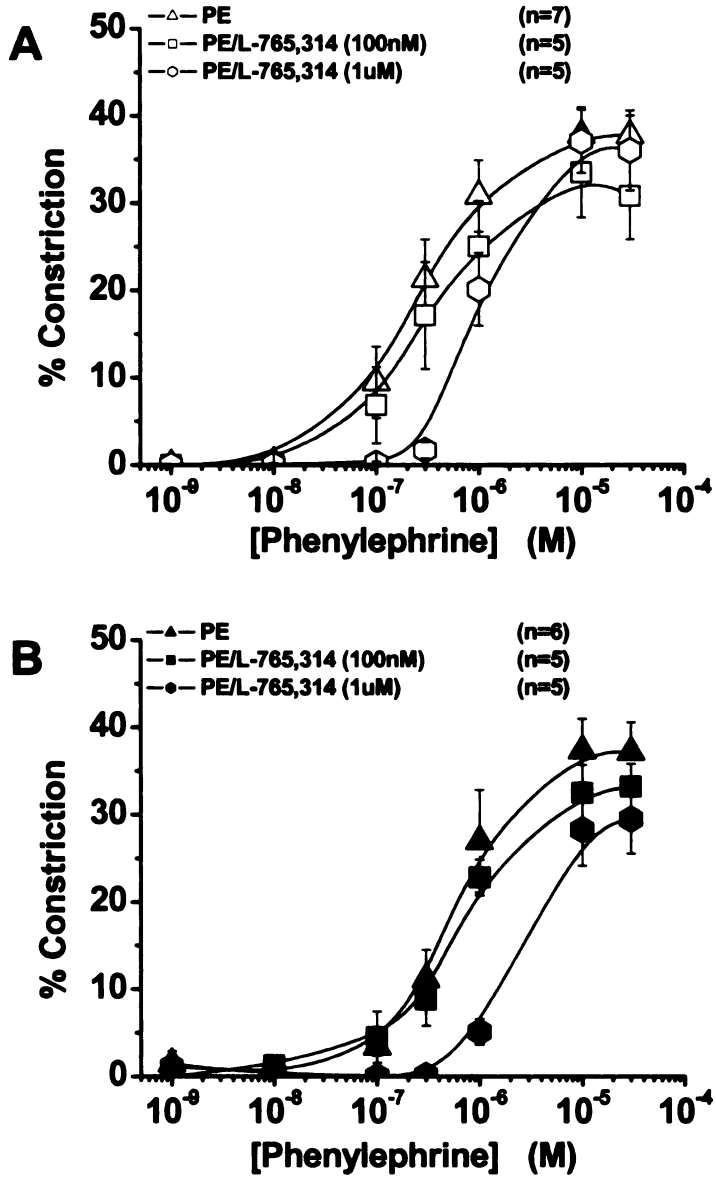

Figure 4. Effects of the selective α_{1B}-AR antagonist L-765,314 on PE-induced constrictions of SHAM (A) and DOCA-salt (B) veins. L-765,314 (100 nM) was not effective in antagonizing responses to PE. However, L-765,314 (1 μM) competitively antagonized PE-induced constrictions in SHAM and DOCA-salt vessels. Data are mean ± SEM from "n" animals.

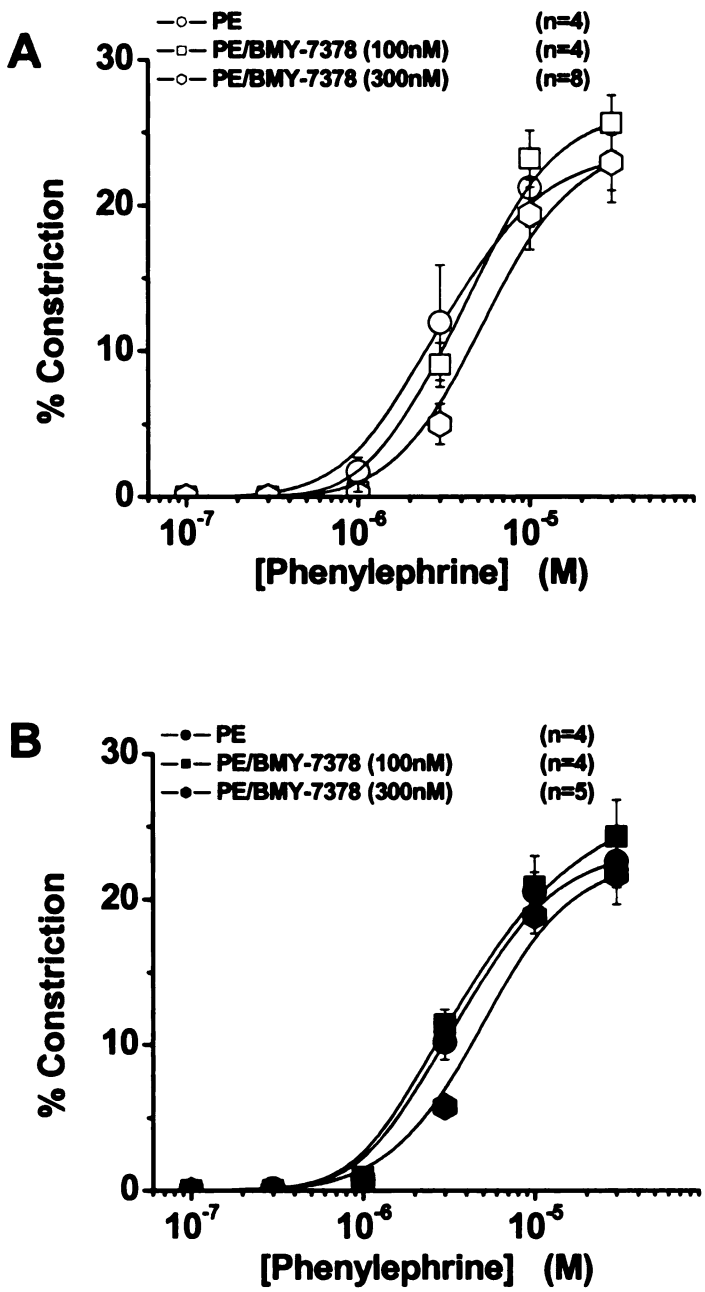

Figure 5. Concentration-response curves for the selective α_1-AR agonist PE in the absence and presence of BMY-7378, a selective α_{1D}-AR antagonist. BMY-7378 did not antagonize PE-induced constrictions of SHAM (A) and DOCA-salt (B) arteries. Data are mean ± SEM from "n" animals.

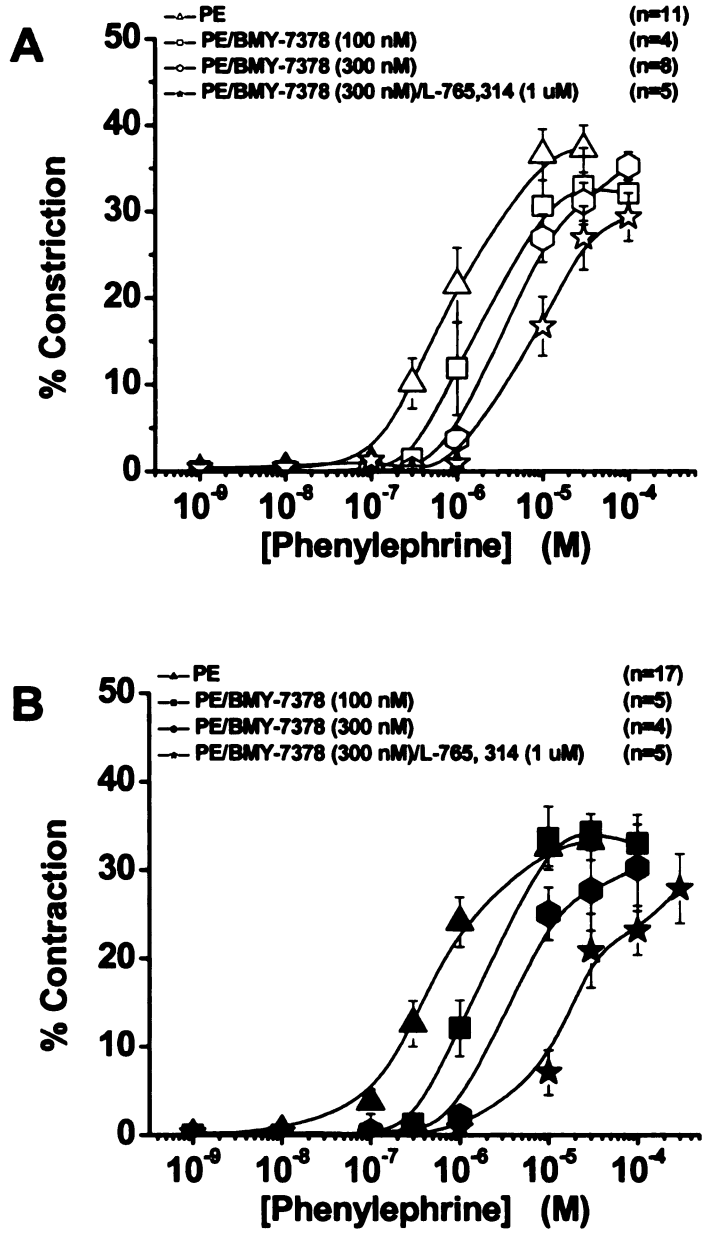

Figure 6. Concentration-response curves for the selective α_1-AR agonist PE in the absence and presence of BMY-7378, a selective α_{1D}-AR antagonist and during combined application with the selective α_{1B}-AR antagonist L-765,314 in SHAM (A) and DOCA-salt veins (B). Data are mean ± SEM from "n" animals.

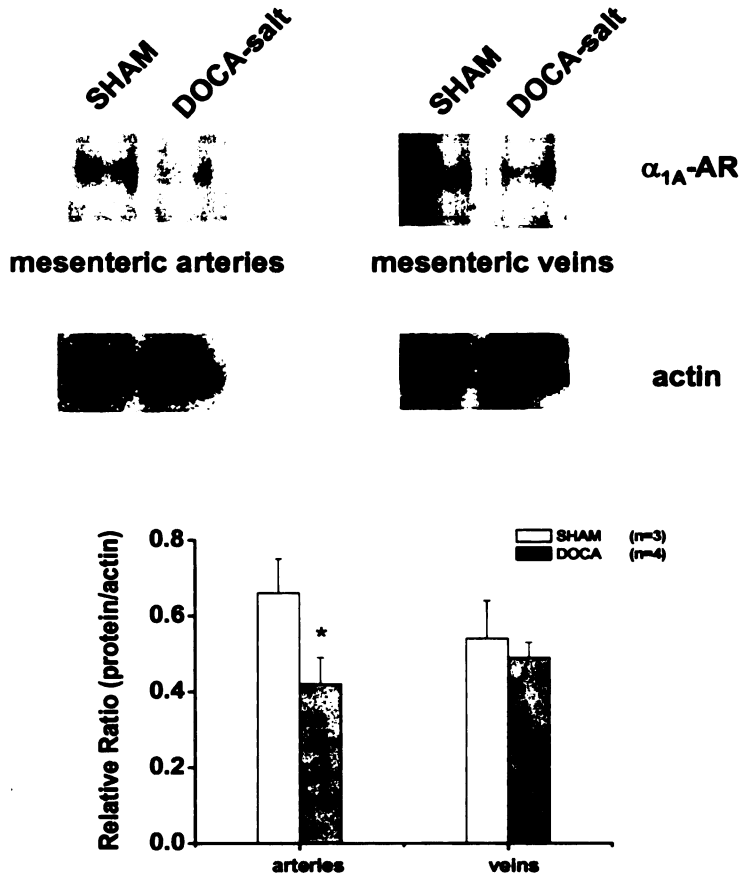

Figure 7. Western analyses demonstrating the presence of the α_{1A}-AR subtype in protein homogenates isolated from mesenteric arteries and veins of SHAM and DOCA-salt mice with their respective alpha-actin controls. Bars represent mean ratios of α_{1A}-AR protein/actin ± SEM from "n" animals. * Statistically significant difference ($p < 0.05$) in α_{1A}-AR protein expression between SHAM and DOCA-salt treatment groups.

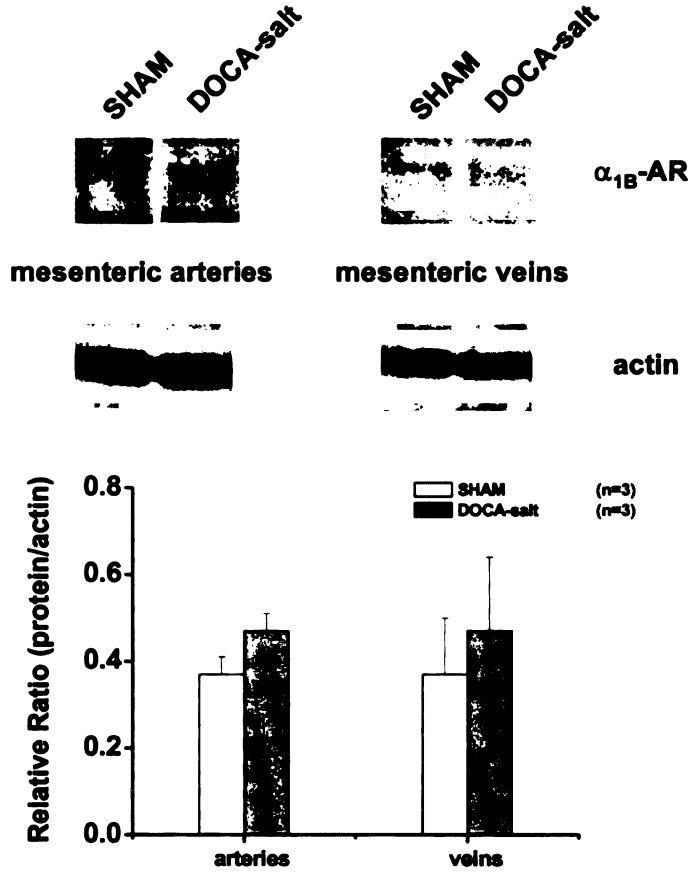

Figure 8. Western analyses demonstrating the presence of the α_{1B}-AR subtype in protein homogenates isolated from mesenteric arteries and veins of SHAM and DOCA-salt mice with their respective alpha-actin controls. Bars represent mean ratios of α_{1B}-AR protein/actin ± SEM from "n" animals.

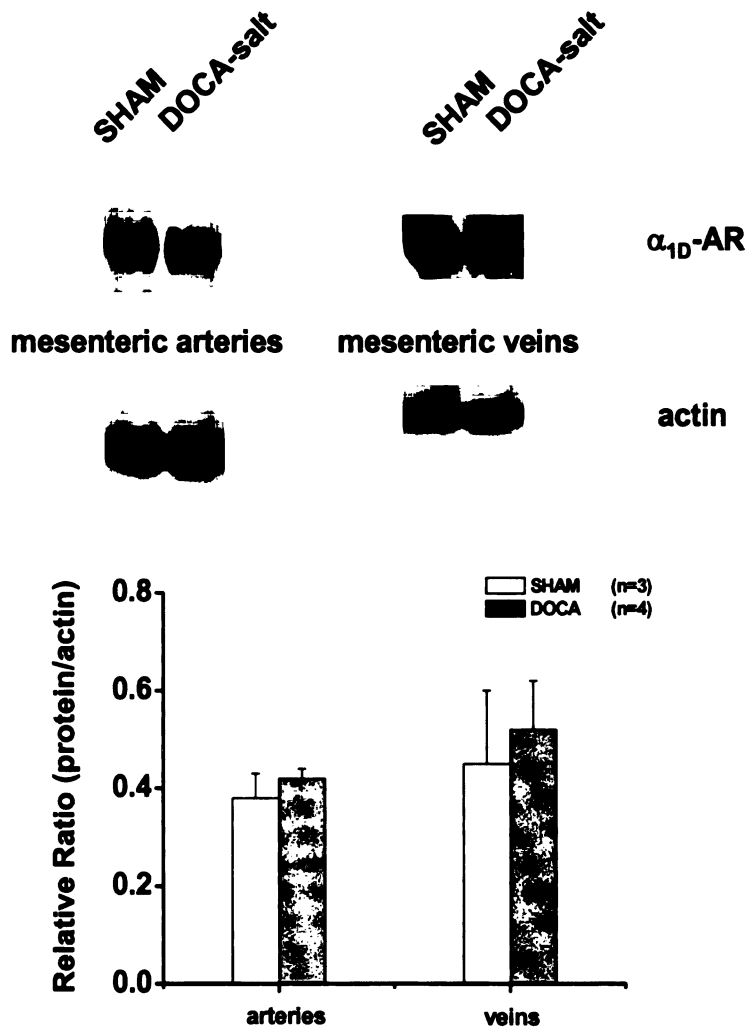

Figure 9. Western analyses demonstrating the presence of the α_{1D}-AR subtype in protein homogenates isolated from mesenteric arteries and veins of SHAM and DOCA-salt mice with their respective alpha-actin controls. Bars represent mean ratios of α_{1D}-AR protein/actin ± SEM from "n" animals.

CHAPTER 5

Differential Contributions of Alpha-1 and Alpha-2 Adrenoceptors to Vasoconstriction in Mesenteric Arteries and Veins of Normal and Hypertensive Mice

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Vascular Pharmacology

INTRODUCTION

α-ARs mediate the actions of NE and epinephrine on blood vessels. α_1-ARs are G-protein coupled receptors that are targets for many therapeutically relevant drugs. Starke et al. showed that pre- and postjunctional α-ARs differ pharmacologically (Starke et al., 1974; Starke et al., 1975a; Starke et al., 1975b) and provided evidence for two subclasses of adrenoceptors. Subsequently, it was proposed that the prejunctional α-AR be named α_2-AR whereas the postjunctional receptor was called α_1-AR (Langer, 1974).

α_1-ARs mediate vascular smooth muscle contraction and they are important regulators of blood pressure and blood flow. α_1-ARs are coupled to PLC activation via pertussis toxin-insensitive G proteins of the $G_{q/11}$ family resulting in phosphoinositide hydrolysis and stimulation of Ca^{++} release from intracellular stores (Guimaraes and Moura, 2001; Piascik et al., 1996 and Piascik and Perez, 2001). However, it has been shown recently that α_1-ARs can activate Ca^{++} influx via voltage-gated Ca^{++} channels (Minneman, 1988, Perez et al., 1993) as well as phospholipase A_2 (Perez et al., 1993). These receptors could also signal through pertussis toxin-sensitive G proteins (Perez et al., 1993).

α_2-ARs were initially thought to mediate exclusively prejunctional inhibition of neurotransmitter release. However, it is now accepted that a subpopulation of postjunctional α_2-ARs is present and regulates vascular tone in conjunction with α_1-ARs in a variety of vascular beds. Vasoconstrictor responses mediated by α_2-ARs involve Ca^{++} entry through voltage-gated Ca^{++} channels as Ca^{++} channel

blockers selectively inhibited α_2-AR mediated pressor responses with little effect on α_1-mediated responses (van Meel et al., 1983; van Zwieten et al., 1983).

α_1- and α_2-ARs are located postjunctionally on many blood vessels and the relative contribution of each receptor type to vasomotor responses is specific to the vascular bed studied. α_1- and α_2-ARs mediate contractions of the rabbit saphenous vein (Daly et al., 1988) and the dog saphenous vein (Fowler et al., 1984). Itoh et al. (1987) demonstrated the presence of postjunctional α-ARs in canine mesenteric arteries and veins. He found that PE, a selective α_1-AR agonist, was a more potent agonist in the mesenteric artery than in the vein. In contrast, the selective α_2-AR agonist UK-14,304 was a more potent agonist in veins than in arteries providing evidence that arteries and veins express different α-ARs. In the mesenteric, splenic, renal and femoral vascular beds there are postjunctional α_1- and α_2-ARs and the contribution of α_2-ARs was more prominent in the mesenteric and femoral beds (Polonia et al., 1986). Maximal contribution for α_1-ARs occurs in renal blood vessels while α_1-ARs make a much smaller contribution in the splenic vascular bed (Polonia et al., 1986).

We have previously shown that murine mesenteric veins are more sensitive than arteries to contractile stimulation mediated by α-AR agonists and are more resistant to desensitization by adrenergic agonists and to α-AR inactivation by PBZ (Pérez-Rivera et al., 2004). In addition, PBZ pre-treated veins became sensitive to desensitization by a continuous challenge with an α-AR agonist. These data provided functional evidence that murine mesenteric veins have an increased α_1-AR reserve compared to arteries (Pérez-Rivera et

al., 2004). This could explain their relative increased adrenergic reactivity and resistance to desensitization compared to arteries.

Given the fact that α_2-ARs can play an important role in contractile responses to adrenergic agonists, a differential role of this adrenoceptor in arteries and veins could contribute to differences in adrenergic reactivity seen in these vessels. In this study, we investigated the presence of functional postsynaptic α_2-ARs in murine mesenteric arteries and veins by using receptor-specific agonists and antagonists. Both mesenteric arteries and veins influence blood pressure by changes in resistance and capacitance, respectively. For that reason, we also examined reactivity differences of postjunctional α_2-ARs in arteries and veins from DOCA-salt hypertensive mice compared to the same vessels from SHAM animals.

