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IMPAIRED PURINERGIC NEUROTRANSMISSION TO MESENTERIC ARTERIES IN SALT-SENSITIVE HYPERTENSION

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ABSTRACT

IMPAIRED PURINERGIC NEUROTRANSMISSION TO MESENTERIC ARTERIES IN SALT-SENSITIVE HYPERTENSION

By

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The sympathetic nervous system regulates blood pressure and its function is altered in animal models and human essential hypertension. Arteries in the splanchnic circulation are critical targets in blood pressure homeostasis. Norepinephrine (NE) and ATP are released from sympathetic nerve terminals and act at α_1-adrenergic receptors (AR) and $P2X_1$ receptors, respectively, on smooth muscle cells (SMC) to cause constriction. Impairments in nerve terminal storage, release or clearance of NE or ATP would alter blood pressure. Increased NE release is associated with high blood pressure. However, alterations in ATP release in hypertension are less clear.

To study alterations in purinergic neurotransmission, deoxycorticosterone-acetate (DOCA)-salt rats were used as a model of salt-sensitive hypertension. Mesenteric arteries (200-300 μm outside diameter) were maintained *in vitro*. Peri-arterial sympathetic nerves were stimulated with a focal stimulating electrode. Intracellular electrophysiological techniques were used to record depolarizations in arterial SMC membrane potential called excitatory junctional potentials (EJPs) that result from ATP release from sympathetic nerve terminals

onto $P2X_1$ ligand-gated ion channel receptors. EJP amplitude, facilitation and rundown were assessed as a measure of ATP release. Treatment of rats with antioxidants was used to study the role of oxidative stress in alterations in purinergic neurotransmission in hypertension.

The results of these studies revealed that purinergic neuroeffector transmission to mesenteric arteries is impaired in DOCA-salt hypertension, but is not due to changes in the function of prejunctional autoreceptors, alterations in calcium handling in the nerve terminal or post-junctional responses to ATP. Rather, there is a decrease in ATP availability in sympathetic nerve terminals in DOCA-salt rats. DOCA-salt hypertension is associated with an increase in vascular oxidative stress which may come from O_2^- generated in sympathetic nerve terminals via NADPH oxidase as two of its subunits were localized to periarterial nerve fibers. Treatment of DOCA-salt rats with antioxidants *in vivo* restores purinergic neuroeffector transmission. These results show for the first time that oxidative stress in salt-sensitive hypertension disrupts purinergic neuroeffector transmission to mesenteric arteries by compromising nerve terminal stores of ATP. These studies reveal the importance of sympathetic nerve terminals as a therapeutic target for the pathophysiology and treatment of salt-dependent hypertension.

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LIST OF ABBREVIATIONS

Acetylcholine	ACh
Adenosine receptor	A_1-R
Adenosine diphosphate	ADP
Adenosine monophosphate	AMP
Adenosine triphosphate	ATP
α_1-adrenergic receptor	α_1-AR
α_2-adrenergic receptor	α_2-AR
Angiotensin	Ang
Angiotensin converting enzyme inhibitor	ACE-I
Autonomic nervous system	ANS
β-adrenergic receptor blocker	β-blocker
Blood pressure	BP
Calcitonin gene related peptide	CGRP
Calcium	Ca^{2+}
Calcium channel inhibitor	CCI
Cardiac output	CO
Celiac ganglia	CG
Celiac ganglionectomy	CGx
Depot pool	DP
Deoxycorticosterone acetate	DOCA
1,3-dipropyl-8-p-sulfophenylxanthine	DPSPX
Dihydroethidium	DHE
Dorsal vagal complex	DVC
Enteric nervous system	ENS
Excitatory junction currents	EJC
Excitatory junction potential	EJP
Excitatory post synaptic potential	EPSP
High performance liquid chromatography	HPLC
High voltage activated	HVA

Human embryonic kidney cells	HEK cells
Hydroxyl radical	H_2O_2
Intermediolateral	IML
Large dense core vesicles	LDCV
Mitogen-activated phosphorylation	MAP
Neuroeffector junction	NEJ
Neuromuscular junction	NMJ
Neuropeptide Y	NPY
Neurotransmitter	NT
Nicotinomide adenine dinucleotide phosphate	NADPH
Nitric oxide synthase	NOS
Noradrenaline/Norepinephrine	NE
Norepinephrine transporter	NET
Nucleus of the solitary tract	NTS
ω-agatoxin IVA	ATX
ω-conotoxin GVIA	CTX
Organum vasculosum lamina terminalis	OVLT
Paraventricular nucleus	PVN
Peripheral nervous system	PNS
Pertussis Toxin	PTX
Reactive oxygen species	ROS
Ready releasable pool	RRP
Renin-angiotensin system	RAS
Rostroventrolateral medulla	RVLM
Small synaptic vesicles	SSV
Smooth muscle cell	SMC
Spontaneously hypertensive rats	SHR
Sprague Dawley	SD
Subfornical organ	SFO
Superoxide	O_2^-
Superoxide dismutase	SOD

Sympathetic nervous system	SNS
Tetrodotoxin	TTX
Total peripheral resistance	TPR
Transmission electron microscopy	TEM
Treatment	Tx
Tyrosine hydroxylase	TH
Vesicular monoamine transporter	vMAT
Voltage dependent calcium channel	VDCC
Water	H_2O

CHAPTER 1

GENERAL INTRODUCTION

Hypertension: A worldwide epidemic

Hypertension, defined as a blood pressure greater than 140/90 mmHg (systolic over diastolic pressures), affects approximately 50 million Americans and approximately 1 billion people worldwide (JNC7, 2003). However, the risk of cardiovascular disease begins at 115/75 mmHg and doubles with each 20 mmHg increment in systolic pressure or 10 mmHg increment in diastolic pressure. Even individuals who are normotensive at age 55 have a 90 percent lifetime risk for developing hypertension (Vasan et al., 2002). Hypertension is a multifactorial disease with both genetic and environmental components complicating treatment regimens. Hypertension control rates, although improving, continue to be low (Hajjar and Kotchen, 2003), and as our population continues to age, the importance of reliable and chronic regulation of blood pressure in patients of all ages, but especially the elderly, is imperative.

With all of the recent advances in cholesterol and blood pressure-lowering medication, why aren't current therapies working? The Healthy People 2010 initiative set a goal of a 50% control rate for hypertensive patients. However, despite treatment rates of 75% or greater, hypertension in only 33 to 50% of patients is controlled to goal levels for BP (Wong et al., 2007). Diet and exercise continue to be the most important first-line therapies for hypertension and is the sole treatment for pre-hypertensive patients (129-139/79-89). Typical pharmacological therapy includes thiazide diuretics alone or in combination with the following: angiotensin-converting enzyme inhibitors (ACE-I), angiotensin

receptor blockers (ARBs), β-adrenergic receptor blockers (β-blockers) or calcium-channel blockers (CCIs).

There are two broad categories of hypertension, secondary, where an underlying cause can be identified and treated, and essential, where the hypertension is due to an unknown cause. 90-95% of hypertensive patients have essential hypertension. A resistant patient is one who has a body mass index (BMI) <24.9, is on three pharmacological therapies and has no underlying condition causing a rise in blood pressure, but continues to be hypertensive. Main causes of this include cost of medication, complicated treatment regimens/polypharmacy or side effects of medication. Therefore, the need for intensified efforts to achieve BP control is imperative.

Neural control of blood pressure

General

Blood pressure (BP) is the product of cardiac output (CO) and total peripheral resistance (TPR). CO, the amount of blood pumped by the heart/minute, is determined by stroke volume and heart rate. TPR is primarily determined by the resistance to blood flow in small arteries and arterioles with a small contribution from small veins and venules. The central nervous system, the autonomic nervous system, humoral factors, and local autoregulation contribute to blood pressure regulation.

The central and peripheral nervous systems play a large role in blood pressure regulation. Alterations in cardiovascular centers in the brain, or in peripheral neurotransmission to vascular tissues correlate with hypertension.

Central nervous system (CNS)

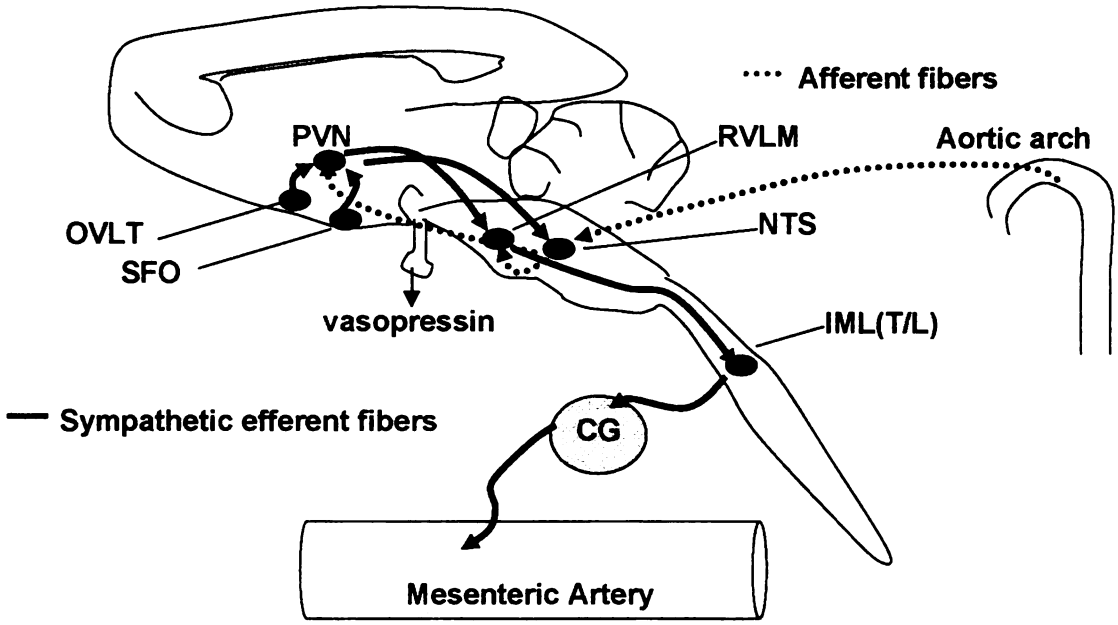
Acute changes in blood pressure are regulated by the baroreflex arc (Cowley et al., 1973). These pathways also contribute to long-term blood pressure regulation. Mechanoreceptors on sensory nerves innervating the carotid artery and aortic arch relay pressure-related information to the nucleus of the solitary tract (NTS), the primary brainstem site for the receipt of cardiovascular information from the periphery. Mechano- and chemosensitive renal afferent nerves also relay peripheral information to autonomic nuclei in the CNS (Stella et al., 1987). The paraventricular nucleus of the hypothalamus (PVN) is the main cardiovascular relay system in the mammalian brain. It receives inputs from the NTS and the rostral ventrolateral medulla (RVLM), a major point of regulation from forebrain and hypothalamic nuclei. The PVN also directly senses cerebral spinal fluid (CSF) and therefore NaCl levels. PVN neurons project back out to the RVLM and NTS, which send axons to the preganglionic neurons in the intermediolateral (IML) column of the spinal cord and the dorsal vagal complex (Brooks et al., 2005a).

Water and salt intake is mainly regulated by the release of vasopressin (AVP), an anti-diuretic, produced by magnocellular neurosecretory cells of the supraoptic nucleus (SON) and PVN. The magnocellular neurons of these nuclei

are regulated by circumventricular organs such as the subfornical organ (SFO) and median preoptic nucleus (MnPO), which have direct access to NaCl levels in the CSF via the ventricles (Denton et al., 1996). These circumventricular tissues are particularly susceptible to changes in plasma sodium, such that small changes in Na^+ levels have a profound effect on blood pressure (Gomez-Sanchez and Gomez-Sanchez, 1995).

Human essential hypertension (Coruzzi et al., 2005) and animal models of hypertension are associated with impaired NaCl sensing in addition to impaired autonomic cardiovascular control. The PVN and the central sympathetic and baroreceptor systems are all involved in the development of mineralocorticoid hypertension in rats (Gomez Sanchez, 1991). Increases in $[Na^+]o$ in the CSF increases sympathetic nerve activity and plasma concentrations of NE (Reid et al., 1975; Lamprecht et al., 1977), and lesions of the PVN ameliorate or prevent the development of salt-sensitive hypertension (Herzig et al., 1991). While this pathway has been well established, the mechanism by which such small increases in $[Na^+]$ can ultimately lead to such large increases in blood pressure are not clear. O'Donaughy and Brooks showed that in the DOCA-salt model sympathoexcitation and hypertension are a result of amplified NaCl signals by DOCA (Brooks et al., 2005b). One possibility for this increase in NaCl sensitivity in the DOCA-salt model is the presence of mineralocorticoid (MC) receptors in the circumventricular areas such as the organum vasculosum of the lamina terminalis (OVLT) and SFO (Pietranera et al., 2001). Activation of MC receptors in these areas by DOCA may amplify the NaCl-induced

Fig. 1



OVLT = organum vasculosum lamina terminalis; PVN = paraventricular nucleus of the hypothalamus; RVLM = rostral ventral lateral medulla; SFO = subfornical organ; NTS = nucleus of the solitary tract; IML = intermediolateral column; T/L = thoracic/lumbar; CG = celiac ganglia

Fig. 1. Schematic illustrating central neural control of the splanchnic circulation. Many cardiovascular nuclei work together in the mammalian brain to regulate blood pressure. Mechanoreceptors located in the aortic arch and carotid arteries send afferent signals to the NTS. NaCl and osmolality of the CSF are monitored through circumventricular organs (eg. SFO, OVLT). The messages received by these nuclei are integrated in hypothalamic nuclei (eg. PVN) and brainstem nuclei (eg. RVLM and NTS). Released vasopressin, an anti-diuretic, will increase fluid volume, and raise blood pressure. The RVLM sends axons to the periphery via the intermediolateral (IML) column (sympathetic). (Parasympathetic signals are relayed through the vagal nucleus (not shown). Mesenteric arteries are solely innervated by the sympathetic branch of the autonomic nervous system. Preganglionic sympathetic neurons synapse in prevertebral ganglia (eg. CG) onto postganglionic nerve fibers. Sympathetic nerve terminals innervate smooth muscle cells of blood vessels to regulate their tone.

sympathoexcitation response to NaCl and enhance pressor and sympathoexcitaory effects of increases in NaCl.

Separate from MC activation other possibilities for dramatic increases in blood pressure in response to increased Na^+ levels and osmolality include changes in synaptic plasticity or increased expression of certain genes in response to long-term activation of autonomic nuclei such as the RVLM (Ito et al., 1999). Studies in Sprague Dawley rats placed on high salt diets have given insight into other critical autonomic targets including the NTS and caudal ventrolateral medulla, whose activity were significantly enhanced by moderate Na^+ loading in animals consuming high dietary Na^+. The increased basal activation of neurons in these medullary sites could account for decreased baroreflex-induced bradycardia in the presence of a high salt diet, ultimately increasing blood pressure (Bealer, 2005; Bealer and Metcalf, 2005).

Finally, oxidative stress, found in hypertension, has a direct effect of modulating crucial intracellular signaling cascades in neurons of the NTS, a brain region that plays an important role in cardiovascular processes (Glass et al., 2007). Oxidative stress has a secondary effect of decreasing nitric oxide (NO) availability. In the PVN, NO is sympathoinhibitory. Therefore decreases in its availability due to oxidative stress may be responsible for increased sympathetic nerve activity in the PVN (Li and Patel, 2003) or other brain nuclei (Kishi et al., 2004).

Autonomic nervous system (ANS)

The autonomic nervous system has three branches, the parasympathetic nervous system (PNS), the sympathetic nervous system (SNS) and the enteric nervous system (ENS). The ENS is an intrinsic nervous system primarily found within the wall of the gastrointestinal tract. The PNS and SNS, which primarily oppose each other in action, work in concert to maintain blood pressure within a narrow margin.

The PNS originates from the dorsal vagal complex (DVC) in the brainstem and from sacral nerves. Pre-synaptic nerves release acetylcholine (ACh) onto post-synaptic nerves at ganglia close to the target organ which they innervate. Post-synaptic nerves also release ACh onto target tissues. Of particular importance is the vagus nerve which carries efferent fibers to the heart. When mechanoreceptors detect an increase in blood pressure, vagal nerves are activated which slows the heart and decreases blood pressure.

Arteries and veins in the splanchnic circulation are innervated by the SNS and sensory afferent nerves, but not the PNS. Preganglionic sympathetic nerve fibers originate in the IML column of the thoraco-lumbar regions of the spinal cord. Prevertebral nerves release ACh onto nicotinic ACh receptors on post-synaptic neurons in prevertebral ganglia. Some of these neurons supply the splanchnic vasculature where they release norepinephrine (NE), ATP and neuropeptide Y (NPY). The splanchnic circulation is densely innervated by sympathetic nerves originating from the celiac and superior mesenteric ganglia (which are indistinguishable in the rat), such that celiac ganglionectomy (CGx) protects against hypertension (King et al., 2007). This establishes the importance

of the splanchnic circulation as well as the ANS as critical components in blood pressure regulation.

Neuroeffector junction

Nerve stimulation of peri-arterial sympathetic nerves results in neurotransmitter release into the neuroeffector junction. Neurotransmitter release is a complex, multi-step process which requires a depolarization in the nerve ending and the influx of Ca^{2+} into active zones where synaptic vesicles containing neurotransmitters are docked (Del Castillo and Katz, 1956). The entrance of Ca^{2+} into these active zones causes the fusion of the vesicles with the plasma membrane and release of neurotransmitter into the neuroeffector junction (NEJ). Neurotransmitters bind to postjunctional receptors mediating the appropriate response in an effector cell, such as arterial smooth muscle cells (SMC). Upon stimulation, a portion of excess neurotransmitter is removed from the NEJ via diffusion. In addition, some of the neurotransmitter binds to prejunctional autoreceptors which negatively regulates neurotransmitter release or transporters (ie. norepinephrine transporter (NET)).

Postganglionic sympathetic nerves release norepinephrine (NE) and ATP as primary neurotransmitters and neuropeptide Y (NPY) as a neuromodulator (Donoso et al., 1997) into the NEJ (each discussed separately below). Nerve stimulation results in a biphasic response with ATP mediating a transient depolarization via P2X receptors and NE mediating a sustained response via α_1-adrenergic receptors (α_1ARs). The contribution of co-transmitters to autonomic

vasoconstriction can vary considerably between vascular beds and with different patterns of stimulation as reviewed by Gibbins and Morris (Gibbins and Morris, 2000). Evoked ATP release ranges from 3.76 pmol in the rabbit aorta (EFS: 16Hz; (Sedaa et al., 1990)) to 4-5000 pM/g in rat caudal artery (EFS: 8 Hz, 1440 shocks (Westfall et al., 1987)). In small mesenteric arteries of normal rats, ATP is the primary neurotransmitter in both non-pressurized (Luo et al., 2003) and pressurized vessels (Rummery et al., 2007). In general, mesenteric arteries are highly branched, and as the order of the vessel gets bigger (vessel diameter gets smaller), the role of ATP is increased (Gitterman and Evans, 2001). The vessels used in these studies were third order, and mostly purinergic (Luo et al., 2003).

The vesicular organization of neurotransmitter containing vesicles and their relationship to each other in the sympathetic nerve terminal can be seen directly using transmission electron microscopy (TEM) (Luff et al., 1987; Klemm et al., 1993). TEM photomicrographs have helped to identify the ultrastructure of sympathetic NEJs of mesenteric arteries and veins. The main findings include the presence of specialized junctions which are found in clusters opposed to arteries and veins. In both vessels clefts (< 100 nM) have a single layer of basal lamina and specialized areas with synaptic vesicle aggregated toward the presynaptic membrane (Luff et al., 1987; Klemm et al., 1993). Presumably these are synaptic release sites. This information helps to clarify questions which are hard to answer due to the small size of sympathetic nerve terminals (1 μM). For example, the presence of active zones suggests that the different time course of constriction mediated by NE compared to ATP is due to 2^{nd} messenger proteins

post-junctionally and not due to a long diffusion time of neurotransmitters. Examples of other properties of vesicular release from autonomic nerves we have learned from TEM include: terminal portions of autonomic nerve fibers are varicose, with intervaricose intervals ranging from 2 – 10 μM; in blood vessels nerves are confined to the adventitial side of the medial layer, different from NMJs; there is a lack of postjunctional specializations; Schwann cells are present, covering axons up until the last few varicosities in a nerve fiber; and the distance between sympathetic nerve terminals and effector cells varies greatly (Burnstock, 2008). Interestingly, different types of vesicles, small and large, dense and clear, can be seen in certain cross sections of a single varicosity, as shown in axon profiles within the myenteric plexus of the guinea-pig (Cook and Burnstock, 1976).

This technique can provide snapshots of neuroeffector junctions at any one time. However, TEM photomicrographs have not been used to compare sympathetic nerve endings between control and hypertensive rats. Because currents cannot be measured directly from mesenteric periarterial nerves, photomicrographs may help to provide insight into the number and distribution pattern of synaptic vesicles in nerve endings.

Fig. 2

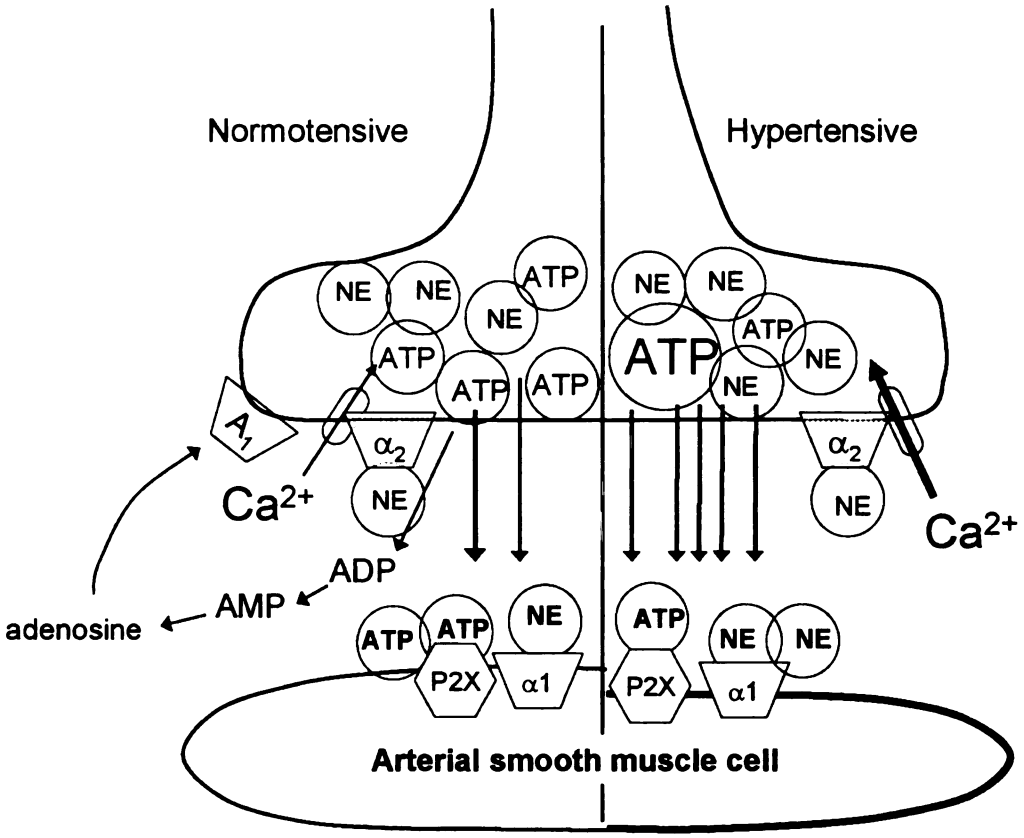


Fig. 2. Schematic depiction of a mesenteric artery neuroeffector junction depicting key components to vesicular release and reuptake of neurotransmitters (*left*) and alterations which occur in salt-dependent hypertension (*right*). Sympathetic nerve terminals innervating arterial smooth muscle cells (SMC) release ATP and NE in response to nerve stimulation and depolarization of the nerve terminal due to Ca^{2+} influx through voltage dependent calcium channels (VDCC). The VDCCs are closely associated with vesicular release apparatus, prejunctional autoreceptors (ie. A_1R and α_2AR) and help to regulate neurotransmission. Postjunctionally, NE binds to G-protein coupled α_1ARs while ATP binds to ligand-gated $P2X_1$ receptors. Upon activation, both receptors increase $[Ca^{2+}]_i$ resulting in constriction. In hypertension, sympathetic nerve endings are altered in several ways including: impaired α_2AR function, increased NE release, decreased ATP release and increased variability of ATP release.

Neural regulation of vascular tone

Adrenergic-mediated constriction

Sympathetic nerves densely innervate mesenteric blood vessels. Upon stimulation, NE is released and it binds to adrenergic receptors on SMCs causing constriction. In the mesenteric arteries and veins, NE acts primarily via α_1-AR. α_1AR couples to activation of a heterotrimeric G protein, Gq, which activates phospholipase C (PLC). PLC in turn hydrolyzes phosphatidylinositol (PIP_2) to diacyl glycerol (DAG) and inositol triphosphate (IP_3). DAG activates protein kinase C (PKC) resulting in the phosphorylation of a cascade of signaling proteins. IP_3 binds to receptors on the smooth endoplasmic reticulum causing the release of Ca^{2+} from intracellular stores. The release of intracellular calcium increases the $[Ca^{2+}]_i$ available to bind to contractile machinery in the SMC resulting in constriction. NE is removed from the NEJ via diffusion or reuptake into prejunctional terminals via specific high-affinity transporters, ie the norepinephrine transporter (NET).

Purinergic-mediated constriction

ATP is a ubiquitous molecule which plays a role in many vital physiologic functions. Its levels in nerve endings are primarily regulated by mitochondrial oxidative phosphorylation which generates ATP from ADP (Sperlagh and Vizi, 1996). In addition to its role as the major energy source for living cells, it has also been identified as a cotransmitter released with other previously identified

neurotransmitters from many nerve endings (Burnstock, 1995). This includes glutamatergic and gabaergic cells in the central nervous system (Pankratov et al., 2006), as well as cholinergic (Dowdall et al., 1974) and sympathetic nerve fiber endings (Stjarne and Stjarne, 1989; Burnstock, 2006) in the peripheral nervous system.