MATERIALS AND METHODS

Animals. C57/BL male mice (25-30g) were obtained from Charles River Labs (Portage, MI). Upon arrival at the animal care facility, mice were maintained according to the standards approved by the Michigan State University All-University Committee on Animal Care and Use. Mice were individually housed in clear plastic cages with free access to standard pelleted chow (Harlan/Teklad 8640 Rodent Diet) and tap water. Mice were housed in temperature and humidity-controlled rooms with a 12 hours on/12 hours off light cycle. Animals were allowed a period of 2-3 days of acclimatization prior to entry into any experimental protocol.

DOCA-salt surgery. Mice were unilaterally nephrectomized under anesthesia using a solution containing ketamine (500 mg/ml) and xylazine (20 mg/ml) in a 9:1 ratio, respectively. Animals within the weight range used (25 – 30g) received about 80 μL of the anesthetic. The skin over the left flank was shaved and a 1.5 cm incision was made through the skin and underlying muscle caudal to the rib cage. The left kidney was exteriorized and removed after ligation of the renal artery and vein with 4-0 silk sutures (Ethicon, Inc, Somerville, NJ). The muscle and skin layers were then closed separately with 4-0 silk sutures. A small area between the shoulder blades was shaved and a 1 cm incision was made through which DOCA-salt pellets were implanted s.c. for a giving dose of 150 mg/kg DOCA. DOCA mice were given water containing 1% NaCl and 0.2% KCl. SHAM mice were also unilaterally nephrectomized, received no DOCA pellet implantation and were given tap water. Both groups of

mice were placed on standard pelleted rodent chow. After recovery, the mice were housed under standard conditions for 4 weeks after which systolic BP was determined by the tail-cuff method.

In-vitro preparation of mesenteric vessels. Mice were euthanized with a lethal dose of pentobarbital (50 mg/kg i.p.). The small intestine with its associated mesenteric vessels was removed from euthanized mice and placed in oxygenated (95% O_2, 5% CO_2) Krebs' solution of the following composition (mmol): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 11. A segment of the intestine with the associated vessels was removed and pinned flat in a silicone elastomer-lined (Sylgard, Dow Corning, Midland, MI) Petri dish. A section of mesentery containing vessels close to the mesenteric border was cut out using fine scissors and forceps. The preparation was transferred to a smaller silicone elastomer-lined recording bath and pinned flat. Second or third-order mesenteric veins or arteries were isolated for study by carefully clearing away the surrounding fat tissue. The recording bath containing the preparation was mounted on the stage of an inverted microscope (Olympus CK-2) and superfused with warm (37°C) Krebs' solution at a flow rate of 7 ml min^{-1}. All preparations were allowed a 20 min equilibration period during which the vessels relaxed to a stable resting diameter.

Video monitoring of vessel diameter. The output of a black and white video camera (Hitachi model KP-111) attached to the microscope was fed to a PC Vision Plus frame-grabber board (Imaging Technology Inc, Woburn, MA)

mounted in a personal computer. The video images were analyzed using Diamtrak software (http://www.diamtrak.com, Adelaide, Australia).

Concentration-response studies. All drugs were added in known concentrations to the superfusing Krebs' solution. Control concentration-response curves were obtained in arteries (10^{-7} M – 3 x 10^{-5} M) and veins (10^{-10} M – 3 x 10^{-6} M) after application of NE (Sigma-Aldrich, St. Louis, MO). Each agonist concentration was applied for 3 min and there was a 20-minute interval between successive applications. The contribution of α_1-ARs to NE constrictor responses was studied by comparing curves in the absence and in the presence of the selective α_1-AR antagonist prazosin (3, 30, 300 nM; Sigma Aldrich, St. Louis, MO). A role for α_2-ARs in mediating contractile responses to NE was studied by comparing curves in the absence and presence of the selective α_2-AR antagonists yohimbine (3, 30, 300 nM; Sigma Aldrich, St. Louis, MO) and rauwolscine (100 nM; Sigma Aldrich, St. Louis, MO). We also directly tested for the presence of contractile α_2-ARs in arteries and veins by challenging blood vessels with the α_2-AR agonists clonidine (10^{-7} M – 10^{-5} M; Sigma-Aldrich, St. Louis, MO) and UK-14,304 (10^{-7} M – 10^{-5} M; Sigma-Aldrich, St. Louis, MO) in the absence or the presence of the cyclooxygenase inhibitor, indomethacin (10 μM), or the nitric oxide synthase inhibitor *N*-nitro-L-arginine (NLA; 100 μM). All antagonists were applied for 20 minutes prior to application of the agonist. A single concentration-response curve was obtained in each preparation.

Data analysis. Constrictions of blood vessels caused by the different treatments are expressed as percentage constriction (percentage reduction from

the resting diameter). Half maximal effective agonist concentration (EC_{50}) and maximum response (E_{max}) were calculated from a least-squares fit of individual agonist concentration response curves using the following logistic function from Origin 7.0 (Microcal Software, Inc, Northampton, MA):

$$Y = \{(E_{min} - E_{max})/[1 + (x/EC_{50})^n]\} + E_{max}$$

where E_{min} is the minimum response (set at 0), n is the slope factor. Data are expressed as mean ± SEM.

The concentration-response curves to agonists in the presence or absence of the antagonists were analyzed by plotting the negative logarithm of the ratio of concentrations of the agonist that produced the same effect (50% maximal response) in the presence and absence of the antagonist minus 1 [log dose ratio (DR) − 1)] against the negative logarithm of the concentration of antagonist (i.e. Schild plot analysis; Arunlakshana and Schild, 1959). The intercept on the X-axis yields the pA_2 value (negative logarithm of the concentration of antagonist that induces a 2-fold rightward shift of the concentration-response to the agonist). A slope close to 1 is considered to be competitive antagonism.

Statistical differences between groups were assessed by Student's two-tailed unpaired t-test. When more than two groups were compared, analysis of variance (ANOVA) was used with Student-Newman-Keuls multiple comparison as a post test. $P < 0.05$ was considered statistically significant. All statistical analyses and 95% confidence interval (CI) calculations were performed using GraphPad InStat for Windows 95 (GraphPad Software, San Diego, CA).

RESULTS

General. Four weeks after the start of DOCA-salt treatment, systolic blood pressure in DOCA-salt (n=93) mice was significantly higher than in SHAM (n=93) mice (122 ± 1 mmHg *vs* 92 ± 1 mmHg, respectively; $p < 0.05$). The initial resting diameter of mesenteric arteries from SHAM and DOCA-salt mice was 138 ± 4 μm and 135 ± 4 μm, respectively ($p > 0.05$). The initial diameter of mesenteric veins from SHAM and DOCA-salt mice was 185 ± 5 μm and 190 ± 7 μm, respectively ($p > 0.05$).

Prazosin inhibits α_1-ARs in mesenteric arteries and veins. We examined contractile responses of arteries and veins in the absence and presence of prazosin (3, 30, 300 nM), a selective α_1-AR antagonist. NE produced a concentration-dependent constriction of arteries (Fig. 1A, Fig. 1B, Table 1). There were no differences in the NE concentration response curves obtained in SHAM and DOCA-salt arteries (Table 1). Prazosin did not change resting diameter of arteries or veins. In SHAM arteries, prazosin produced parallel rightward shifts of the NE concentration-response curve (Fig.1A, Table 1). Prazosin at any concentration did not significantly change E_{max} for NE. The Schild plot (Fig. 1C) gave a line with a slope of 0.9 ± 0.1 (95% CI: 0.6 - 1.1) that was not different from unity. In DOCA-salt arteries (Fig. 1B, Table 1), prazosin produced similar results. Schild analysis (Fig. 1D) yielded a line with a slope of 0.8 ± 0.08 (95% CI: 0.6 – 1.0), including unity).

NE constricted veins in a concentration-dependent manner (Fig. 2A, Fig. 2B, Table 1). There were no differences between SHAM and DOCA-salt veins in

NE reactivity but veins were 10-30 fold more sensitive than arteries to the constricting effects of NE (Table 1). Prazosin antagonized NE-induced constrictions in both SHAM (Fig. 2A, Table 1) and DOCA-salt (Fig. 2B, Table 1) veins. However, prazosin effects on NE concentration response curves in veins differed markedly from the effects seen in arteries. In SHAM veins, prazosin did not produce evenly spaced rightward shifts in the concentration-response curves (Fig. 2A). Similar results were obtained in DOCA-salt veins (Fig. 2B). As a consequence, Schild plots for SHAM veins (Fig. 2C) had a slope of 0.3 ± 0.2 significantly less than 1 (95% CI: 0.2 – 0.8). Similarly, the Schild plot in DOCA-salt veins also had a slope (0.5 ± 0.2) that was significantly less than 1 with a 95% CI between 0.1 – 0.9.

Clonidine and UK-14,304 do not constrict arteries or veins. Clonidine, an α_2-AR agonist, was used to directly test whether or not there are contractile α_2-ARs in smooth muscle cells of murine mesenteric arteries and veins. Clonidine (10^{-7} – 10^{-5} M) caused < 10% maximal constriction in arteries and veins (data not shown). To corroborate the results obtained with clonidine, we also looked at contractile responses in arteries and veins with the α_2-AR agonist UK-14,304 (10^{-7} – 10^{-5} M). UK 14,304 also did not cause more than 10% constriction of arteries or veins (data not shown).

Stimulation of endothelial α_2-ARs by α_2-AR agonists results in endothelium-dependent vasorelaxation (Bockman et al., 1996; Figueroa et al., 2001) that could antagonize any contractile effects of these agonists on vascular smooth muscle. For this reason, contractile responses in arteries and veins to

clonidine and UK-14,304 were determined in the presence of the cyclooxygenase inhibitor, indomethacin (10 μM), or the nitric oxide synthase inhibitor *N*-nitro-L-arginine (NLA; 100 μM) to inhibit endothelial-mediated release of vasodilatory cyclooxygenase derivatives and nitric oxide, respectively. Even in the presence of these inhibitors, contractile responses to clonidine and UK-14,304 were minimal (< 10% constriction; data not shown).

Yohimbine and rauwolscine inhibit α_2-ARs in veins but not arteries. In order to further examine the contractile role of α_2-ARs in mesenteric vessels and to corroborate the results obtained with the α_2-AR agonists, contractile responses to NE were examined in the absence or presence of α_2-AR selective antagonists. Yohimbine (3, 30, 300 nM) did not alter the resting diameter of arteries and it did not affect NE-induced constrictions of SHAM (Fig. 3A, Table 2) and DOCA-salt (Fig. 3B, Table 2) arteries. However, yohimbine antagonized NE-induced contractile responses in mesenteric veins as shown by the rightward shift in the NE concentration-response curve of SHAM (Fig. 4A, Table 2) and DOCA-salt (Fig. 4B, Table 2) vessels. Nevertheless, yohimbine did not cause concentration-dependent and parallel rightward shifts in the NE concentration response curve. As a consequence, this resulted in non-linear Schild plots in SHAM (Fig. 4C) and DOCA-salt veins (Fig. 4D).

These results were not consistent with our agonist data showing that clonidine and UK-14,304 do not constrict veins. Therefore, it could be argued that the inhibition we observed in veins with yohimbine was due to effects of this antagonist at the α_1-AR. To address this latter possibility, concentration-response

curves to PE, a selective α_1-AR agonist, were obtained in SHAM and DOCA-salt veins in the absence and presence of yohimbine. Yohimbine (30 nM) did not antagonize contractile responses to PE in SHAM (Fig. 5A, Table 3) or DOCA-salt (Fig. 5B, Table 3) veins.

To determine whether the inhibition of NE-induced contraction in veins but not arteries was specific for yohimbine, we examined NE-induced contractile responses in arteries and veins in the absence and presence of rauwolscine (100 nM). As seen with yohimbine, preincubation of SHAM (Fig. 6A, Table 3) and DOCA-salt (Fig. 6B, Table 3) arteries with rauwolscine (100 nM) did not antagonize NE-induced constrictions. However, rauwolscine did cause rightward shifts of NE concentration-response curves of SHAM veins (Fig. 7A, Table 3) and DOCA-salt veins (Fig. 7B, Table 3).

DISCUSSION

α_1-ARs mediate constriction of mesenteric arteries and veins. Prazosin competitively antagonized contractile responses to NE in mesenteric arteries. *In vitro* studies have consistently shown that α_1-ARs predominantly mediate contraction of mesenteric arteries in rat (Hussain and Marshall, 2000) and mouse (Yamamoto and Koike, 2001). α_1-ARs are involved in NE-induced contractile responses in mesenteric veins as well. Prazosin antagonized contractile responses to NE in mesenteric veins as suggested by the rightward shifts in the NE concentration-response curves. These data agree with previous reports demonstrating the involvement of α_1-ARs in contractile responses of rat (Luo et al., 2003) and mouse (Pérez-Rivera et al., 2004) mesenteric veins. It should be noted that Schild analysis revealed a pattern not typical of competitive antagonism suggesting, that perhaps, a single receptor population is not responsible for contractile responses in veins. It could be argued that other factors, such as insufficient time for equilibration, could be a reason for the lack of a competitive antagonism pattern. However, we believe that this is unlikely as arteries were examined under the same protocol used for veins and we were able to demonstrate that prazosin competitively antagonized contractile responses to NE.

It is important to note that we did not find any differences in adrenergic reactivity between DOCA-salt arteries and veins compared to their SHAM counterparts. This is in contrast to the studies by Luo et al. (2003) who showed a decreased reactivity of DOCA-salt veins but no difference in reactivity between

SHAM and DOCA-salt arteries. Other studies performed in DOCA-salt rats have found that mesenteric arterial adrenergic reactivity is enhanced (Longhurst et al., 1988; Perry and Webb, 1988). Potential reasons for the discrepancies seen are differences in size of the vessels studied or the different methods used to assess vascular reactivity. It should also be noted that despite significant increases in blood pressure in mice subjected to DOCA and salt treatment, the degree of hypertension in mice is much less than that reported for rats (Johns et al., 1996). Therefore, increases in blood pressure seen in DOCA-salt mice may have not been large enough to alter vascular adrenergic reactivity.

Indirect contribution of α_2-ARs to constriction in veins but not arteries. Clonidine and UK 14,304 did not constrict mesenteric arteries or veins from SHAM and DOCA-salt mice. These data agree with our previous results in murine mesenteric arteries and veins (Pérez-Rivera et al., 2004) where we also showed an inability of α_2-AR agonists to stimulate a contractile response. Similar results have been obtained in rat mesenteric vessels (Luo et al., 2003).

These results suggest that α_2-ARs do not contribute to NE-induced constrictions of murine mesenteric arteries or veins. Specifically, our data suggest that contractile responses of mesenteric arteries and veins are not mediated by direct stimulation of α_2-ARs. However, a vasoconstrictor role for α_2-ARs has been shown in other blood vessels (Civantos Calzada and Aleixandre de Artinano, 2001). McCafferty et al. (1999) showed that in the pithed mouse, the α_{2B}-AR mediates pressor responses to α_2-AR agonists. Alternatively, it could be that α_2-AR contractile mechanisms may be active *in vivo* but not *in vitro*.

We further clarified the role played by α_2-ARs in NE-induced contractile responses by examining concentration-response curves in the absence or presence of the α_2-AR antagonist, yohimbine. Yohimbine did not antagonize contractile responses to NE in arteries from SHAM or DOCA-salt mice. This was in complete agreement with data obtained with the α_2-AR agonists. It appears that in murine mesenteric arteries, α_2-ARs are not involved in contractile responses to NE.

α_2-ARs contribute to NE-induced venous contractile responses as yohimbine competitively antagonized NE responses. These results were puzzling in light of the failure of clonidine and UK-14,304 to constrict veins. However, the high potency of yohimbine as an antagonist, as well as the fact that yohimbine did not antagonize PE responses, suggests that yohimbine-mediated inhibition of NE-induced constrictions is due to selective antagonism of α_2-ARs. In addition, inhibition of venous but not arterial contractile responses by rauwolscine confirms the involvement of α_2-ARs in NE-induced contractility of mesenteric veins. The lack of rauwolscine antagonism in arteries provides further evidence that α_2-ARs are not involved in contractile responses to NE in arteries.

Increased reactivity of murine mesenteric veins to adrenergic stimulation; are α_2-ARs the cause? The fact that α_1- and α_2-ARs contribute to NE-induced venous constriction while arteries employ just α_1-ARs could have important physiological consequences for control of vasomotor function. In these studies we were able to corroborate that veins were more sensitive than arteries

to the contractile effects of NE. Data from the present study suggest that a contribution of α_2-ARs to NE-induced constriction of veins but not arteries is an additional factor that contributes to greater noradrenergic reactivity of veins compared to arteries. However, we could not discard at this point a differential role of α_1-ARs as veins were also more sensitive to the contractile effects of PE, a selective α_1-AR agonist. This was in agreement with previous studies in rats (Luo et al., 2003) and mice (Pérez-Rivera et al., 2004) that showed an increased reactivity of mesenteric veins to stimulation by exogenous adrenergic agonists.

Is there a crosstalk between α_1- and α_2-ARs? Agonists at α_2-ARs did not constrict veins but antagonism of α_2-ARs inhibited contractile responses to NE. These results suggest that in order to see a contribution of α_2-ARs to contractile responses in veins, co-activation of both α_1- and α_2-ARs is necessary. Similar cross-talk mechanisms have been described in heterologous expression systems for α_1-ARs and specifically, the α_{2A}-AR subtype (Reynen et al., 2000). In those studies, NE did not increase $[Ca^{++}]_i$ in chinese hamster lung fibroblast cells which express α_1-ARs. However, NE increased $[Ca^{++}]_i$ in these cells when they were transfected with the α_{2A}-AR. NE stimulatory effects were antagonized by subtype selective concentrations of both α_1- and α_2-AR antagonists. Selective agonists of α_1- and of α_2-ARs did not have any effect on $[Ca^{++}]_i$ release but when added together induced a robust stimulation of $[Ca^{++}]_i$. As Reynen et al. (2000) stated, this phenomenon could be of physiological importance in vascular smooth muscle cells (in particular venous smooth muscle cells) that have been consistently found to express functional α_1- and α_2-ARs. Our data provide

evidence that this functional interaction can occur in cells (venous smooth muscle cells) that normally co-express α_1- and of α_2-ARs and express the receptors at physiologically relevant levels. This is not always the case in heterologous expression systems.

An important question is how this receptor interaction might occur. Is it due to a direct physical interaction between α_1- and α_2-ARs or an interaction involving the signaling cascades activated by both receptors? It is known that α-ARs could interact with other receptor systems in ways that are receptor-specific. For example, in mouse atria, angiotensin and bradykinin receptors interact with α_2-AR (Cox et al., 2000; Trendelenburg et al., 2003). It appears that protein kinase C is involved in this interaction as determined by experiments in hearts of newborn rats (Mota and Guimaraes, 2003). More detailed experiments are necessary to determine whether or not α_1- and α_2-ARs could actually interact with each other and what are the molecular mechanisms behind this apparent crosstalk between α_1- and α_2-AR in mesenteric veins.

Conclusions. We have provided pharmacological evidence that there are different α-AR contractile mechanisms in murine mesenteric arteries and veins: α_1-ARs mediate constriction in arteries and veins whereas α_2-ARs do so in veins but not arteries. This difference in adrenoceptor contractile mechanisms could explain the enhanced responses to sympathetic nervous system activity in mesenteric veins and points to the notion of a possible crosstalk between α_1- and α_2-ARs in veins. Abnormalities in these mechanisms do not appear to participate in the development of DOCA-salt hypertension in mice.

Table 1. Properties of NE concentration response curves in arteries and veins from SHAM and DOCA-salt mice in the absence and presence of prazosin. Data are expressed as mean ± SEM. Numbers in parentheses refer to the number of animals from which the data were obtained. E_{max} is the maximum constriction based on data fitted to a logistic equation. EC_{50} is the negative logarithm of the molar concentration of agonist producing half maximal constriction. *: $p < 0.05$ –vs- control.

	E_{max} (%)		EC_{50} (- log M)	
	ARTERY	*VEIN*	*ARTERY*	*VEIN*
SHAM				
NE (control)	25.7 ± 3.1 (5)	38.8 ± 4.7 (8)	5.7 ± 0.08 (5)	7.2 ± 0.2 (8)
NE/Prazosin (3 nM)	23.9 ± 2.9 (4)	32.6 ± 2.4 (6)	5.1 ± 0.2* (4)	6.3 ± 0.3* (6)
NE/Prazosin (30 nM)	24.0 ± 7.0 (4)	32.0 ± 3.2 (7)	4.5 ± 0.1* (4)	6.1 ± 0.3* (7)
NE/Prazosin (300 nM)	23.4 ± 2.3 (4)	28.8 ± 3.0 (7)	3.5 ± 0.2* (4)	5.7 ± 0.3* (7)
DOCA-salt				
NE (control)	28.1 ± 2.9 (5)	33.3 ± 2.8 (12)	5.8 ± 0.06 (5)	7.4 ± 0.2 (12)
NE/Prazosin (3 nM)	19.4 ± 2.5 (4)	26.2 ± 3.8 (8)	5.1 ± 0.09* (4)	6.3 ± 0.2* (8)
NE/Prazosin (30 nM)	18.6 ± 3.8 (4)	28.5 ± 3.0 (7)	4.5 ± 0.06* (4)	5.8 ± 0.3* (7)
NE/Prazosin (300 nM)	23.8 ± 1.3 (4)	29.8 ± 8.5 (5)	3.7 ± 0.1* (4)	5.4 ± 0.2* (5)

Table 2. Response of mesenteric arteries and veins from SHAM and DOCA-salt mice to NE in the absence or presence of yohimbine. Data are expressed as mean ± SEM. Numbers in parentheses refer to the number of animals from which the data were obtained. E_{max} is the maximum constriction based on data fitted to a logistic equation. EC_{50} is the negative logarithm of the molar concentration of agonist producing half maximal constriction. *: $p < 0.05$ – vs- control.

	E_{max} (%)		EC_{50} (- log M)	
	ARTERY	*VEIN*	*ARTERY*	*VEIN*
SHAM				
NE (control)	25.7 ± 3.1 (5)	38.8 ± 4.7 (8)	5.7 ± 0.08 (5)	7.2 ± 0.2 (8)
NE/Yohimbine (3 nM)	26.8 ± 2.6 (5)	33.9 ± 2.6 (4)	5.8 ± 0.05 (5)	6.4 ± 0.2* (4)
NE/Yohimbine (30 nM)	20.5 ± 2.8 (4)	30.4 ± 4.0 (5)	5.6 ± 0.05 (4)	5.3 ± 0.2* (5)
NE/Yohimbine (300 nM)	28.8 ± 1.1 (4)	31.1 ± 3.1 (6)	5.6 ± 0.06 (4)	5.1 ± 0.3* (6)
DOCA-salt				
NE (control)	28.1 ± 2.9 (5)	33.3 ± 2.8 (12)	5.8 ± 0.06 (5)	7.4 ± 0.2 (12)
NE/Yohimbine (3 nM)	29.5 ± 5.3 (4)	32.8 ± 4.5 (4)	5.7 ± 0.1 (4)	6.4 ± 0.2 (4)
NE/Yohimbine (30 nM)	33.6 ± 6.6 (4)	30.4 ± 2.7 (6)	5.8 ± 0.1 (4)	6.4 ± 0.5 (6)
NE/Yohimbine (300 nM)	35.6 ± 5.1 (4)	26.4 ± 6.1 (5)	5.7 ± 0.09 (4)	5.4 ± 0.7* (5)

Table 3. Response of mesenteric arteries and veins from SHAM and DOCA-salt mice to NE in the absence or presence of rauwolscine and to the selective α_1-AR agonist PE in the absence and presence of yohimbine. Data are expressed as mean ± SEM. Numbers in parentheses refer to the number of animals from which the data were obtained. E_{max} is the maximum constriction based on data fitted to a logistic equation. EC_{50} is the negative logarithm of the molar concentration of agonist producing half maximal constriction. *: $p < 0.05$ –vs- control.

	E_{max} (%)		EC_{50} (- log M)	
	ARTERY	*VEIN*	*ARTERY*	*VEIN*
SHAM				
NE (control)	25.7 ± 3.1 (5)	40.0 ± 3.0 (5)	5.7 ± 0.08 (5)	7.5 ± 0.2 (5)
NE/Rauwolscine (100 nM)	26.1 ± 2.5 (6)	28.7 ± 2.6* (6)	5.7 ± 0.08 (6)	7.0 ± 0.05* (6)
PE (control)	n.d.	36.5 ± 2.8 (9)	n.d.	6.5 ± 0.1 (9)
PE/Yohimbine (30 nM)	n.d.	39.7 ± 3.1 (9)	n.d.	6.6 ± 0.1 (9)
DOCA-salt				
NE (control)	28.1 ± 2.9 (5)	38.9 ± 1.2 (8)	5.7 ± 0.07 (5)	7.2 ± 0.2 (8)
NE/Rauwolscine (100 nM)	25.3 ± 1.4 (5)	24.7 ± 4.0* (7)	5.8 ± 0.04 (5)	6.7 ± 0.1* (7)
PE(control)	n.d.	34.9 ± 5.4 (6)	n.d.	6.6 ± 0.1 (6)
PE/Yohimbine (30 nM)	n.d.	42.2 ± 6.4 (5)	n.d.	6.4 ± 0.03 (5)

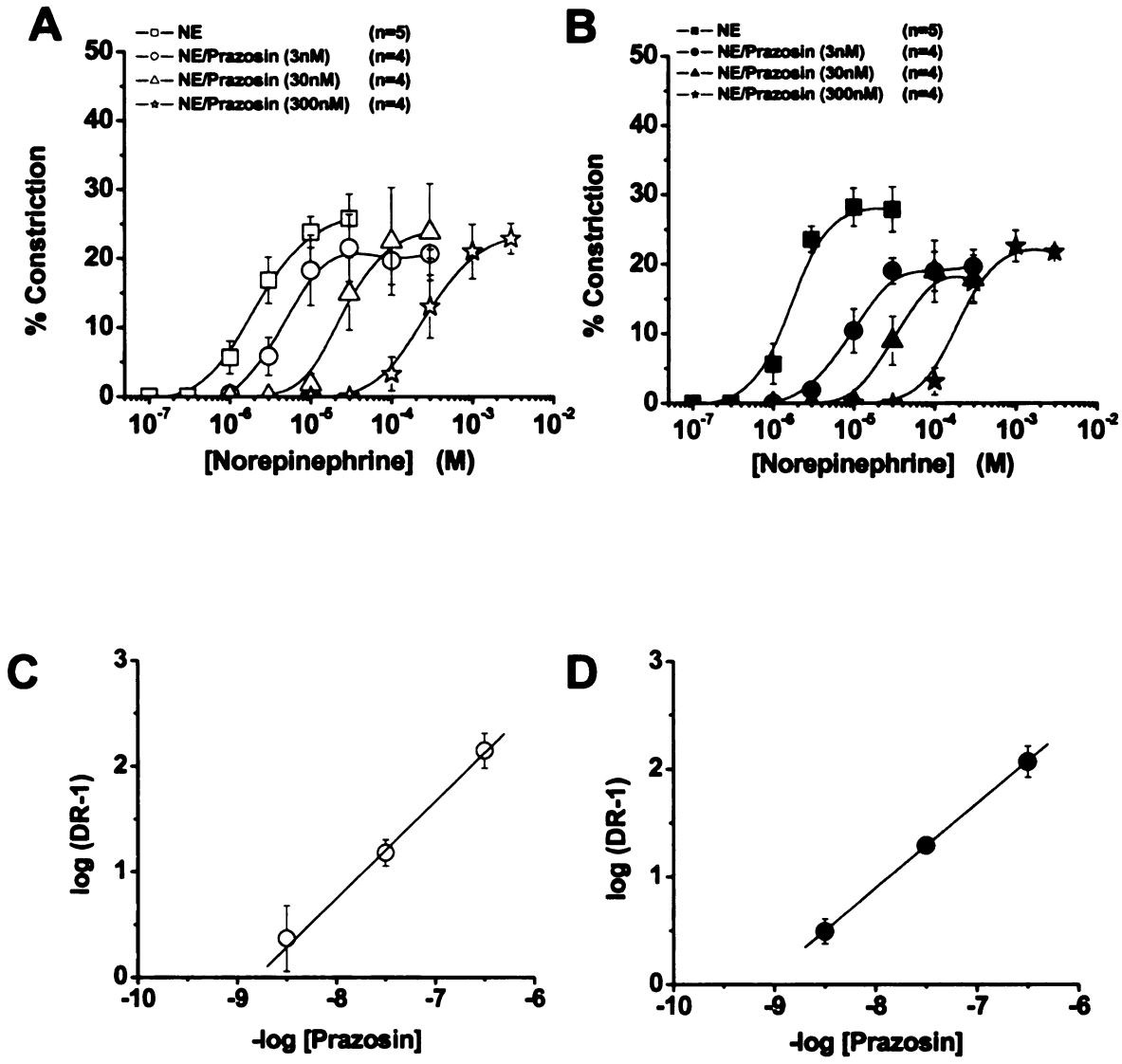

Figure 1. Effect of prazosin on NE-induced constrictions of SHAM (A) and DOCA-salt (B) mesenteric arteries. Prazosin produced concentration-dependent and parallel rightward shifts in the NE-concentration-response curve of SHAM and DOCA-salt arteries with no changes in maximal response among treatment groups. Schild plots for prazosin antagonism of NE-induced contractile responses in SHAM (C) and DOCA-salt (D) mesenteric arteries. Data are mean ± SEM. N indicates the number of animals from which preparations were obtained.

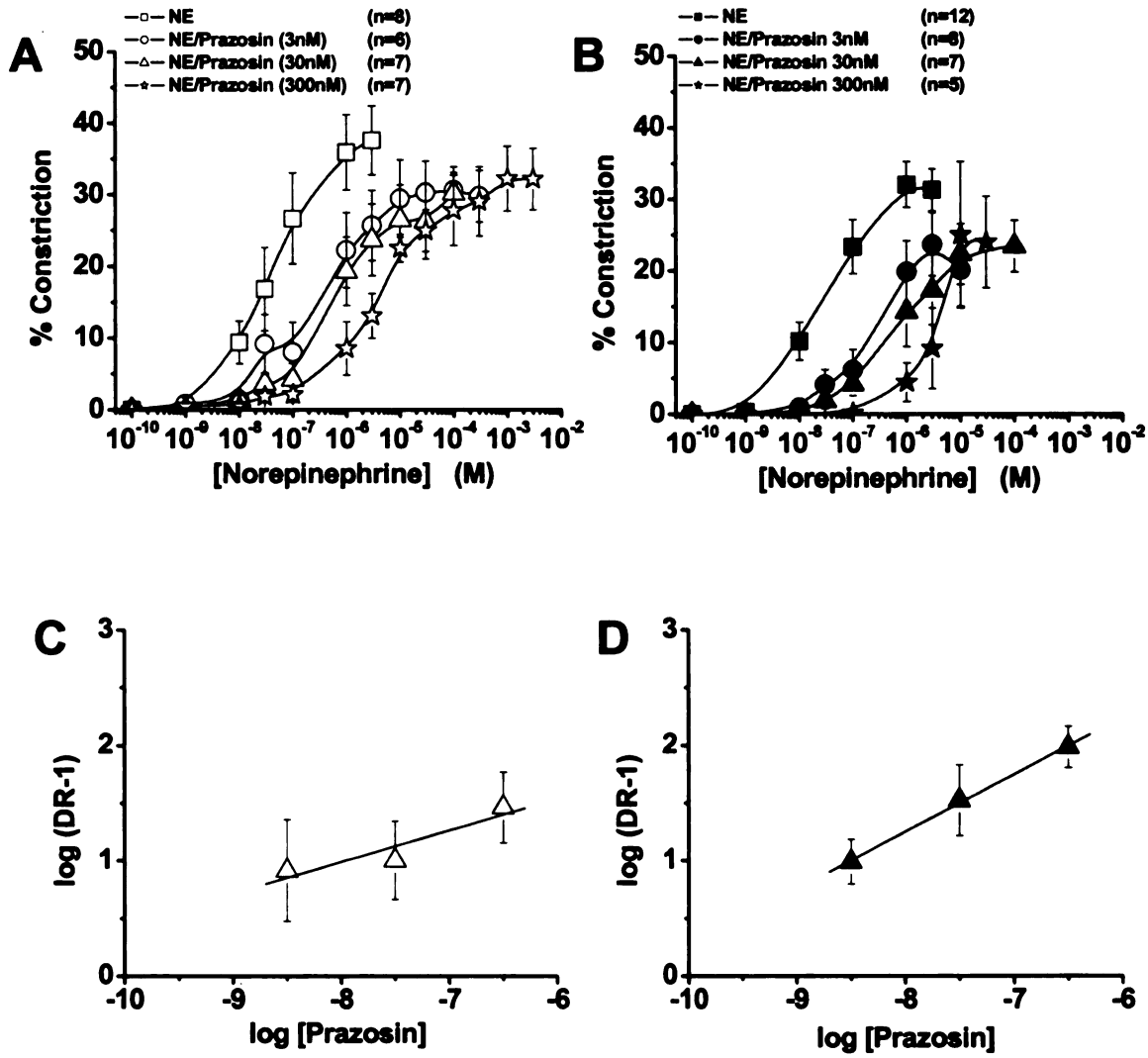

Figure 2. Effect of prazosin on NE- induced constriction of SHAM (A) and DOCA-salt (B) mesenteric veins. All prazosin concentrations produced significant rightward shifts in NE concentration-response curves in SHAM and DOCA-salt veins with no change in maximal response among treatment groups. Schild plots for the prazosin antagonism of NE-induced contractile responses in SHAM (C) and DOCA-salt (D) mesenteric veins. Data are mean ± SEM. N indicates the number of animals from which preparations were obtained.

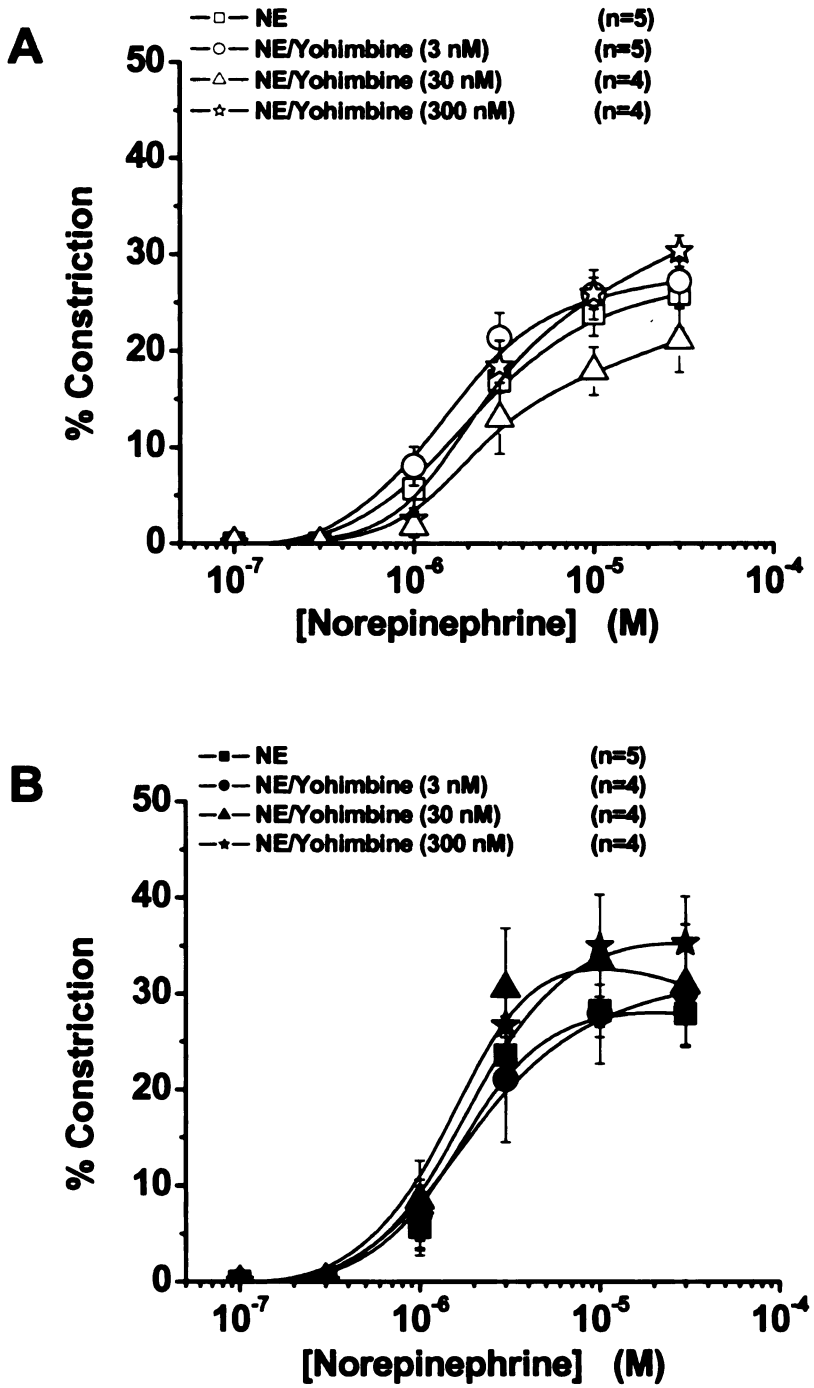

Figure 3. Yohimbine did not affect NE concentration response curves in SHAM (A) or DOCA-salt (B) mesenteric arteries. Data are expressed as mean ± SEM. N indicates the number of animals from which preparations were obtained.

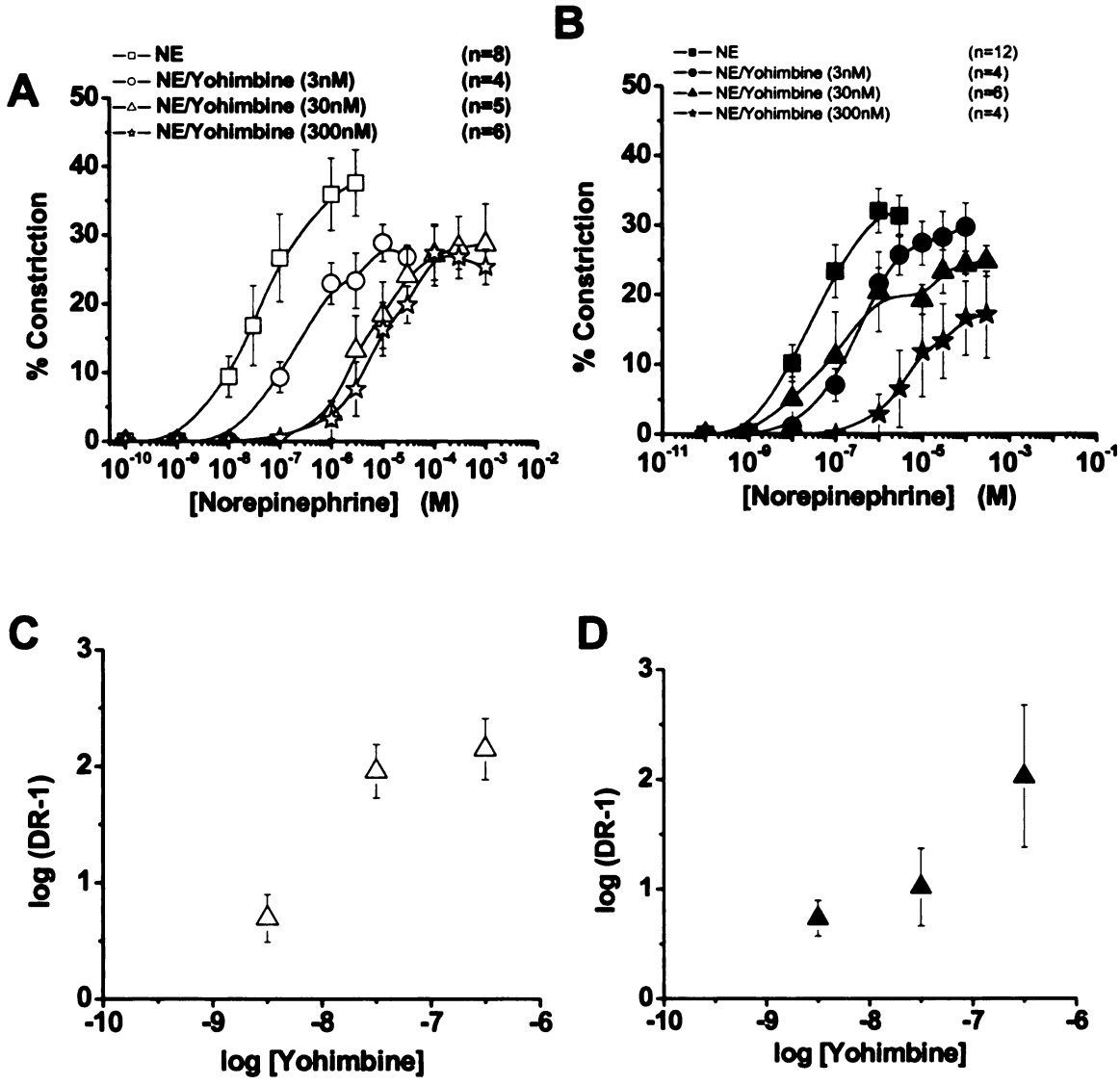

Figure 4. Effect of yohimbine on NE-induced constriction of SHAM (A) and DOCA-salt (B) mesenteric veins. Yohimbine produced a significant rightward shift in the concentration-response curve of SHAM and DOCA-salt veins. Agonist contractile responses are expressed as percentage constriction. Schild plots for the yohimbine antagonism of NE-induced contractile responses in SHAM (C) and DOCA-salt (D) mesenteric veins revealed a non-linear relation. Data are mean ± SEM. N indicates the number of animals from which preparations were obtained.