Purinergic neurotransmission has been studied in the vas deferens (Stjarne, 2001; Westfall et al., 2002) and vascular SMCs (Ralevic, 2000; Smyth et al., 2000b). Purinergic receptors which mediate ATP effects are classified into two categories based on their pharmacological properties. P1 receptors are activated by adenine nucleosides and their derivatives, and P2 receptors are bound by adenine nucleotides, such as ATP. P2 receptors are further divided into two subgroups: P2X and P2Y. P2X receptors have 2-TM domains with both the N- and C- terminus located intracellularly. They are ligand-gated ion channels that are permeable to Ca^{2+}, Mg^{+} and Na^{+} ions and primarily mediate vasoconstriction. P2Y receptors are G-protein coupled receptors that have 7-TM domains with an intracellular C-terminus and an extracellular N-terminus. P2Y receptors couple to Gq-proteins in smooth muscle and endothelial cells contributing to both constriction (Galligan et al., 2001) and vasodilation (Ralevic, 2000). In the mesenteric vasculature there is subtype-specific localization of the two types of receptors. $P2X_1$ receptors are found on arterial SMCs where, upon activation, cation flux mediates a transient postjunctional response which accounts for 60-80% of constriction at low frequencies (Sjoblom-Widfeldt et al., 1990; Gitterman

and Evans, 2001; Luo et al., 2003). Ca^{2+} influx through $P2X_1$ receptors mediates transient constriction of arterial SMCs.

In response to nerve stimulation ATP is released and then quickly degraded by nucleotide triphosphate diphosphorylases (NTPDases; aka. ecto-ATPase, ecto-pyrase or ectonucleotidases) which are released with ATP (Todorov et al., 1997; Westfall et al., 2000b; Westfall et al., 2000a). Interestingly, it has been suggested that proteins carrying the enzyme activity originate from ATP-storage vesicles as opposed to catecholamine vesicles due to the time course and modulation of release (Mihaylova-Todorova et al., 2001), suggesting separate storage of the two neurotransmitters. The breakdown of ATP can be slowed by inhibiting the nucleotidases with suramin and ARL 67156 (Westfall et al., 2000a). The nature of the enzymes released are not fully understood but seem to have similarities with members of the mammalian ecto-ATPase CD39 (ENTPDases) family as well as AMPases. This may allow cooperative breakdown of extracellular ATP as it is released from sympathetic nerves (Westfall et al., 2002). Adenosine, the product of the enzymatic breakdown of ATP, is a major regulator of neurotransmitter release via the A_1 receptor found on the nerve terminal (discussed below).

The purinergic component of neurotransmission can be effectively monitored via intracellular recordings from SMCs. Excitatory junction potentials (EJPs) are $P2X_1$-mediated responses as they can be inhibited by both tetrodotoxin (TTX; a Na^+ channel antagonist) and PPADS (a drug that can block $P2X_1$-

receptors). EJP peak amplitude can be assessed as an indirect, but semi-quantitative measurement of ATP release.

The role of purinergic neurotransmission in vivo

Few studies have assessed the role of purinergic neurotransmission *in vivo*. One of the roles for ATP as a cotransmitter from sympathetic nerves includes blood pressure homeostasis, which may occur via both prejunctional and postjunctional mechanisms. The role of purinergic transmission in blood pressure regulation *in vivo* varies depending on the tissue bed and species being examined. In general, it appears that ATP has its greatest role in mediating vascular tone in small vessels like tertiary branches of mesenteric blood vessels (Gitterman and Evans, 2001) and arterioles in the kidney. For example, autoregulatory mechnanisms of afferent arterioles in the kidney have been attributed to locally released ATP via activation of $P2X_1$ receptors (Inscho, 2001). Purinergic neurotransmission to kidney arterioles is impaired in a model of hypertension induced by chronic angiotensin II infusion (Ang-II) (Zhao et al., 2005). In the mesentery, resistance vessels play a substantial role in blood pressure regulation and contain a relatively large percentage of cardiac output at any one time (up to 70%). ATP also can modulate NE release and the release of endothelium derived hyperpolarizing factor (EDFH) via $P2Y_2$ receptors (Thapaliya et al., 1999). Therefore, the role of purinergic neurotransmission to these tissues is likely to have profound effects on blood pressure.

In vivo, blocking postjunctional P2X receptors with PPADS decreased MAP in one study (Tarasova et al., 1998), but had no effect in others (Emonnot et al., 2006). P2X receptor blockade increases blood pressure variability suggesting that ATP plays a role in transient homeostatic hemodynamic control. This was not the case when adrenergic neurotransmission was blocked (Golubinskaya et al., 1999). This suggests purinergic neurotransmission is important for stabilization of MAP.

The ATP metabolite, adenosine, regulates blood pressure via the prejunctional adenosine autoreceptor (A_1-R). The prejunctional effects of A_1-R blockade has profound consequences on blood pressure as exemplified by the hypertension model produced by A_1-R antagonist, DCNX (discussed below).

Neuropeptide Y (NPY)

NPY is tyrosine-rich peptide in the brain (Tatemoto, 1982). It is also released as a neuromodulator from peripheral autonomic synapses onto blood vessels (Donoso et al., 1997; Smyth et al., 2000a; Pablo Huidobro-Toro and Veronica Donoso, 2004). NPY increases the vasoconstrictor action of ATP and NE mainly via the Y_1 receptor subtype (Westfall et al., 1995). The Y_2 subtype is primarily found on prejunctional membranes and it plays an inhibitory role on the release of neurotransmitters in the central nervous system as well as sympathetic perivascular nerve terminals (Uddman et al., 2002; Pablo Huidobro-Toro and Veronica Donoso, 2004).

The role of NPY in hypertension is controversial. Administration of the selective NPY Y_1 receptor antagonist does not lower BP (Zhao et al., 1997). Furthermore, Y_1 knock-out mice have normal blood pressure and the basal blood pressures and heart rates of transgenic rats with increased levels of NPY in cardiovascular tissues was also normal (Michalkiewicz et al., 2001). However, transgenic rats had increased total vascular resistance and elevated blood pressure responses to NE injections (Michalkiewicz et al., 2001). Furthermore, at least one study in humans found a significant increase in plasma ir-NPY levels in patients with severe hypertension as compared to age-matched controls (Erlinge et al., 1992). The role of NPY as a direct constrictor via Y_1 receptors and as a neuromodulator of the actions of ATP and NE is continuing to unfold. Future studies may identify a use of Y1 receptor antagonists for treatment of hypertension.

Prejunctional autoregulation of sympathetic neurotransmission

α_2-adrenergic receptors (α_2AR)

Some of the NE released from the NEJ will bind to prejunctional α_2-ARs. α_2ARs are 7-transmembrane domain autoreceptors which couple to $G_{i/o}$ proteins. They inhibit neurotransmitter release by decreasing adenylate cyclase activity and cAMP production, inhibiting N- and P/Q-type Ca^{2+} channels, and activating presynaptic K^+ channels. There are three human subtypes of α_2ARs: α_{2A}, α_{2B} and α_{2C}. A fourth, α_{2D}, found in rats, may be an orthologue of the α_{2A} receptor

(Docherty, 1998). The α_{2A} and α_{2C} subtypes are thought to be located on presynaptic terminals and their activation inhibits neurotransmitter release from sympathetic nerves in the heart, the central nervous system and from perivascular sympathetic nerves (Docherty, 1998; Hein et al., 1999). The actions of prejunctional α_2-ARs can be modified by circulating neuronal and humoral substances. Possible modifiers include O_2^- (Nishida et al., 2000), ATP, adenosine and bradykinin.

Previous studies have shown α_2AR function is impaired in human essential hypertension (Tsuda et al., 1989; Damase-Michel et al., 1992; Damase-Michel et al., 1993) and in DOCA-salt hypertension (Tsuda et al., 1989; Moreau et al., 1995; Luo et al., 2004), and clinically, α_2-agonists are used to treat high blood pressure in hypertensive patients (Brede et al., 2004). However, the exact mechanism which renders this receptor dysfunctional is not known.

Conventionally, α_2AR was thought only to regulate NE, but since Burnstock purposed the idea of co-transmission, the role of the α_2AR on regulating ATP release has been investigated. Through a combination of electrophysiological, electrochemical and pharmacological experiments, the role of the α_2AR in regulating not only NE, but also ATP has been revealed. Blocking the α_2AR at the sympathetic NEJ results in increased NE and ATP overflow (Driessen et al., 1993). Long trains of nerve stimulation with and without yohimbine, an α_2AR agonist, increases ATP release in addition to NE release (Bobalova and Mutafova-Yambolieva, 2001) in canine mesenteric arteries and veins. In guinea pig mesenteric arteries phentolamine and yohimbine enhanced

EJPs at low but not high frequency stimulation (Mutafova-Yambolieva and Keef, 2001). In addition, idazoxan another α_2AR agonist, increased the amplitude of EJPs evoked during trains of low frequency stimulation, even more than it increased facilitation of NE release (Dunn et al., 1999). This provides substantial evidence for the co-regulation of NE and ATP by the α_2AR, even though the efficiency of regulation may of each transmitter may differ and may be tissue specific.

A_1-adenosine receptors

ATP released from perivascular nerves is enzymatically degraded by ecto-5'nucleotidases into ADP, AMP and finally adenosine (Todorov et al., 1997). Adenosine binds to prejunctional A_1-Rs and inhibits neurotransmitter release via Gi/Go linked second messenger systems.

Adenosine acting at the A_1-R in the mesenteric vasculature bed has been shown to have effects on blood pressure. This has been illustrated best with the development of the 1,3-dipropyl-8-sulphophenylxanthine (DPSPX)-treated rat model of hypertension. The chronic inhibition of A_1-Rs with DPSPX, a non-selective antagonist of adenosine receptors results in increased blood pressure and increased purinergic and adrenergic neurotransmission indicating that adenosine plays a regulatory role in maintaining blood pressure via prejunctional autoreceptors (Karoon et al., 1995). This model also suggests the importance of ATP availability in sympathetic nerve terminals on blood pressure homeostasis.

This model of hypertension also identifies cross-regulatory mechanisms whereby NE release is regulated by ATP and adenosine via A_1-Rs. ATP also regulates NE release via prejunctional P2X receptors (Illes and Norenberg, 1987; Morikawa et al., 2007). Other prejunctional receptors responsible for purinergic mediated inhibition of NE have been identified, including a xanthine-sensitive ATP receptor (Morikawa et al., 2007), a P2 receptor (Tanaka et al., 2004) and an unidentified purinoceptor that is blocked not only by P2-receptor antagonists but also by P1-receptor antagonists (Shinozuka et al., 1988).

Others have shown that, *in vivo*, endogenous adenosine does not modulate noradrenergic neurotransmission (Jackson, 1987; Kuan and Jackson, 1988). However, Jackson et al. also showed that spontaneously hypertensive rats are less sensitive to the inhibitory effects of exogenous adenosine on adrenergic neurotransmission compared to WKY controls suggesting that enhanced noradrenergic neurotransmission in the SHR is not due to defective modulation of neurotransmission by adenosine (Jackson, 1987). The effects of exogenous adenosine in DOCA-salt hypertensive rats have not been examined.

Identification of the dual role of neurotransmitters as neuromodulators is becoming more common in the literature, although the details of this process are still under investigation. ATP, adenosine, NE and NPY all have prejunctional autoreceptors which modulate their own release in addition to the other co-transmitters in the terminal. NPY also is known to modulate ATP and NE at the postjunction. In addition, other neuromodulators have been identified, in adrenal medullary chromaffin cells. Catestatin, an active peptide cleaved from

chromogranin A inhibits the release of NE, ATP and NPY from these cells (Mahapatra et al., 2006). Interestingly, the secretory prohormone chromogranin A (CHGA) is overexpressed in essential hypertension, while its neurotransmitter release-inhibitory fragment catestatin is diminished (Mahapatra et al., 2005). These complex neuromodulatory pathways make understanding neural regulation of blood vessels even more diverse and complex

Cotransmission

Storage, regulation and release of ATP, NE and NPY

The relative contribution of each of the neurotransmitters in mediating constriction varies greatly depending on vascular bed, species and frequency of stimulation, and the regulation of different neurotransmitters is not clear. Four possible vesicle distribution patterns are depicted in figure 3. Panel A depicts the differential packaging of ATP and NE, with vesicles homogenously mixed. Evidence for this model includes the results that EJPs can be elicited with nerve stimulation after tissues have been treated with reserpine, a drug which depletes NE stores in guinea-pig mesenteric artery (Mutafova-Yambolieva and Keef, 2001). NE and ATP overflow from sympathetic nerves innervating guinea-pig vas deferens has also been studied. In this preparation evoked overflow of ATP exceeded that of NE, also suggesting differential storage of vesicles. Finally, the overflow of NE is tonic while the overflow of ATP and other purines is phasic (Todorov et al., 1996).

Fig. 3

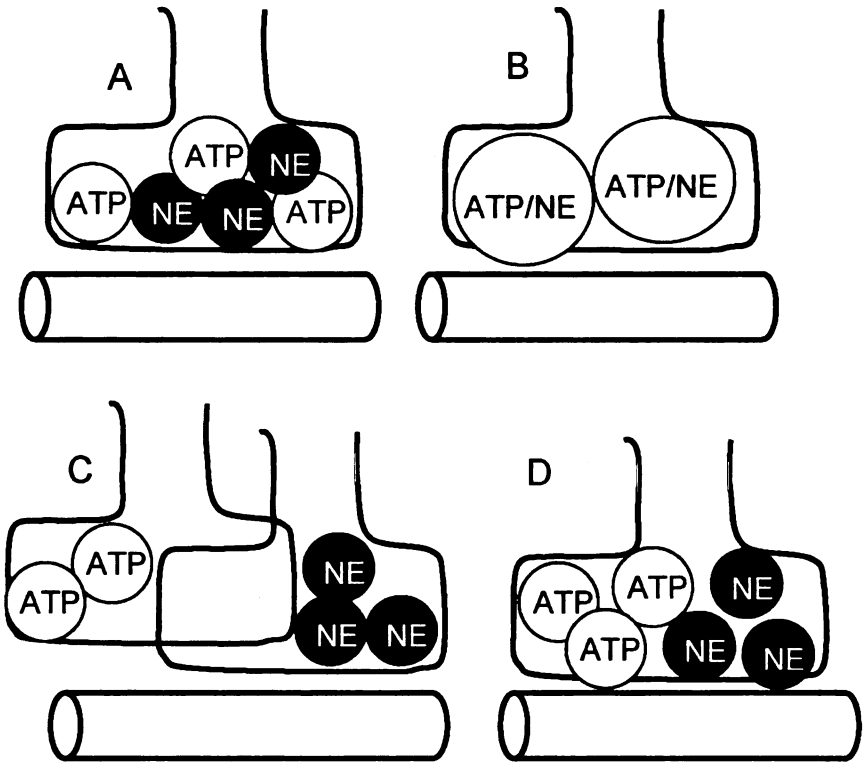


Fig. 3. Diagrammatic depictions of four hypotheses for cotransmission of neurotransmitters from sympathetic nerve terminals. Panel A depicts the theory that ATP and NE are stored and released from separate vesicles, and that these vesicles are arranged in a homogeneous mixture. Panel B shows the hypothesis that ATP and NE are co-stored and co-released from the same vesicle. Panel C depicts the hypothesis that ATP and NE are released from separate nerve terminals. Panel D shows the two neurotransmitters arranged in a manner which allows them to be released and regulated individually. The four hypotheses described above were considered as possible models for neurotransmitter release when analyzing data in these studies.

However because many pharmacological manipulations affect NE and ATP to a similar extent, differential storage may not be the case in all preparations. Stjarne's work supports the hypothesis shown in Figure 2B, where ATP and NE are co-stored and co-released. For example, a study used α_2AR agonists and antagonists in combination with excitatory junctional currents (EJC) measurements and amperometric techniques to measure ATP and NE simultaneously. Although NE and ATP measurements responded differently in some cases, the authors concluded that these responses could be explained in terms of "the known post-secretory effects of these agents" (Msghina et al., 1992). These results suggest that ATP and NE may be regulated in the same manner. In a paired pulse analysis of ATP and NE release from sympathetic nerves of rat tail artery and mouse vas deferens, pharmacological blockade of K^+ channels depressed excitatory junctional currents (ATP) and NE oxidation current to a similar extent, also suggesting that ATP and NE are released in parallel (Msghina et al., 1998).

Fig. 3C depicts the hypothesis that different neurotransmitter compositions exist in different nerve fibers. This idea is true for fibers innervating mesenteric arteries and veins. These fibers run close together from sympathetic ganglia to the mesentery. However those innervating arteries are veins originate from separate neurons in the ganglia and innervate different tissues (Browning et al., 1999).

Finally, there is growing evidence for the differential distribution of NE and ATP containing vesicles in the nerve ending as depicted in Panel D.

Differential regulation of these pools of transmitters may provide functional roles such as "chemical coding" (Furness and Costa, 1987). This may be necessary for precise regulation of blood pressure, creating more transient or tonic control of the vessel as needed. Differential storage and vesicle localization has been established for small and large vesicles. For example NE and NPY are found in LDCV, while NE, but not NPY is found in SSV (Roden et al., 2007). ATP has been reported in both SSVs and LDCVs. It is widely accepted that the release of SSVs and LDCVs are regulated differently. SSVs are coupled to voltage-dependent calcium channels and LDCVs, further from release zones, respond to Ca^{2+} which has diffused through the cytoplasm (Matteoli et al., 1988; Langley and Grant, 1997).

Differential release of the same size vesicles is more controversial. With more and more evidence of differential storage and release of ATP and NE from sympathetic nerves, their possibilities for differential regulation are being explored. Relative appropriation to Ca^{2+} channels would be one way in which vesicles of the same size could be differentially regulated and released (Waterman, 2000). Differences in the kinetics of Ca^{2+} binding between vesicle pools could also allow for neurotransmitters to be released at different rates (Voets, 2000). Additionally, the various isoforms of vesicular release components such as the SNARE proteins and Ca^{2+} sensors, are also candidates for the differential regulation of cotransmitters (Moore et al., 2006).

Pharmacological studies targeting prejunctional autoreceptors have been used to answer questions of storage and release of co-transmitters. Differences in

the α_2ARs ability to regulate NE and ATP release have been demonstrated. For example, in the canine mesenteric artery a higher concentration of idazoxan was needed to increase ATP release compared to that needed to increase NE release. Furthermore, ATP release is only altered in the presence of α_2AR blockers at high frequencies where NE is increased at low and high frequencies (Bobalova and Mutafova-Yambolieva, 2001). This suggests that NE release is more closely coupled to the α_2AR than ATP. When NE and ATP release were measured from the rat mesenteric artery NEJ using amperometry and intracellular recordings, respectively, the opposite was true. Using these techniques, α_2AR blockade resulted in greater facilitation of EJPs compared to the facilitation of current (Dunn et al., 1999). Besides the use of different species in these studies, different techniques may account for the contradictory results. However, taken together these data support the differential storage and regulation of ATP and NE. These differences may play an important role in the control of blood flow and volume distribution in splanchnic circulation and therefore blood pressure.

Furthermore, it appears that the alterations to the NEJ in disease processes such as hypertension can be reflected in changes in the relative contribution of each neurotransmitter released (Kolo et al., 2004a). However the role for altered regulation of neurotransmitter release in hypertension has mainly focused on changes in NE release. Because of the overlap in regulation between neurotransmitters and their metabolites, an intricate balance of neurotransmitters must be maintained. There is still a lot to understand about regulation of multiple

transmitters found in sympathetic nerve endings, and how that may be altered in hypertension.

Properties of purinergic neurotransmission

The first intracellular recordings of EJPs were recorded from the guinea pig vas deferens in response to stimulation of the hypogastric nerve (Burnstock and Holman, 1961). The term purinergic neurotransmission was coined by Burnstock in 1972 (Burnstock, 1972). EJPs are similar to end plate potentials (EPPs) recorded from the somatic neuromuscular junction, such that EJPs are quantal, can summate and eventually mediate action potentials and contraction of smooth muscle. Then in 1976 Burnstock wrote the controversial review entitled, "Do some nerve cells release more than one transmitter?" which paved the way for the principle of co-transmission (Burnstock, 1976).

It is now clear that ATP serves as a co-transmitter in sympathetic neurotransmission in the rat tail artery (Sneddon and Burnstock, 1984), rabbit ear artery (Morris et al., 1998) and rat and mouse mesenteric artery (Donoso et al., 1997). The physiological role of ATP as a neurotransmitter is complex, and the details remain elusive. Importantly, the properties of EJPs change in pathological states such as diabetes (Gunes et al., 2005) and hypertension (Brock and Van Helden, 1995). As sympathetic nerves fire at rates ranging from 0.04 to 10 Hz (Barman and Kenney, 2007) experiments were designed within these parameters

to study purinergic neurotransmission to resistance arteries from DOCA-salt hypertensive rats.

Facilitation of EJPs

Facilitation, or short term synaptic enhancement, as discussed by Fisher et al., is characterized by an increase in the number of quantal packets released per spike or frequency of spontaneous miniature endplate potentials (fMEPP) without an increase in quantal amplitude – the postsynaptic response to a single quantum of neurotransmitter released (Fisher et al., 1997). The way that this definition translates and the mechanisms that underlie EJP facilitation at the sympathetic neuroeffector junction remains poorly understood. It is likely that facilitation, regardless of synapse or junction, involves an increase in the level of free and/or bound Ca^{2+} (residual Ca^{2+}) in the nerve terminal during the period of stimulation as first described by Katz and Miledi in 1968 (Katz and Miledi, 1968; Hardy and Brock, 2001).

Ca^{2+} influences neurotransmission and downstream effects in many ways ranging from vesicle mobilization, docking and priming, to long term facilitation in the central nervous system. The actual distribution of vesicles and their distance from Ca^{2+} channels in sympathetic nerve terminals is also important for release properties. Long term potentiation, which has been studied extensively in the CNS, occurs in response to a Ca^{2+} dependent processes including increased cAMP acitivity leading to increased cAMP-dependent protein kinase (PKA (Trudeau, L 1996)), phosphorylation of SNAP-25 and vesicular movement (Nagy

2002 and 2004). Alterations in Ca^{2+} handling in sympathetic nerve terminals could play a role in altered neurotransmission in disease states such as hypertension.

Facilitation of post-synaptic responses is dependent on more than just Ca^{2+} influx. The probability of release is also an important factor in determining the amount of facilitation. Alterations in facilitation can manifest from a change in the probability of release (p), a change in the number of releasable quanta (N), or both (Fisher et al., 1997). In general, a facilitating synapse is one in which there is a low probability of initial transmitter release. It is not necessarily dependent on the size of the readily releasable pool as once thought (Stevens and Tsujimoto, 1995). In fact in the crustacean motor neurons increasing the number of docked vesicles did not result in a higher synaptic release probability (Millar et al., 2002).

The readily releasable pool (RRP) plays a role in facilitation, but definitions of the different vesicle pools are not consistent in the literature, with two to four distinct pools being described. Most studies have been conducted in unusually large nerve endings such as the calyx of Held or large neuromuscular junctions. Recently however better techniques including flash photolysis of DM-Nitrophen-EGTA and amperometric recordings from neuroendocrine cells have helped to better characterize vesicle pools. Becherer and Rettig define four pools according to their release kinetic and their spatial distribution: a RRP, slowly releasable pool (SRP), an unprimed pool (UP) and a depot pool (DP) (Becherer and Rettig, 2006). Others have differentiated the RRP into an immediately releasable pool (IRP), found in the immediate vicinity of Ca^{2+} channels (Neher,

1998) and a highly Ca^{2+}-sensitive pool, which is released in response to Ca^{2+} levels < 10uM (Yang et al., 2002). The primed pools are released within 20-200ms, where the unprimed pools are only released in response to a sustained stimuls and increased $[Ca^{2+}]_i$. The DP, > 200nm from the membrane, is the largest pool of vesicles in adults in most synapses studied (Sorensen 2004). The docked, RRP and DPs can be seen in transmission electron microscopy (TEM).

It should be noted that the information which is available in the literature, and discussed above, has been gathered almost entirely from CNS synapses or large neuromuscular junctions (NMJ). However, many differences exist between synapses, typical NMJs and NEJs, were smooth muscle cells are the effector tissue (Burnstock, 2008). While it is known that EJPs in sympathetic neuroeffector junctions facilitate (Hardy and Brock, 2001), the presynaptic terminals are too small to determine the mechanistic details with the techniques available. Indirect techniques have been used to record excitatory junctional currents (ECJs) from single, or a group of, visualized varicosities of sympathetic nerve terminals innervating the vas deferens (Brock and Cunnane, 1988; Lavidis and Bennett, 1992). This allows for the recording of quantal secretion from nerve terminals. In addition, the increased time and spatial resolution of microelectrodes coupled to amperometry have increased our knowledge of neurotransmitter release from adrenochromaffin cells.

Purinergic neurotransmission from sympathetic nerve terminals is just beginning to be addressed in detail (Morris et al., 1998) . Hardy and Brock found adenosine and angiotensin II increased the rate of facilitation by modifying Ca^{2+}

entry into the nerve terminal, but that isoprenaline's effects were not solely based on modifications in Ca^{2+} entry, suggesting other mechanisms besides Ca^{2+} are involved in facilitation of EJPs (Hardy and Brock, 2001). However not many studies have furthered these novel findings. The use of amperometry and perhaps recordings of EJCs from single varicosities will extend our knowledge of single vesicle mobilization and release properties and other aspects of facilitation.

Facilitation of EJPs from DOCA-salt rats and controls in response to low and high frequency trains can be used to study alterations in the number of vesicles in each of the described pools, their location in sympathetic nerve terminals in respect to Ca^{2+} channels, and their mobilization. Furthermore alterations in Ca^{2+} concentration may help to answer questions concerning Ca^{2+} dependence of ATP release. Alterations in prejunctional autoregulation of neurotransmitter release and availability of ATP in the nerve endings can also be probed using various electrophysiological and pharmacological techniques.

Depletion of neurotransmitter (rundown)

With high frequency trains of stimulation, the potential for depleting NT stores increases. Neither replacement with reserve pools, nor recycling of vesicles cannot keep up with the rate at which the RRP is depleted and excitatory post-synaptic potentials (EPSPs) (or post-junctional responses) become smaller. This is known as synaptic (or junctional) rundown. Vesicles will eventually be replenished with time, although the recovery time constant (τ) varies depending on synapse and degree of rundown. The recovery rate depends on trafficking

between vesicle pools and can vary from 5-8 s at synapses between hippocampal CA3 and CA1 pyramidal neurons (Debanne et al., 1996) to 2.8 s at rat superior cervical ganglion synapses (Lin et al., 2001). Pathological conditions and increased ROS can also play a role in modulating synaptic rundown and recovery in hippocampal cells (Pellmar, 1987).

Due to the important role of these processes in learning and memory, extensive studies in CNS pyramidal cells have used either hypertonic solution (sucrose) or nerve stimulation to examine the stores of vesicles, synaptic facilitation and depression (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). Studies of single hippocampal boutons have shown that frequent repetitive stimulation resulted in a decreased incidence of excitatory postsynaptic currents (EPSCs) similar to that found upon stimulation of several nerve endings. Furthermore, the decreased response was reversible, supporting the case that rundown is due to exhaustion of a pool of available vesicles (Liu and Tsien, 1995).