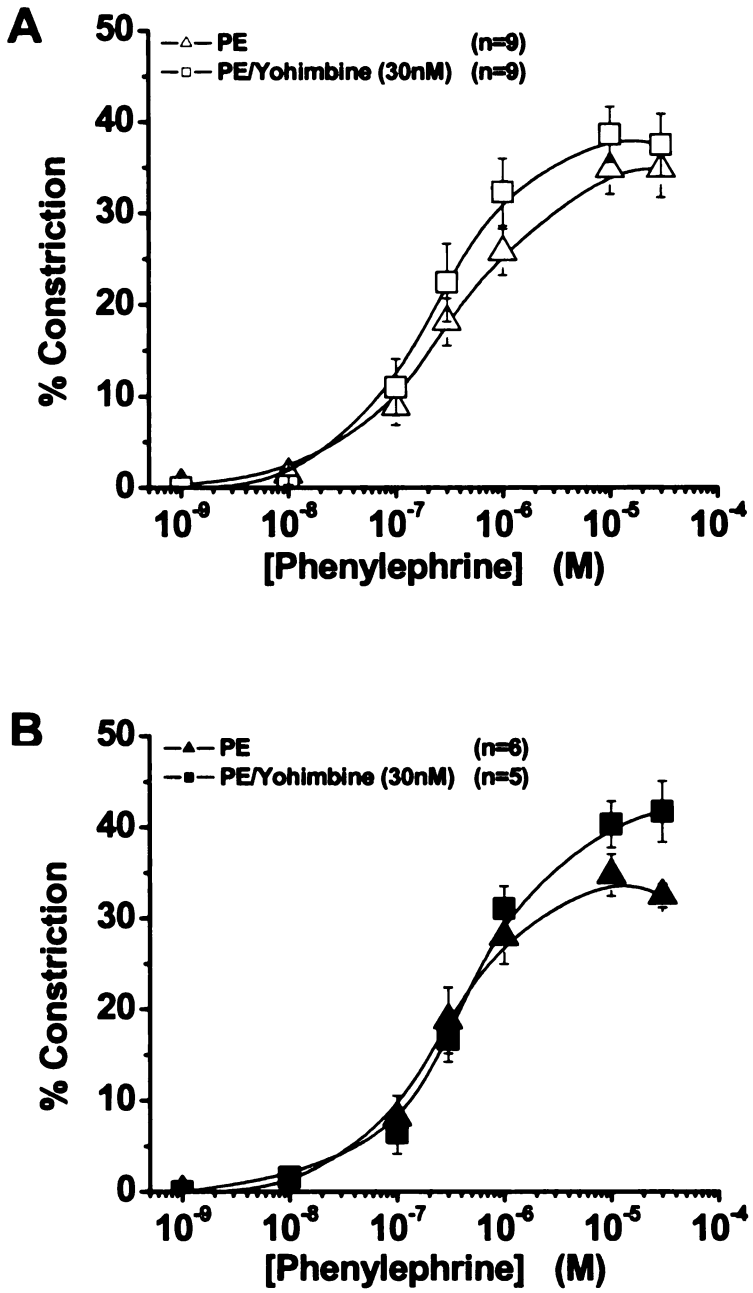

Figure 5. Yohimbine did not affect constrictions induced by phenylephrine (PE) in SHAM (A) or DOCA-salt (B) mesenteric veins. PE responses are expressed as percentage constriction. Data are mean ± SEM. N indicates the number of animals from which preparations were obtained.

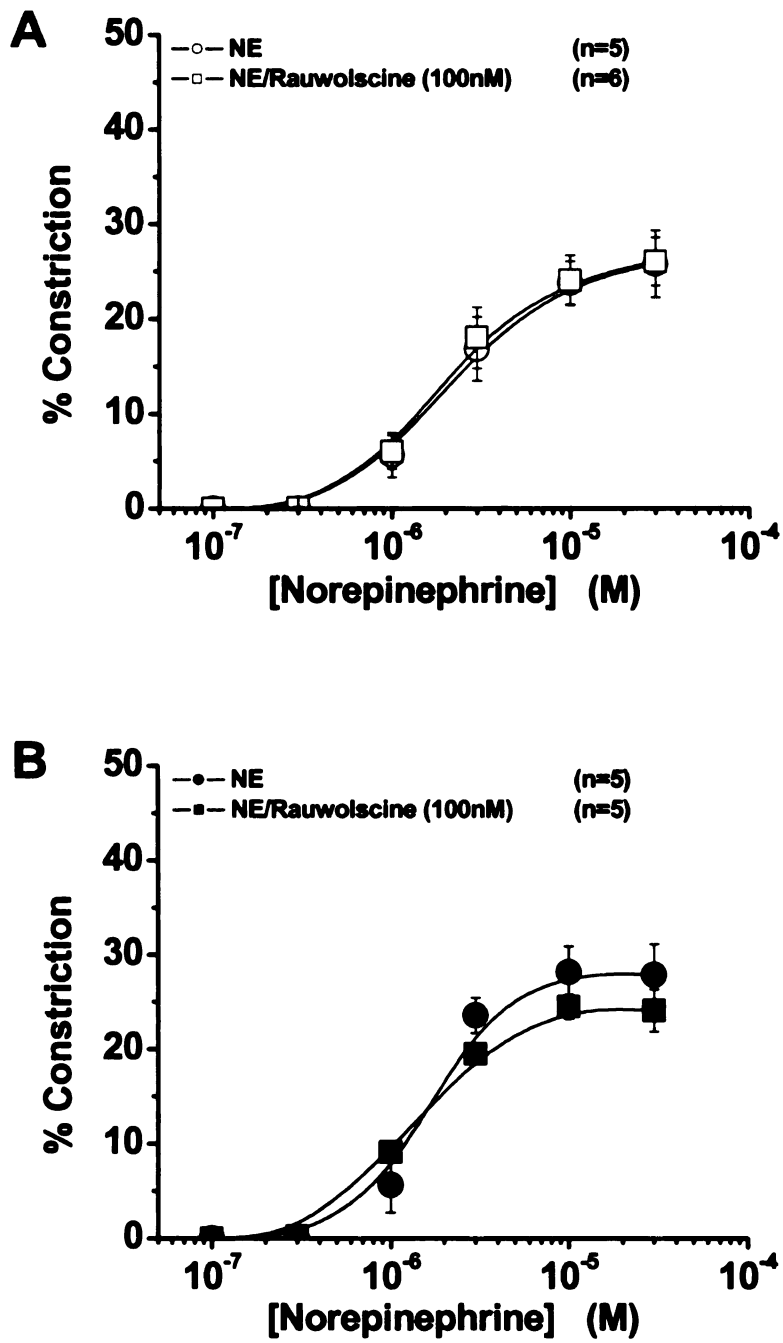

Figure 6. Rauwolscine did not affect NE-induced constriction of SHAM (A) and DOCA-salt (B) mesenteric arteries. NE-induced responses are expressed as percentage constriction. Data are mean ± SEM. N indicates the number of animals from which preparations were obtained.

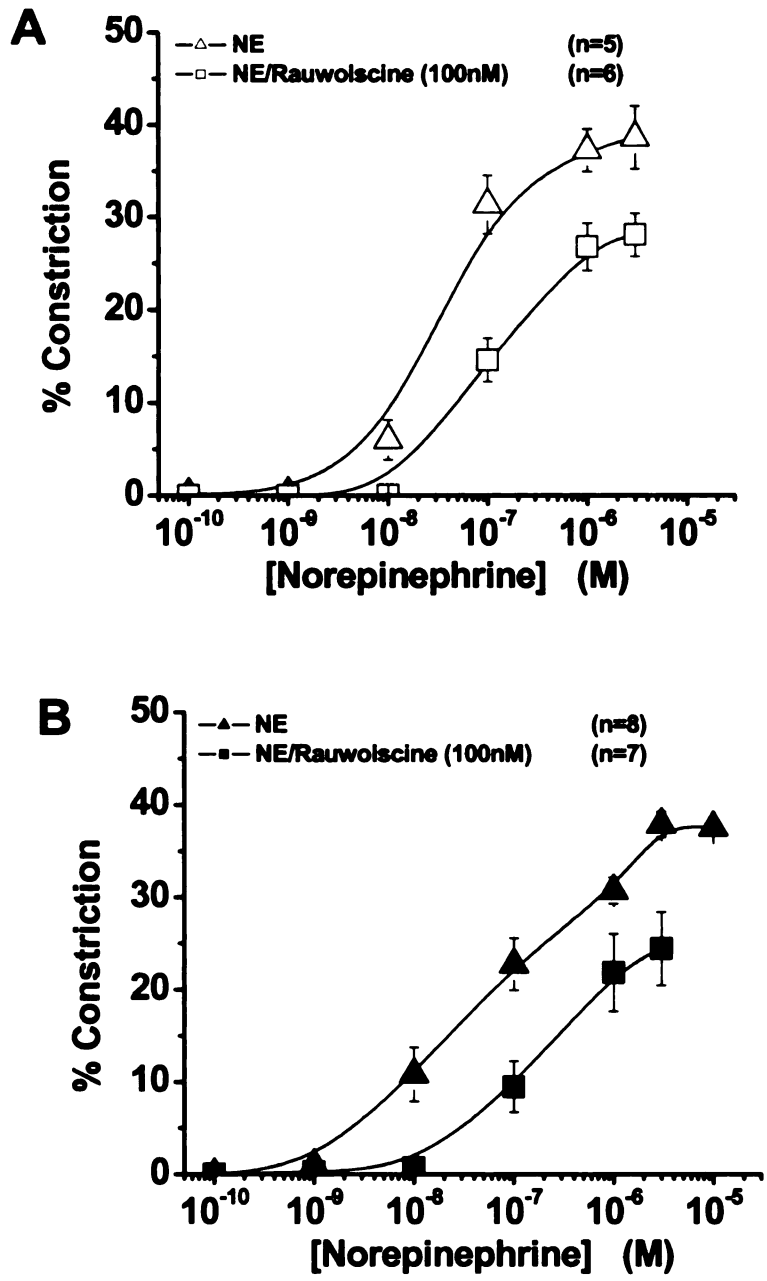

Figure 7. Effect of rauwolscine on NE-induced constriction of SHAM (A) and DOCA-salt (B) mesenteric veins. Rauwolscine produced a significant rightward shift in the concentration-response curve of veins from both treatment groups. Data are expressed as mean ± SEM. N indicates the number of animals from which preparations were obtained.

CHAPTER 6

Alpha-1B Adrenoceptors Mediate Neurogenic Constriction in Mesenteric Arteries of Normotensive and DOCA-salt Hypertensive Mice

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Autonomic Neuroscience: Basic and Clinical

INTRODUCTION

The SNS is an important regulator of systemic blood pressure in health and disease. Most effects of sympathetic activation on effector organs are due to the action of Epi, secreted mostly by the adrenal medulla and NE, released from sympathetic postganglionic fibers in the periphery (Lefkowitz et al., 1990). α_1-ARs are G-protein coupled receptors that mediate the actions of NE and Epi (Guimaraes and Moura, 2001; Piascik and Perez, 2001). α_1-ARs located in vascular smooth muscle cells regulate total peripheral resistance and systemic arterial blood pressure. Three genes encode distinct α_1-AR subtypes (Lomasney et al., 1991; Schwinn et al., 1990). These subtypes are named α_{1A}-, α_{1B}-, and α_{1D}-ARs (Hieble et al., 1995).

Pharmacological studies using receptor subtype-specific antagonists suggest that different α_1-AR subtypes mediate the contractile actions of exogenous catecholamines in different vascular beds. The α_{1A}-AR mediates agonist induced contractions of rat renal (Hrometz et al., 1999) and caudal arteries (Piascik et al., 1997). The α_{1A}-AR also contributes to agonist-induced constrictions of the murine tail and mesenteric arteries (Daly et al., 2002). The α_{1D}-AR mediates contractions of rat femoral (Hrometz et al., 1999; Piascik et al., 1997), iliac (Piascik et al., 1997) superior mesenteric artery (Piascik et al., 1997), and aorta (Piascik et al., 1997). Contractions of murine aorta are predominantly α_{1D}-mediated (Chalotorn et al., 2003; Daly et al., 2002). The α_{1B}-AR appears to play just a minor role in mediating agonist-induced constriction of the rat mesenteric artery (Piascik et al., 1997) and the mouse aorta, mesenteric, carotid

and caudal arteries (Daly et al., 2002). Gene K/O approaches have also been used to complement pharmacological studies of α_1-ARs in blood pressure regulation (Philipp and Hein, 2004). Experiments carried out in α_{1A}- (Rokosh and Simpson, 2002) α_{1B}- (Cavalli et al., 1997) and α_{1D}-AR KO mice (Tanoue et al., 2002b) have demonstrated that all three subtypes contribute to blood pressure regulation.

In this series of studies we specifically studied the relative contribution of α_1-AR subtypes to sympathetic neurogenic vasoconstriction of mesenteric arteries of normotensive and hypertensive mice. We used transmural stimulation to evoke contractile responses in mesenteric arteries in the absence and in the presence of subtype-selective α_1-AR antagonists. We focused on mesenteric arteries because they make a major contribution to total peripheral resistance and blood pressure regulation. Furthermore, there is an increase in sympathetic nerve activity in the DOCA-salt model of high blood pressure (de Champlain, 1990). For this reason, we also looked at adrenergic neurotransmission in mesenteric arteries taken from DOCA-salt hypertensive mice to determine whether there are hypertension-associated changes in the α_1-AR subtypes mediating neurogenic constrictions.

MATERIALS AND METHODS

Animals. C57/BL male mice (25 - 30g) were obtained from Charles River Labs (Portage, MI). Upon arrival at the animal care facility, mice were maintained according to the standards approved by the Michigan State University All-University Committee on Animal Care and Use. Mice were individually housed in clear plastic cages with free access to standard pelleted chow (Harlan/Teklad 8640 Rodent Diet) and tap water. Mice were housed in temperature and humidity-controlled rooms with a 12-h on/off light cycle. Animals were allowed 2-3 days of acclimatization prior to entry into any experimental protocol.

DOCA-salt surgery. Mice were unilaterally nephrectomized under anesthesia using a solution containing ketamine (500 mg/ml) and xylazine (20 mg/ml). Mice (25 – 30g) were injected with 80 μL of the anesthetic. The skin over the left flank was shaved and a 1.5 cm incision was made through the skin and underlying muscle caudal to the rib cage. The left kidney was exteriorized and removed after ligation of the renal artery and vein with 4-0 silk sutures (Ethicon, Inc, Somerville, NJ). The muscle and skin layers were then closed separately with 4-0 silk sutures. A small area between the shoulder blades was shaved and a 1 cm incision was for implanting DOCA pellets that provided a giving dose of 150 mg/kg DOCA. DOCA mice were given water containing 1.5 % NaCl and 0.2% KCl. SHAM mice were also unilaterally nephrectomized, but they did not receive a DOCA pellet and they were given tap water. All mice were placed on standard pelleted rodent chow. After recovery, the mice were housed

under standard conditions for 4 weeks after which systolic BP was determined by the tail-cuff method.

In-vitro preparation of mesenteric vessels. At 4 weeks post-surgery, mice were euthanized with a lethal dose of pentobarbital (50 mg/kg i.p.). The small intestine with its associated mesenteric vessels was removed from euthanized mice and placed in oxygenated (95% O_2, 5% CO_2) Krebs' solution (pH: 7.35 – 7.45) of the following composition (mmol/l): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 11. A segment of the intestine with the associated vessels was removed and pinned flat in a silicone elastomer-lined (Sylgard, Dow Corning, Midland, MI) petri dish. A section of mesentery containing vessels close to the mesenteric border was cut out using fine scissors and forceps. The preparation was transferred to a smaller silicone elastomer-lined recording bath and pinned flat. Second or third-order mesenteric veins or arteries were isolated for study by carefully clearing away the surrounding fat tissue. The recording bath containing the preparation was mounted on the stage of an inverted microscope (Olympus CK-2) and superfused with warm (37°C) Krebs' solution at a flow rate of 7 ml min^{-1}. All preparations were allowed a 20 minute equilibration period during which the vessels relaxed to a stable resting diameter.

Video monitoring of vessel diameter. The output of a black and white video camera (Hitachi model KP-111) attached to the microscope was fed to a PC Vision Plus frame-grabber board (Imaging Technology Inc, Woburn, MA)

mounted in a personal computer. The video images were analyzed using Diamtrak software version 3.5 (http://www.diamtrak.com, Adelaide, Australia).

Transmural stimulation of perivascular nerves. Two silver/silver chloride electrodes connected to a Grass Instruments stimulator (S88) were placed parallel to the longitudinal axis of mesenteric arteries. Parameters for nerve stimulation were the following: 60 stimuli, 1 msec duration of stimuli, frequency from 0.5, 1, 5, 10, 20 and 30 Hz and 150 V. The neurogenic origin of constrictions caused by electrical stimulation was verified in each preparation by demonstrating that a constriction caused by 20 Hz stimulation was blocked by tetrodotoxin (TTX; 0.3 μM). Preparations in which the contraction evoked by an initial 20 Hz stimulation was not blocked by TTX were discarded.

Control (no antagonist) frequency-stimulation curves were obtained in an arterial preparation by measuring stimulation-evoked contractile responses. Then in the same tissues, the contribution of α_1-, P2X, α_2-, α_{1A}, α_{1B}-, and α_{1D}-AR to neurogenic constrictions was assessed by examining frequency-response curves in the presence of either prazosin (0.1 μM), PPADS (10 μM), yohimbine (1 μM) , 5-MU (0.1 μM), L-765,314 (1 μM) and BMY-7378 (0.3 μM); selective antagonists at α_1-, P2X, α_2-, α_{1A}, α_{1B}-, and α_{1D}-AR, respectively. Preparations were pretreated for 20 minutes with the antagonist before starting the second frequency-response curve and tissues were exposed to the antagonist throughout the experimental procedure. Only one antagonist was tested in a single arterial preparation.

Glyoxylic acid fluorescence histochemical method. One-centimeter segments of mesenteric arteries were taken from the mesentery of SHAM and DOCA-salt mice. Fat and connective tissue were carefully removed. Tissue samples were incubated for 15 minutes in a 2 % glyoxylic acid in 0.1 M phosphate buffer solution. Following incubation, vessel segments were stretched on microscope slides and were dried in an oven (TempCon Oven, Baxter Scientific Products) for 10 minutes at 80°C, mounted in mineral oil, and coverslipped. Samples were immediately examined and photographed under epifluorescent illumination using a Nikon fluorescence microscope equipped with filter cubes. Five fields per specimen were photographed (40x magnification) for analysis.

Measurement of NE levels in mesenteric arteries. One centimeter segments of mesenteric arteries were obtained from SHAM and DOCA-salt mice. After all fat and connective tissue were removed carefully, tissue samples were placed in microtubes with 50 μL 0.1 N perchloric acid and stored in a freezer at -80°C until assayed. Samples were centrifuged for 10 seconds in order to collect a tissue pellet. Tissue pellets were then sonicated using a tissue sonicator (Heat Systems-Ultrasonic, Plainview, NY) in order to disperse the tissue into the supernatant. Samples were re-centrifuged for 30 seconds to separate supernatant from the protein pellet. The supenatant was drawn up with a microsyringe and brought to a final volume of 65 μL with perchloric acid and transferred into another set of microtubes prior to HPLC analysis. NE content was measured using HPLC with electrochemical detection. Supernatant (50 μL)

was injected into a C-18 reverse-phase analytical column (5-μm spheres; 250×4.6 nm; Biophase ODS, Bioanalytical Systems), which was protected by a precolumn cartridge filter (5-μm spheres; 30×4.6 nm). The HPLC column was coupled to a single colorimetric electrode-conditioning cell in series with dual-electrode analytical cells. The conditioning electrode potential was set at +0.4 V; the analytical electrodes were set at +0.12 and −0.31 V, respectively, relative to the reference electrodes. The HPLC mobile phase consisted of 1.0 M phosphate–citrate buffer, pH 2.7, with 0.1 mM EDTA, 0.35% sodium octylsulfate and 20% methanol. The amount of NE in the samples was determined by comparing peak heights (determined by a Hewlett Packard Integrator, model 3393A) with those obtained from standards ran on the same day. To the remaining tissue pellets, 200 μL 1.0 N NaOH was added. The protein content of the tissue pellet was measured using the method of Lowry et al. (1951).