Less is known about rundown at the neuroeffector junction of perivascular nerves, although several studies have examined vesicle replenishment, both through recycling and vesicle mobilization from reserve pools, in the myenteric plexus (Ren and Galligan, 2005) and superior cervical ganglion (Lin et al., 2001). Lin et al. found that blocking prejunctional autoreceptors did not restore depression, while decreasing the concentration of external calcium or increasing the exogenous adenosine concentration increased depression suggesting that

depletion of the docked vesicles leads to depression of transmitter release during a high frequency train of impulses (Lin et al., 2001).

Using intracellular recordings, the effect of high frequency trains of stimuli on EJPs can be assessed. Decreased EJP amplitude can be attributed to depletion of ATP stores from presynaptic storage sites. However, other factors could also explain depleted NT stores. Examples include postjunctional desensitization, negative feedback from prejunctional autoreceptors, decreased calcium sensitivity, and alterations in reuptake and release mechanisms. Studies will be outlined to test each of these mechanisms for increased run-down in mesenteric arteries from control and DOCA-salt rats.

NE detection using amperometry

Until recently NE overflow with subsequent HPLC or radioligand detection were standard for measuring NE release in response to nerve stimulation *in vivo*. Recently, the application of continuous amperometric detection of NE with carbon-fiber microelectrodes to biological systems has made it possible to detect NE with much better spatial and temporal resolution compared to overflow techniques (Park et al., 2006, 2007). This technique takes advantage of the electroactive properties of catecholamines such as NE. Nerve stimulation causes synaptic vesicles release of NE into the neuroeffector junction. NE can bind to post-junctional receptors on SMCs, prejunctional autoreceptors on nerve terminals, recaptured by the NE transporter (NET) or it can diffuse away from the

NEJ.. The NE that escapes from the NEJ, will come in contact with the surface of a carbon-fiber microelectrode which can oxidize NE at the appropriate electrode potential. The resulting oxidation current is proportional to the number of molecules of NE adsorbed to the surface of the electrode. The number of NE molecules on the electrode is proportional to the number of NE molecules released and therefore reflects the concentration of endogenous NE at the blood vessel surface (Teschemacher, 2005). At an applied potential of 500mV, NE can be selectively oxidized, as ATP, adenosine and NPY, are either not electroactive or are not oxidized at this potential (El-Nour and Brajter-Toth, 2003).

Amperometric studies have been especially useful in elucidating neurotransmitter release and recovery properties from nerves at high temporal and spatial resolutions.

This technique has been previously used to study NE release from sympathetic nerve endings in both rat tail artery and mesenteric arteries and veins (Bao et al., 1993; Dunn et al., 1999; Park et al., 2007). Previous studies in hypertension have used overflow techniques to show increased NE overflow in response to nerve stimulation, however these techniques do not distinguish between release and reuptake of NE.

Voltage dependent calcium channels (VDCC)

Structure and function

Neurotransmission from central and peripheral nerves depends on the influx of Ca^{2+} through VDCCs (Katz and Miledi, 1968; Smith and Augustine,

1988) for migration, fusion and exocytosis of synaptic vesicles. Neurotransmitter is released into the junction when Ca^{2+} interacts with soluble NSP attachment protein (SNAP) receptor proteins on synaptic vesicles (v-SNARE) and on nerve terminal membranes (t-SNARE) resulting in fusion of vesicles with the plasma membrane (Sollner et al., 1993). In order for synaptic vesicles to fuse with the prejunctional membrane relatively high (50-100 μM) internal calcium concentrations are required (Matthews, 1996). VDCCs are therefore concentrated within microdomains of the terminal for more precise regulation of calcium in the synaptic ending (Fejtova and Gundelfinger, 2006). Immunohistochemical studies by Westenbroek *et al* co-localized the α_{1A} subunit of the P/Q-type calcium channel with syntaxin I, an important protein involved in the vesicular release pathway, in the same synaptic terminals (Westenbroek et al., 1995). Furthermore, synaptic responses are reduced with fast calcium chelators such as BAPTA, but not by slow calcium chelators such as EGTA. This is consistent with a localized active zone containing prejunctional calcium channels closely associated with synaptic vesicle machinery (Adler et al., 1991).

Synaptic transmission is inhibited in part by blocking VDCCs. Upon activation, prejunctional autoreceptors interact with VDCCs to block Ca^{2+} entry and therefore decrease NT release (Smith and Cunnane, 1998; Bian and Galligan, 2007).

Tissue Distribution

There are six high voltage activated (HVA) calcium channels (L-, N-, P/Q- and R-types) and one low voltage activated channel (T-type). HVA Ca^{2+} channels are multimeric complexes formed by a pore-forming α_1 subunit and several auxiliary subunits (β, α_2/δ and γ). It is the α_1 subunit which confers the pharmacological and functional properties of the calcium channel subtype.

Calcium channel subtypes are not homogenously distributed throughout nervous tissue, but vary depending on species of animal or vascular tissue bed studied. In general, the N-type and P/Q-type VDCCs appear to play predominant roles in neurotransmitter release from presynaptic terminals of central and peripheral autonomic and motor neurons (Currie and Fox, 1997). The P/Q-type calcium channel is the predominant VDCC involved in acetylcholine (ACh) release from the neuromuscular junction, while the N-type VDCC is the most prevalent subtype mediating neurotransmission at sympathetic nerve terminals.

However, N-type and P/Q-type VDCCs can coexist at a single release site and contribute jointly to local Ca^{2+} transients (Reid et al., 2003) and neurotransmission (Smith and Cunnane, 1997). In hippocampal neurons, it was shown that a mixture of N-type and P/Q-type channels varies markedly between terminals on the same afferent (Reid et al., 1997). Multiple channel subtypes in autonomic nerve endings may differentially regulate neurotransmitter release from vasodilator and vasoconstrictor neurons innervating the bladder (Waterman, 1996; Morris et al., 2001, 2002). However, other studies from the same group have not been able to reproduce these findings of differential regulation of neurotransmission from sympathetic nerves in this model (Morris et al., 2004).

Multiple Ca^{2+} subtypes have also been show to regulate NE and ATP release in the mouse vas deferens (Waterman, 1997) and ATP release from rat anococcyngeous muscle (Smith and Cunnane, 1997).

It seems probable that there is a heterogeneous profile expression pattern of VDCC subtypes in the nerve endings of most neuroeffector junctions. It has been hypothesized that subtypes of VDCCs could exhibit different efficacies in their ability to trigger and control the secretory process. The relative importance of this hypothesis in sympathetic nerves innervating mesenteric arteries is not known. Very few studies have examined sympathetic nerves innervating the rat mesenteric tissue bed. Intracellular electrophysiological and organ bath studies suggest small mesenteric arteries contain both N and "non-N" VDCC subtypes (Pruneau and Angus, 1990). It appears that at low frequencies the N-type Ca^{2+} channel solely regulates neurotransmitter release, but at high frequencies (24 Hz), only 80% of the response can be inhibited by ω-CTX (Wright and Angus, 1996). In periarterial mesenteric sympathetic nerve endings from the rat, it has been reported that N- and L-type calcium channels jointly regulate NT release (Rittenhouse and Zigmond, 1999) or that a combination of N- and P/Q- type Ca^{2+} channels are involved (Tanaka et al., 1999). However the exact identity of the VDCC subtypes responsible for the N-type Ca^{2+} channel resistant component in mesenteric vessels is unknown (Waterman, 2000). Furthermore, regulation of neurotransmitter release from sympathetic nerves in DOCA-salt hypertension has not been studied. Changes in the distribution of VDCCs may contribute to altered neurotransmission in disease states such as hypertension (Aldea et al., 2002).

Pharmacology

Several toxins have been isolated which bind to specific Ca^{2+} channel subtypes with high affinity and therefore have become useful tools to study neurotransmitter release. Because Ca^{2+} must enter the nerve terminal for vesicle fusion and neurotransmitter release, these toxins can be used *in vivo* and *in vitro* to block neurotransmitter release from nerve endings. N-type calcium channels and P/Q-type calcium channels have been implicated in sympathetic neurotransmission from post-ganglionic nerves. ω-conotoxin GVIA (CTX), a specific and irreversible N-type VDCC blocker in neurons, was originally retrieved from the venom of *Conus geographus* (marine snails) (McCleskey et al., 1987). ω-CTX binds to macrosites on the extracellular surface of the receptor and without the involvement of second messenger systems (McCleskey et al., 1987). P- and Q- type VDCCs are splice variants of a single gene. ω-agatoxin IVA (ATX), isolated from the funnel-web spider *Agelenopsis aperta*, blocks both variants irreversibly, but can be distinguished by their sensitivities (Olivera et al., 1994).

Clinical relevance

Both P/Q-type and N-type Ca^{2+} channels play a pivotal role in regulating neurotransmitter release at neuromuscular and sympathetic neuroeffector junctions (Waterman, 1997). Clinically, channelopathies account for many genetic disorders including cerebellar ataxias, Lambert-Eaton Syndrome,

abnormalities in pain responses, deafness and dysregulation of cardiac pacemaker activity. Blood pressure regulation is also highly dependent on Ca^{2+} channel function. For example, the α_1c subunit of the L-type Ca^{2+} channel is up-regulated in hypertension (Sonkusare et al., 2006). N-type Ca^{2+} channel deficient mice, display increased MAP and heart rate, which could not be attenuated with w-CTX (30 mg/kg) suggesting abnormalities in regulation of cardiovascular function (Ino et al., 2001).

Ca^{2+} channel diversity at sympathetic nerve endings may also be implicated in differential regulation and release of co-transmitters such as NE and ATP, a concept that is under current debate. Exploring physiological and pathophysiological properties prejunctional Ca^{2+} channels may reveal certain subtypes as potential therapeutic targets for the treatment of hypertension.

Oxidative stress and hypertension

Reactive oxygen species (ROS) are O_2 molecules with an unpaired electron including superoxide (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH-). In the vasculature, ROS are signaling molecules which can modulate vascular tone and structure (Griendling et al., 2000b; Griendling et al., 2000a). In physiological conditions, these molecules are kept at relatively low levels via endogenous anti-oxidants including superoxide dismutase (SOD),

Fig. 4

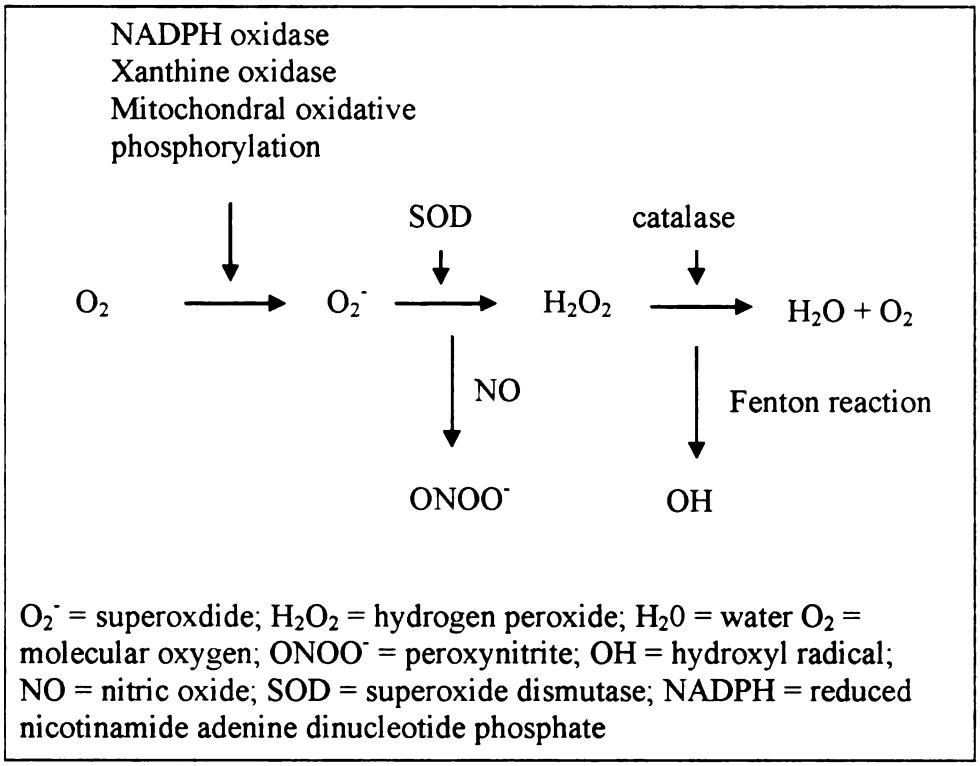


Fig. 4. Example pathways for the production of reactive oxygen species. There are many endogenous oxidant and antioxidant pathways, which participate in cellular signaling, some of which are shown here. In several enzymatic pathways, oxygen accepts a single electron, to produce superoxide (O_2^-), the parent reactive oxygen species. O_2^- is extremely reactive and is reduced to hydrogen peroxide (H_2O_2) either spontaneously or with the help of superoxide dismutase (SOD). O_2^- can also react with nitrogen species to produce another extremely reactive molecule peroxynitrite ($ONOO^-$). The hydroxyl radical (OH) is produced from H_2O_2 via the Fenton reaction while water is produced from H_2O_2 in a reaction mediated by catalase. An increase in prooxidants relative to antioxidants results in oxidative stress which occurs in response phosphorylation of the membrane-bound $p47^{phox}$ subunit.

catalase and glutathione peroxidase. However, in many pathological situations there is increased ROS which causes an upregulation of several signaling pathways resulting in smooth muscle cell growth, altered vasomotor tone and inflammatory responses (Dai et al., 2004; Gunes et al., 2005; Cao et al., 2007). Very high levels of ROS results in oxidative stress. Oxidative stress causes cell damage as a result of the oxidation of nucleic acids and proteins, cell loss owing to apoptosis, and phenotypic alteration as a result of the activation of abnormal gene programs (Hare, 2004).

A major source of O_2^- is a multi-subunit enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expressed by endothelial cells, vascular smooth muscle cells, fibroblasts (Sorescu and Griendling, 2002) and neural tissue (Glass et al., 2006). NADPH is a multi-subunit enzyme with both cytosolic and membrane-bound components, which vary depending on cell type. In the prototypical phagocytic cell, $p47^{phox}$ is found intracellularly, while the catalytic core, cytochrome b558 is made up of membrane bound subunits, NOX-1/$gp91^{phox}$ and $p22^{phox}$.(Lambeth, 2004). In order for NADPH oxidase to be active, the cytosolic subunits must translocate to the membrane

Additionally, cytosolic ADP-rac must be converted to ATP-rac which subsequently binds to its target of $p67^{phox}$ (Takeya and Sumimoto, 2003). Together these steps result in the massive production of O_2^- and oxidative burst of the phagocytic cell. Ca^{2+} influx can be a trigger for NADPH oxidase activity with subsequent phosphorylation of translocation of cytosolic subunits to the

membrane (Cai et al., 2003), while in some cases however, NADPH oxidase is constitutively active (Li and Shah, 2002).

NADPH oxidase derived O_2^- has been implicated in the pathogenesis of several cardiovascular diseases including hypertension (Cai and Harrison, 2000; Cai et al., 2003). In human essential hypertension and animal models of hypertension there is increased ROS production. ROS produced in vascular and endothelial tissue produces paracrine and/or autocrine effects by inactivating and decreasing the availability of NO (Kolo et al., 2004a) and thereby promoting vasoconstriction. Although it is well known that NO and O_2^- are important signaling molecules in central and peripheral catecholaminergic pathways (Smythies and Galzigna, 1998; Kuhn and Geddes, 2002; Kolo et al., 2004b; Mueller et al., 2005) much of the work until recently has focused on vascular and endothelial tissue as producers and targets of oxidative stress. Recently however several papers have demonstrated that neural tissue also produces and is affected by ROS. Notably, Glass et al., using immunogold electron microscopic cytochemistry, localized $p47^{phox}$, $gp91^{phox}$ and $p22^{phox}$ to neuronal and astrocytic processes in the rat mNTS, where in many cases these subunits either co-localized with or apposed to TH containing neurons. There were also several cases where NADPH oxidase labeled astrocytes apposed vascular tissue (Glass et al., 2006). Together these data were the first to show directly that NADPH oxidase-containing neural tissue is likely to regulate postjunctional responses.

In central autonomic nuclei O_2^- generated by NADPH oxidase may be involved in the detrimental effects of neurogenic hypertension (Girouard et al.,

2006; Glass et al., 2007) independent on activation of angiotensin receptors (Glass et al., 2007). In small (<1 μM) dendritic processes, chronic infusion of both Angiotensin (Ang) II and phenylephrine produced a decrease in intracellular $p47^{phox}$ labeling selectively in dorsal medial NTS (dmNTS) neurons suggesting that systemic hypertension may produce alterations in the trafficking of NADPH oxidase in central autonomic neurons, thus revealing a potentially important neurogenic component of free radical production and systemic blood pressure elevation (Glass et al., 2007). In the peripheral autonomic ganglia, there increased ROS as a result of increased NADPH oxidase activity in hypertension (Dai et al., 2004). Furthermore, there is an increase in protein expression of $p22^{phox}$, and translocation of $p47^{phox}$ from the cytosol to the membrane in the prevertebral sympathetic ganglia in DOCA-salt hypertension (Cao et al., 2007).

One potential trigger for increased NADPH oxidase activity is the increased calcium signaling found in both vascular and neural tissues in hypertension (Sonkusare et al., 2006) and other diseases associated with NADPH oxidase-derived ROS (Abramov et al., 2004). In hypertension NADPH oxidase activity may increase in response to several factors including shear stress, increased calcium signaling, and in response to Ang II signaling. In fact, many of the down stream effects of Ang II occur as a result of NADPH activation. In DOCA-salt hypertension however, where the RAS is suppressed, ROS is still elevated and NADPH oxidase activity is increased in sympathetic ganglia (Cao et al., 2007). Potential activators of NADPH oxidase in the sympathetic nerves of DOCA-salt hypertensive rats include increased Ca^{2+} influx into the nerve terminal

but this has not been well characterized. In several animal models, including DOCA-salt hypertension, chronic antioxidant treatment in rat models *in vivo* have proved to be therapeutic as blood pressure lowering agents (Schnackenberg and Wilcox, 1999; Beswick et al., 2001; Xu et al., 2004; Hu et al., 2006). Therefore more research needs to be done to uncover mechanisms which promote subunit translocation and NADPH oxidase activity.

NADPH oxidase is only one of several mechanisms by which ROS can be produced. Other mechanisms include xanthine oxidase, mitochondrial respiration, inflammatory responses and uncoupled nitric oxide synthase (NOS). Several of these mechanisms have been implicated in hypertension as well. For example, mitochondrial generated ROS (mROS) is the downstream effector molecule that translates receptor-mediated Ca^{2+} signals into proinflammatory signaling. ROS have been shown to target Gi/Go proteins (Nishida et al., 2000), which are known to be dysfunctional in DOCA-salt hypertension (Tsuda et al., 1989; Luo et al., 2004). Somers et al. showed increased ROS in the DOCA-salt model of hypertension plays a role in altered vascular function (Somers et al., 2000).

Using techniques such as dihydroethidium (DHE) staining and lucigenin-enhanced chemiluminescence, relative increases or decreases in O_2^- can be measured (Munzel et al., 2002). There are several pharmacological tools that can be used to elucidate the role of ROS in hypertension and pathological conditions associated with disease *in vitro* and *in vivo*. Such tools include tempol, an SOD

mimetic, apocynin, an NADPH inhibitor and diphenylene iodonium (DPI), a flavoprotein inhibitor (Sercombe et al., 2004).

The deoxycorticosterone-acetate (DOCA)-salt model of hypertension

In these studies DOCA-salt hypertension in rats was used to model salt-sensitive hypertension. Deoxycorticosterone is a steroid hormone produced by the adrenal gland that possesses mineralocorticoid activity and acts as a precursor to aldosterone. Endogenously, aldosterone activity is stimulated by an increase in angiotension II, adrenocorticotropic hormone (ACTH) and high K^+ levels as well as a decrease in stretch detected by stretch receptors in the atrium. As a result, the effects of aldosterone are two-fold. It acts at mineralocorticoid receptors on principal cells in the distal convoluted tubule to increase permeability of the luminal membrane to sodium and potassium, while at the same time activating basolateral Na^+/K^+ pumps. This leads to the reabsorption of Na^+ and H_20 into the blood and secretion of K^+ into the urine. Secondly, aldosterone stimulates H^+ secretion through its activation of Na^+/K^+ pumps. The end effect is an increase in blood volume and blood pressure.

The DOCA-salt treatment, uninephrectomy and saline drinking solution results in a rise in systolic blood pressure (SBP) which is significantly greater than sham controls. This is accompanied by pathological changes occurring both centrally and peripherally. Increases in peripheral norepinephrine turnover,

elevated plasma NE content and increased catecholamine content in the brain stem and hypothalamic nuclei of hypertensive animals have been observed. Previous studies have shown that central catecholaminergic neurons are important in the initiation of DOCA-salt hypertension (Lamprecht et al., 1977). Moreover, depletion of central adrenergic neurons with 6-hydroxydopamine prior to the initiation of DOCA treatment prevents hypertension (Okuno et al., 1983). However others argue that there was no difference in noradrenergic activity in DOCA-salt hypertensive animals as compared to sham controls in central cardiovascular structures (Qualy and Westfall, 1995).

Importantly, there are also changes at the neuroeffector junction. Significant to my studies is an alteration in the prejunctional α_2AR. Previous studies show that increased NE release from the sympathetic nerve endings in DOCA-salt hypertension may be due in part to impaired presynpatic α_2AR-mediated inhibition of neurotransmitter release (Tsuda et al., 1989; Luo et al., 2004). Changes occur at the postjunction as well. For example L-type VDCCs are upregulated due to a depolarized membrane potential in hypertension. This can further increase calcium influx, and arterial tone.

It is interesting to note however that the sickest, most hypertensive rats are the most sensitive to superfused agonists such as ATP and NE, however are almost unresponsive to direct nerve stimulation. This phenomenon called degenerative hypersensitivity points to a disease originating in nerve fiber loss which then causes super-sensitivity of the vasculature.

CHAPTER 2

RESEARCH GOALS AND SPECIFIC AIMS

Research Goals

Hypertension is a multi-factorial disease which is a major health concern in the United States. Furthermore, uncontrolled hypertension is a risk factor for life-threatening complications such as stroke, myocardial infarctions and end-organ damage for which there are no cures. There are various strategies for lowering blood pressure, however the autonomic nervous system quickly adapts to these interventions and blood pressure often returns to pretreatment levels. The proposed studies will investigate alterations in peri-arterial sympathetic nerve fibers and endings in hypertension. Upon stimulation, norepinephrine (NE) and ATP are released from sympathetic nerve terminals and subsequently act on postjunctional α_2-adrenergic receptors (AR) and $P2X_1$ receptors respectively. Previous studies have shown impaired regulation of NE release in human essential and animal models of hypertension while changes in purinergic neurotransmission are less clear. Impaired neurotransmission to mesenteric arteries would effect blood vessel constriction and may be an underlying cause or may contribute to the maintenance of increased blood pressure.

These studies will focus the deoxycorticosterone acetate (DOCA)-salt model of hypertension in rats. The DOCA-salt model is associated with increased release of epinephrine and NE, indicating a hyperactive sympathetic nervous system. However alterations in ATP release from sympathetic nerves are not known. DOCA-salt hypertension is also associated with increased oxidative stress in the mesenteric vascular bed and this may contribute to alterations in

sympathetic nervous system function in hypertension. One explanation for increased neurotransmitter release is that there is a disruption in adrenergic and/or purinergic neurotransmission to mesenteric arteries. At the vascular neuroeffector junction, prejunctional α_2ARs regulate NE and ATP release. Alterations in the function of proteins which regulate neurotransmitter release, such as the α_2AR, would impair the negative feedback loop which ordinarily controls NE and ATP release. Identifying changes which are exclusive to purinergic neurotransmission would identify novel pharmaceutical targets and might lead to a policy-changing approach to the control and treatment of hypertension. My specific interest in this comprehensive study includes characterizing changes in the purinergic component of neurotransmitter release at the neuroeffector junction of mesenteric resistance vessels in hypertension. **I hypothesize that DOCA-salt hypertension is associated with impairment of the purinergic component of sympathetic neurotransmission in DOCA-salt rats as a result of increased reactive oxygen species.** The proposed work will address this hypothesis in the context of three specific aims:

Specific Aim 1: Determine properties of ATP release from sympathetic peri-arterial nerves in control and hypertensive conditions. 1a. Electrophysiological recordings of excitatory junction potentials (EJPs) from arterial smooth muscle cells in the presence and absence of α_2AR agonists and antagonists will be used to study purinergic neurotransmission in hypertension.

1b. Facilitation and rundown of EJPs will be studied to assess ATP bioavailability in control and DOCA-salt rats.

Specific Aim 2: Test the hypothesis that NADPH oxidase subunits are found in sympathetic and sensory nerve fibers innervating mesenteric arteries. 1a. Antibodies raised against NADPH oxidase subunits, $p47^{phox}$ and $p22^{phox}$, will be incubated with anti-tyrosine hydroxylase (TH) and anti-neuropeptide Y (NPY), markers for sympathetic nerves, to determine if NADPH oxidase subunits are localized to sympathetic nerve fibers and endings. **1b.** anti-$p47^{phox}$ and anti-$p22^{phox}$ will be incubated with anti-calcitonin gene-related peptide (CGRP) to determine if NADPH oxidase is localized to sensory nerve fibers.

Specific Aim 3: Test the hypothesis that increased oxidative stress impairs the function of purinergic neurotransmission from peri-arterial nerves in hypertensive rats. 3a. Apocynin, an NADPH oxidase inhibitor will be superfused over tissue *in vitro* to asses the role of reactive oxygen species (ROS) on blood vessel contractility and purinergic neurotransmission. **3b.** Apocynin and tempol, as superoxide dismutase mimetic, will be given *in vivo* to assess the role of oxidative stress on blood pressure. **3c.** Effects of chronic apocynin and tempol treatments on properties of purinergic and adrenergic neurotransmission will be assessed using electrophysiological and electrochemical techniques.

CHAPTER 3

IMPAIRED PURINERGIC NEUROTRANSMISSION FROM MESENTERIC PERIARTERIAL NERVES IN DOCA-SALT RATS

Abstract

Sympathetic nerves release norepinephrine (NE) and ATP onto mesenteric arteries. In deoxycorticosterone acetate (DOCA)-salt hypertensive rats, there is increased arterial sympathetic neurotransmission due in part to impaired α_2-adrenergic receptor (α_2AR) function and impaired prejunctional regulation of NE release. Prejunctional regulation of the purinergic component of sympathetic neuroeffector transmission in hypertension is less well understood. We hypothesized that α_2AR dysfunction alters purinergic neurotransmission to arteries in DOCA-salt hypertensive rats. Mesenteric artery preparations were maintained *in vitro* and intracellular electrophysiological methods were used to record excitatory junction potentials (EJPs) from smooth muscle cells (SMCs). EJP amplitude was reduced in SMCs from DOCA-salt (4 ± 1 mV) compared to control arteries (9 ± 1 mV; $P<0.05$). When using short trains of electrical stimulation (0.5 Hz, 5 pulses), the α_2AR antagonist, yohimbine (1 μM), potentiated EJPs in control more than in DOCA-salt arteries (180 ± 35 % vs. 86 ± 7 %; $P<0.05$). NE (0.1 – 3 μM), the α_2AR agonist UK 14,304 (0.001-0.1 μM), the A_1 adenosine receptor agonist cyclopentyladensosine (CPA, 0.3 – 100 μM) and the N-type calcium channel blocker ω−conotoxin GVIA (0.0003 – 0.1 μM) decreased EJP amplitude equally well in control and DOCA-salt arteries. Trains of stimuli (10 Hz) depleted ATP stores more completely and the latency to EJP recovery was longer in DOCA-salt compared to control arteries. These data indicate that there is reduced purinergic input to mesenteric arteries of DOCA-salt

rats. This is not due to increased inhibition of ATP release via prejunctional α_2ARs or A_1 receptors, but rather a decrease in ATP bioavailability in sympathetic nerves. These data highlight the potential importance of altered neural regulation of resistance arteries as a therapeutic target for drug treatment of hypertension.

Introduction

The nervous system plays an essential role in short and long-term blood pressure regulation and sympath(Zugck, Lossnitzer et al. 2003)etic nerves innervating the splanchnic circulation are particularly important in blood pressure regulation (King, Osborn et al. 2007). In the peripheral vasculature, norepinephrine (NE), adenosine 5' triphosphate (ATP) and NPY are released from post-ganglionic, sympathetic nerve endings at the vascular neuroeffector junction (Donoso, Steiner et al. 1997), and smooth muscle cells (SMC) in arteries express receptors for these transmitters which contribute to the vasoconstrictor effect of sympathetic nerve activity. NE mediates a prolonged arterial constriction via an action at α_1-adrenergic receptors (ARs) which are G-protein coupled receptors. ATP acts at $P2X_1$ receptors, which are ligand-gated cation channels. ATP acting at $P2X_1$ receptors causes rapidly developing but transient depolarizations of the arterial SMC membrane potential called excitatory junction potentials (EJPs) (Burnstock and Holman 1961). EJPs that reach the potential at which L-type calcium channels activate cause calcium influx and SMC contraction (Bao, Gonon et al. 1993). NPY is a neuromodulator co-stored in perivascular nerve terminals with NE and ATP (Lundberg, Terenius et al. 1983). Upon release, NPY acts at Y_1 receptors on vascular tissues, potentiating the effects of NE and ATP (Pablo Huidobro-Toro and Veronica Donoso 2004). The relative contribution of NE, ATP and NPY to arterial constriction varies depending on the tissue, species and age of the animal and in different

pathophysiological conditions (Burnstock 1995).

Alterations in central and peripheral neural mechanisms contribute to increased sympathetic drive in both human essential hypertension and in animal models of hypertension (Masuyama, Tsuda et al. 1986; Damase-Michel, Tavernier et al. 1992; Damase-Michel, Tran et al. 1993; Ferrier, Jennings et al. 1993). This has been established through measurements of splanchnic NE spillover (Bouvier and de Champlain 1985) and direct amperometric measurements of NE release from sympathetic nerves associated with mesenteric blood vessels (Park, Quaiserova-Mocko et al. 2008). NE overflow in response to nerve stimulation is increased in isolated mesenteric vasculature beds of deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Masuyama, Tsuda et al. 1986; Luo, Hess et al. 2003) indicating that increased adrenergic tone occurs at least in part, from changes at the sympathetic neuroeffector junction. However, there are far fewer studies which focus on alterations in the regulation of purinergic neurotransmission at the neuroeffector junction in hypertension. These studies will focus on changes in purinergic neurotransmission at the neuroeffector junction of mesenteric arteries in the DOCA-salt model of hypertension. This model is a well-established salt-sensitive model, characterized by an increase in NE release from sympathetic nerve endings (Moreau, Drolet et al. 1995; Luo, Fink et al. 2004) while renin activity is markedly suppressed (Gavras, Brunner et al. 1975).

The aim of these experiments was to use sharp electrode intracellular recordings to directly identify alterations in purinergic neurotransmission in

DOCA-salt hypertension. It was hypothesized that dysfunction of prejunctional inhibitory receptors, α_2AR and A_1R result in the altered regulation of purinergic neurotransmission. It was also hypothesized that decreased ATP bioavailability may be responsible for the decrease in purinergic neurotransmission in DOCA-salt hypertension. Mechanisms responsible for decreased purinergic neurotransmission from sympathetic nerves to mesenteric arteries were explored using pharmacological tools in addition to low and high frequency trains of nerve stimulation. Importantly, these studies shed light on the importance of prejunctional regulation of ATP release in both normotensive and hypertensive states.

Methods

Animals

All experiments were done using Sprague-Dawley rats from Charles River Laboratories (Portage, MI). Upon arrival at the animal care facility, animals were maintained according to standards approved by the Institutional Animal Care and Use Committee at Michigan State University. All experimental procedures were carried out in accordance with the "Guiding Principles in the Care and Use of Animals" of the American Physiological Society. Rats were acclimated for 2-3 days before entry into any experimental protocol. Pelleted rat chow (Harlan/Teklad 8640 Rodent Diet) and distilled water were provided *ad libitum*. Rats were housed in temperature- and humidity- controlled rooms with a 12:12-h light-dark cycle.

DOCA-salt hypertension

Male Sprague-Dawley rats (250-275g) were anesthetized with 3% Isoflurane. The skin over the left lateral abdominal wall was shaved and prepared with chlorhexadine. A 1.5-cm vertical 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, vein, and ureter with 6-0 silk sutures and the incision was closed with 4-0 monofilament nylon sutures. A 3 x 1.5-cm rectangular area between the shoulder blades of the back was shaved and disinfected for subcutaneous implantation of a Silastic (Dow Corning) sheet containing DOCA-salt (Sigma; 150mg/kg) pellet under a 1.5-cm incision. The

skin was closed with 4-0 nylon sutures. Sham-operated rats underwent left kidney removal only. Surgery was performed on a heated pad, and rats recovered in a heated box. After recovery, rats were housed under standard conditions for four weeks. DOCA-implanted rats received standard pelleted rat chow and salt water (1% NaCl + 0.2% KCl) ad libitum, whereas sham rats received standard pelleted rat chow and distilled water. Blood pressures were measured by tail-cuff plethysmography. On day 28, rats were removed from their cage and warmed under a heat lamp. Three readings were averaged to attain a final systolic arterial blood pressure (SBP). Rats with SBP equal to or higher than 150 mmHg were considered hypertensive.

Tissue preparation

The week following blood pressure measurements, rats were euthanized with an intrapertioneal injection of sodium pentobarbital (50 mg/kg). The mesentery was surgically removed. Tertiary branches of mesenteric arteries were dissected out, cleaned of adipose and connective tissue and pinned taut using stainless steel pins (50 μM diameter) in a perfusion chamber coated with Sylgard® (Dow Corning, Midland, MI). Tissues were superfused with Krebs' solution of the following composition (mM): NaCl, 117; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; dextrose, 11. Nifedipine (1μM) (L-type VDCC antagonist) and prazosin (0.1μM) (α_1AR antagonist) were added to the buffer to attenuate vessel constriction during trains of electrical nerve stimulation (see below). The buffer was heated to 37° C and bubbled with 95% O_2 and 5%

CO_2 and the tissue was allowed to equilibrate for 30 minutes before beginning electrophysiological studies.

Electrophysiological recording

Intracellular recordings from individual SMCs were obtained using glass microelectrodes filled with 2M KCl (100-200 MΩ tip resistance). Impalements were only accepted if the following criteria were satisfied: (1) the cell penetration was abrupt (2) the membrane potential increased to -50 mV or higher and (3) the membrane potential was stable for at least 5 minutes. Individual recordings from a single cell lasted for at least 20 minutes and up to 2 hours. An amplifier (IX2-700 dual intracellular preamp, Dagan) was used to record membrane potential in the current clamp mode. Signals were filtered using a HumBug 50/60 Hz Noise Eliminator (Digitimer Research Instruments, Quest Scientific, North Vancouver, BC, Canada). Impalements were accepted if the resting membrane potential dropped quickly to more that -50 mV. EJPs were evoked using a Krebs' solution-filled, bipolar, focal stimulating electrode containing two parallel silver/silver chloride wire electrodes connected to a Grass Instruments S88 stimulator (Grass Technologies, Astro-Med, Inc, West Warwick, RI). The stimulating electrode was positioned perpendicular to the tissue and directly across from the recording electrode. For most experiments, a stimulation frequency of 0.5 Hz, 0.5 ms pulse width at the lowest voltage (50-120V) which produced a maximal amplitude EJP was used. Signals were recorded using an analog to digital converter (Digidata 1200, Axon Instruments, Foster City CA) and Axoscope 9.0 software (Axon

Instruments). A digital average of five sweeps was used to measure the amplitude of EJPs under control and treatment conditions unless otherwise noted. Data were analyzed using Clampfit 9.0 software (Axon Instruments) and a laptop computer.

Drug application

Drugs were either added to the physiological buffer or superfused directly onto the tissue using a VC-8 Valve Controller application system (Warner Instruments, Hamden, CT). There was a ~1 minute delay between the onset of drug application and the onset of drug effect and tissues were exposed to the drug 5 minutes before testing for drug effects. Control responses were paired with a drug response in each experiment.

Immunohistochemistry

Tertiary mesenteric arteries were excised, cleaned of excess adipose and connective tissue. Vessels were perfused with saline to remove blood from the lumen. Isolated tissues were placed in Zamboni fixative (2% [vol/vol] formaldehyde and 0.2% [vol/vol] picric acid in 0.1M phosphate buffered saline, PBS) overnight (4 °C). The next day, the tissues were washed 3x with 0.1M PBS and then incubated in PBS with triton-X100 (0.5%) for 1 hour. Tissues were then co-incubated overnight at 4 °C in diluted (1:200, in triton-PBS) anti-$Ca_v2.2$ (rabbit polyclonal, Alomone labs, Jerusalem, Israel) and anti-tyrosine hydroxylase (TH, mouse monoclonal, Calbiochem, San Diego, CA). Next, tissues were washed 3x in 0.1M PBS buffer and then incubated for 1 hour in a dark,

humidified chamber at room temperature in diluted fluorescene isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:40) to visualize TH, and Cy3-conjugated goat anti-rabbit IgG (1:150) to visualize $Ca_V2.2$ (N-type voltage-dependent calcium channel subunit) staining (Jackson ImmunoResearch Laboratories, Inc.). Vessels were then washed 3x with 0.1 M PBS at 5-minute intervals and coverslipped with Prolong Gold anti-fade reagent (Molecular Probes (Invitrogen) Eugene, OR) for fluorescence microscopy. Specimens were viewed using a Nikon fluorescence microscope (model TE 2000-U), and images were acquired and analyzed using MetaMorph software.

Drugs

The following drugs used in these studies were obtained from Sigma chemical (St. Louis, MO): nifedipine, prazosin, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS) tetrodotoxin (TTX), 5-bromo-6-(2-imidazolin-2-yl-amino)-quinoxaline tartrate (UK 14,304), yohimbine and cyclopentyladenosine (CPA). ω-Conotoxin GVIA (CTX) was obtained from Alomone labs (Jerusalem, Israel). All drugs were diluted in deionized water except for prazosin and nifedipine which were dissolved in 95% ethanol to make a concentrated stock solution. Working solutions of nifedipine and prazosin contained <0.01% ethanol. Final solutions were made in Krebs' buffer on the day of the experiment.

Statistics

Data are mean ± SE and n values are the number of animals from which the data were obtained. Concentration-response data were fitted using non-linear regression and the Hill equation (Graphpad Prism, San Diego, U.S.A). Results from other experiments were tested with either one-way ANOVA or Student's t-test for paired or unpaired data, as appropriate. Differences were considered significant when $P < 0.05$. For each cell, EJP peak amplitude is the mean of 5 individual EJPs computed by a signal-averaging program (Sneddon, Westfall et al. 2000).

Results

EJPs in mesenteric arteries from control and DOCA-salt rats

Systolic blood pressures of DOCA-salt rats were elevated (196 ± 7 vs. 138 ± 2 mmHg; $P < 0.001$) and body weights lower (359 ± 14 vs. 450 ± 11 g; $P < 0.001$) in DOCA-salt rats compared to control rats.

Fig. 5 shows representative EJPs recorded from mesenteric arteries from control and DOCA-salt rats. EJPs from DOCA-salt and control animals were blocked by tetrodotoxin (TTX 0.3 μM), a Na^+ channel blocker, which verified that the EJPs were neurogenic. EJPs were also blocked by PPADS (10 μM), the P2 receptor antagonist indicating that the EJPs were purinergic. EJPs were blocked similarly in arteries from DOCA-salt and control rats (Fig. 5B). The resting membrane potential (RMP) of SMCs from DOCA-salt rats was depolarized compared to control rats (Fig. 5C, *left*; $P < 0.05$) and EJPs were smaller in DOCA-salt compared to control arteries (Fig. 5C, *right*; $P < 0.05$). Hyperpolarizing current was injected through the intracellular recording microelectrode to SMCs from DOCA-salt treated animals to test the possibility that decreased EJP amplitude in DOCA-salt rats was due to a decreased driving force for the transmembrane Na^+ and K^+ movement responsible for the EJP. However, adjusting the RMP did not change EJP amplitude (4 ± 1 mV vs. 4 ± 1 mV, DOCA-salt vs. DOCA-salt adjusted; $N = 9$).

EJP amplitude could be smaller in DOCA-salt arteries because of a decreased reactivity of SMCs to neurally released ATP. We tested this possibility by comparing concentration-response curves for ATP (3 - 300 μM)-induced

constriction of mesenteric arteries. We found that there were no differences in ATP reactivity in mesenteric arteries from control and DOCA-salt rats (data not shown). These data are identical to those reported previously (Luo, Hess et al. 2003).

α_2AR and A_1 adenosine autoreceptors regulate ATP release, and this is not altered in DOCA-salt hypertension

Concentration-response curves for agonists at prejunctional α_2AR and A_1 adenosine autoreceptors were obtained in tissues from control and DOCA-salt rats (fig. 6). IC_{50} values and Hill-slopes are reported in Table 1. NE (0.01 – 3 μM) and UK 14,304 (0.001 – 0.1 μM), an α_2AR agonist, reduced EJP peak amplitudes in a concentration-dependent manner equally well in control and DOCA-salt tissues (fig. 6A and B). These data suggest that the prejunctional α_2AR inhibits ATP release from mesenteric sympathetic nerve terminals, and this effect is not impaired in DOCA-salt hypertension. Similarly, cyclopentyladenosine (CPA; 0.003 – 0.1 μM), an A_1-adenosine receptor agonist, also inhibited EJP peak amplitude equally well in control and DOCA-salt tissues, suggesting that A_1 receptor function is not impaired in DOCA-salt rats (Fig. 6C). Complete inhibition of EJPs by both UK 14,304 and CPA indicated that ATP release is coupled to both the α_2AR and A_1 receptors.

N-type calcium channel function is not altered in DOCA-salt

hypertension

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in NE synthesis, and TH labeling was used as a marker for sympathetic nerves (Fig. 7A). Previous studies have shown that the N-type calcium channel is the predominate calcium channel involved in neurotransmitter release from sympathetic nerves innervating mesenteric arteries (Waterman 2000). We used an antibody raised against the α1B calcium channel pore forming subunit, a marker for the N-type calcium channel ($Ca_v2.2$; Fig. 7B). There was substantial overlap between the nerve fibers containing TH labeling with those containing the N-type calcium channel (Fig. 7C), however we did not detect any differences in the intensity or distribution of labeling for either antigen in tissues from control or DOCA-salt rats.

ω-Conotoxin GVIA (CTX), a specific and irreversible inhibitor of N-type calcium channels, was used to test the functionality of the channel in peri-arterial sympathetic nerve fibers from DOCA-salt and control animals. CTX (0.001 – 0.1 μM) caused a concentration-dependent inhibition of peak EJP amplitude. CTX concentration response curves were not different between DOCA-salt and control blood vessels and CTX produced complete inhibition of the EJP (Fig. 7D).

Impaired facilitation of purinergic neuroeffector transmission in DOCA-salt arteries

Facilitation occurs throughout the nervous system during short bursts of synaptic activity. Synaptic facilitation is a successive increase in the amplitude of a postsynaptic potential due to an increase in transmitter release as calcium enters

the nerve terminal faster than it can be cleared. Previous work has shown that EJPs recorded from mesenteric arteries facilitate during short trains of stimulation (Burnstock and Holman 1961) and we investigated the effects of DOCA-salt hypertension on this response. In these studies, the degree of facilitation was compared in tissues from control and DOCA-salt rats using 5 stimuli applied at a frequency of 0.5 Hz in the presence of yohimbine. Previous studies have reported impaired prejunctional α_2ARs in DOCA-salt hypertension. Therefore, yohimbine (1 μM) was used to study facilitation to assure that hypertension associated changes in the function of this receptor were not confounding our results. We used low frequency stimulation in order to mimic physiological levels of sympathetic nerve activity (Gilbey 2007) and to minimize constriction.

Fig. 8A shows representative tracings of EJPs elicited by 0.5 Hz trains of nerve stimulation in arteries from control and DOCA-salt treated animals (*left:* before yohimbine; *right:* with yohimbine). We used the amplitude of the 5th EJP vs. the amplitude of the 1st EJP in the train to calculate percent facilitation. In control arteries in the absence of yohimbine, there was little change in EJP amplitude during the short train of stimulation. However, in the presence of yohimbine, there was a substantial increase in EJP amplitude during the train of stimulation in control arteries (Fig. 8A). These data indicate that α_2AR autoreceptor activation suppresses ATP release during bursts of sympathetic nerve activity and in the absence of α_2AR autoreceptor function, the sympathetic neuroeffector junction exhibits facilitation similar to synapses in the central and peripheral nervous systems. EJPs recorded from DOCA-salt arteries were smaller

than those recorded from control arteries (Fig. 8A; lower traces), as discussed above, and no facilitation was observed in the absence of yohimbine in these tissues. Even in the presence of yohimbine, EJP facilitation was markedly impaired in DOCA-salt arteries. These data are summarized in Fig. 8B which shows mean EJP amplitudes of the 1^{st} and 5^{th} EJP, with or without yohimbine, in tissues from control and DOCA-salt rats. EJPs do not facilitate at 0.5 Hz without yohimbine (solid bars). The addition of yohimbine does not affect the first EJP in the train (Fig. 8B, *left*). The fifth EJP amplitude is significantly greater with yohimbine than without yohimbine in control animals, but not DOCA-salt animals (Fig. 8B, *right*). Facilitation of EJPs recorded from DOCA-salt tissues (86 ± 7% mV; n = 12) was significantly less than in control tissues (180 ± 35%; n = 13, $P < 0.05$).

EJP rundown in arteries from DOCA-salt rats

High frequency trains of nerve stimulation can be used to deplete stores of neurotransmitter (Lin, Graham et al. 2001). The rate of decline in the amplitude of the postsynaptic or postjunctional response can be used as a measure of the amount of transmitter stored in the presynaptic or prejunctional nerve terminals. The reduced amplitude of the EJP recorded from DOCA-salt arteries rats could be due to reduced nerve terminal stores of ATP in DOCA-salt hypertension. Therefore, we measured EJP amplitudes during 10 s trains of 10 Hz stimulation. Because we used a relatively high frequency of stimulation, EJPs summated to produce a sustained depolarization until ATP stores were depleted from the

sympathetic nerve endings. Fig. 9A shows representative experiments in control and DOCA-salt tissues. It is apparent that tracings from control animals remain depolarized for a longer period of time compared to tracings from DOCA-salt rats. Mean EJP amplitudes were analyzed for the first 5 EJPs in the train and then the 10^{th}, 20^{th} and 30^{th} EJP (Fig. 9C). EJP facilitation peaked between the 4th and 6th stimuli in control and DOCA-salt tissues. Peak facilitation was significantly attenuated in the DOCA-salt compared to control tissues (Fig. 9D; *top*; $P < 0.05$). The extent of run-down was compared between DOCA-salt and control tissues. This was measured by dividing the amplitude of the largest EJP occurring early in train by the amplitude of the EJP recorded after 3 s of 10 Hz stimulation (the 30^{th} EJP). All amplitudes were assessed from the original baseline to account for differences in the amount of facilitation between control and hypertensive animals. Rundown was greater in the DOCA-salt compared to control tissues (Fig. 9D; *bottom*; $P < 0.05$).

Impaired recovery of purinergic transmission after depletion of nerve terminal ATP stores

Increased run-down of ATP in DOCA-salt hypertension suggests a decreased bioavailablity of vesicular ATP at the neuroeffector junction. This may be due to an impairment of vesicular filling mechanisms that pump ATP into synaptic vesicles. To test this hypothesis, he time to recovery of EJP amplitude was assessed after rundown. Restoration of EJP amplitude after rundown was used as an indication of the efficiency of vesicular refilling with ATP. After

stimulating sympathetic nerves with a train of stimuli (10 Hz for 10 s), a single EJP was elicited. The latency between the end of the train and the single stimulus was varied between 0.2 and 2 s. A ratio of the initial EJP amplitude in the train to the recovery EJP amplitude was calculated as an index of recovery from rundown. Fig. 10A shows a representative tracing obtained in a control artery, showing 10 s of stimulation followed by 2 s recovery time before a single recovery stimulus. Mean data from control and DOCA-salt animals is shown in Fig. 10B. EJP amplitudes from control animals were not different from baseline at 0.2 s suggesting that restoration of vesicular stores of ATP is very efficient in control animals. In addition, at 1 and 2 s after the end of the train, EJP amplitudes were significantly greater than baseline indicating that facilitation is sustained after a long train of nerve stimulation. ATP stores in sympathetic nerves from DOCA-salt rats were significantly lower than baseline after 0.2 s, and did not fully recover until 2 s after the end of the stimulus train. The recovery ratio was significantly different at 0.5, 1.0 and 2.0 seconds latency between DOCA-salt and control animals (Fig. 10B; &P < 0.05 DOCA-salt vs. control).

Table 1: Agonists for prejunctional autoreceptors at the sympathetic neuroeffector junction

Drug	Group	$logIC_{50}$	hillslope	N
UK (1nM -100nM)	D	-8.2±0.2	-0.8±0.4	9
	C	-8.3±0.3	-1.3±0.5	9
NE (10nM - 3μM)	D	-7.2±0.1	-0.9±0.2	6
	C	-7.3±0.3	-0.8±0.4	4
CPA (.3nM - 100nM)	D	-8.3±0.2	-1.0±0.2	5
	C	-8.3±0.2	-1.0±0.2	5

Table 1. Effects of NE, α_2AR agonist, UK 14,304 and A_1-R agonist cyclopentyladensosine (CPA) on ATP release from sympathetic nerves in control and DOCA-salt rats. IC_{50}, Hill-slope and N-values are reported for UK 14,304 (1 -100 nM), NE (10 – 3 μM) and CPA (0.3 – 100 nM) for control and DOCA-salt rats. Data were obtained from the indicated number of animals (N) and are means ± SE. D = DOCA-salt; C = control.

Fig. 5

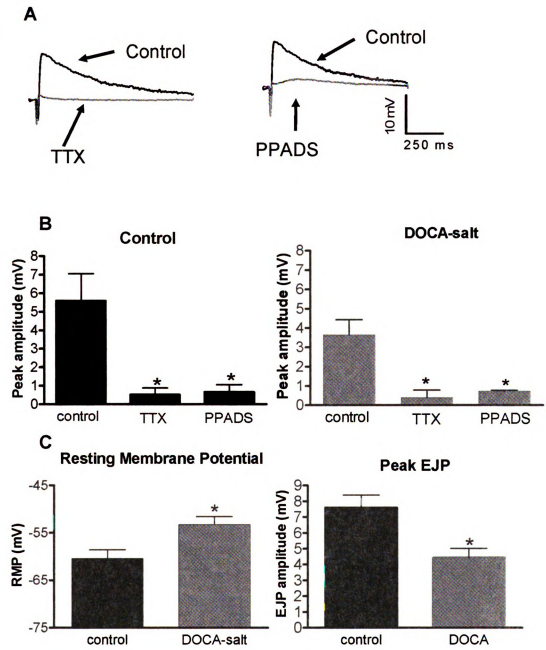


Fig. 5. Electrophysiological properties of mesenteric arterial smooth muscle cells from control and DOCA-salt rats. A. Representative recordings showing EJPs are blocked by TTX (0.3 μM) or PPADS (10 μM). **B**. EJPs were blocked similarly by TTX and PPADS in control and DOCA-salt arteries (n=4; $P > 0.05$) indicating that they were neurogenic and mediated by activation of P2X receptors. **C**. Resting membrane potential (RMP) of mesenteric arterial smooth muscle cells (SMC) were depolarized in DOCA-salt compared to control tissues (-53 ± 2 mV vs. -60 ± 2 mV; n = 22; * = $P < 0.05$; DOCA-salt vs. control). EJPs in arteries from DOCA-salt rats were smaller than those in control arteries (4 ± 1 mV vs 8 ± 1 mV; n = 22; * = $P < 0.05$; DOCA-salt vs. control).