Data analysis. Constrictions of blood vessels caused by sympathetic nerve stimulation in the absence and presence of receptor-specific antagonists are expressed as percentage reduction from the resting diameter. Half maximal effective stimulation frequency (S_{50}) and maximum response (E_{max}) were calculated from a least-squares fit of individual frequency-response curves using the following logistic function from Origin 7.0 (Microcal Software, Inc, Northampton, MA):

$$Y = \{(E_{min} - E_{max})/[1 + (x/S_{50})^n]\} + E_{max}$$

where E_{min} is the minimum response (set at 0), n is the slope factor. Data are expressed as mean ± SEM. Statistical differences between groups were

assessed by Student's two-tailed paired t-test. When more than two groups were compared, analysis of variance (ANOVA) was used with Student-Newman-Keuls multiple comparison as a post test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad InStat for Windows 95 (GraphPad Software, San Diego, CA).

RESULTS

General. Four weeks after the start of DOCA-salt treatment, systolic blood pressure in DOCA-salt (n=57) mice was higher than systolic blood pressure in SHAM (n=50) mice (143.0 ± 2.0 mmHg –vs- 108.5 ± 3.3 mmHg, respectively; $p < 0.05$). The inner diameter of mesenteric arteries from SHAM and DOCA-salt mice was 159.9 ± 3.4 μm and 162.7 ± 4.1 μm, respectively ($p > 0.05$).

Adrenergic contribution to neurogenic constrictions of mesenteric arteries. Frequency-response curves were obtained in the absence and in the presence of prazosin (0.1 μM) or in the absence or presence of prazosin (0.1 μM) with the P2X receptor antagonist PPADS (10 μM). Electrical field stimulation produced frequency-dependent constrictons of SHAM arteries that were inhibited by prazosin (Fig. 1A, Table 1). Subsequent addition of PPADS to the prazosin-containing solutions did not produce further reductions in neurogenic constrictions (Fig. 1A, Table 1). Furthermore, PPADS alone did not alter neurogenic responses in SHAM arteries (Fig. 1C, Table 1).

Control neurogenic responses in DOCA-salt arteries exhibited a frequency-dependency pattern as well (Fig. 1B, Table 2). Prazosin inhibited neurogenic contractile responses in these vessels (Fig. 1B, Table 2). In the presence of prazosin and PPADS, there was a non-significant tendency for a further inhibition of E_{max} (Fig. 1B, Table 2). PPADS alone did not alter neurogenic contractile responses in DOCA-salt mesenteric arteries (Fig. 1D, Table 2).

Prejunctional α_2-ARs in mesenteric arteries. We looked at neurogenic-mediated contractile responses in mesenteric arteries from SHAM and DOCA-

salt mice in the absence or presence of the selective α_2-AR antagonist yohimbine (1 μM). Yohimbine potentiated neurogenic constrictions in both SHAM (Fig. 2A, Table 1) and DOCA-salt (Fig. 2B, Table 2) as indicated by significant leftward shifts in the frequency-response curves. However, yohimbine did not change E_{max} values.

α_1-AR subtypes mediating neurogenic constrictions in mesenteric arteries. Contribution of the α_{1A}-AR subtype to sympathetic-mediated constriction was assessed in the absence or presence of 5-MU (0.1 μM), a selective α_{1A}-AR antagonist. In SHAM arteries, 5-MU produced a small, but significant, reduction in the E_{max} (Fig. 3A, Table 1). In contrast, neurogenic responses in DOCA-salt arteries were not changed by 5-MU (Fig. 3B, Table 2). L-765,314 (1 μM), a selective α_{1B}-AR antagonist, also reduced E_{max} values in SHAM (Fig. 4A, Table 1) and DOCA-salt (Fig. 4B, Table 2) arteries.

Frequency-response curves in SHAM (Fig. 5A, Table 1) and DOCA-salt (Fig. 5B, Table 2) arteries preincubated with the selective α_{1D}-AR antagonist, BMY-7378 (0.3 μM), were not changed compared to control curves. This provided pharmacological evidence that the α_{1D}-AR is not involved in contractile responses to endogenously released NE in mesenteric arteries from both SHAM and DOCA-salt mice.

Catecholamine fluorescence in mesenteric arteries. Glyoxylic acid induced fluorescence of neuronal stores of catecholamines was used to evaluate the disposition of sympathetic nerves associated with mesenteric arteries from SHAM and DOCA-salt mice. There was a dense network of noradrenergic nerve

fibers in SHAM (Fig. 6A) and DOCA-salt (Fig. 6B) arteries but there was no difference in adrenergic nerve density or pattern of distribution.

NE content in SHAM and DOCA-salt arteries. HPLC with electrochemical detection was used to measure NE content of SHAM and DOCA-salt arteries. Analysis showed that there were no differences in NE content when DOCA-salt hypertensive arteries were compared to their respective SHAM controls (Fig. 7).

DISCUSSION

Neurogenic constrictions in SHAM and DOCA-salt mesenteric arteries. The present data show that in SHAM arteries prazosin blocked neurogenic constrictions while PPADS did not alter these responses. These results suggest that NE is the dominant vasoconstrictor transmitter released by periarterial sympathetic nerves in normotensive mice. In DOCA-salt arteries, prazosin also inhibited neurogenic constrictions. While co-application of prazosin and PPADS produced a somewhat greater inhibition of contractile responses in DOCA-salt arteries this effect was not statistically significant. In addition, PPADS alone did not affect neurogenic responses in DOCA-salt arteries suggesting that there is not a substantial purinergic contribution to neurogenic responses under our experimental conditions.

Several studies have shown that NE and ATP are co-released from sympathetic nerves associated with mesenteric arteries in guinea-pig (Bobalova and Mutafova-Yambolieva, 2001), rabbit (Starke et al., 1991; von Kugelgen and Starke, 1985) and rat (Donoso et al., 1997). Our studies in murine mesenteric arteries suggest that ATP is not involved in sympathetic neurotransmission. In agreement with our results in mesenteric arteries, it was found that in the guinea-pig mesenteric artery (Smyth et al., 2000), in the rat femoral resistance arteries (Zacharia et al., 2004), and in the human gastroepiploic artery (Fukui et al., 2005), NE exclusively mediates the contractile response to sympathetic nerve stimulation.

It is been suggested that at least in rat mesenteric arteries there is a differential contribution of the purinergic and adrenergic components to neurogenic responses: the P2X receptor-mediated constriction dominates in small mesenteric arteries like the ones examined in these studies whereas the adrenergic constriction dominates in the larger arteries (Gitterman and Evans, 2001). This rat-mouse difference in the relative contributions of adrenergic and purinergic components to neurogenic constrictions should not come as a surprise as these two species differ considerably in many cardiovascular parameters related to autonomic nerve activity.

However, a recent study by Vial and Evans (2002) looked at the purinergic contribution to vasoconstriction of mouse mesenteric arteries. In their studies, PPADS reduced the nerve stimulation-evoked constriction of mesenteric arteries by approximately 50% while in $P2X_1$ receptor-deficient mice vasoconstriction induced by nerve stimulation was unaffected by PPADS leading the authors to conclude that a purinergic component is partly responsible for the contractile responses upon nerve stimulation. It looks that at least differences seen in this study were not due to a rat-mouse difference as far as the relative contribution of the P2X receptor is concerned.

However, experimental conditions were somewhat different and could help explain the differences seen. Their trains of electrical field stimulation (100 pulses at 10 Hz, 50 V, 0.25 ms pulse width) were different from ours (60 stimuli, 150 V, 1 ms pulse width, frequency from 0.5, 1, 5, 10, 20 and 30 Hz). It could be concluded that at least under our experimental conditions a purinergic

contribution to contractile responses of mice mesenteric arteries could not be uncovered but probably under another set of conditions it could be suggested that ATP plays a role as a cotransmitter with NE. More detailed studies looking specifically at the purinergic component of sympathetic transmission are definitely a possibility for future studies.

Prejunctional α_2-ARs mediate negative feedback inhibition of NE release. Presynaptic α_2-ARs regulate NT release via a negative feedback mechanism (Langer, 1974). We tested for a role of these receptors in neurogenic constrictions in mice by comparing frequency-response curves in the absence or presence of the selective α_2-AR antagonist yohimbine. Our data show that α_2-ARs mediate negative feedback inhibition of NE as blockade of these receptors potentiated neurogenic constrictions in mesenteric arteries. Potentiation of responses was seen in SHAM and DOCA-salt arteries suggesting presynaptic α_2-AR function is not impaired in this animal model. There is impaired function of prejunctional α_2-ARs in the mesenteric vasculature of DOCA-salt rats (Luo et al., 2004; Tsuda et al., 1989).

Despite significant increases in blood pressure in DOCA-salt mice, the degree of hypertension is less than that occurring in rats as also shown by others (Johns et al., 1996). Therefore, increases in blood pressure seen in DOCA-salt mice may have not been large enough to result in dysfunction of presynaptic α_2-ARs. However, whether the dysfunction of presynaptic α_2-ARs in hypertension is a pressure-dependent effect is not known.

α_{1B}-ARs mediate neurogenic constriction of mesenteric arteries. In the present study, we show that the α_{1B}-AR and to a lesser extent the α_{1A}-AR mediate neurogenic constrictions of the normotensive mouse mesenteric artery. This conclusion is based on the observation that the α_{1B}-AR antagonist, L-765,314 and the α_{1A}-AR antagonist, 5-MU, inhibited neurogenic constrictions while these responses were unaffected by BMY-7378, a selective α_{1D}-AR antagonist. Our results obtained using a pharmacological approach complement those of Townsend et al. (2004) who used a genetic approach to determine the adrenoceptor subtype mediating vascular sympathetic neurotransmission. They found that contractile responses elicited by nerve stimulation *in vitro* were markedly depressed in α_{1B}-AR KO mice. This was not due to a generalized decrease in adrenergic reactivity as contractile responses to exogenous NE were similar in α_{1B}-AR KO and control mice.

Previous work has shown that α_{1A}-ARs largely mediate exogenous agonist-induced contractions of murine mesenteric arteries while α_{1B}-ARs make a minor contribution (Daly et al., 2002). In this study, we found that α_{1B}-ARs are largely responsible for neurogenic constrictions with little or no contribution from α_{1A}-ARs. Altogether, this suggests that exogenous and nerve-released NE act at different receptor populations to constrict mesenteric arteries in mice. Differential vascular responses to α_1-AR agonists could result from these receptors being present mainly at junctional vs. extrajunctional sites (Mallard et al., 1992; Vargas et al., 1994; Yang XP and Chiba S, 2001; Zacharia J et al., 2004). This could be a factor determining the α_1-AR subtype involved in neurogenic versus agonist-

induced vasoconstriction. In rat femoral arteries, Zacharia et al. (2004) showed that α_{1A}-ARs mediate constrictions caused by exogenous and nerve-released NE whereas α_{1D}-ARs are activated only by nerve-released NE. Yang and Chiba (2001) showed that in perfused canine splenic arteries, NE released by sympathetic nerves acts at α_{1B}- and to a lesser extent α_{1D}-ARs. Exogenously administered NE, on the other hand, produced its contractile effects *via* an action at α_{1A}-ARs.

It is also important to note that hypertension may be associated with changes in α_1-AR expression at neuroeffector junctional vs. extrajunctional sites. Our study has shown that in SHAM arteries 5-MU, the α_{1A}-AR antagonist, reduced neurogenic constrictions while these responses were unaffected by 5-MU in DOCA-salt arteries. This result is consistent with previous findings from our group that demonstrated downregulation of α_{1A}-AR in mesenteric arteries from DOCA-salt mice. The physiological significance of these changes remains to be elucidated.

In addition, the physiological significance of the findings pointing to heterogeneity of α_1-AR subtypes at junctional vs. extrajunctional sites is not yet clear. One possibility is that nerve-released and circulating catecholamines modulate vascular tone by acting at different α_1-AR subtypes and at different sites. The possibility that α_{1B}-ARs and, to a lesser extent, the α_{1A}-AR are present at the mesenteric arterial sympathetic neuroeffector junction where they mediate the effects of nerve-released NE strongly agree with our findings and those of Townsend et al. (2004). Whether the different α_1-AR subtypes differ significantly

in their affinity for NE and if that is an important factor determining junctional vs. extrajunctional localization is worth exploring. For example, low affinity receptors would be localized to the junction where the concentration of nerve-released NE would be expected to be the highest while high affinity receptors would be extrajunctional where the "spillover concentration" of NE from the junction would be low.

DOCA-salt hypertension does not change sympathetically-mediated neurogenic constriction. Our studies showed that frequency-response curves of mesenteric arteries to sympathetic nerve stimulation were not different between SHAM and DOCA-salt mice. This contrasts with data obtained in the mesenteric and tail arteries of SHRs (Muir and Wardle, 1989) and in the mesenteric arteries of DOCA-salt rats (Luo et al., 2003; Tsuda et al., 1989) where it was shown that responses to electrical stimulation were greater in hypertensive vessels compared to controls. This may be due to species differences in sympathetic neuroeffector transmission between in the mouse and the rat. Alternatively, the increases in blood pressure in DOCA-salt mice may be insufficient to alter vascular adrenergic reactivity.

We have previously shown a downregulation of the α_{1A}-AR in murine DOCA-salt arteries as assessed by Western blotting. In these studies we showed that despite a downregulation of α_{1A}-ARs in DOCA-salt arteries, as we have seen before, neurogenic reactivity in these vessels is not compromised. However, by Western blotting we could not distinguish between membrane-bound and intracellular α_{1A}-ARs. These leads to the possibility there is a selective alteration

in the ratio of intracellular versus membrane-bound receptors in DOCA-salt arteries. It could also be argued that sympathetic constriction is not compromised in DOCA-salt hypertension because of a greater affinity of NE for the remaining receptors. It is also possible that there is increased activation of post receptor events in DOCA-salt arteries.

However, the lack of a differential neurogenic effect seen in murine DOCA-salt arteries is supported by our anatomical data showing the density of adrenergic nerve fibers was not different between tissues from SHAM and DOCA-salt mice. In addition, no differences were seen in the NE content of mesenteric arteries from SHAM and DOCA-salt mice. Although release was not measured directly in these studies, our data suggest that NE release from sympathetic nerves associated with mesenteric arteries in DOCA-salt mice is not altered compared to that in SHAM mice.

Conclusions. NE mediates sympathetic neurotransmission in murine SHAM mesenteric arteries by acting at α_{1B}-AR and to a lesser extent α_{1A}-AR. In DOCA-salt arteries, neurogenic constrictions are mediated by α_{1B}-ARs. The α_{1D}-AR does not play a significant role in neurogenic constrictions of both SHAM and DOCA-salt arteries. No evidence was found for ATP acting as a NT in arteries from SHAM or DOCA-salt mice. No changes in neurogenic adrenergic reactivity were seen in arteries taken from DOCA-salt hypertensive mice. These functional findings agree with the fact that there was no difference in the adrenergic nerve fiber innervation or in the NE content in arteries from both treatment groups. These studies point to the possibility of junctional versus extrajunctional

localization of α_1-AR subtypes. The physiological significance and whether or not this differential localization of α_1-AR subtypes, as determined by functional studies, could be exploited clinically remains to be considered.

Table 1. Maximal response (E_{max}) and half-maximal stimulation frequency (S_{50}) in mesenteric arteries from SHAM control mice in the absence (control) and presence of prazosin, PPADS, yohimbine, 5-methylurapidil, L-765,314 and BMY-7378; selective antagonists at α_1-, P_2, α_2-, α_{1A}, α_{1B}-, and α_{1D}-AR, respectively. Data are expressed as mean ± SEM. Numbers in parentheses refer to the number of animals from which the data were obtained. *: $p < 0.05$ –vs- control.

	E_{max} (%)	S_{50} (Hz)
SHAM		
Control	21.0 ± 2.7 (10)	11.2 ± 1.0 (10)
Prazosin (0.1 μM)	5.3 ± 1.7* (5)	6.4 ± 1.3 (5)
Prazosin (0.1μM)/PPADS (10 μM)	6.5 ± 2.6* (5)	8.8 ± 2.0 (5)
Control	16.7 ± 2.1 (5)	10.1 ± 1.1 (5)
PPADS (10 μM)	17.7 ± 1.9 (5)	8.6 ± 1.1 (5)
Control	21.4 ± 2.2 (5)	11.3 ± 0.8 (5)
Yohimbine (1 μM)	16.2 ± 2.3 (5)	5.6 ± 1.2* (5)
Control	19.7 ± 1.6 (11)	9.9 ± 1.3 (11)
5-methylurapidil (0.1 μM)	15.2 ± 1.4* (11)	8.4 ± 1.6 (11)
Control	17.7 ± 1.3 (4)	8.1 ± 0.3 (4)
L-765,314 (1 μM)	10.3 ± 1.6* (4)	6.9 ± 1.1 (4)
Control	18.6 ± 2.5 (6)	9.6 ± 1.0 (6)
BMY-7378 (0.3 μM)	19.1 ± 2.0 (6)	8.3 ± 1.7 (6)

Table 2. Maximal response (E_{max}) and half-maximal stimulation frequency (S_{50}) in mesenteric arteries from DOCA-salt hypertensive mice in the absence (control) and presence of prazosin, PPADS, yohimbine, 5-methylurapidil, L-765,314 and BMY-7378; selective antagonists at α_1-, P_2, α_2-, α_{1A}, α_{1B}-, and α_{1D}-AR, respectively. Data are expressed as mean ± SEM. Numbers in parentheses refer to the number of animals from which the data were obtained. *: $p < 0.05$ –vs- control.

	E_{max} (%)	S_{50} (Hz)
DOCA-salt		
Control	24.9 ± 2.2 (14)	10.6 ± 0.8 (14)
Prazosin (0.1 μM)	14.0 ± 3.5* (6)	9.0 ± 1.3 (6)
Prazosin (0.1 μM)/PPADS (10 μM)	7.1 ± 1.7* (8)	8.7 ± 0.7 (7)
Control	23.0 ± 1.0 (9)	8.4 ± 0.7 (9)
PPADS (10 μM)	21.1 ± 1.8 (9)	9.8 ± 0.9 (9)
Control	25.0 ± 4.0 (5)	12.0 ± 1.0 (5)
Yohimbine (1 μM)	22.5 ± 3.3 (5)	5.0 ± 0.4* (5)
Control	21.6 ± 3.0 (7)	9.0 ± 1.2 (7)
5-methylurapidil (0.1 μM)	17.2 ± 2.4 (7)	7.3 ± 1.8 (7)
Control	22.7 ± 1.4 (4)	10.0 ± 1.4 (4)
L-765,314 (1 μM)	11.4 ± 1.7* (4)	7.5 ± 1.7 (4)
Control	21.3 ± 0.9 (5)	7.7 ± 1.4 (5)
BMY-7378 (0.3μM)	19.7 ± 1.3 (5)	5.7 ± 1.7 (5)

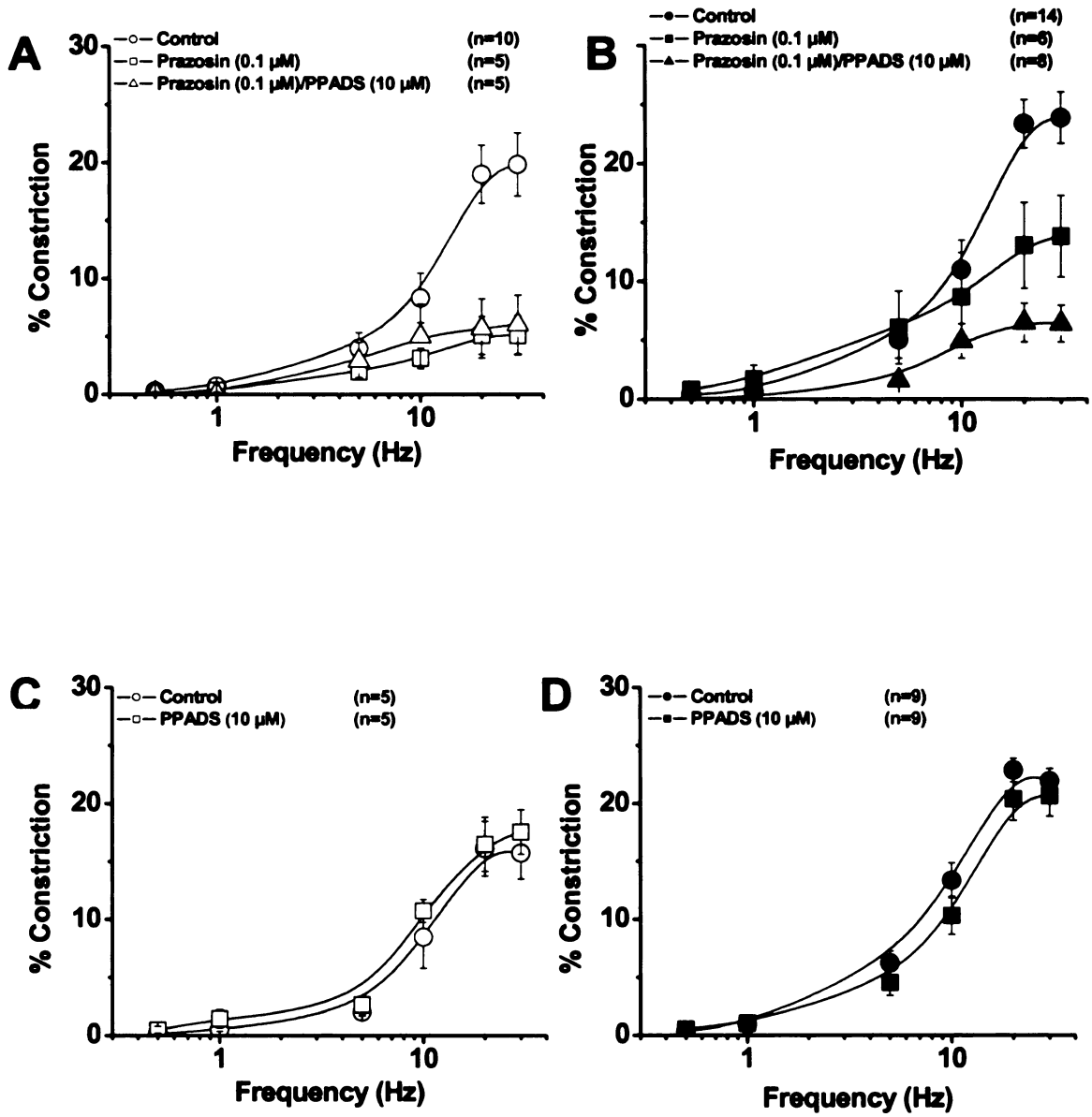

Figure 1. Frequency-response curves obtained before (control) and after application of the selective α_1-AR antagonist prazosin or after combined application of prazosin and the selective P2 receptor antagonist PPADS in mesenteric arteries from SHAM (A) and DOCA-salt (B) mice. Neurogenic responses of SHAM (C) and DOCA-salt (D) arteries in the absence or presence of the selective P2 receptor antagonist PPADS. Data are mean ± SEM from "n" animals.

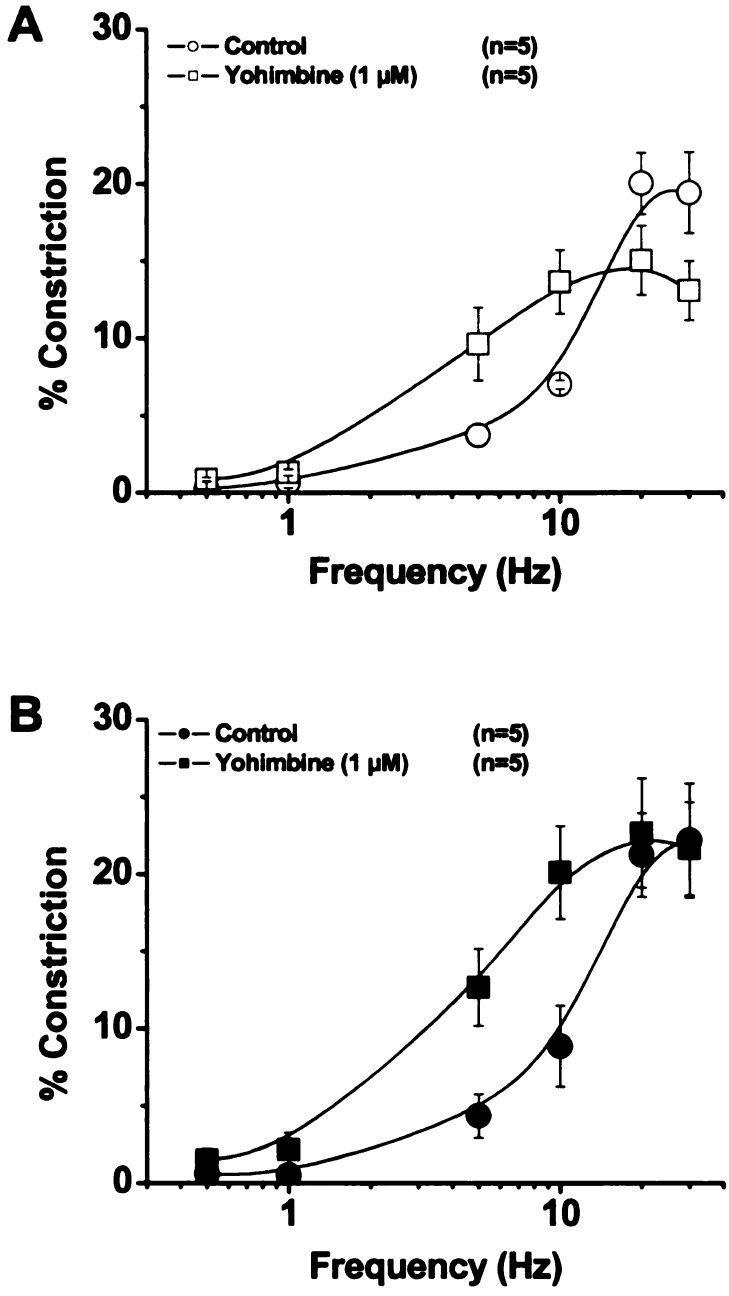

Figure 2. Contribution of α_2-AR to neurogenic constrictions of mesenteric arteries from SHAM (A) and DOCA-salt (B) mice. Frequency-response curves were obtained before (control) and after application of the selective α_2-AR antagonist yohimbine. Data are mean ± SEM from "n" animals.

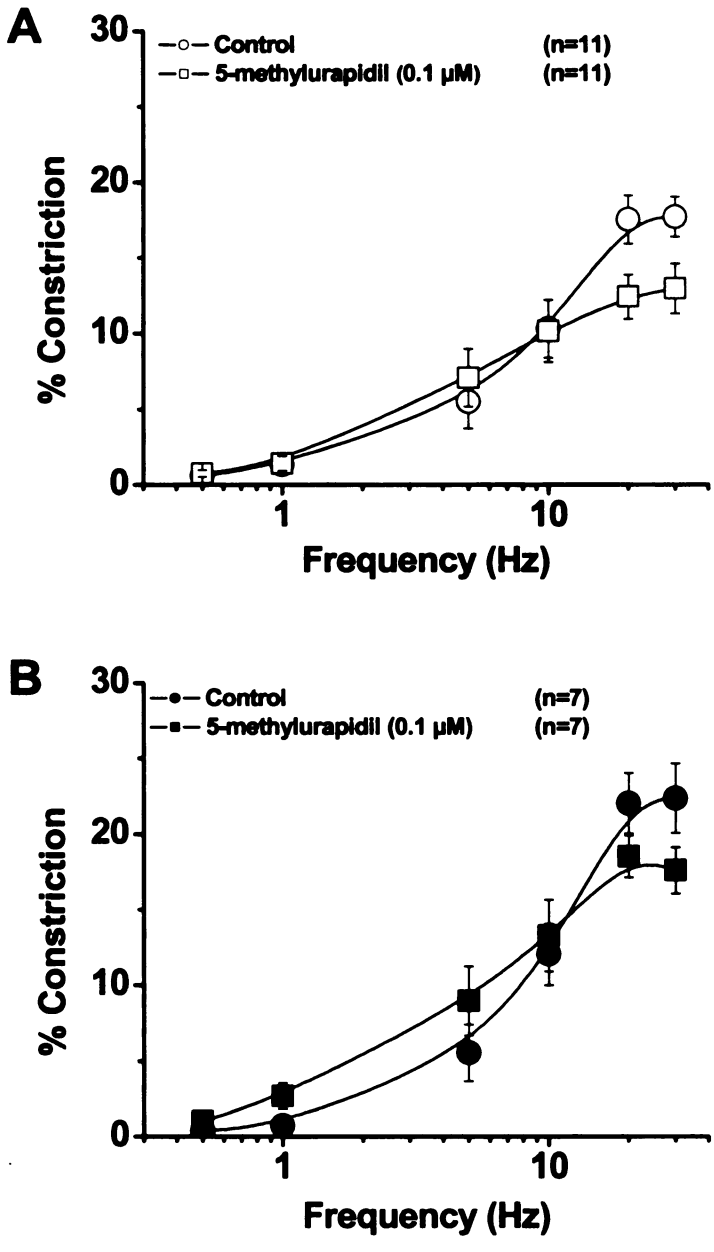

Figure 3. Contribution of the α_{1A}-AR subtype to neurogenic constrictions of mesenteric arteries from SHAM (A) and DOCA-salt (B) mice. Frequency-response curves were obtained before (control) and after application of the selective α_{1A}-AR antagonist 5-methylurapidil. Data are mean ± SEM from "n" animals.

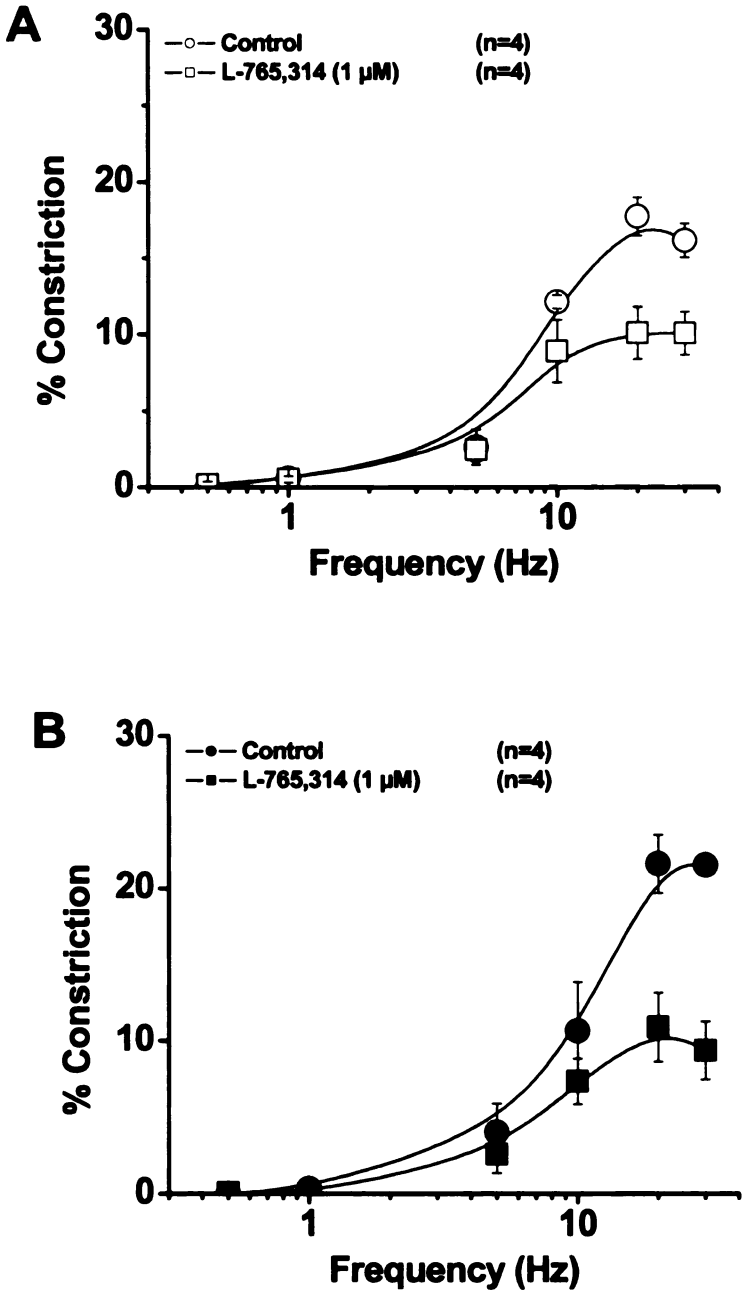

Figure 4. Contribution of the α_{1B}-AR subtype to neurogenic constrictions of mesenteric arteries from SHAM (A) and DOCA-salt (B) mice. Frequency-response curves were obtained before (control) and after application of the selective α_{1B}-AR antagonist L-765,314. Data are mean ± SEM from "n" animals.

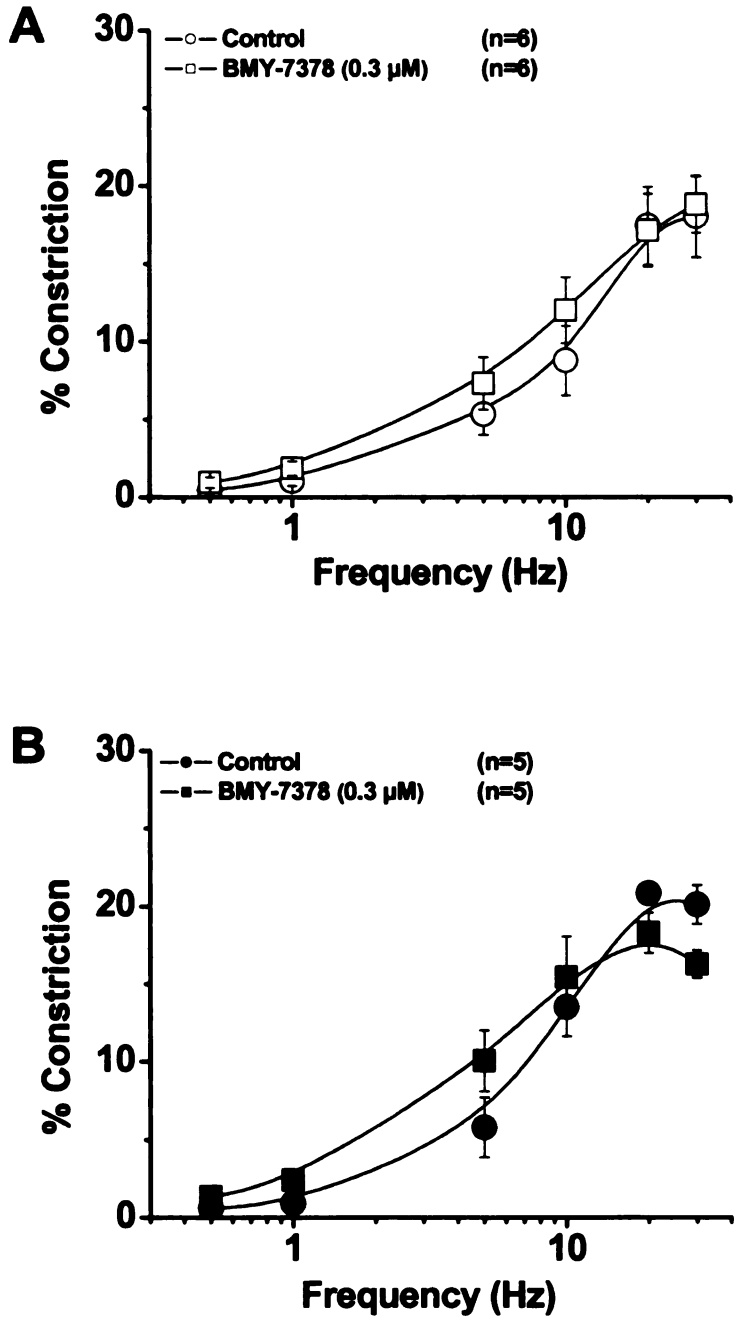

Figure 5. Contribution of the α_{1D}-AR subtype to neurogenic constrictions of mesenteric arteries from SHAM (A) and DOCA-salt (B) mice. Frequency-response curves were obtained before (control) and after application of the selective α_{1D}-AR antagonist BMY-7378. Data are mean ± SEM from "n" animals.

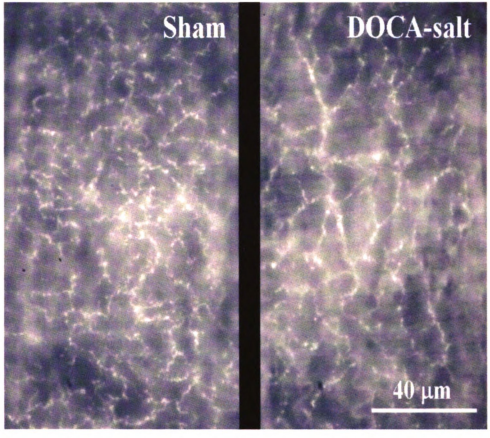

Figure 6. Representative photos obtained with the glyoxilic acid method showing innervation density of adrenergic nerve fibers in mesenteric arteries from SHAM (A) and DOCA-salt (B) arteries.

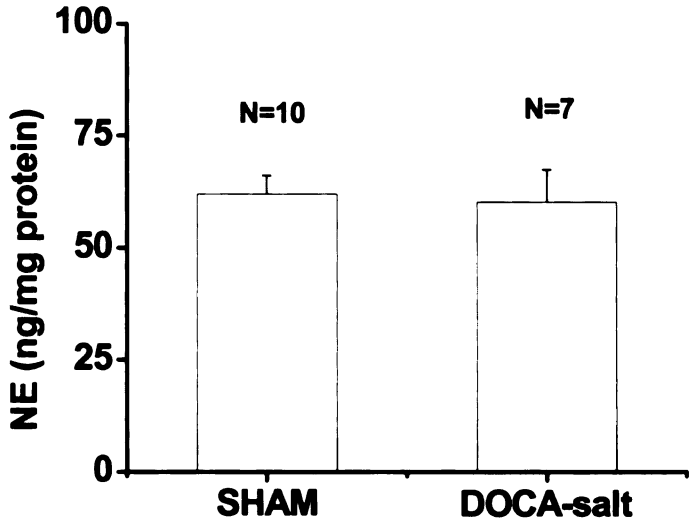

Figure 7. Norepinephrine content in mesenteric arteries from SHAM and DOCA-salt mice as determined by high performance liquid chromatography with electrochemical detection. N indicates the number of mice from which the tissues were obtained.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

The objective of my initial set of studies was first, to characterize acute vascular reactivity and time-dependent desensitization of mesenteric arteries and veins in a murine model of DOCA-salt hypertension. I selected this particular hypertension model because it is a salt-sensitive, low-renin experimental model where SNS activity has been found to play an important role (de Champlain, 1990). In addition, venous capacitance has been shown to be decreased by the SNS as determined by changes in MCFP (Fink et al., 2000) making it a relevant model for the studies that I performed. Small mesenteric arteries and veins were chosen as these small vessels are important players in blood pressure regulation as they are the major determinants of total peripheral resistance and vascular capacitance, respectively. Below is a general summary and discussion of the main findings of my studies.

1. Comparison of α_1-AR reserve in murine mesenteric arteries and veins.

1a. Greater α_1-AR reserve in veins compared to arteries: pharmacological and functional evidence. Veins were more sensitive than arteries to the contractile effects of the adrenergic agonist NE. The increased sensitivity of veins to adrenergic agonists compared to arteries led me to suggest that a larger α_1-AR concentration in veins is a likely explanation for the observed results. Experiments with PBZ provided functional and pharmacological evidence to this effect. PBZ reduced the initial NE-elicited constriction in SHAM and DOCA-salt veins in a concentration-dependent manner. In contrast, all PBZ

concentrations used completely inhibited NE responses in arteries suggesting that there is a larger α_1-AR reserve in veins than arteries. The fact that veins exhibited a more sustained contractile response that was more resistant to desensitization by continuous exposure to an adrenergic agonist also suggest to the existence of an increased α_1-AR population in veins. This idea was tested by hypothesizing that decreasing the α_1-AR reserve in veins with PBZ would render them more susceptible to desensitization by adrenergic agonists. This, in fact, was the case. PBZ-treated veins showed a partial desensitization to NE exposure, similar to that seen in arteries.

There were some differences in the desensitization characteristics of SHAM and DOCA-salt arteries. When exposed to NE, DOCA-salt arteries were relatively resistant to desensitization compared to control arteries. However, this difference was not seen when arteries were preincubated with PE. It could be argued that the differential effects of NE on SHAM and DOCA-salt arteries are due to activation of α_2-ARs in a greater fashion in DOCA-salt arteries than in SHAM vessels. However, I showed that α_2-ARs do not play a contractile role in constriction of murine mesenteric arteries to adrenoceptor agonists. Upregulation of α_1-ARs is an unlikely case as I failed to see the same effect with PE, the selective α_1-AR agonist. It looks that the differences I documented in arteries from both treatment groups, are due to events occurring not at the receptor but more at a post-receptor level. Further experimentation is, therefore, needed to clarify this point.

All these pharmacological and functional data showing: 1) an increased reactivity of murine mesenteric veins to the contractile effects of the adrenergic agonists NE and PE; 2) complete inhibition of contractile responses in mesenteric arteries but not veins upon alkylation of the α_1-AR with PBZ; 3) resistance to desensitization exhibited by mesenteric veins; 4) but susceptibility to it after pretreatment with PBZ, suggest that murine mesenteric veins express more α_1-ARs compared to arteries and that this differences in receptor number could be the reason behind the differential reactivity seen between arteries and veins in this animal model.