Fig. 6

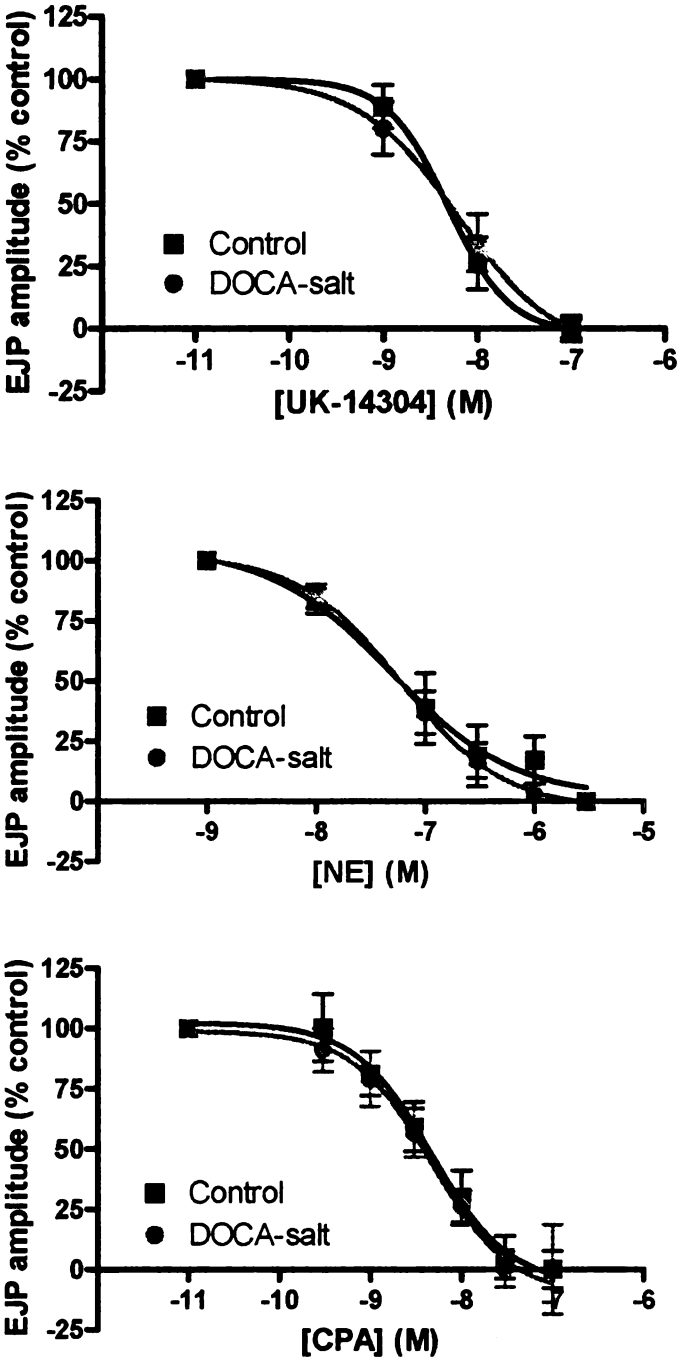


Fig. 6. Prejunctional α_2AR and A_1-R, regulate ATP release from sympathetic nerves in control and DOCA-salt rats. Concentration response curves for **A**, UK 14,304 (n = 9), an α_2-AR agonist, **B**, norepinephrine (NE, n = 4) and **C**, cyclopentyladensosine (CPA, n=5), an A_1-adenosine receptor agonist were not different in tissues from control and DOCA-salt rats. Data points are mean ± SEM. Curves are non-linear fits of the Hill equation to the data points.

Fig. 7

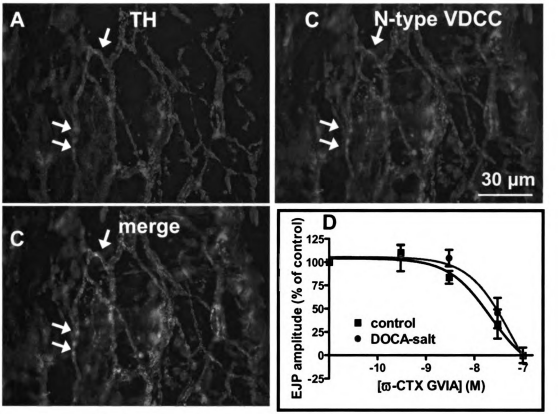


Fig. 7. N-type voltage-dependent calcium channel (VDCC) on peri-arterial sympathetic nerve fibers. A. Sympathetic nerve fibers were co-labeled with an anti-tyrosine hydroxylase (TH) antibody and an anti-α1B calcium channel (N-type calcium channel) subunit antibody. Immunoreactivity for TH and N-type calcium channels (B) were observed in peri-arterial nerve fibers and nerve endings in the mesentery. **C**. Overlay of photomicrographs in A and B showing co-localization (yellow labeling) of TH and N-type calcium channels. Scale bar = 30 µM. **D**. CTX causes a concentration-dependent inhibition EJP amplitude ($P > 0.05$; control vs. DOCA-salt). Data points are mean ± SEM. Lines are non-linear fits of the Hill equation to the data points.

Fig. 8

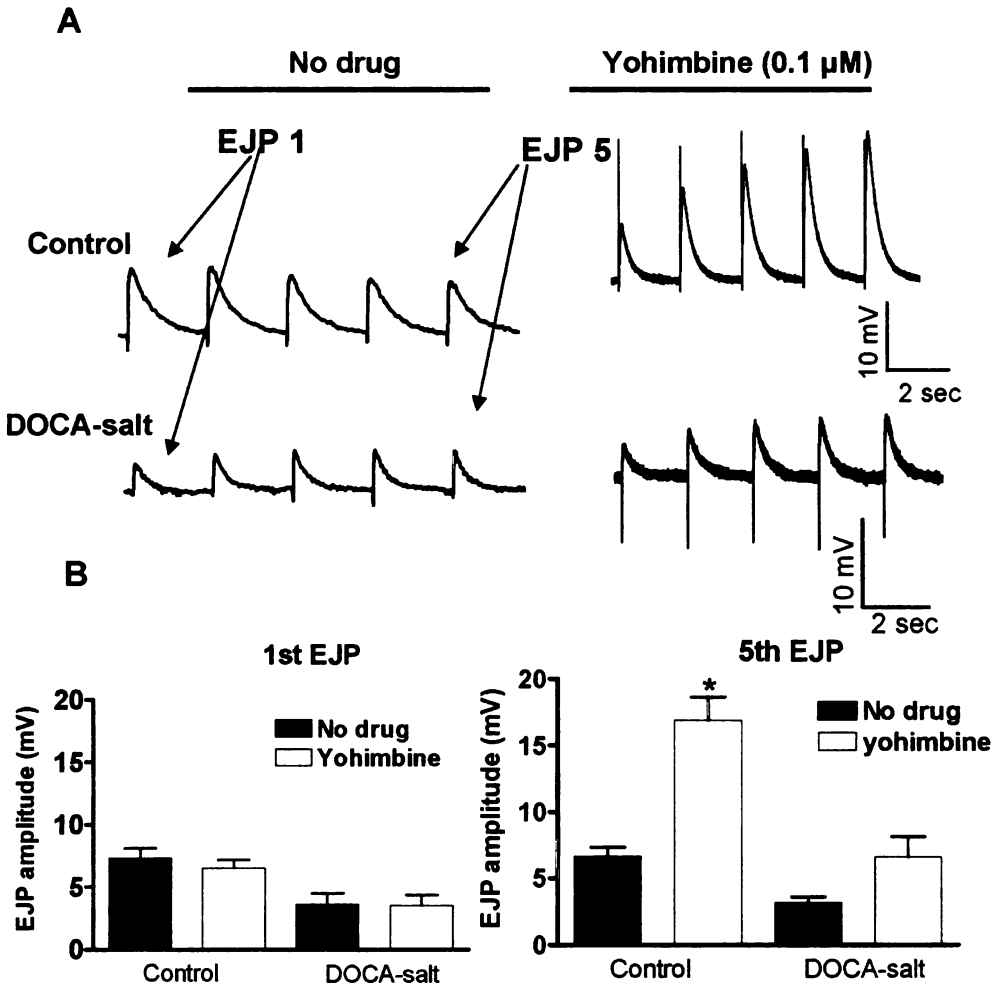


Fig. 8. EJP facilitation in arteries from control and DOCA-salt rats. EJPs were evoked using a short train (0.5 Hz, 10 s) of stimulation with or without yohimbine (0.1 μM). **A**. Representative traces show EJPs recorded from control and DOCA-salt treated arteries. **B**. Mean EJP amplitude in arteries from control and DOCA-salt rats without and with yohimbine (* = $P < 0.05$; control *vs.* control with yohimbine).

Fig. 9. EJP rundown caused by trains of stimulation in arteries from DOCA-salt and control rats. A train of stimulation (10 Hz) causes EJP facilitation and then rundown. **A**. Representative recordings of EJPs elicited by nerve stimulation for 5 seconds are shown for arteries from control (*left*) and DOCA-salt (*right*) rats. At this frequency of stimulation, individual EJPs summate to cause a sustained depolarization. EJPs rundown in arteries from DOCA-salt rats and the sustained depolarization declines to baseline level during the train of stimulation. **B**. Mean data from experiments similar to that shown in "A". **C**. Summary data for EJP facilitation in DOCA-salt (gray bars) and control (black bars) arteries (* = $P < 0.05$; DOCA-salt *vs.* control). The ratio of peak EJP amplitude to the EJP amplitude at 30 s was calculated. This ratio was compared between control (black bars) and DOCA-salt animals (**D**; gray bars; * = $P < 0.05$).

Fig. 9

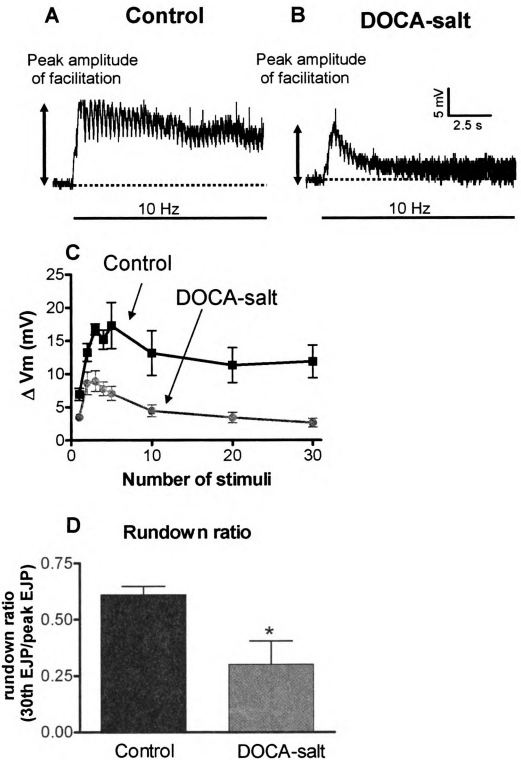

A
Control
B
DOCA-salt
Peak amplitude of facilitation
Peak amplitude of facilitation
5 mV
2.5 s
10 Hz
10 Hz
C
Control
DOCA-salt
Δ Vm (mV)
Number of stimuli
D
Rundown ratio
rundown ratio (30th EJP/peak EJP)
Control
DOCA-salt

Fig. 10

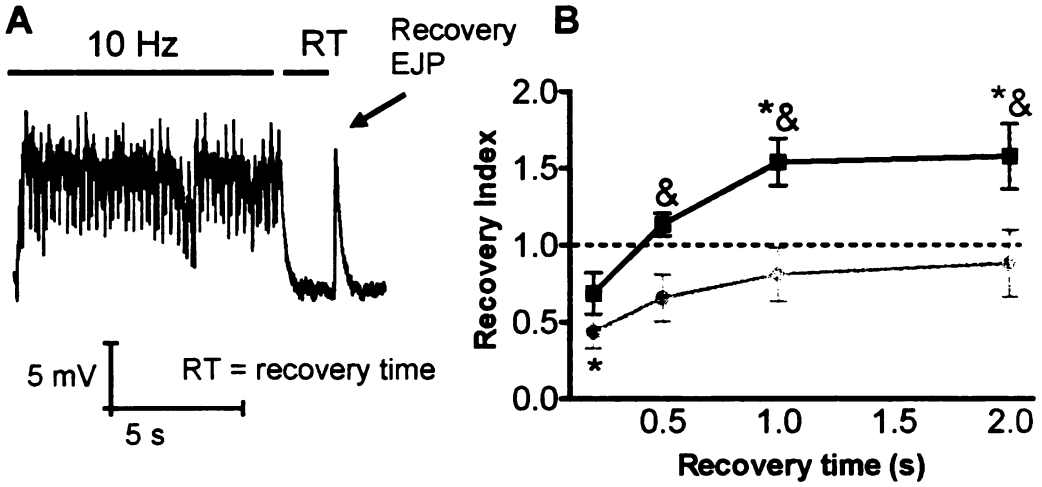


Fig. 10. Impaired recovery of EJP amplitude after rundown in arteries from DOCA-salt rats. A single stimulus was given at 0.2, 0.5, 1 and 2 seconds after the end of a 10 Hz train (10 s). **A**. Representative recordings from a control artery showing complete recovery of EJP within 1s after the end of the 10 Hz train of stimulation. **B**. Summary data from control and DOCA-salt animals. In control arteries EJP amplitude at 0.2 s was no different from the initial EJP amplitude in the train. At 1 and 2 seconds after the end of the train, the EJP amplitudes were significantly greater than the initial EJP (* = $P < 0.05$; compared to initial amplitude). In DOCA-salt arteries the EJP at 0.2 s was significantly smaller than the initial EJP (* = $P < 0.05$; as compared to initial amplitude). EJP amplitudes during the recovery period in DOCA-salt arteries were significantly smaller than control arteries at 0.5, 1.0 and 2.0 s ($^{\&}$ = $P < 0.05$; DOCA-salt vs. control).

Discussion

It is well established that NE and ATP are co-transmitters released from sympathetic nerves supplying mesenteric and other arteries (Sneddon and Burnstock 1984; Sneddon and Westfall 1984; Donoso, Steiner et al. 1997). However, there have been few studies that have examined hypertension associated changes in the purinergic component of sympathetic neuroeffector transmission in arteries (Brock and Van Helden 1995). Previous studies measured higher concentrations of NE and ATP overflow solutions from DOCA-salt compared to control arteries when long trains of stimulation were used to evoke transmitter release from sympathetic nerves (Tsuda, Tsuda et al. 1989; Luo, Fink et al. 2004). Few studies have used intracellular recordings to measure purinergic neurotransmission in hypertension. When ATP binds to ligand-gated $P2X_1$ receptors on SMCs cations transverse the membrane resulting in a transient depolarization or EJP. Intracellular recordings of EJPs from arterial SMCs provide a very localized assessment of ATP release from neuroeffector junctions compared to measurements made in overflow solutions. Increased sympathetic nerve activity resulting in increased rates of neurotransmitter release (Schlaich, Lambert et al. 2004), increased vasoconstriction and increased adrenergic neurotransmission (Luo, Hess et al. 2003) all occur in hypertension. However, changes associated with purinergic neurotransmission at the neuroeffector junction in the DOCA-salt model of hypertension have not been considered. In our studies, EJPs recorded from arterial SMCs were used as a measure of ATP release from sympathetic nerve endings. Intracellular recordings from SMCs

revealed a decrease in EJP amplitude in mesenteric arteries from DOCA-salt hypertensive rats indicating impaired ATP release. This is a novel finding. Previously, EJPs were measured in spontaneously hypertensive rats (SHRs), a genetic model of hypertension. Brock and Van Helden found that on average, EJPs recorded in mesenteric arteries were increased in this model of hypertension (Brock and Van Helden 1995). Another study reported similar results in the prostatic portion of vas deferens in SHRs. The authors concluded that the increased postjunctional responsiveness was responsible for the enhanced purinergic transmission (Guitart, Jimenez et al. 2002). In our study, the resting membrane potential of SMCs from DOCA-salt rats was depolarized compared to control arteries. However, this and previous studies showed that there is no difference in the concentration response curve for ATP-induced constriction of control and DOCA-salt arteries (Luo, Hess et al. 2003). Furthermore, hyperpolarizing SMCs in DOCA-salt arteries to a level similar to that in control arteries did not increase EJP amplitudes. These data suggest that in DOCA-salt rats, changes in EJP amplitude are probably not due to postjunctional changes and instead, DOCA-salt hypertension alters either storage or release of ATP by perivascular sympathetic nerves. Decreases in EJPs from DOCA-salt hypertension but increased EJPs found in SHRs can be explained by differences in the models of hypertension. One study found that enhancement of EJPs in rat mesenteric arteries is mediated by the angiotensin AT1 receptor (Ziogas and Vessey 2001). While the SHR model is characterized by increased renin-angiotensin system (RAS) signaling, the DOCA-salt model has a suppressed RAS

activity (Gavras, Brunner et al. 1975).

Changes in neural regulation vascular tone in hypertension are important because altered regulation of neurotransmitter release from sympathetic nerves coincides with increased blood pressure. One important mechanism for regulating neurotransmitter release at the neuroeffector junction is via prejunctional autoreceptors. NE binds to prejunctional α_2ARs which negatively regulate NE and ATP release (Todorov, Mihaylova-Todorova et al. 1999). Sympathetic nerves release ATP along with ecto-ATPases and 5'-nucleotidases which quickly degrade ATP into ADP, AMP and adenosine (Todorov, Mihaylova-Todorova et al. 1997). Adenosine acts at prejunctional autoreceptors (A_1) which also negatively regulate NE and ATP release (Ekas, Steenberg et al. 1983). Both A_1- and α_2ARs couple to pertussis toxin sensitive Gi/Go-proteins. They inhibit release of neurotransmitter by opening K^+ channels, blocking voltage-dependent Ca^{2+} channels (VDCCs) and inhibiting adenylate cyclase. Importantly, impaired prejunctional α_2AR function is associated with hypertension in animals (Tsuda, Tsuda et al. 1989; Moreau, Drolet et al. 1995; Zugck, Lossnitzer et al. 2003; Luo, Fink et al. 2004) and humans (Damase-Michel, Tavernier et al. 1992; Damase-Michel, Tran et al. 1993; Rana, Insel et al. 2007).

Previously it was found that prejunctional α_2ARs are impaired in DOCA-salt hypertension resulting in increased NE release from perivascular sympathetic nerves (Luo, Fink et al. 2004). Since the α_2AR also regulates ATP release

(Todorov, Mihaylova-Todorova et al. 1999), we tested the hypothesis that the α_2AR may also be involved in altered purinergic neurotransmission. However, application of either UK 14,304 or exogenous NE inhibited EJP amplitudes equally well in DOCA-salt and control animals. The prejunctional adenosine (A_1) receptor regulates ATP release. ATP is quickly degraded by ectonucleotidases into adenosine, which then can bind to the A_1 receptor. We therefore tested the hypothesis that the A_1 autoreceptor was dysfunctional. CPA, an agonist at the prejunctional A_1-R, showed that EJP amplitudes were inhibited equally well in control and hypertensive rats. While previous studies have shown that the α_2AR is impaired in its regulation of NE, these data are the first to show that α_2AR regulation of ATP is normal in DOCA-salt hypertension. These data correspond with previous reports that the α_2AR is more tightly coupled to regulating NE compared to ATP release. Furthermore, these data supports previous findings that ATP and NE release are regulated differentially by the α_2AR (Dunn, Brock et al. 1999).

Because direct inhibition of nerve terminal calcium channels is one way in which autoreceptors decrease neurotransmitter release we also investigated the function of prejunctional calcium channels. Immunohistochemical techniques were used to identify the predominant subtype of prejunctional calcium channels found on the mesenteric peri-arterial nerve fibers. Antibodies directed at the N-type calcium channel were used along with an antibody against TH, a marker for sympathetic nerves. Co-localization of the N-type calcium channels was

prominent suggesting that sympathetic neurotransmission is coupled to calcium entry through the N-type calcium channel. Therefore we used ω-CTX GVIA, a specific and irreversible inhibitor of this channel to test its function. ω-CTX GVIA completely inhibited EJPs in DOCA-salt and control arteries at 0.1 μM, a concentration specific for the N-type channels (Whorlow, Angus et al. 1996) indicating that other calcium channel subtypes do not contribute to ATP release in control of DOCA-salt arteries. Together, these data suggest that alterations in the α_2AR, A_1-R or N-type calcium channels are not responsible for impaired purinergic neurotransmission in DOCA-salt hypertension.

Decreased ATP bioavailability in DOCA-salt hypertension

We next tested the hypotheses that either decreased ATP bioavailability in the nerve terminal or dysfunction in vesicular refilling of ATP was responsible for decreased purinergic neurotransmission in DOCA-salt hypertension. It was expected that if there was decreased ATP bioavailability in nerve terminals from DOCA-salt rats, then high frequency trains of stimuli would deplete ATP stores faster in tissues from DOCA-salt rats as compared to controls. We found that depletion of ATP stores, or run-down, did occur at a faster rate and more completely in peri-arterial nerve fibers from DOCA-salt rats compared to control rats. There are several possible explanations for these data.

One possibility is that there is a decrease in prejunctional ATP stores. Mitochondrial damage is both a result of and a contributing factor for increased production of reactive oxygen species in hypertension (ROS; oxidative stress

(Szeto 2006)). While mitochondrial damage in itself would directly result in decreased ATP production, increased ROS may also reduce vesicular ATP stores and alter neurotransmission. ROS alter neurotransmission in animal models of diabetes mellitus. For example, ROS impair sympathetic neurotransmission to the vas deferens of streptozotocin-diabetic rats (Gunes, Ceylan et al. 2005). Another possibility for decreased purinergic neurotransmission is increased ATP degradation due to an increase in metabolic demands of sympathetic nerves in hypertensive rats. Sympathetic nerve activity is increased in DOCA-salt rats (Iriuchijima, Mizogami et al. 1975) and this would result in increased nerve terminal Ca^{2+} concentrations, vesicular recycling and neurotransmitter release, all of which are ATP dependent. Thus ATP stores could quickly be depleted in the nerve endings in hypertension.

Decreased purinergic neurotransmission could be a result of increased NE release. Previous studies have shown interplay between the regulation of ATP and NE stores (Mutafova-Yambolieva and Keef 2001; Morikawa, Tanaka et al. 2007). Because we know that there is an increase in NE release in hypertension, this could in itself inhibit ATP release. Alternatively, a decrease in ATP, could possibly increase NE release. More studies must be done to elucidate if any or all of these reasons contribute to decreased purinergic neurotransmission. However, we showed that blocking the prejunctional α_2AR with yohimbine, does not recover EJP amplitude. Therefore, increased NE release is most likely not the cause for decreased ATP release.

Finally, we tested the possibility that impaired vesicular filling played a

role in decreased purinergic neurotransmission. The mechanism for the accumulation of ATP into secretory vesicles in the periphery has not been entirely elucidated. Two proposed mechanisms include a vesicular ATP transporter, initially discovered in the electric organ of *Torpedo marmorata* (Luqmani 1981), or alternatively, ATP may enter vesicles by passive diffusion through non-specific anion channels (Lange and Brandt 1993). Considering the later, the ability for refilling would be compromised with an overall decrease in ATP bioavailability in the nerve terminals. To examine vesicular refilling, we analyzed time to recovery after run-down. The latency to recovery was longer in DOCA-salt hypertensive arteries. This indicates that impaired vesicular re-filling with ATP after depletion is a potential mechanism for decreased purinergic neurotransmission in DOCA-salt hypertension.

Perspectives

The novel finding of decreased ATP bioavailability in the nerve terminals of hypertensive rats may have several important implications in the pathogenesis of hypertension. The decrease in ATP combined with an increase in NE release suggests a phenotypic switch from NE to ATP as the primary mediator of mesenteric arterial constriction in DOCA-salt hypertension. Very different pathways are responsible for the post-junctional effects of ATP and NE. For example, while the effects of ATP are transient, due to both desensitization of $P2X_1$ receptors and fast degradation of nucleotides, the effects of NE at the α_1AR on arterial SMCs are long lasting. α_1ARs on arterial SMCs do not desensitize,

and furthermore α_1ARs couple to initiation of a cascade of events resulting from G-protein coupled receptor activation including sustained calcium release and phosphorylation of proteins. A decline in purinergic transmission will further disinhibit NE release and cause increased vascular tone. The end result is a feed-forward effect resulting in hypertension. These studies suggest the importance of prejunctional regulation of neurotransmitter release as a target for future drug therapies for hypertension.

CHAPTER 4

MECHANISMS OF IMPAIRED REGULATION OF PURINERGIC NEUROTRANSMISSION TO MESENTERIC ARTERIES IN DOCA-SALT HYPERTENSIVE RATS

Abstract

Sympathetic innervation of mesenteric arteries contributes to blood pressure regulation. Alterations in the function of sympathetic nerves supplying mesenteric arteries occur in hypertension. Norepinephrine (NE) and ATP are released from sympathetic nerve, where they act at α_1-adrenergic (AR) and $P2X_1$ receptors respectively to constrict arteries. While NE release is increased in hypertension due to impaired prejunctional α_2AR function, less is known about purinergic neurotransmission in hypertension. $P2X_1$ receptors are ion channel receptors permeable to cations. Activation of P2X1 receptors causes a transient depolarization (excitatory junction potential, EJP) of arterial smooth muscle membrane potential. I hypothesized that altered purinergic transmission in DOCA-salt hypertension would cause increased EJP amplitude variability and increased rundown of EJPs during bursts of nerve activity. Intracellular recordings of EJPs in response to low and high frequency trains of nerve stimulation were obtained from mesenteric arteries *in vitro*. EJP amplitudes were more variable in DOCA-salt compared to control arteries. Although α_2AR function was impaired, increased EJP rundown in DOCA-salt arteries was independent of α_2AR function or changes in calcium sensitivity ATP release. Guanethidine concentration response curves for inhibition of ATP release measured as EJP amplitude were more shallow in DOCA-salt compared to sham arteries, suggesting that one or more pools of neuronal ATP is depleted. I conclude that impaired purinergic neurotransmission in DOCA-salt hypertension

is independent of calcium handling and autoreceptor activity and may be due to depletion of synaptic stores of ATP.

Introduction

Blood pressure regulation is highly dependent on sympathetic neurotransmission to the splanchnic circulation (King et al., 2007). Alterations in sympathetic nerves innervating the mesenteric vasculature are associated with hypertension in several animal models (Iriuchijima et al., 1975; Masuyama et al., 1986; Mathias, 1991; Brock and Van Helden, 1995; Luo et al., 2004). The splanchnic vasculature is densely innervated by sympathetic nerves which release norepinephrine (NE), adenosine 5'-triphosphate (ATP) and neuropeptide Y (NPY) from peri-arterial sympathetic nerves, (Donoso et al., 1997) and smooth muscle cells (SMC) have receptors where neurotransmitters bind and exert vasoconstrictor effects. While ATP is found ubiquitously throughout the central and peripheral nervous system as the primary cellular energy source, it is also released as a neurotransmitter (Burnstock, 1972, 1995). The regulation and release of ATP-containing vesicles is dependent on calcium influx through voltage-gated calcium channels (Katz and Miledi, 1968; Waterman, 1997; Hardy and Brock, 2001).

$P2X_1$ receptors are cation channels that when activated by ATP mediate an influx of Ca^{2+} into the SMC and a depolarization of the resting membrane potential (RMP) called an excitatory junction potential (EJP). Intracellular recordings from SMCs detect EJPs which are used as an indirect measurement of ATP release. Ectonucleotidases released with ATP quickly degrade ATP to ADP,

AMP and adenosine (Todorov et al., 1997; Westfall et al., 2002). Prejunctional regulation of ATP release occurs via the binding of ATP to P2X receptors, adenosine to A_1 receptors (Shinozuka et al., 1988; Morikawa et al., 2007) and NE to α_2-adrenergic autoreceptors (α_2ARs) on sympathetic nerve endings (Driessen et al., 1993). These receptors are coupled to pertussis-toxin sensitive second messenger systems which inhibit neurotransmitter release in part through calcium (Ca^{2+}) dependent mechanisms.