In recent years, the cloning of three genes that encode distinct α_1-AR subtypes (Lomasney et al., 1991; Schwinn et al., 1990) and the subsequent classification of α_1-AR into α_{1A}-, α_{1B}-, and α_{1D}-AR subtypes (Hieble et al., 1995) has sparked a major interest in the regulatory actions of these adrenoceptor subtypes. It is known that α_1-AR subtypes have different susceptibilities to desensitization induced by sustained NE stimulation (Chalotorn et al., 2002; Zhang et al., 1997). As there are differences in desensitization and internalization properties of α_1-ARs, a potential reason for the differences I saw between mesenteric arteries and veins could be that arteries and veins express different functional α_1-AR subtypes with different desensitization and internalization characteristics or that they differ in their sensitivity to PBZ.

For that reason, the objectives of my second set of experiments were to determine the relative contribution of individual α_1-AR subtypes in mediating the

vasoconstriction of mesenteric arteries and veins from SHAM control and DOCA-salt mice and to compare α_1-AR subtype protein expression between normotensive and DOCA-salt hypertensive vessels to see if the suggested increased α_1-AR reserve suggested for veins in our pharmacological and functional studies could be correlated with molecular approaches.

2. Specific contractile regulation in arteries and veins by α_1-AR subtypes.

2a. α_{1A}-ARs are involved in contractile responses in arteries whereas α_{1D}-ARs are involved in PE-induced constriction in mesenteric veins. My studies showed that the selective α_{1A}-AR antagonist 5-MU inhibited PE responses in arteries but not veins with a high affinity. This has been suggested by other investigators (Daly et al., 2002). This subtype-selective effect of α_{1A}-ARs in small resistance arteries suggests that this particular adrenoceptor subtype is an important regulator of blood pressure by alterations in peripheral resistance. I provided evidence that α_{1D}-ARs mediate constrictions of veins but not arteries as the selective α_{1D}-AR antagonist BMY-7378 did not affect PE contractile responses in arteries but competitively antagonized PE-induced constrictions in mesenteric veins. The α_{1B}-AR subtype does not seem to play a fundamental function in contractile responses to catecholamines in murine mesenteric vessels.

These findings suggesting a subtype-specific regulation of contractile responses in murine mesenteric vessels have a very important implication in terms of cardiovascular and blood pressure regulation. First of all, the fact that

the α_{1A}-AR selectively affected arterial but not venous tone suggests that vascular α_1-AR may preferentially affect resistance in small vessels via α_{1A}-AR as reviewed by Philipp and Hein (2004) since no evidence of α_{1A}-AR mediated constriction has been found in large compliance arteries. In these large vessels, α_{1D}-ARs are the major contractile isoform. This suggests that the main role of α_{1A}-ARs in mice may be to alter blood flow via changes in peripheral resistance. In addition, it points to the possibility of selectively targeting this adrenoceptor with subtype-specific antagonists in an effort to control elevated blood pressures associated with changes in total peripheral resistance.

It should be known that α_{1A}-ARs are important regulators of blood pressure in vivo as determined by experiments with genetically-modified mice. α_{1A}-AR K/O mice were hypotensive under resting conditions compared to wild type controls (Rokosh and Simpson, 2002). α_{1D}-ARs are also important key regulators of blood pressure. Mice genetically modified to lack this particular α_1-AR subtype showed a significantly lower basal systolic and mean arterial blood pressure (Tanoue et al., 2002b). In addition, contractile responses of the aorta and pressor responses of the perfused mesenteric arterial bed were decreased.

Moreover, the α_{1D}-AR has also been implicated in the pathogenesis of hypertension, particularly salt-induced hypertension (Tanoue et al., 2002a; Hosoda et al., 2005). This brings a very important point. I showed that α_{1D}-ARs mediate contractile responses to PE in murine mesenteric veins but not arteries and several studies have linked this adrenoceptor subtype in the pathogenesis of

salt-induced hypertension (Tanoue et al., 2002a; Hosoda et al., 2005). This suggests that veins could also be important blood pressure regulators.

Historically, the arterial side of the circulation has been given the most attention as it is been thought that because of their predominant role as resistance vessels, they are the major modifiers of systemic blood pressure by changes in vascular tone. However, the splanchnic mesenteric vascular bed contains up to 30% of blood volume (Greenway, 1983). This capacitance function largely resides in veins and venules. A reduction in capacitance of systemic veins will shift blood from peripheral vascular beds toward the thoracic cavity (Ricksten et al., 1981) leading to increases in CO; one of the determinants of systemic blood pressure. In this way, catecholamine-induced stimulation of α_{1D}-ARs in mesenteric veins could affect blood pressure.

An important question to ponder is what is the physiological rationale for having different α_1-AR subtypes controlling contractile responses in different vascular beds. At this point this is a very obscure area as little is known regarding this phenomenon. So far it looks that vascular α_1-AR subtypes may differentially affect compliance of large arteries, peripheral resistance of small arteries and capacitance of veins via different adrenoceptor subtypes. Therefore, this could be one mechanism underlying the differential function (resistance versus capacitance) performed by mesenteric arteries and veins. In addition, as suggested by Philipp and Hein (2004), the fact that NE has a lower affinity for the α_{1A}- than for the α_{1D}-AR subtype could suggest that nerve-released NE (that can achieve high circulating levels) may primarily control α_{1A}-AR whereas circulating

catecholamines may primarily affect compliance of large arteries via α_{1D}-ARs. However, this should not be taken as an universal phenomenon as there could be species or vascular bed-related differences. Definitively, further research is needed to better understand the mechanisms behind this subtype-selective regulation of contractile responses in mesenteric vessels.

2b. α_1-AR subtype expression in murine mesenteric arteries and veins. I looked at α_1-AR subtype protein expression in murine mesenteric arteries and veins by Western immunoblotting. Analysis revealed that arteries and veins express the α_{1A}-AR protein. However, there were differences in α_{1A}-AR expression between SHAM control and DOCA-salt hypertensive arteries. It was noted that α_{1A}-AR protein expression was downregulated in arteries from DOCA-salt hypertensive mice. The most likely reason for the downregulation seen for this particular adrenoceptor subtype is an increase in sympathetic nerve activity. An increased sympathetic nerve activity is a common feature in experimental models of hypertension, like the DOCA-salt model (de Champlain, 1990; Oparil, 1986) that can cause adrenoceptor downregulation. However, I did not measure directly whether or not there is an increased sympathetic activity in DOCA-salt mice compared to SHAM controls. So, this still remains a speculation.

Of interest is that arterial contractile responses were not compromised in DOCA-salt arteries despite α_{1A}-AR downregulation. The most likely reason for this maintained reactivity is enhanced postreceptor events involved in the signal transduction once the α_{1A}-AR is activated by PE in arteries.

The α_{1B}- and the α_{1D}-AR are also ubiquitously expressed in mesenteric arteries and veins. The fact that all three adrenoceptor subtypes are expressed in mesenteric vessels but not necessarily are involved in contractile responses suggest that examination of receptor expression alone is not enough to examine the regulatory activities of a given receptor. So, we should be aware that expression of a given protein itself is not a determinant of functional activity as I demonstrated here that expression does not necessarily link a particular adrenoceptor subtype to functional contractile effects.

The notion that the α_{1A}-AR is the only adrenoceptor subtype whose expression changed upon DOCA-salt treatment in mice points again to an important role of this adrenoceptor subtype in blood pressure regulation as stated above. However, I can not exclude the possibility that α_{1B}- and α_{1D}-AR are also important players as it is known that all three adrenoceptor subtypes play important roles in blood pressure homeostasis as well as determined in experiments with K/O animals (Tanoue et al., 2002b).

2c. Differential α_1-AR subtype function and expression: correlation with vascular reactivity. In my first series of studies I showed that murine mesenteric veins were more sensitive to the contractile effects of adrenergic agonists. Pharmacological data provided functional evidence pointing to the fact that there could be a difference in the α_1-AR reserve in veins as oppose to arteries: veins having an increased reserve. I was not able to correlate the functional evidence obtained from pharmacological studies with the molecular findings obtained by using Western immunoblotting. However, I was not able to

specifically determine expression of membrane as opposed to intracellular proteins. It is know that agonists of G-protein coupled receptors produce their effects by specifically interacting with membrane-bound proteins. There is a possibility that expression of membrane-bound α_1-ARs in veins is higher in veins than in arteries, supporting my functional evidence to the fact that veins express more functional α_1-ARs than arteries. Protein determination by Western immunoblotting takes into account both membrane-bound as well as intracellular α_1-ARs which could explain why I did not see any differences in expression between arteries and veins despite the fact that functional data provide evidence to a difference in receptor population between both sets of vessels.

I showed that α_{1A}-ARs are the predominant contractile isoform in murine mesenteric arteries whereas the α_{1D}-AR mediates contractile responses in veins. Whether PBZ has a greater affinity for the α_{1A}- than for the α_{1D}-AR is a possibility that could explain why arterial responses were easily inhibited by preincubation with PBZ. As far as I am aware there are no reports testing the relative sensitivity of α_1-AR subtypes to PBZ.

Several reports have showed that α_{1D}-ARs are constitutively active (Gisbert et al., 2000; Gisbert et al., 2002; Gisbert et al., 2003). As the name implies, a constitutively active receptor will show spontaneous activity even in the absence of an agonist. Therefore, a possible reason behind the increased reactivity seen in mesenteric veins could be due to the fact that in these vessels the major contractile α_1-AR subtype is the α_{1D}-AR subtype, which is constitutively

active in nature as opposed to arteries in which the α_{1A}-AR but not the α_{1D}-AR plays a major contractile role.

It is now known that there is a postjunctional α_2-AR population that regulates vascular tone in conjunction with α_1-ARs in a variety of vascular beds (Daly et al., 1985; Fowler et al., 1984; Itoh et al., 1987; Polonia et al., 1986). Therefore, the possibility of a differential α_2-AR activity in veins as opposed to arteries is a possibility that I explored in order to explain the differences in adrenergic reactivity between mesenteric arteries and veins.

3. Role of α_1- and α_2-ARs in contractile responses of mesenteric arteries and veins.

3a. α_1-ARs mediate constriction in mesenteric arteries and veins. I demonstrated an important effect of α_1-ARs in contractile responses in mesenteric arteries and veins as the α_1-AR antagonist prazosin competitively antagonized contractile responses to NE with high affinity. This is consistent with the fact that in numerous opportunities, α_1-ARs have been shown to play a prdominant role in rat (Hussain and Marshall, 2000) and mouse (Yamamoto and Koike, 2001) mesenteric arteries and also agrees with more recent studies showing that α_1-ARs are also involved in contractile responses in rat (Luo et al., 2003) and mouse (Pérez-Rivera et al., 2004) mesenteric veins as well.

The novel finding of these studies is not the fact that α_1-ARs are involved in contractile responses of mesenteric vessels but the pattern of inhibition exhibited by arteries and veins in the presence of prazosin, the α_1-AR antagonist. In arteries, prazosin produced parallel and even rightward shifts in the

concentration-response curves. Although there was an inhibition of contractile responses in veins in the presence of prazosin, the displacements seen in the concentration-response curves were not as parallel and even as those seen for arteries. Therefore, the pattern of inhibition seen in veins did not strictly follow the model of simple competitive antagonism. This led me to postulate that perhaps, other receptors could be involved in contractile responses in mesenteric veins, perhaps the α_2-AR, that could explain the atypical inhibitory pattern seen with the α_1-AR antagonist alone.

3b. α_2-ARs mediate constriction in mesenteric veins but not arteries. The α_2-AR agonists clonidine and UK-14,304 did not contract mesenteric arteries and veins. Initially, these results suggested that α_2-ARs are not involved in the contractile responses to NE in murine mesenteric vasculature. In order to examine in greater detail whether or not α_2-ARs are involved in contractile responses, inhibition of contractile responses to NE in the absence or presence of selective α_2-AR antagonists was examined.

Yohimbine, a selective α_2-AR antagonist, did not have any effect on the contractile responses of arteries from both SHAM and DOCA-salt mice. Therefore, data obtained in arteries is in complete agreement with experiments where the α_2-AR agonists were used that rejected a contractile role of α_2-AR in murine mesenteric arteries. These data and the previous data where prazosin elicited parallel and even shifts of the NE concentration-response curves suggest that α_1- but not α_2-ARs are involved in the contractile responses to catecholamines in murine mesenteric arteries.

The results obtained with yohimbine in veins showed that α_2-ARs are involved in contractile responses as it competitively antagonized contractile responses to NE with high affinity. At first, these results were puzzling as previously we showed the failure of clonidine and UK-14,304 in stimulating a contractile response in mesenteric veins. Nonetheless, the fact that yohimbine acted with high affinity for its receptors points to a α_2-AR selective effect. In addition, the lack of antagonism seen in arteries contracted with PE, a selective α_1-AR antagonist suggest that yohimbine-mediated inhibition of contractile responses in veins is due to its selective effects at α_2-AR and not at other receptors, such as the α_1-AR. Therefore, pharmacological data obtained with yohimbine suggest that in murine mesenteric veins both α_1- and α_2-ARs serve a contractile function as opposed to arteries where just α_1-ARs are found. This also explains why prazosin-mediated shifts in the NE concentration-response curve were not as even and parallel as those in arteries and did not follow a model of simple competitive antagonism. These findings were corroborated with another α_2-AR antagonist, rauwolscine which showed an inhibition of venous but not arterial contractile responses to NE and provided more evidence of a differential contribution of α_1- and α_2-ARs to vasoconstriction in murine mesenteric arteries and veins.

3c. Involvement of α_2-ARs in mesenteric veins: correlation to adrenergic vascular reactivity. Comparison of adrenergic reactivity between arteries and veins has consistently shown that veins are more sensitive to the contractile effects of NE (Luo et al., 2003; Pérez-Rivera et al., 2004). In my first

set of studies, pharmacological analysis with the alkylating agent PBZ supported the notion that a potential reason behind the differential reactivity behind murine mesenteric arteries and veins is a difference between α_1-AR reserve between arteries and veins; veins having a larger adrenoceptor reserve than arteries (Pérez-Rivera et al., 2004).

We have shown in these studies that α_1-ARs mediate contractile responses in both mesenteric arteries and veins whereas the α_2-AR is an important mediator of the contractile responses to catecholamines in veins but not arteries. Perhaps, the increased reactivity documented in murine mesenteric veins relative to arteries is due to the presence of contractile α_2-ARs in the former but not the latter.

It looks that abnormalities in these regulatory contractile mechanisms involving α_1 and α_2-ARs do not take place in DOCA-salt hypertension in mice. This could explain why adrenergic reactivity is not changed in hypertensive vessels compared to their control counterparts. Other studies in DOCA-salt rats have found that α-AR reactivity is altered (Luo et al., 2003; Longhurst et al., 1988; Perry and Webb, 1988). An important difference between the rat and mouse models of DOCA-salt hypertension is that in mice the degree of hypertension, although significant, is much less than that reported for rats. In other words, mice do not become as hypertensive as rats undergoing the same treatment protocol. It could be that the huge dramatic increases in blood pressure seen in DOCA-salt rats are the cause for these differences in adrenergic reactivity reported in the literature whereas in mice increases in blood pressure

may have not been dramatic enough to alter vascular adrenergic reactivity in DOCA-salt vessels.

3d. Potential cross talk between α_1- and α_2-ARs. The studies presented here suggest that in order to see a contribution of α_2-ARs to contractile responses in veins, co-activation of both α_1- as well as α_2-ARs is necessary. A recent report by Reynen et al. (2000) have described a similar mechanism to the one just described in these set of studies. In their studies, Reynen et al. (2000) specifically described a cross talk between α_1-AR and specifically, the α_{2A}-AR subtype. Whether the α_{2A}-AR is the particular α_2-AR subtype involved in cross talk with α_1-AR in murine mesenteric veins is not known as I did not used subtype-selective α_2-AR antagonists. The fact that the phenomenon I described in veins is similar to the one described by Reynen et al. (2000) in a heterologous expression system particularly expressing the α_{2A}-AR subtype strongly points to the α_{2A}-AR as the major candidate for interaction with α_1-AR. However, at this point a role for the other two α_2-AR subtypes (α_{2B}-, α_{2C}-) could not be excluded yet.

4. Adrenoceptor subtypes mediating neurogenic vasoconstriction in mesenteric arteries.

The data discussed so far have supported the notion that arteries and veins differ in the adrenergic mechanisms controlling their responses to a contractile stimulus. In that regard, it could be said that differential adrenergic reactivity of murine mesenteric arteries and veins could be due to a certain number of factors, like differences in α-AR receptor number, subtype-specific

contributions to vasoconstriction in arteries as opposed to veins, differential contribution of α_2-ARs to contractile responses in these vessels, among other factors.

An important factor that may influence vasoconstriction physiologically is the mode of receptor activation, in other words, whether different α_1-AR subtypes are activated by circulating catecholamines or by sympathetic nerve-released NE. For that reason, in my last set of studies I looked at contractile responses of mesenteric arteries to perivascular nerve stimulation and compared those responses to the ones obtained with exogenous application of α-AR agonists.

4a. Adrenergic but not a purinergic contribution to neurogenic vasoconstriction. Studies with receptor-specific antagonists suggested that NE is the dominant NT released by periarterial sympathetic nerves in normotensive mice. There is no contribution of ATP to neurogenic contractile responses in this animal model. It has been widely accepted that sympathetic nerves could release other substances along with NE as cotransmitters when stimulated. Particularly, evidence for cotransmission is abundant with respect to NE and ATP. However, it could be that as shown in the guinea-pig mesenteric artery (Smyth et al., 2000), in the rat femoral resistance arteries (Zacharia et al., 2004), and in the human gastroepiploic artery (Fukui et al., 2005), NE exclusively mediates the contractile response to sympathetic nerve stimulation in murine mesenteric arteries as well with a minimal purinergic contribution to sympathetic nerve stimulation (Smyth et al., 2000).

Adrenergic antagonists did not completely blocked neurogenic responses. There is still a possibility that other cotransmitters could be released with NE when periarterial sympathetic nerves are stimulated. In addition to ATP, there is a growing list of other substances, particularly peptides that have been found on the adrenal medulla, nerve fibers or autonomic ganglia and that have been postulated as potential cotransmitters. These include the enkephalins, substance P, somatostatin, calcitonin gene-related peptide, vasoactive intestinal peptide and neuropeptide Y (Lefkowitz et al., 1996). Therefore, the possibility that other substances besides ATP are cotransmitted with NE in perivascular sympathetic nerves associated with murine mesenteric arteries is a possibility that will have to be addressed.

4b. The α_{1B}- and the α_{1A}-AR subtypes mediate neurogenic responses. The particular α_1-AR subtypes involved in these responses were examined by analyzing frequency-response curves in the absence and presence of subtype-specific antagonists. The α_{1A}-AR antagonist 5-MU caused a reduction in the maximal contractile response to nerve stimulation. In contrast, neurogenic responses in DOCA-salt arteries were not affected by 5-MU pretreatment suggesting that α_{1A}-AR participate in neurogenic responses of SHAM but not DOCA-salt arteries. The physiological significance of this differential role of the α_{1A}-AR in SHAM but not DOCA-salt arteries is not yet clear but completely agrees with previous findings (see Chapter 2) suggesting a downregulation of α_{1A}-AR in mesenteric arteries from DOCA-salt mice.

The α_{1B}-AR antagonist L-765,314 provided clear evidence that this adrenoceptor subtype mediates neurogenic responses in mesenteric arteries. Maximal contractile responses were significantly decreased in vessels preincubated with this antagonist. This provided pharmacological evidence that the α_{1B}-AR is the predominant adrenoceptor subtype involved in transmission at the sympathetic neuroeffector junction. Townsend et al. (2004) showed that the in vitro mesenteric contractile response elicited by electrical nerve stimulation was depressed in α_{1B}-AR K/O mice, therefore, in agreement with our pharmacological studies in mice. α_{1D}-ARs appear not to be involved in sympathetic transmission in the murine mesenteric arterial neuroeffector junction as responses to electrical nerve stimulation were unaffected by BMY-7378, a selective α_{1D}-AR antagonist.

The results presented in Chapter 4 showed that α_{1B}-ARs play no contractile role in the responses to the exogenously applied α_1-AR agonist PE. In contrast, neurogenic responses were blocked by the selective α_{1B}-AR antagonist. Other studies have provided evidence that there is heterogeneity with respect of the α_1-AR subtypes involved in contractile responses to nerve-released and exogenously applied catecholamines. In rat femoral resistance arteries, α_{1A}-ARs have the predominant role in contractions due to exogenous and nerve-released NE. In addition, α_{1D}-ARs are involved in nerve-mediated but not contractile responses to exogenous NE (Zacharia et al., 2004). In the canine splenic artery, NE released from sympathetic nerves exerts its effects via activation of α_{1B}- and to a lesser extent the α_{1D}-ARs whereas the α_{1A}-AR mediates the contractile

effects of exogenous NE leading Yang and Chiba (2001) to postulate that there may be different α_1-AR subtypes in the sympathetic neurovascular junction and extrajunctional region.

Although my results point to that possibility, it could not be proven conclusively by this set of functional studies and other factors such as equilibrium conditions during NT release, uptake mechanisms, etc could also explain the differential role of α_1-AR subtypes in responses to endogenous and exogenous catecholamines. In any case, it will be interesting to demonstrate that modulation of vascular tone by nerve-released and hormonal catecholamines occurs through different α_1-AR subtypes.