Trains of nerve stimulation cause facilitation of EJPs (Surprenant, 1980). Facilitation of EJPs in the autonomic nervous system has been best characterized in the vas deferens (Brain and Bennett, 1997; Hardy and Brock, 2001) and rat tail artery (Msghina et al., 1998). Similar mechanisms are thought to apply to EJP facilitation in the mesenteric arterial vascular bed (Surprenant, 1980). In large neuromuscular junctions, prejunctional nerve endings can be studied directly, and the basic principles of facilitation can be addressed (Logsdon et al., 2006). In these and other synapses, differences in pools of vesicles (ie. readily releasable pool (RRP), depot pool (DP), etc.) and probability of release are the primary factors in determining facilitation and/or depression of postjunctional responses (Fisher et al., 1997; Wong et al., 2003). Similarly, when sympathetic nerves are stimulated with a train of stimuli, EJPs facilitate in a calcium-dependent process (Burnstock and Holman, 1961; Brain and Bennett, 1997). Rundown occurs when the RRP has been depleted. At peripheral synapses, high frequency trains of nerve stimulation result in rundown of acetylcholine (ACh) at neuromuscular junctions (Moyer and van Lunteren, 2001; Ren and Galligan, 2005) and ATP and ACh at synapses in the myenteric plexus (Ren and Galligan, 2005). However not

much is known about neurotransmitter depletion, or rundown, at sympathetic neuroeffector junctions.

In human hypertension and animal models of hypertension, altered regulation of neurotransmitter release, due in part to impaired α_2ARs, results in increased NE release (Bouvier and de Champlain, 1985; Moreau et al., 1995; Luo et al., 2004). However, the mechanisms for impaired regulation of ATP release in hypertension are not clear. I hypothesized that increased vulnerability to ATP depletion in response to high frequency trains of nerve stimulation are an indicator for impaired regulation of ATP in sympathetic nerve endings. The mechanism for altered regulation of ATP in hypertension is not known, but could be due to 1) alterations in the handling of ATP in the nerve terminal; 2) changes in ATP responsiveness at the SMC or 3) decreased ATP availability. In these studies tertiary branches of mesenteric arteries were isolated *in vitro* and SMCs were impaled for intracellular recordings of EJPs. EJP amplitudes were assessed in control and deoxycorticosterone acetate (DOCA)-salt rats. High frequency trains (10 Hz) of nerve stimulation were used to deplete neuronal stores of ATP in periarterial nerves from DOCA-salt rats and pharmacological tools were used to isolate mechanisms likely to result in increased rundown. Together these experiments give insight into impairment of the neuroeffector junction including altered regulation of ATP release from sympathetic nerve terminals.

Methods

Animals

All experiments were done using Sprague-Dawley rats from Charles River Laboratories (Portage, MI). Upon arrival at the animal care facility, animals were maintained according to standards approved by the Institutional Animal Care and Use Committee at Michigan State University. All experimental procedures were carried out in accordance with the "Guiding Principles in the Care and Use of Animals" of the American Physiological Society. Rats were acclimated for 2-3 days before entry into any experimental protocol. Pelleted rat chow (Harlan/Teklad 8640 Rodent Diet) and distilled water were provided *ad libitum*. Rats were housed in temperature- and humidity- controlled rooms with a 12:12-h light-dark cycle.

DOCA-salt hypertension

Male Sprague-Dawley rats (250-275g) were anesthetized with 3% Isoflurane. The skin over the left lateral abdominal wall was shaved and prepared with chlorhexadine. A 1.5-cm vertical 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, vein, and ureter with 6-0 silk sutures and the incision was closed with 4-0 monofilament nylon sutures. A 3 x 1.5-cm rectangular area between the shoulder blades of the back was shaved and disinfected for subcutaneous implantation of a Silastic (Dow Corning) sheet

containing DOCA-salt (Sigma; 150mg/kg) pellet under a 1.5-cm incision. The skin was closed with 4-0 nylon sutures. Sham-operated rats underwent left kidney removal only. Surgery was performed on a heated pad, and rats recovered in a heated box. After recovery, rats were housed under standard conditions for four weeks. DOCA-implanted rats received standard pelleted rat chow and salt water (1% NaCl + 0.2% KCl) ad libitum, whereas sham rats received standard pelleted rat chow and distilled water. Blood pressures were measured by tail-cuff plethysmography. On day 28, rats were removed from their cage and warmed under a heat lamp. Three readings were averaged to attain a final mean arterial blood pressure (MAP). Rats with MAP equal to or higher than 150 mmHg were considered hypertensive.

Tissue preparation

The week following blood pressure measurements, rats were euthanized with an intrapertioneal injection of sodium pentobarbital (50 mg/kg). The mesentery was surgically removed. Tertiary branches of mesenteric arteries were dissected out, cleaned of adipose and connective tissue and pinned taut using stainless steel pins (50 μM diameter) in a perfusion chamber coated with Sylgard® (Dow Corning, Midland, MI). Tissues were superfused with Krebs' solution of the following composition (mM): NaCl, 117; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; dextrose, 11. Nifedipine (1μM) (L-type VDCC antagonist) and prazosin (0.1μM) (α_1-AR antagonist) were added to the buffer to attenuate vessel constriction during trains of electrical nerve stimulation (see below). The buffer was heated to 37° C and bubbled with 95% O_2 and 5%

CO_2 and the tissue was allowed to equilibrate for 30 minutes before beginning electrophysiological studies.

Electrophysiological recording

Intracellular recordings from individual SMCs were obtained using glass microelectrodes filled with 2M KCl (100-200 MΩ tip resistance). Impalements were only accepted if the following criteria were satisfied: (1) the cell penetration was abrupt (2) the membrane potential increased to -50 mV or higher and (3) the membrane potential was stable for at least 5 minutes. Individual recordings from a single cell lasted for at least 20 minutes and up to 2 hours. An amplifier (IX2-700 dual intracellular preamp, Dagan Inc, Minneapolis, MN) was used to record membrane potential in the current clamp mode. Signals were filtered using a HumBug 50/60 Hz Noise Eliminator (Digitimer Research Instruments, Quest Scientific, North Vancouver, BC, Canada). Impalements were accepted if the resting membrane potential dropped quickly to $\geq$ -50 mV. EJPs were evoked using a Krebs' solution-filled, bipolar, focal stimulating electrode containing two parallel silver/silver chloride wire electrodes connected to a Grass Instruments S88 stimulator (Grass Technologies, Astro-Med, Inc, West Warwick, RI). The stimulating electrode was positioned perpendicular to the tissue and directly across from the recording electrode. For most experiments, a stimulation frequency of 10 Hz, 0.5 ms pulse width at the lowest voltage (50-120V) which produced a maximal amplitude EJP was used. Signals were recorded using an analog to digital converter (Digidata 1200, Molecular Devices Inc., Foster City

CA) and Axoscope 9.0 software (Molecular Devices Inc). Data were analyzed using Clampfit 9.0 software (Molecular Devices Inc) and a laptop computer.

Drugs

The following drugs used in these studies were obtained from Sigma chemical (St. Louis, MO): αβ-methylene ATP (αβ-met ATP), nifedipine, prazosin, pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS), 5-bromo-6-(2-imidazolin-2-yl-amino)-quinoxaline tartrate (UK 14,304), yohimbine, pertussis toxin (PTX) and Bafilomycin A1. All drugs were diluted in deionized water except for prazosin and nifedipine which were dissolved in 95% ethanol to make a concentrated stock solution. Working solutions of nifedipine and prazosin contained <0.01% ethanol. Final solutions were made in Krebs' buffer on the day of the experiment.

Statistics

Data are mean ± SE and n values are the number of animals from which the data were obtained. Concentration-response data were fitted using non-linear regression and the Hill equation (GraphPad Prism, San Diego, U.S.A). Results from other experiments were tested with either one-way ANOVA or Student's t-test for paired or unpaired data, as appropriate. Differences were considered significant when $P < 0.05$.

Results

Four weeks after surgery, DOCA-salt rats had elevated systolic blood pressures (196 ± 7 vs. 138 ± 2 mmHg; $P < 0.001$) and lower body weights (359 ± 14 vs. 450 ± 11 g; $P < 0.001$) compared to control rats.

Variability of EJP amplitude is increased in DOCA-salts

EJP amplitudes recorded from DOCA-salt arteries had greater variance compared to controls (F-test for equal variances, $P < 0.05$) (Fig. 11A). However, the SMC RMPs were not different between groups (Fig. 11B). These data suggest a dysregulation in purinergic neurotransmission in sympathetic nerve terminals.

Autoreceptor function regulates EJP facilitation but not rundown in arteries from DOCA-salt rats

EJP amplitudes facilitate during 10 Hz trains of nerve stimulation (Fig. 12A) and these EJPs are blocked by the P2X receptor antagonist, PPADS (10 μM), suggesting EJPs are due only to ATP acting at $P2X_1$ receptors. Mean EJP amplitudes in response to 10 Hz nerve stimulation were averaged at different time points over 30 s before and after autoreceptor blockade in DOCA-salt and control arteries (Fig. 12B,C). EJP amplitudes facilitated to a similar extent, but rundown was increased in DOCA-salt rats. Yohimbine (1 μM; Fig. 12D,F), an α_2AR antagonist, and pertussis toxin (PTX, 2 μg/mL; Fig. 12E,G), a Gi/Go inhibitor, were used to block signaling from prejunctional receptors inhibiting ATP release,

to determine if increased rundown was the result of autoinhibition in DOCA-salt hypertension. Treatment with yohimbine had no effect on facilitation in DOCA-salt rats but significantly increased peak facilitation in control rats suggesting the α_2-AR is impaired in its regulation of ATP release in DOCA-salt rats (Fig. 12D). Yohimbine had no effect on EJP amplitude at 3 s in either control or DOCA-salt rats (Fig. 12F). After yohimbine, rundown was still significantly greater in DOCA-salt rats compared to control. This suggests that the α_2AR does not play a significant role in mediating EJPs at 3 s of a high frequency train, and that rundown in DOCA-salt is not due to altered regulation by the α_2AR.

The α_2AR is only one of several possible inhibitory receptors on the sympathetic nerve terminal. Therefore, mesenteric arteries were incubated with PTX (2ug/mL) for 2 hours at 37°C to determine if any Gi/Go-coupled receptors were responsible for increased rundown in DOCA-salt rats. Peak facilitation was not different between control and DOCA-salt rats before or after treatment (Fig. 12E). Although PTX tended to increase peak EJP amplitude in response to nerve stimulation in controls, there was no significant difference in either DOCA-salt or control rats. PTX did not attenuate rundown in DOCA-salt arteries (Fig.12F). To verify the effectiveness of the PTX treatment, vessels were treated with UK 14,304, an α_2AR agonist. EJP amplitudes in response to nerve stimulation were assessed in UK 14,304 treated vessels before and after PTX treatment. It was expected that treatment with PTX would attenuate the inhibitory effects of UK 14,304 on EJP amplitude. Interestingly, inhibition of EJPs by UK 14,304 was attenuated after PTX treatment in DOCA-salt but not in control rats. This data is consistent with previous reports that the α_2AR is dysfunctional in DOCA-salt

hypertensive rats (Luo et al., 2004), so that PTX was effective in DOCA-salt rats because not all Gi/Go proteins were working to begin with, and therefore there were less to inhibit.

UK 14,304 decreased peak amplitude and increased time to peak, but did not affect rundown

Tissues were incubated with UK 14,304 (30 nM), a selective α_2AR agonist, to test the role of α_2AR on facilitation peak amplitude, time to peak and rundown in response to high frequency trains in control and DOCA-salt sympathetic nerves. Fig. 13A shows mean EJP amplitude in response to 10 Hz nerve stimulation up to 1 s. EJPs facilitate to a less extent and the time to peak facilitation is delayed after treatment with UK 14,304. Fig. 13B shows mean EJP amplitudes over 30 s. The facilitation and rundown pattern of EJPs in response to 10 Hz nerve stimulation (3 s) is shown, before and after treatment with UK 14,304. UK 14,304 had profound effects on the first part of the train, where it decreased EJP amplitude at 3 s in arteries from both control and DOCA-salt rats compared to their no-drug control (fig. 13C). In addition, the time to peak facilitation was delayed in both control and DOCA-salt rats (Fig. 13D). These data suggest that α_2ARs are activated early in the train, reducing individual EJP amplitudes, as well as total facilitation. UK 14,304 did not affect the extent of rundown in arteries from DOCA-salt rats, supporting previous findings that the α_2AR does not regulate ATP release at 3 s in high frequency trains (fig. 13E).

EJP amplitudes and facilitation are equally sensitive to changes in extracellular calcium ($[Ca^{2+}]_e$) in DOCA-salt and control rats

Miledi and Katz first discovered that neurotransmission was dependent on calcium influx into presynaptic terminals (Katz and Miledi, 1968). Ca^{2+} also plays a role in facilitation of EJPs during trains of stimulation of sympathetic nerves (Hardy and Brock, 2001), but its role in rundown at the neuroeffector junction is not known. The role of calcium sensitivity on purinergic neurotransmission from sympathetic nerves was compared between control and DOCA-salt rats by decreasing the $[Ca^{2+}]_e$ from 2.5 - 0.625 mM and then stimulating the nerves with low frequency trains (0.5 and 1 Hz) for 10 seconds. Yohimbine (1μM) was present in all experiments to minimize the role of prejunctional α_2-ARs in EJP facilitation. Linear regression analysis of the amplitudes of the first (fig. 14A) and last (fig. 14B, C) EJPs in response to various Ca^{2+} concentrations was compared between arteries from DOCA-salt and control rats. The slope of the line was similar between arteries from DOCA-salt and control rats at 0.5 and 1.0 Hz. Furthermore, increasing the concentration of calcium to 5.0 mM did not increase EJPs further in arteries from DOCA-salt rats (data not shown). These data suggest that alterations in purinergic neurotransmission, including rundown is not due to an increased sensitivity to $[Ca^{2+}]_e$ in DOCA-salt hypertension

ATP handling and storage in DOCA-salt rats

Little is known about how ATP, a negatively charged molecule, gets through the hydrophobic membrane of synaptic vesicles. As for NE uptake into vesicles, vacuolar (or vesicular) ATPase (vATPase) uses ATP to pump hydrogen ions (H^+) into the vesicles to create a concentration gradient and assure a low pH inside the vesicles. Hydrogen ions are then exchanged for NE via the vesicular monoamine transporter (vMAT), and NE moves into the vesicles. We tested the hypothesis that ATP is also transported into vesicles using the H^+ gradient set up by vATPase by using, bafilomycin A1, a selective vATPase inhibitor (Bowman and Bowman, 2005), as a tool to inhibit vesicular filling of synaptic vesicles with ATP. Vessels were incubated with bafilomycin (1 μM) for 30 and 120 m and then EJP amplitude in response to a single stimulus from control and DOCA-salt arteries was recorded (Fig. 15 A and B). There was no change in EJP amplitude in either control or DOCA-salt rats up to 120 minutes suggesting that either vesicular storage of ATP in sympathetic nerve terminals is not dependent on vATPase or that the time of incubation in bafilomycin A1 was not sufficient. Incubating the tissues longer was not practical, due to loss of tissue viability.

Guanethidine is taken up into sympathetic nerve terminals by the norepinephrine transporter (NET) and subsequently prevents reuptake of NE and ATP into vesicles (Meehan et al., 1991). Guanethidine (0.1 – 3.0 μM), inhibited EJPs and the EC_{50}'s were similar between DOCA-salt and control arteries. However, the concentration-response curve in DOCA-salt arteries had a lower Hill slope compared to controls (fig 15C).

Affect of P2X receptor blockade *in vivo*

To examine the role of the purinergic component of neurotransmission on hemodynamic measurements *in vivo*, rats were implemented with catheters in their femoral artery, for blood pressure measurements, and femoral vein, for IV drug infusion. This allowed for drug delivery in a way which was minimally invasive and non-stressful to the rat. An αβ-met ATP (P2X receptor agonist; 20 μg/kg) challenge resulted in dramatic heart rate and blood pressure changes which were blocked by PPADS (20 mg/kg), a P2X receptor antagonist. This suggests that this was an effective dose for P2X receptor blockade *in vivo* (data not shown). However, a single IV injection of PPADS (20 mg/kg) did not alter blood pressure or heart rate up to 1 h (Fig. 16A and B). Interestingly, figures 16C and D show that blocking the adrenergic component of neurotransmission with prazosin (0.5 mg/kg, IV) only transiently lowered blood pressure and the addition of PPADS did not affect blood pressure. This suggests that there are many factors besides the peripheral nervous system which work together to maintain blood pressure in the non-anesthetized rat.

Table 2: Summary of statistical analysis of control and DOCA-salt EJP amplitudes

	N-value	Mean	Variance	Kurtosis	A^2
Control	41	7.0	9.8	0.2	1.46
DOCA-salt	50	6.5	18.1*	1.5	0.76

* $P < 0.05$ vs. control

Table 2. Statistical analysis of EJP amplitude distribution. Mean, variance, kurtosis and A^2, descriptive indices of the normalcy of a distribution, were compared between DOCA-salt and control animals.

Fig. 11

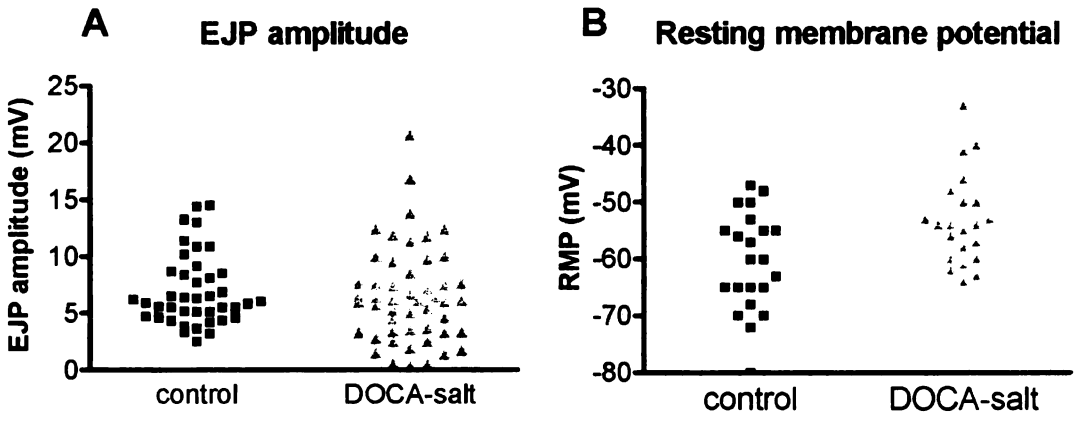


Fig 11. Increased variability of EJP amplitudes but not RMPs in DOCA-salt arteries. A. Individual data points representing EJP amplitude (mV) for DOCA-salt and control arteries. Coefficient of variation for control and DOCA-salt rats is 45 and 65% respectively. An F-test for equal variances was used to determine that EJPs from control and DOCA-salt arteries have unequal variances ($F = 0.54$; $p < 0.05$). **B**. Coefficient of variation of RMPs was similar between control and DOCA-salt rats (14 vs. 15 %).

Fig. 12

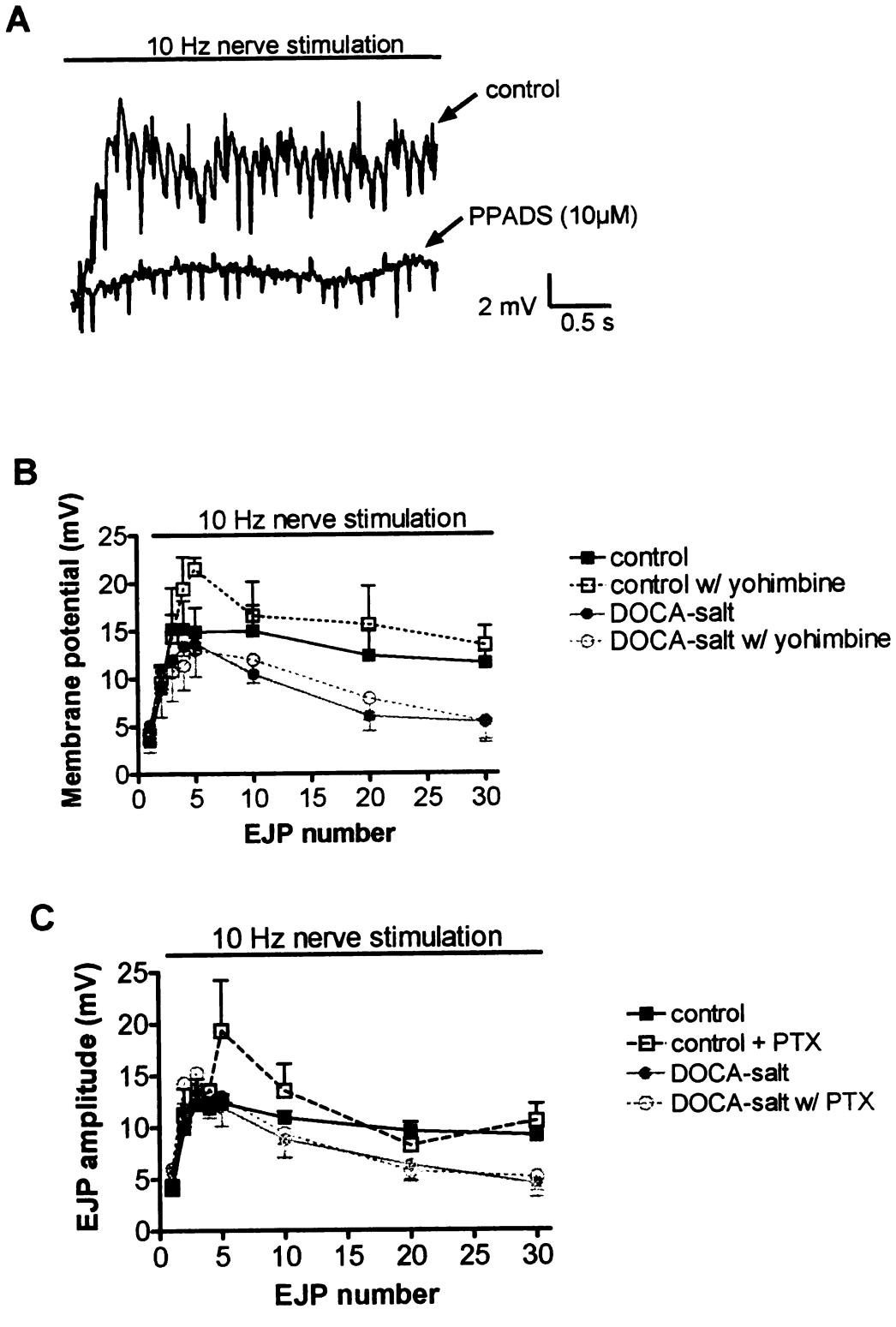

A
10 Hz nerve stimulation
control
PPADS (10μM)
2 mV
0.5 s
B
10 Hz nerve stimulation
Membrane potential (mV)
EJP number
control
control w/ yohimbine
DOCA-salt
DOCA-salt w/ yohimbine
C
10 Hz nerve stimulation
EJP amplitude (mV)
EJP number
control
control + PTX
DOCA-salt
DOCA-salt w/ PTX

Fig. 12 continued

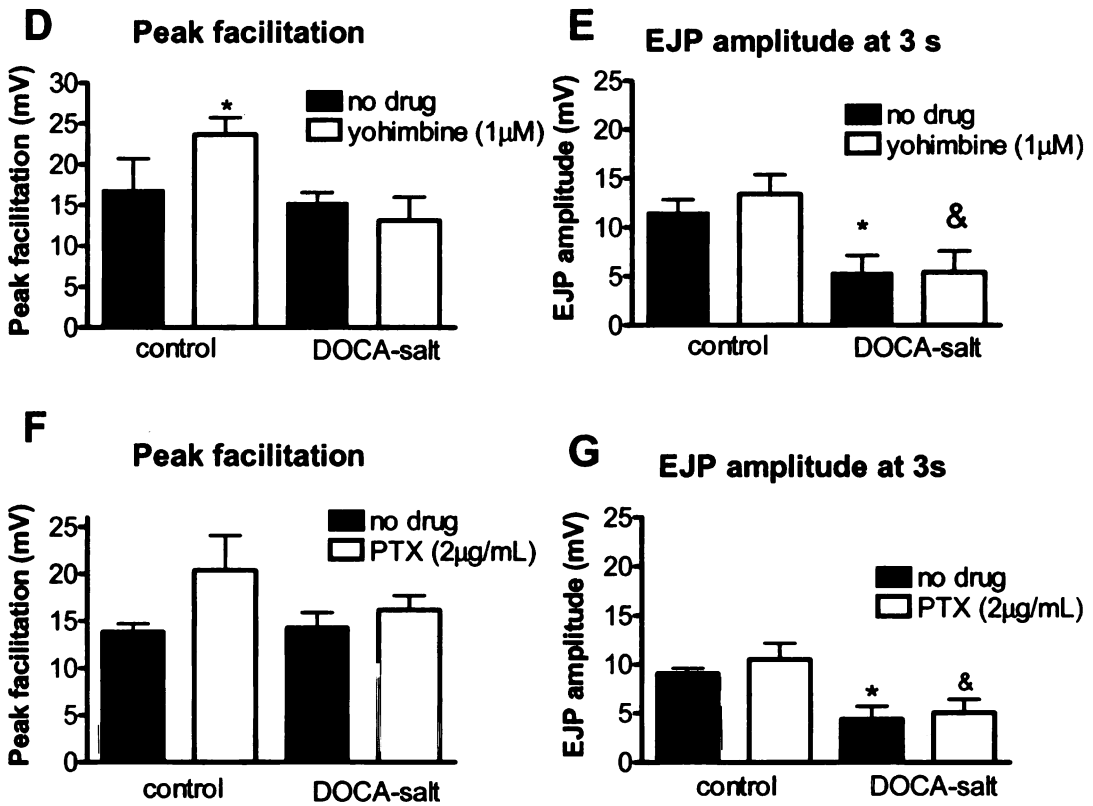


Fig. 12. EJP rundown in arteries from DOCA-salt rats persists in the presence of autoreceptor blockade. A 10 Hz train of nerve stimulation causes EJPs to rundown in arteries from DOCA-salt rats. Peak EJP amplitude and EJP amplitude at 3 s (30^{th} stimuli) were compared for control and DOCA-salt rats before and after autoreceptor blockade. **A.** A representative tracing of 10 Hz nerve stimulation for 3s in a control animal, with and without PPADS (10 μM), a $P2X_1$ receptor blocker confirms the responses recorded are purinergic. **B.** Mean data for control and DOCA-salt rats before and after yohimbine, an α_2-AR antagonist (N = 5-6). **D.** After treatment with yohimbine, peak EJP amplitude increased in control (17 ± 4 vs 24 ± 2 mV; *P < 0.05), but not DOCA-salt arteres (15 ± 1 vs. 13 ± 3 mV). **F.** At 3s, DOCA-salt EJPs are smaller than control (12 ± 1 vs. 5 ± 2 mV; *P < 0.05 DOCA-salt vs. control, no drug) and are not restored with yohimbine (13 ± 2 vs. 5 ± 1 mV; &P < 0.05 DOCA-salt vs. control, with yohimbine (1μM). **C.** Mean data for EJPs from control and DOCA-salt arteries before and after pertussis toxin (PTX, 2μg/mL), a Gi/Go protein antagonist (N = 6-7). The effectiveness of PTX treatment was determined by assessing the % change in response of a single stimulus to UK 14,304. % inhibition of EJPs was significantly attenuated in DOCA-salt arteries (P < 0.05), but not controls (data not shown). **E.** After treatment with PTX, peak amplitudes tended to increase more in controls (14 ± 1 mV vs. 20 ± 4) than DOCA-salt arteries (14 ± 2 vs. 16 ± 2), but were not significantly different in either group. **G.** At 3s, DOCA-salt EJP amplitudes are smaller than controls (9 ± 1 vs. 4 ± 1; *P < 0.05 control vs. DOCA-salt, no drug) and are not restored with PTX (11 ± 2 vs. 5 ± 1; &P < 0.05 control vs. DOCA-salt, with drug).