It could then be hypothesized that determination of what α_1-AR subtypes are located in the junctional versus extrajunctional areas could prove valuable from the clinical standpoint. There are a number of cardiovascular diseases, where increases in sympathetic nerve activity have been correlated to the pathological events. Therefore, selective targeting of α-AR subtypes, particularly α_{1B}- and to a lesser extent the α_{1A}-AR as suggested by the functional studies just described, could be a potential clinical means of treating sympathetic-mediated contribution to hypertension and other cardiovascular disorders.

4c. Unaltered neurogenic vascular reactivity between SHAM and DOCA-salt arteries. An important finding is that neurogenic responses of mesenteric arteries to sympathetic nerve stimulation were not different between SHAM control and DOCA-salt hypertensive mice. A typical characteristic in DOCA-salt hypertension in rats is the enhanced reactivity of vessels to

contractile agonists (Muir and Wardle, 1989; Luo et al., 2003; Tsuda et al., 1989). As shown throughout this dissertation, mice are not merely small rats as there are profound differences in several cardiovascular parameters between these two species. Species differences between the mouse and the rat or increases in blood pressure in DOCA-salt mice that may have not been large enough to alter vascular adrenergic reactivity in DOCA-salt arteries are potential reasons behind this lack of differential neurogenic responses between SHAM and DOCA-salt arteries. However, the lack of a differential neurogenic contractile effect seen in murine DOCA-salt arteries is supported by our anatomical data showing that density of adrenergic nerve fibers was not different between tissues from SHAM and DOCA-salt mice and that NE content of mesenteric arteries from both treatment groups did not differ. I previously showed that there is no difference in reactivity of mesenteric arteries to contractile stimulation to adrenergic agonists agreeing with the fact of no imbalances in sympathetic neurotransmission in murine mesenteric arteries in DOCA-salt hypertension.

5. Overall conclusions and implications.

The overall goal of my dissertation studies was to assess the reasons why veins are more sensitive to adrenergic stimulation. I tested several potential mechanisms behind this differential reactivity:

a. **differences in α_1-AR reserve:** functional studies supported the notion that murine mesenteric veins could have a greater adrenoceptor reserve compared to arteries.

b. **α_1-AR subtype-selective contractile regulation of arteries and veins:** the fact that different α_1-AR subtypes mediate constrictions in arteries and veins support the notion of a differential sympathetic regulation of arteries and veins. This is not only important in explaining differences in adrenergic reactivity between these vessels but in sympathetic-mediated regulation of arterial resistance and venous capacitance by distinct mechanisms and receptors.

c. **α_2-AR mediated-contractile responses in arteries and veins:** I was able to show that α_2-ARs mediate constrictions in veins but not in arteries providing evidence that this differential effect of α_2-ARs could help explain the increased reactivity to adrenergic agonists seen in veins when compared to arteries.

d. **neurogenic-mediated contractile responses in mesenteric arteries:** the α_1-AR subtype responsible for vasoconstriction to nerve-released NE in arteries (α_{1B}-AR) was different from the adrenoceptor subtype mediating constrictions to exogenously applied catecholamines (α_{1A}-AR). Whether this suggest differential localization of α_1-AR subtypes in junctional and extrajunctional areas or that α_1-AR subtypes are activated preferentially by either nerve-released or circulating catecholamines is a possibility that will have to be addressed in the future.

So far, I have listed three potential reasons behind the increased reactivity of murine mesenteric veins to adrenergic agonists: 1) differences in α_1-AR

number; 2) differential role of α_1-AR subtypes in contractile responses of arteries and veins; and 3) selective constriction mediated by α_2-ARs. For a summary of these findings, see Figures 1, 2, 3, 4. All these are mechanisms focus on a receptor level. However, still there is the possibility that an increased activation of post-receptor events in veins as opposed to arteries is the reason behind the increased reactivity of murine mesenteric veins.

Regardless, these studies suggest that by virtue of their increased reactivity, mesenteric veins are also important and critical mediators of blood pressure regulation. Therefore, increases in sympathetic nerve activity or in α-AR activation will lead preferentially to changes in venomotor tone with subsequent reductions in capacitance resulting in shifting of blood from peripheral vascular beds toward the thoracic cavity (Ricksten et al., 1981) leading to increases in CO. The venous system, therefore, could also be an important target of adrenergic agonists and antagonists in an effort to target venous-mediated effects in hypertension and perhaps, other cardiovascular diseases that take away so many lives every year.

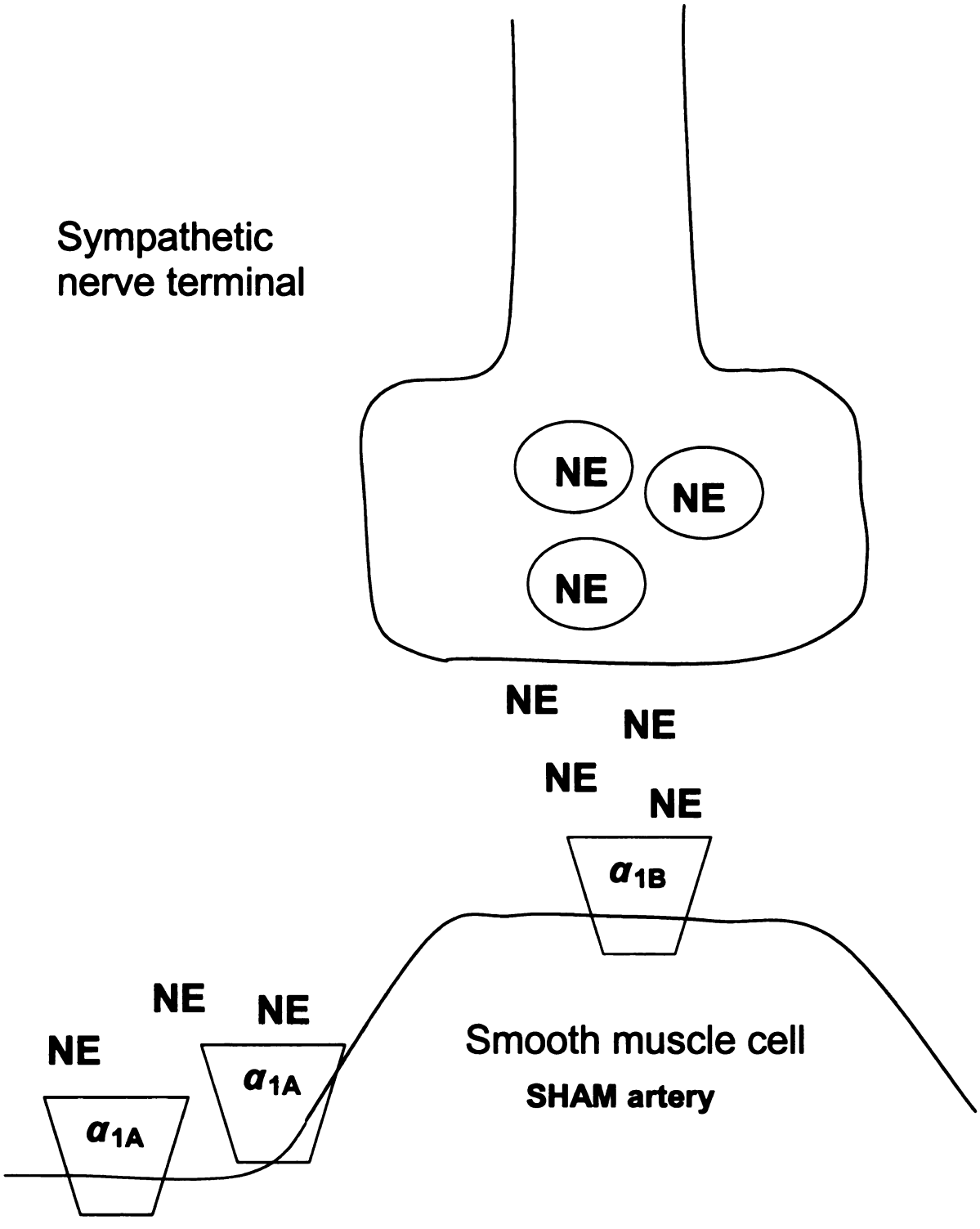

Figure 1: Schematic diagram summarizing the adrenergic mechanisms involved in contractile responses of SHAM normotensive arteries as determined by experiments in this dissertation. Stimulation of sympathetic nerves associated with mesenteric arteries potentially results in a contractile response due to stimulation of α_{1B}-ARs whereas contractile responses due to exogenous catecholamines involve the α_{1A}-ARs.

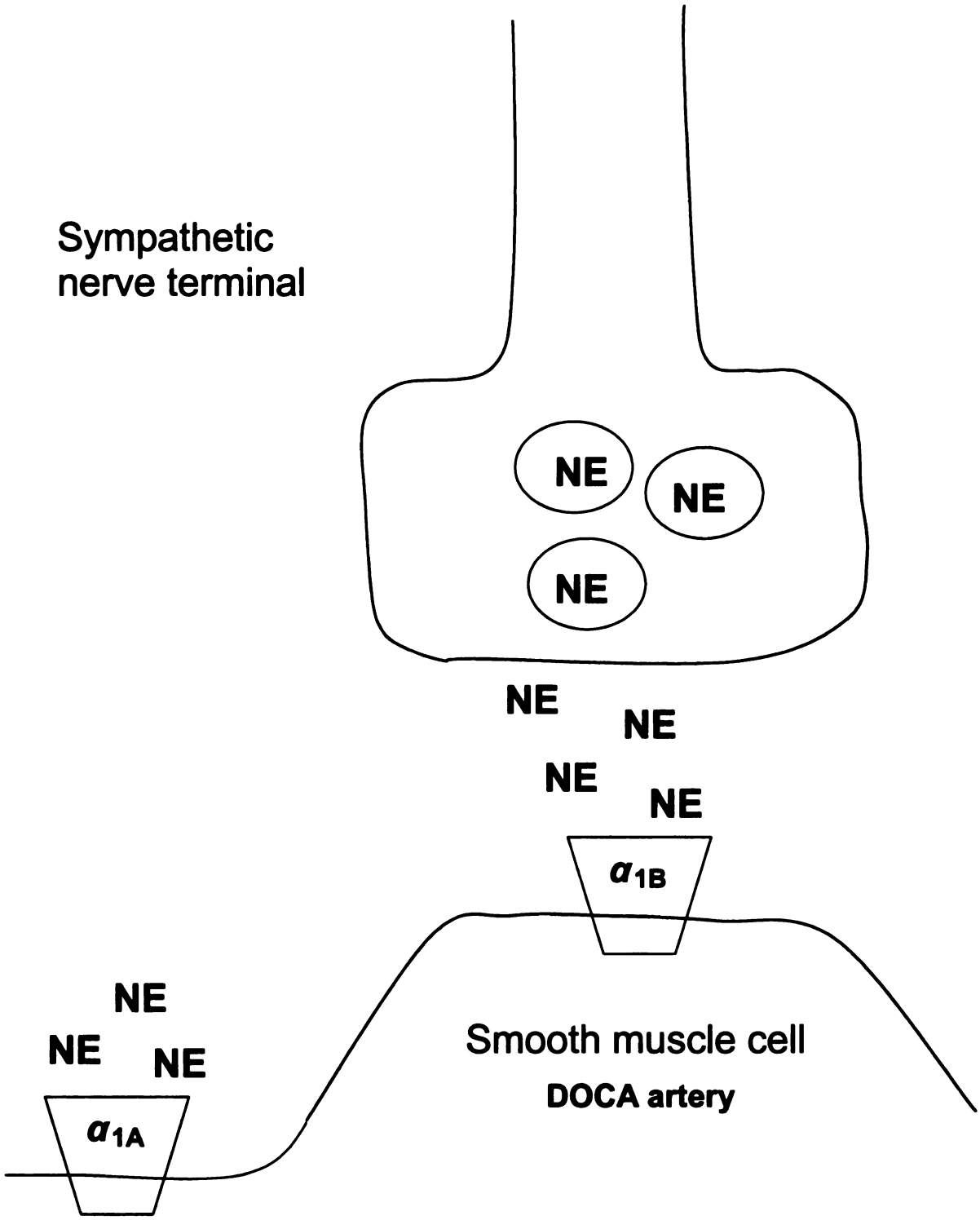

Figure 2: Schematic diagram summarizing the adrenergic mechanisms involved in contractile responses of DOCA-salt hypertensive arteries as determined by experiments in this dissertation. Stimulation of sympathetic nerves associated with mesenteric arteries potentially results in a contractile response due to stimulation of α_{1B}-ARs whereas contractile responses due to exogenous catecholamines involve the α_{1A}-AR which is downregulated.

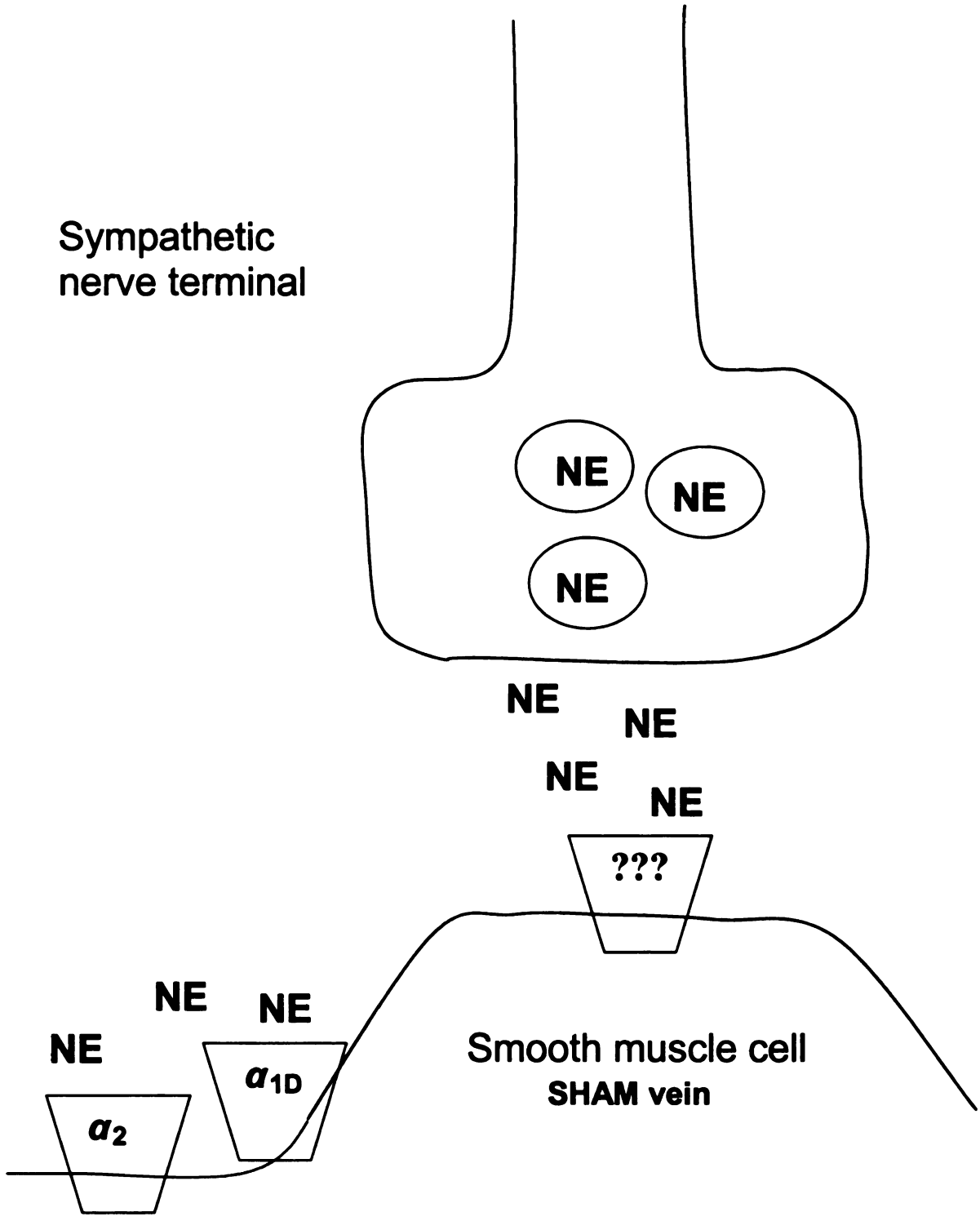

Figure 3: Schematic diagram summarizing the adrenergic mechanisms involved in contractile responses of SHAM normotensive veins as determined by experiments in this dissertation. Stimulation of sympathetic nerves associated with mesenteric veins potentially results in a contractile response due to stimulation of a yet unknown adrenoceptor. Contractile responses due to exogenous catecholamines involves the α_{1D}-AR but also α_2-ARs.

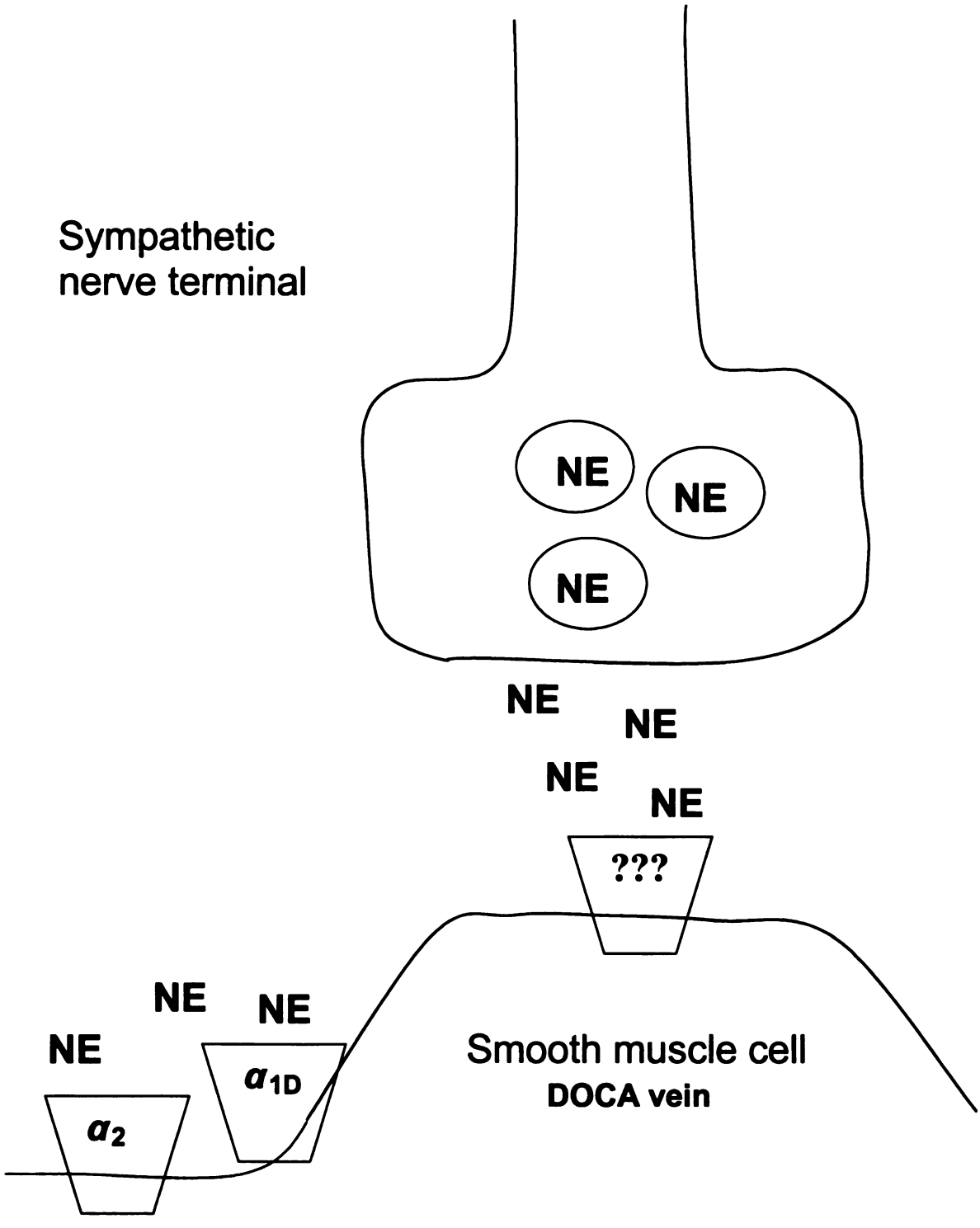

Figure 4: Schematic diagram summarizing the adrenergic mechanisms involved in contractile responses of DOCA-salt hypertensive veins as determined by experiments in this dissertation. Stimulation of sympathetic nerves associated with mesenteric veins potentially results in a contractile response due to stimulation of a yet unknown adrenoceptor. Contractile responses due to exogenous catecholamines involves the α_{1D}-AR but also α_2-ARs.

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