Fig. 13

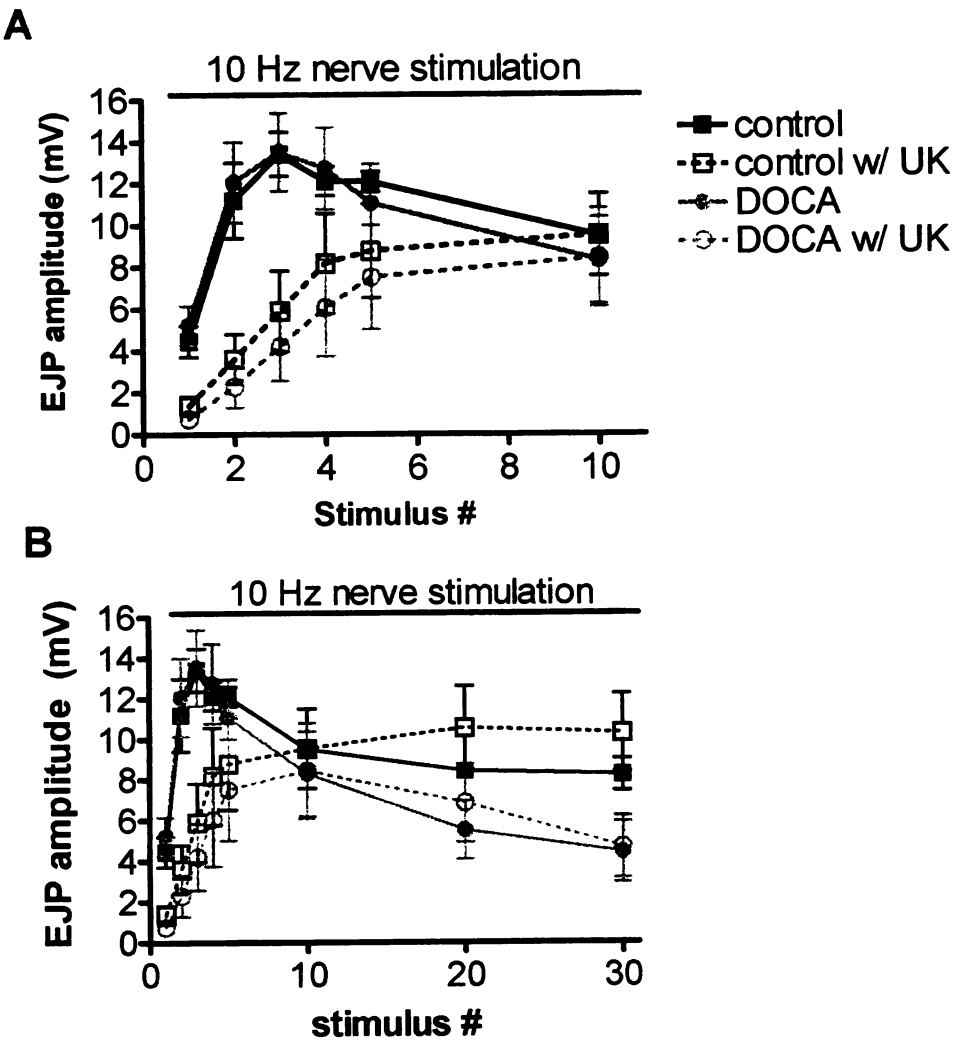

A
10 Hz nerve stimulation
EJP amplitude (mV)
Stimulus #
control
control w/ UK
DOCA
DOCA w/ UK
B
10 Hz nerve stimulation
EJP amplitude (mV)
stimulus #

Fig. 13 continued

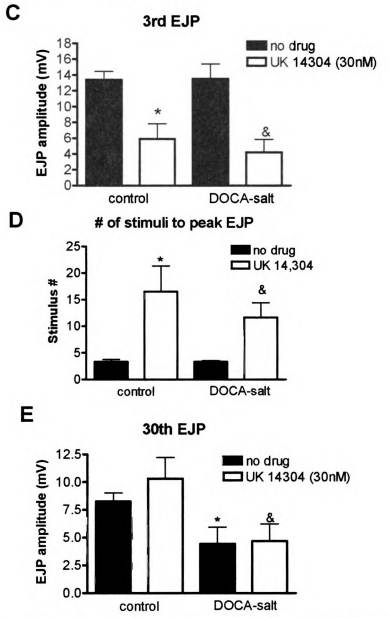


Fig. 13. UK 14,304 reduces peak facilitation and time to peak facilitation, but not rundown in control and DOCA-salt rats. Mesenteric arteries were stimulated with a 10 Hz train of nerve stimulation for 3s before and after treatment with UK14,304 (30 nM) to assess the role of the α_2-AR in regulating neurotransmitter release in response to high frequency trains of stimulation in control and DOCA-salt arteries. Mean data for control and DOCA-salt rats before and after UK 14,304 (N = 6) up to 1 **(A)** and 3 s **(B)**. **C.** After treatment with UK 14,304, peak amplitudes were significantly lower in control (13 ± 1 mV vs. 6 ± 2 mV; *****P < 0.05, control no drug vs. drug) and DOCA-salt rats (14 ± 2 vs. 4 ± 2 mV; &P < 0.05 vs. DOCA-salt no drug vs. drug). **D**. After UK14304 treatment, the time to peak facilitation was delayed in control (0.3 vs 1.5 s, *****P < 0.05 control, before and after drug), and DOCA-salt rats (0.3 vs. 1.1 s, *****P <0.05 DOCA-salt, before and after drug). **E**. At 3s, DOCA-salt EJP amplitudes are smaller than control rats before (8 ± 2 vs. 4 ± 1 mV *****P < 0.05 control vs. DOCA-salt, no drug) and after UK 14,304 (10 ± 2 vs. 5 ± 2 mV; &P< 0.05 control vs. DOCA-salt, with UK 14304).

Fig. 14

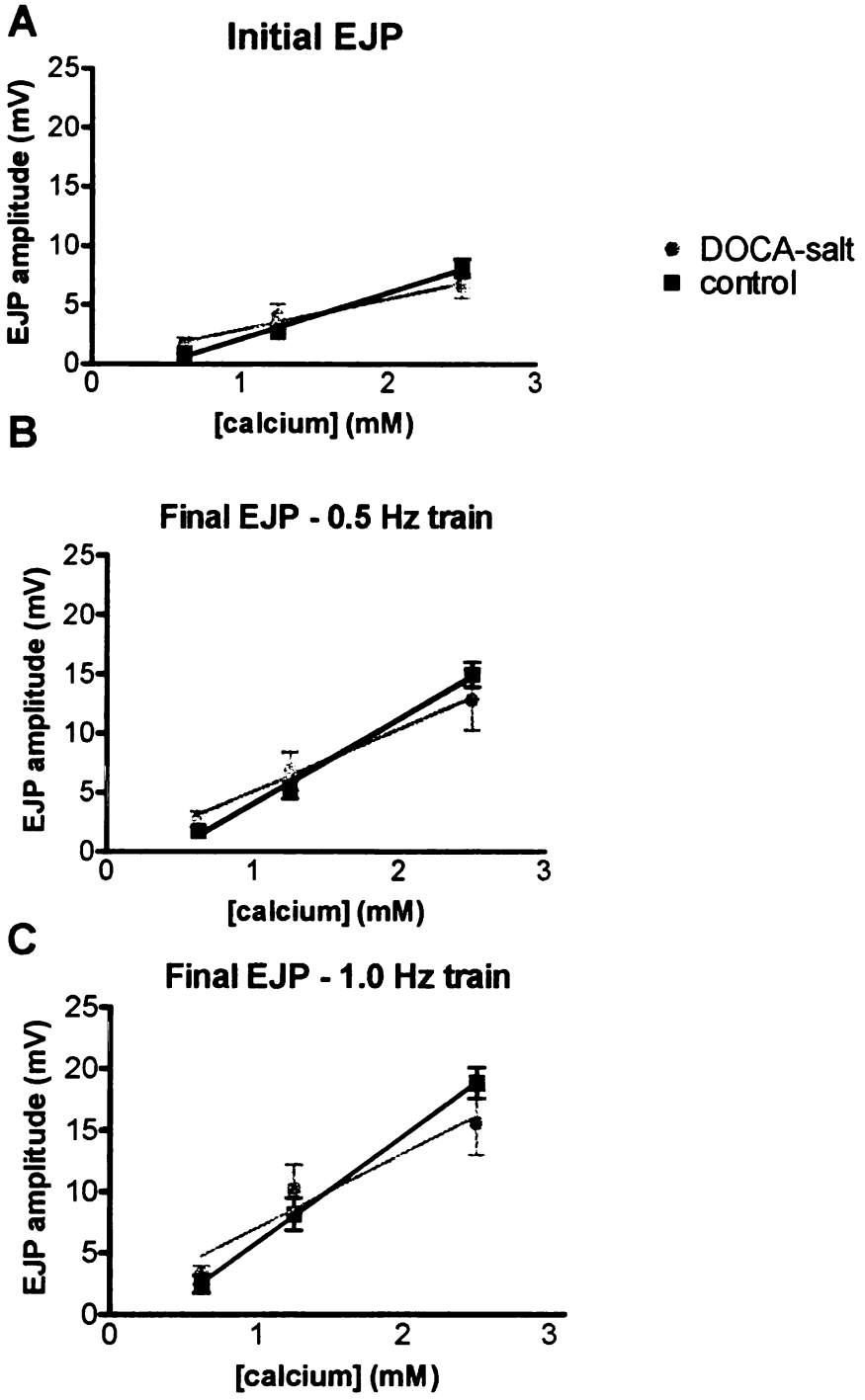
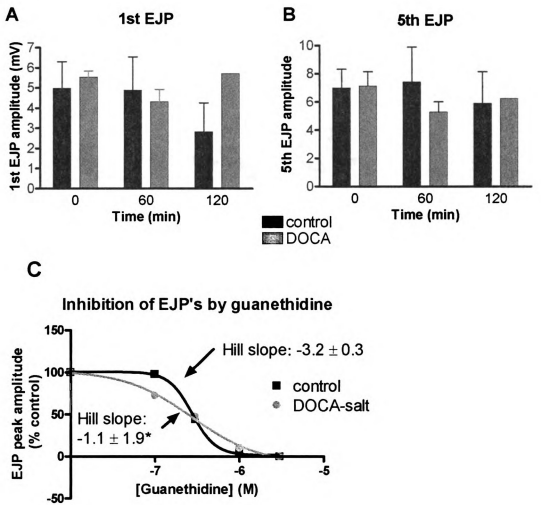


Fig. 14. Decreases in calcium reduce EJP amplitude similarly in control and DOCA-salt rats. Calcium in the physiological buffer was reduced from 2.5 - 0.625mM and responses to nerve stimulation were assessed by measuring the first and last EJP amplitude in the presence of yohimbine (1 μM). The responses were similar for control and DOCA-salt animals at the first peak (**A**) and last peak in response to 0.5 (**B**) and 1.0 (**C**) Hz nerve stimulation. Increasing the Ca^{+} concentration to 5.0 mM did not increase EJP amplitudes further in either control or DOCA-salt rats (data not shown).

Fig. 15

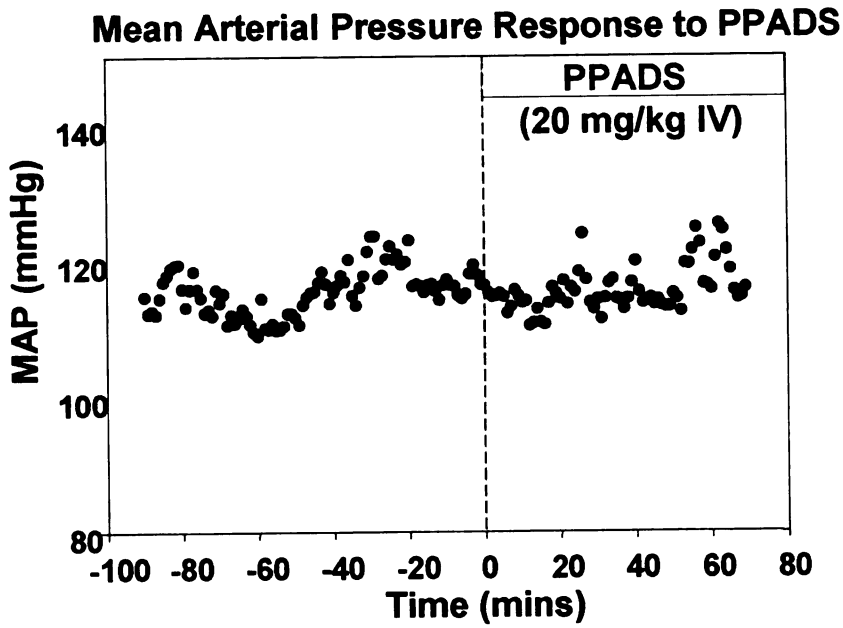


* = p < 0.05; Hill slope DOCA-salt vs. control

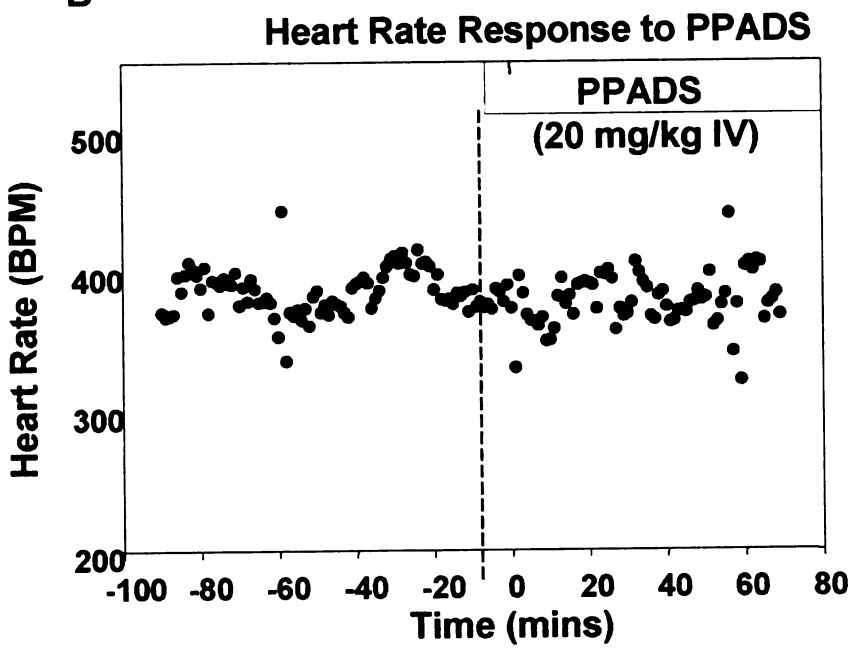
Fig. 15. Handling of ATP is altered in sympathetic nerve terminals in DOCA-salt rats. Mesenteric arteries and perivascular nerves from control and DOCA-salt rats were treated with Bafilomycin A1 for 30 or 120 minutes *in vitro*, and EJP amplitude in response to a 0.5 Hz train (10s) was assessed. There was no difference in the amplitude of the 1st EJP **(A)** or 5th EJP **(B)** in response to 0.5 Hz nerve stimulation after Bafilomycin A1 treatment. **C.** A concentration-response curve with guanethidine, an inhibitor of neurotransmission from adrenergic nerves, had differential effects on EJP amplitude in control (-3.2 ± 1.9) and DOCA-salt rats (-1.1 ± 0.3; * = P < 0.05; Hill slope, DOCA-salt vs. control).

Fig. 16

A


Mean Arterial Pressure Response to PPADS
PPADS
(20 mg/kg IV)
MAP (mmHg)
140
120
100
80
-100 -80 -60 -40 -20 0 20 40 60 80
Time (mins)

B


Heart Rate Response to PPADS
PPADS
(20 mg/kg IV)
Heart Rate (BPM)
500
400
300
200
-100 -80 -60 -40 -20 0 20 40 60 80
Time (mins)

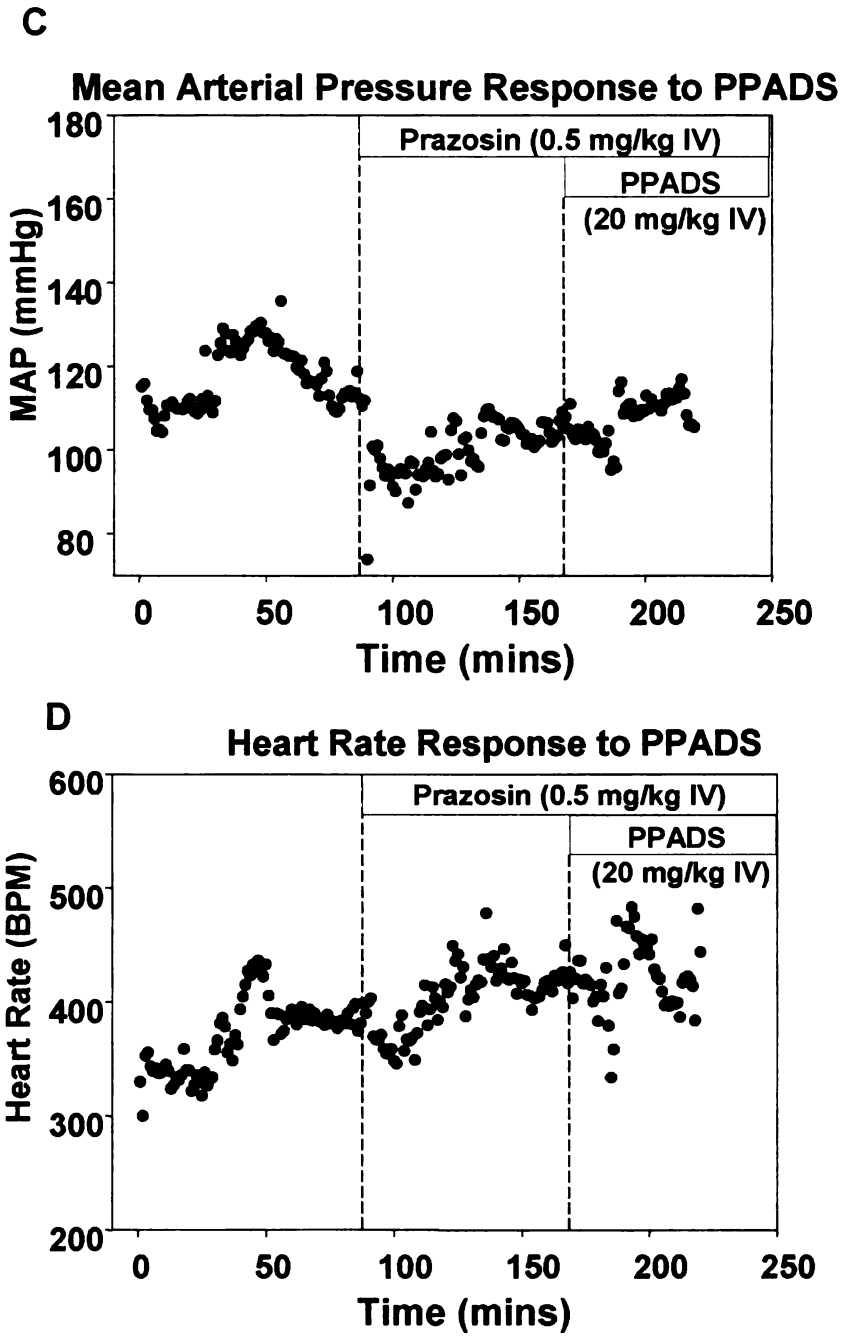


Fig. 16. PPADS effect on hemodynamic measurements *in vivo*. A and B. After one hour of baseline MAP and heart rate measurements, PPADS (20 mg/kg, IV), a P2X receptor antagonist, was injected IV (t = 0). Neither MAP nor heart rate was affected by this dose of PPADS, a dose that blocked αβ-met ATP responses. **C and D.** After one hour of baseline measurements, prazosin (0.5 mg/kg, IV), an α_1 adrenergic receptor antagonist, was injected, followed one hour later by PPADS. Prazosin lowered blood pressure transiently, while PPADS had no effect.

Discussion

In hypertension changes in vascular tone are due in part to alterations in sympathetic nerves including impaired regulation of adrenergic (Ferrier et al., 1993; Karoon et al., 1995; Luo et al., 2004) and purinergic (Brock and Van Helden, 1995) neurotransmission. Previously I showed that stimulating periarterial nerve fibers with trains of nerve stimulation results in decreased EJP amplitudes and increased rundown of EJPs in DOCA-salt rats. In these studies I explored possible mechanisms for altered purinergic neurotransmission, and increased rundown in particular, in the DOCA-salt model of hypertension. I found that there is increased variability of EJP amplitudes in DOCA-salt hypertension, but that the RMPs, while depolarized, have equal variances between groups. I also showed that the prejunctional α_2ARs are impaired in their ability to regulate ATP release during the early phase of high frequency trains of nerve stimulation, but that prejunctional autoreceptors to not play a role in increased rundown seen in DOCA-salt hypertension. These findings in DOCA-salt rats are novel and have important implications for explaining the increased vascular tone in salt-sensitive hypertension (Fink et al., 2000).

I previously reported increased rundown in DOCA-salt hypertension, and in these studies we used pharmacological probes to investigate possible mechanisms for faster depletion of ATP from sympathetic nerves. I found that using either PTX or yohimbine to block prejunctional autoreceptors that might regulate ATP release increased EJP facilitation, but did not block rundown. This

suggests that prejunctional receptors that use PTX sensitive G-proteins in their signaling pathway are not responsible for the decline in EJP amplitude that occurs during a 3 s, 10 Hz train of nerve stimulation. The results confirmed previous studies demonstrating synaptic rundown is independent of G-protein coupled receptor mediated prejunctional inhibition (Lin et al., 2001; Ren and Galligan, 2005).

Yohimbine increased EJP facilitation in controls, but not DOCA-salt arteries, suggesting that α_2AR function is impaired in these blood vessels. The result corroborates previous findings that increased NE release from sympathetic nerves in tissues from DOCA-salt rats is due in part to impaired α_2AR function (Tsuda et al., 1989; Moreau et al., 1995; Luo et al., 2004). Because there are several autoreceptors found on sympathetic nerve terminals which interact with each other, we used PTX to confirm that rundown of ATP in DOCA-salt rats is not due to changes in the function of any autoreceptors which regulate ATP. However PTX treatment did not change EJP facilitation in control arteries. One reason for this may be incomplete inhibition of Gi/Go proteins with PTX. UK 14304 is an a_2AR agonist and a_2ARs use a PTX sensitive G-protein to link to inhibition of neurotransmitter release in sympathetic nerves. I used UK 14,304 induced inhibition of EJPs as a measure of the effectiveness of PTX in inactivating prejunctional G-proteins. UK 14,304 induced inhibition of EJPs in DOCA-salt rats was reduced 50% by PTX pretreatment however UK 14,304 response in control arteries were not significantly reduced. However, although EJP inhibition by UK 14,304 was reduced by PTX pretreatment in DOCA-salt arteries EJP rundown was not changed. The PTX protocol incubation protocol

used here has been shown to disrupt α_2AR mediated presynaptic inhibition in the submucosal plexus of the guinea pig ileum (Surprenant and North, 1988; Ren and Galligan, 2005). In the present study, I found that longer PTX incubation periods were associated with a decline in overall tissue viability. It is possible that the G-protein reserve on the nerve terminals of control arteries is large and that PTX treatment can not reduce this pool below a threshold required to reduce α_2AR mediated prejunctional inhibition of ATP release. DOCA-salt hypertension is associated with impairment of α_2AR function and this might be due to a reduction in the concentration of function of G-proteins linked to α_2ARs. PTX treatment of DOCA-salt arteries can reduce the available G-proteins below a critical threshold needed for UK 14,304-mediated inhibition of EJPs.

Increased Ca^{2+} sensitivity was also tested as a mechanism for impaired purinergic neurotransmission. Facilitation of EJPs in the presence of yohimbine is impaired in DOCA-salt hypertension (Chapter 3). Studies of autonomic neurotransmission have shown the importance of Ca^{2+} in EJP facilitation in the densely innervated vas deferens *in vitro* (Brain and Bennett, 1997; Hardy and Brock, 2001). In the studies presented here, EJP amplitude and facilitation were assessed in response to 0.5 and 1.0 Hz trains of nerve stimulation with various $[Ca^{2+}]_e$. The results of these studies confirmed that EJPs are sensitive to $[Ca^{2+}]_e$, but that Ca^{2+} sensitivity of the sympathetic nerve terminals is not altered in DOCA-salt rats. Altered Ca^{2+} handling has been studied in other synapses where neurotransmission is decreased due to presynaptic changes. For example H_2O_2 causes a decrease in the population spike in response to nerve stimulation in

hippocampal cells via mechanisms independent of Ca^{2+} entry (Avshalumov et al., 2000).

ATP handling in sympathetic nerve terminals of DOCA-salt rats is altered. I suggest that there are 3 possible explanations remain: 1) ATP is in the nerve terminal, but its storage in vesicles is not regulated effectively, 2) mobilization of ATP-containing vesicles is impaired, or 3) there is less ATP available resulting in greater fluctuations in EJPs. To study these possibilities, pharamacological tools were used to try and deplete vesicular stores of ATP. ATPases pump H^+ into the vesicle, acidifying it, and creating an electrochemical gradient which favors neurotransmitter influx into the vesicle (Bowman and Bowman, 2005). Bafilomycin A1 is a vacuolar ATPase (vATPase) inhibitor, which has been shown to reduce ACh (Cordeiro et al., 2006) and glutamate release from central synapses (Nishizaki, 2004). However it did not reduce EJP amplitudes or facilitation in either DOCA-salt or control animals after incubations up to two hours. This suggests that ATP storage into vesicles is not dependent on Bafilomycin A1-sensitive vATPases. It is also possible that the treatment was not effective. However, over time, EJP amplitudes decay, and therefore incubation times longer than 120 m were not feasible. Handling of ATP was also assessed with guanethidine. Guanethidine displaces NE from sympathetic varicosities, and also blocks sympathetic adrenergic neurotransmission by activating potassium channels in the neural membrane (Fabiani and Story, 1996). Guanethidine inhibited ATP release from sympathetic nerves from control and DOCA-salt animals equally well, however the Hill-slopes between the two groups were significantly different. This suggests that cooperativity of mechanisms which

store neurotransmitter is reduced. Because guanethidine gets into the nerve terminal via norepinephrine transporter (NET), changes in NET in hypertension may be altered. The shallow concentration response curve for guanethidine could be due to reduced transport of guanethidine into the nerve terminal and therefore there is a lower guanethidine concentration inside the nerve terminal to bind to the synaptic vesicle.

Finally, experiments using high trains of nerve stimulation may result in rundown due to decreased ATP bioavailability in one or more of the ATP-containing vesicle stores in terminals in DOCA-salt hypertensive animals. With increased neurotransmission, increased vesicle recycling and calcium dependence (Masuyama et al., 1984; Tsuda et al., 1986) ATP stores may be depleted. Additionally, increased reactive oxygen species in sympathetic nerves (Campese et al., 2004; Cao et al., 2007) could also disrupt mitochondria and deplete ATP stores (Puddu et al., 2007). Therefore, the variability in purinergic neurotransmission seen with hypertension may be early signs of neuronal damage as mitochondrial synthesis of ATP is disrupted and decreased availability follows. This conclusion may also explain the increased variability of EJP amplitudes between rats.

Together these data indicate that the purinergic component of sympathetic neuroeffector transmission in mesenteric arteries is disrupted in DOCA-salt hypertension. *In vivo* blockade of P2X receptors with PPADS does little to MAP and HR mean values. However previous studies have shown increase variability of MAP during P2X receptor blockade, suggesting that ATP functions in an immediate and transient way to maintain blood pressure homeostasis (Tarasova et

al., 1998; Golubinskaya et al., 1999a; Golubinskaya et al., 1999b). Changes in purinergic neurotransmission are involved on other disease processes as well. For example, diabetic neuropathy results in erectile dysfunction in part due to impaired purinergic neurotransmission. In this case, decreased ATP release acting at $P2Y_4$ receptors on cavernous smooth muscle results in impaired relaxation (Calvert et al., 2008). Characterization of purinergic neurotransmission to the vasculature and its alterations in disease processes highlight novel therapeutic targets for the treatment of several diseases including hypertension.

Chapter 5

APOCYNIN LOWERS BLOOD PRESSURE AND RESTORES α_2-ADRENERGIC AUTORECEPTOR FUNCTION IN SYMPATHETIC NERVES SUPPLYING MESENTERIC ARTERIES OF DOCA-SALT HYPERTENSIVE RATS.

Abstract

Hypertension is associated with elevated vascular O_2^- and increased vascular tone. Increased sympathetic nerve activity, impaired function of prejunctional α_2-adrenergic autoreceptors, increased norepinephrine (NE) release and depleted vesiclular stores of ATP occur in DOCA-salt hypertensive rats. However, the relationship between O_2^-, sympathetic neurotransmission and blood pressure regulation has not been investigated. The aim of these studies was to 1) determine if subunits for the O_2^- generating enzyme, NADPH oxidase are expressed by periarterial nerves and 2) determine if chronic antioxidant treatment can lower blood pressure and normalize sympathetic neurotransmission to mesenteric arteries. DOCA-salt rats were treated for four weeks with tempol (1 mmol/L) or apocynin (1 or 2 mmol/L) in 1% NaCl drinking solution. Telemetric pressure transducers fixed to femoral arterial catheters were surgically implanted in the rats. Apocynin and tempol lowered blood pressure compared to untreated DOCA-salt rats. Antioxidants reduced O_2^- measured using dihydroethidium (DHE) fluorescence in sympathetic ganglia. Depressor responses caused by the ganglion blocking drug, hexamethonium, were reduced in apocynin and tempol-treated rats. Sympathetic neurotransmission to mesenteric arteries was studied *in vitro* using intracellular microelectrodes to record purinergic excitatory junction potentials (EJPs) and amperometry to measure norepinephrine release. EJP amplitude was highly variable and α_2-autoreceptor function was impaired in DOCA-salt rats; antioxidant treatment restored purinergic and adrenergic neurotransmission. These data indicate that O_2^- disrupts sympathetic

neurotransmission to mesenteric arteries in DOCA-salt rats by impairing neuronal stores of ATP and α_2-autoreceptor function. Sympathetic nerves are a target for the deleterious effects of O_2^- in hypertension.

Introduction

Reactive oxygen species (ROS) are important signaling molecules affecting vascular tone in health and disease (Griendling et al., 2000b; Griendling et al., 2000a). Superoxide anion (O_2-), one of several ROS, is produced through the donation of one electron to molecular oxygen (O_2) which occurs through various mechanisms including the mitochondrial electron transport chain, uncoupled nitric oxide synthase (NOS), xanthine oxidase and reduced nicotinamide-adenine dinucleotide phosphate (NAPDH) oxidase. $O_2^{\cdot}$ is converted to hydrogen peroxide (H_2O_2) either spontaneously or by superoxide dismutase (SOD). H_2O_2 is more stable than $O_2^{\cdot}$ and is able to cross cell membranes where it can modulate several intracellular pathways (Avshalumov et al., 2000; Tabet et al., 2004; Thakali et al., 2006). Catalase converts H_20_2 to water (H_2O) and O_2. A delicate balance of pro- and anti-oxidant mechanisms exists to maintain homeostasis.

Although first discovered in neutrophils, NADPH oxidase is a significant source of ROS in many cell types including smooth muscle cells (SMC)(Westenbroek et al., 1995; Zugck et al., 2003) endothelial cells (Ago et al., 2005) and fibroblasts (Jones et al., 1994). NADPH oxidase is localized to neurons and astrocytes in the solitary tract nucleus (NTS), an area of the brain important in autonomic function (Glass et al., 2006), sympathetic and sensory neurons (Tammariello et al., 2000; Cao et al., 2007) cortical neurons and astrocytes (Noh and Koh, 2000).

Increased ROS production and increased NADPH activity is associated with many cardiovascular diseases, including hypertension (Westenbroek et al., 1995; Zugck et al., 2003), diabetes (Gunes et al., 2005) and senescence (Chakravarti and Chakravarti, 2007). Increased ROS occurs in experimental models of hypertension (Kerr et al., 1999; Schnackenberg and Wilcox, 1999; Somers et al., 2000; Beswick et al., 2001a);(Somers et al., 2000; Virdis et al., 2004; Zhang et al., 2005; Pech et al., 2006) and in human hypertension (Touyz and Schiffrin, 2001). NADPH oxidase-derived ROS in particular plays a role in the pathogenesis of several animal models of hypertension (Li et al., 2003; Zhang et al., 2005; Girouard et al., 2006) (Zugck et al., 2003).

A large amount of literature has reported that the pathogenic role of oxidative stress lies in altered in vascular and endothelial signaling (Griendling et al., 2000b; Griendling et al., 2000a). For example, ROS generation in smooth muscle cells is associated with hypertrophy of arterial smooth muscle through mitogen-activated phosphorylation (MAP) kinase pathways (Griendling et al., 2000b).

The detrimental effects of ROS have been attributed to their ability to decrease nitric oxide (NO) availability, endothelial nitric oxide synthase (eNOS) activity or endothelial function (Banday et al., 2007). Additionally, the beneficial effects of antioxidants have been attributed restoring NO. However, other studies have found that antioxidants, such as tempol, have blood pressure lowering effects that were independent of NO (Xu et al., 2002, 2004). Therefore ROS may have a direct effect on proteins which would result in increased blood pressure,

separate from their effects on NO. However, increased sympathetic nerve activity also plays an important role in modulating vascular tone in hypertension. Sympathetic nerves innervating the splanchnic circulation play a critical role in regulating blood pressure (King et al., 2007). Campese et al. showed a connection between increased ROS and increased central and peripheral sympathetic nervous system activity in hypertension. However, the location of NADPH oxidase was not specified (Campese et al., 2004), and a neuronal ROS component was not identified. ROS has also been implicated in increasing renal nerve activity in hypertension (Nishiyama et al., 2001). Few studies have looked at the effects of oxidative stress on the function of post-ganglionic sympathetic nerves supplying arteries (Ma et al., 2006; Cao et al., 2007). Furthermore, there have been no studies of the localization of NADPH oxidase subunits to sympathetic nerve endings.

NADPH oxidase is multi-subunit enzyme containing an integral membrane protein (flavocytochrome $b_{558;}$ consisting of $gp91^{phox}$ and $p22^{phox}$) and four cytosolic protein components ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and a small GTP-binding protein (Rac) . However the composition of the subunits vary depending on the cell type (Zalba et al., 2005). In SMCs and epithelial cells Nox1, a homologue of $gp91^{phox}$ is found (Lassegue and Clempus, 2003). In the superior cervical ganglia of neonatal rats NOX2/$gp91^{phox}$ and $p22^{phox}$ were localized to the membrane and $p47^{phox}$and $p67^{phox}$ were expressed intracellularly (Hilburger et al., 2005). Expression of these subunits and activity of NADPH oxidase is increased in sympathetic ganglia in DOCA-salt hypertension (Cao et al., 2007).

However surprisingly few studies have looked at post-ganglionic sympathetic nerves which resistance vessels (Ma et al., 2006; Cao et al., 2007), and no studies have reported localization of NADPH oxidase subunits to sympathetic nerve endings.

Therefore, the first aim of these studies was to conduct a series of immunohistochemical experiments to localize membrane-bound $p22^{phox}$ and cytosolic $p47^{phox}$ subunits to mesenteric periarterial nerve fibers. Sympathetic nerve fibers course within the same axon bundle as sensory nerves to reach the peripheral blood vessels and organs. Therefore distinguishing between the two is difficult. The close proximity of the sensory and sympathetic fibers may allow for signaling between them as is seen in the rat ear artery (Maynard et al., 1990). We therefore co-localized NADPH oxidase subunits with markers for both sympathetic and sensory fibers. Whole-mount mesenteric arteries containing perivascular nerve fibers and endings were isolated from rats and incubated with NADPH oxidase subunit antibodies together with markers for sympathetic and sensory nerves. We hypothesized that NADPH oxidase would localize to peri-arterial sympathetic and sensory nerve fibers and endings.

In many cases the detrimental effects of ROS have been attributed to their ability to decrease nitric oxide (NO) availability, endothelial nitric oxide synthase (eNOS) activity or endothelial function (Banday et al., 2007). Additionally, the beneficial effects of antioxidants have been attributed to the restoration of NO. However, other studies have found that antioxidants, such as tempol, have blood pressure lowering effects that were independent of NO (Xu et al., 2002, 2004).

Therefore ROS may have a direct effect on proteins which would result in increased blood pressure, separate from their effects on NO.

Norepinephrine (NE) and ATP are released from periarterial sympathetic nerves (Donoso et al., 1997). NE binds to α_1ARs, which act through G-protein coupled receptors to induce constriction in arterial SMCs. In DOCA-salt hypertension NE release and clearance are impaired (Moreau et al., 1995; Somers et al., 2000; Luo et al., 2003; Luo et al., 2004). Increased NE release from sympathetic nerves is due in part to impairment of the function of prejunctional α_2ARs (Moreau et al., 1995; Luo et al., 2004). ATP, released with NE at the neuroeffector junction, binds to ligand-gated $P2X_1$ receptors on arterial SMCs, which results in a transient depolarization in the membrane potential, called an excitatory junction potential (EJP). ATP handling is impaired in DOCA-salt hypertension via unknown mechanisms. Therefore the second aim of these studies was to study the effects of ROS on sympathetic neurotransmission to mesenteric arteries in DOCA-salt hypertension.

In human hypertension (Kumar and Das, 1993) and a model of hypertension induced by chronic angiotensin II (Ang II) infusion, there is increased production which has been attributed to increased circulating levels of Ang II (Nishiyama et al., 2001). The deoxycorticosterone acetate (DOCA)-salt model is a salt-dependent model of hypertension with a suppressed renin-angiotensin system (RAS) (Gavras et al., 1975). Importantly, despite low levels of renin, the DOCA model also has increased ROS (Somers et al., 2000; Beswick

et al., 2001b; Dai et al., 2004) and can therefore be used to study ROS independent of RAS.

We hypothesized that alterations in neuroeffector transmission to mesenteric arteries in DOCA-salt hypertension are in part do to detrimental effects of ROS on prejunctional storage, release and regulatory mechanisms of purinergic and noradrenergic neurotransmission. We used chronic treatment of DOCA-salt hypertensive rats with the superoxide dismutase mimetic, tempol and the NADPH oxidase inhibitor, apocynin to test the hypothesis that reductions in oxidative stress could normalize sympathetic neuroeffctor transmission to mesenteric arteries. To test this hypothesis, EJP amplitude and facilitation in response to low and high frequency trains of nerve stimulation was used as an indirect measurement of ATP release. Microelectrodes coupled to electroanalytical techniques were used to measure NE release from sympathetic nerve terminals. Pressure transducers were implanted for four weeks *in vivo* to compare hemodynamic measurements between DOCA-salt (control), DOCA-salt + apocynin (2mmol/L), and DOCA-salt + tempol (1mmol/L) treatment groups. Inferior mesenteric ganglia were labeled with dihydroethidium, *in vitro*, to measure O_2^- levels after antioxidant treatment.

Methods

Animals

All experiments were done using Sprague-Dawley rats from Charles River Laboratories (Portage, MI). Upon arrival at the animal care facility, animals were maintained according to standards approved by the Michigan State University All-University Committee on Animal Use and Care. All experimental procedures were carried out in accordance with the "Guiding Principles in the Care and Use of Animals" of the American Physiological Society. Rats were acclimated for 2-3 days before entry into any experimental protocol. Pelleted rat chow (Harlan/Teklad 8640 Rodent Diet) and distilled water were provided ad libitum. Rats were housed in temperature- and humidity- controlled rooms with a 12:12-h light-dark cycle.

Surgical procedures

Capsaisin-treated (cap-tx) rats: Briefly, on days 1 and 2 of life, neonatal Wistar rats received capsaicin (50 mg/kg) SC as described previously (Wang et al., 1998). Control rats were treated with equal volumes of vehicle solution (5% ethanol, 5% Tween 80 in saline). All treatments were performed with rats under ether anesthesia (Wang and Li, 1999).

Celiac ganglionectomized (CGx) animals: CGx was performed by locating the celiac plexus in between the aorta, celiac artery, and cranial mesenteric artery; dissecting it free from surrounding tissue; and removing it. Any additional nerves

along these vessels in the area of the celiac ganglion were also dissected free and transected (King et al., 2007). Treatments were performed in anesthetized rats.

DOCA-salt rat preparation and telemetry implantation: 35 male Sprague-Dawley rats (250-275g) were anesthetized with 3% Isoflurane in oxygen delivered by nose cone. The skin over the left lateral abdominal wall was shaved and prepared with Chlorhexadine. A 1.5-cm vertical 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, vein, and ureter with 6-0 silk sutures and the incision was closed with 4-0 monofilament nylon sutures. Rats were instrumented with a radiotelemetry transmitter (TA11PAC40, Data Sciences International) to measure arterial pressure via an incision near the left inguinal region. A catheter was inserted into the left femoral artery. Surgery was performed on a heated pad, and rats recovered in a heated box. After recovery, rats were housed singly under standard conditions for four weeks. Rats received standard pelleted rat chow and salt water (1% NaCl + 0.2% KCl) ad libitum. Seven days after uninephrectomy and telemetry implantation, rats were briefly anesthetized. A 3 x 1.5-cm rectangular area between the shoulder blades of the back was shaved and disinfected for subcutaneous implantation of a Silastic (Dow Corning) sheet containing DOCA-salt (Sigma; 150mg/kg) pellet under a 1.5-cm incision. The skin was closed with 4-0 nylon sutures.

Immunohistochemistry

At the end of the study, rats were euthanized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The mesentery was surgically removed and maintained in 0.1M phosphate-buffered saline (PBS). Mesenteric arteries were cleaned of adipose and connective tissue and cleared of blood via an intravascular PBS bolus. Tertiary branches were excised and isolated tissues were placed in Zamboni fixative (2% [vol/vol] formaldehyde and 0.2% [vol/vol] picric acid in 0.1M phosphate buffered saline, PBS) overnight (4 °C). The next day, the tissues were washed 3x with 0.1M PBS and then incubated in PBS with blocking serum (donkey) diluted in triton-X100 (1.0 %) for 1 hour. Tissues were then co-incubated for 2 hours at 37 °C in diluted primary antibodies (in triton-PBS) raised against $p22^{phox}$ or $p47^{phox}$ together with a marker for either sympathetic or sensory nerve fibers (anti-tyrosine hydroxylase (TH), neuropeptide Y (NPY) or calcitonin gene related peptide (CGRP)). For sources, host pieces and dilutions of primary antibodies refer to Table 1. Next, tissues were washed 3x in 0.1M PBS buffer and then incubated for 1 hour in a dark, humidified chamber at room temperature in diluted fluorescene isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (1:40) or Cy3-conjugated donkey anti-rabbit IgG (1:300) (Jackson ImmunoResearch Laboratories, Inc.). For host species and dilutions of secondary antibodies refer to Table 2.Vessels were then washed 3x with 0.1 M PBS at 5-minute intervals and coverslipped with Prolong Gold anti-fade reagent (Molecular Probes (Invitrogen) Eugene, OR). Tissues were examined using either a Leica TSL laser confocal microscope (Leica Microsystems Inc., Bannockburn, IL). Excitation wavelength of 488 was used to elicit FITC staining and 543 to elicit

Cy3. Some of the images (specified in text) were taken using a conventional fluorescence microscope (Nikon TE2000-U inverted microscope). Images were taken using a SPOT Insight Color Mosaic Camera (Mager Scientific, Inc.) with Metaimaging software.

Intracellular electrophysiological recording

Four weeks after the start of DOCA-salt treatment, rats were euthanized with an i.p.injection of sodium pentobarbital (50 mg/kg). The mesentery was then surgically removed. Tertiary branches of mesenteric arteries were dissected out, cleaned of adipose and connective tissue and pinned taut using stainless steel pins (50 μm diameter) in a perfusion chamber coated with Sylgard® (Dow Corning, Midland, MI). Tissues were superfused with Krebs' buffer solution of the following composition (mM): NaCl, 117; KCl, 4.7; CaCl2, 2.5; MgCl2, 1.2; NaHCO3, 25; NaH2PO4, 1.2; dextrose, 11. Nifedipine (L-type VDCC antagonist, 1 μM) and prazosin (α_1-AR antagonist, 0.1μM) were added to the buffer to inhibit vessel constriction. The buffer was heated to 37°C and bubbled with 95% O_2 and 5% CO_2 and the tissue was allowed to equilibrate for 30 minutes prior to the start of an experiment.

Intracellular recordings from individual smooth muscle cells were carried out using glass microelectrodes (100-200 MΩ resistance) filled with 2M KCl. An amplifier (IX2-700 dual intracellular preamp, Dagan Inc, Minneapolis, MN) was used to record membrane potential in the current clamp mode. Signals were filtered using a HumBug 50/60 Hz Noise Eliminator (Digitimer Research

Instruments, Quest Scientific, North Vancouver, BC, Canada). Impalements were accepted if the resting membrane potential dropped quickly to more that -50 mV. EJPs were evoked using a Krebs' solution-filled, bipolar, focal stimulating electrode containing two parallel silver/silver chloride wire electrodes connected to a grass Instruments stimulator (S88, Quincy, MA). The stimulating electrode was positioned perpendicular to the tissue and directly across from the recording electrode. For most experiments, a stimulation frequency of either 0.5 Hz or 10 Hz, 0.5 ms pulse width at the lowest voltage which produced a maximal amplitude was used. The signal was recorded on an oscilloscope (Gould 20ms/sec Digital Recording Oscilloscope). A digital average of five sweeps was used to measure the amplitude of EJPs under control and treatment conditions unless otherwise noted. The data was digitized using a Digidata 1200 analog/digital converter and were acquired using Axoscope 9.0 software (Axon Instruments, Foster City, CA). Data were analyzed offline using Clampfit 9.0 software (Axon Instruments) and a laptop computer.

Dihydroethidium (DHE) staining

Inferior mesenteric ganglia (IMG) were surgically removed from euthanized rats in chilled Krebs-Ringers-HEPES (KRH) solution or the following composition (mM): NaCl 130, KCl 1.3, Ca_2Cl_2 2.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, HEPES 1.0, glucose 0.09 (pH = 7.4). Freshly dissected tissues were allowed to sit at room temperature before incubation with 2 μM DHE solution in a light-protected tube at 37° C for 60 min. Following DHE incubation, IMGs were

washed 3x with KRH solution and mounted with Fluoromount G (Southern Biotechnology Associates) mounting medium. Confocal fluorescent images were were obtained (543 nm excitation; wavelengths > 560 were collected; Zeiss, LSM 5 Pascal). Neurons were counted and fluorence intensity in each sample was measured offline using Image-Pro Plus 2.

Amperometric measurement of NE

NE release evoked by focal electrical stimulation was measured using continuous amperometry with carbon fiber microlectrodes (Park et al., 2007). Prior to use carbon fiber microelectrodes were rinsed with distilled isopropyl alcohol (IPA; Ranganathan *et al.* 1999). The microelectrode was then affixed to a micromanipulator (MP-1, Narishige Instruments, Japan), and lowered to a parallel position with regards to the vessel. The electrode was pressed gently against the artery so that the electrode maintained contact with the artery during stimulation evoked constrictions. A platinum wire counter and a commercial 'no leak' Ag–AgCl (3 M KCl, model EE009, Cypress Systems Inc., USA) reference electrode were also mounted in the bath to complete the electrochemical cell. Amperometric measurements were made with an Omni 90 analog potentiostat (Cypress Systems Inc.), a mini digi analog-to-digital converter (Labmaster 125) and a computer running Axoscope 9.0 (Axon Instruments, Union City, CA, USA). Data were acquired at a sampling rate of 1 kHz. An applied potential of 500 mV was used to detect NE current. This voltage allows for NE to be oxidized at a mass-transfer limited rate, but assures adenosine, also electroactive, will not be

oxidized. Current recordings were low-pass filtered at a time constant of 200 ms (5 Hz) before being digitized using an A/D converter at a sampling rate of 50 Hz. The data were then stored on a computer for off line analysis.

Drugs

All chemicals were purchased from Sigma-Aldrich (St Louis, MO), except apocynin (Calbiochem).

Statistics

Blood-pressure and heart rate data were analyzed with a two-way ANOVA. Other measures were compared with a one-way ANOVA with Bonferroni's post hoc test for the DHE staining and Neuman-Kewls for the electrophysiology and electrochemistry studies. $P < 0.05$ was considered significant. GraphPad Prism and Minitab software were used for statistical analysis.

Results

NADPH oxidase subunits, $p47^{phox}$ and $p22^{phox}$ co-localize to sympathetic periarterial nerve fibers.

In order to determine if NADPH oxidase subunits are localized to sympathetic nerve endings, tertiary branches of mesenteric arteries were fixed and labeled with an antibody raised against NPY as a marker for sympathetic perivascular nerves (Lundberg et al., 1983), and either NADPH oxidase subunit: anti-$p22^{phox}$ or anti-$p47^{phox}$. The top rows in Figs. 17 and 18 are representative images of low magnification showing periarterial nerves (Park et al., 2007). The bottom row of Figs. 17 and 18 show representative high magnification images focused on a single peri-vascular nerve fiber. The images show an example of NPY and either p47 or $p22^{phox}$ in the same fibers. $P47^{phox}$ (red; fig. 17 A,D), is found in the same nerve fibers as NPY (green; fig. 17 B,E) although the localization of the staining within the nerve fiber is variable. NPY staining is varicose while $p47^{phox}$ is present throughout the nerve fibers. The merged images (fig. 17 C,F) show that $p47^{phox}$ and NPY are found in the same nerve fiber bundles (yellow). Immunostaining for $p22^{phox}$ (red; fig. 18 A,D), is also found in some, but not all, of the same nerve fibers as NPY (green; fig. 18 B,E), suggesting that there are perivascular nerves which do not contain $p22^{phox}$. The merged images (fig. 18 C,F) show that $p22^{phox}$ is found in some, but not all NPY-containing nerve fibers. This is different from vascular cells, where $p22^{phox}$ is a critical component of NADPH oxidase derived O_2^- (Ushio-Fukai et al., 1996), and suggests that

subunits in nerve fibers may be different from those in vascular and/or endothelial cells.

Tyrosine hydroxylase (TH), the rate limiting enzyme in NE synthesis, is another marker for sympathetic nerves. Figure 19 shows NADPH oxidase subunit immunoreactivity in TH-containing nerves. $P47^{phox}$ (fig. 19B) and $p22^{phox}$ (Fig. 19E) immunoreactivity is found in sympathetic nerve bundles with TH (fig. 19C,F), showing similar patterns as NPY-containing nerves discussed above.

NAPDH oxidase subunits $p47^{phox}$ and $p22^{phox}$ are in perivascular sensory nerve fibers.

To determine if NADPH oxidase subunits are located in peri-arterial sensory nerves, tertiary mesenteric arteries were fixed and labeled with anti-calcitonin gene-related peptide (CGRP), a marker for sensory nerves, and either anti-$p47^{phox}$ or anti-$p22^{phox}$. Top rows in figs 20 and 21 are representative images of CGRP immunostaining at low magnification showing the pattern of sensory nerves in mesenteric arteries (Westenbroek et al., 1995; Zugck et al., 2003). Bottom rows in figs. 20 and 21 are higher power magnifications which focus on a single peri-vascular nerve bundle showing examples of CGRP and either p22 or $p47^{phox}$ in the same fibers. $p47^{phox}$ immunostaining is shown in red (fig. 20A, D), and CGRP immunostaining is shown in green (fig. 20B,E). The merged images (fig 20C, F) suggest that $p47^{phox}$ co-localizes to afferent sensory nerve fibers (yellow), and merged images (fig 21C,F) show $p22^{phox}$ also co-localizes with sensory nerve fibers (yellow). The $p47^{phox+}$ and $p22^{phox+}$ fibers, which do not co-

localize with CGRP are most likely located in either sympathetic or intestinofugal fibers.

Perivascular NADPH oxidase immunostaining remains after capsaicin treatment (cap-tx).

Sensory and sympathetic nerve fibers are present in the same para- and perivascular nerve bundles. Therefore, when using immunohistochemical methods and light microscopy it is difficult to definitively determine if proteins are contained in different or closely aligned nerve fibers. Therefore, tissues obtained from rats that had been treated neonatally with capsaicin in order to destroy periarterial sensory nerve fibers were used (Wang and Li, 1999). This enables an evaluation of the localization of NADPH oxidase subunits to sympathetic nerves in tissues when sensory nerves had been destroyed. $P47^{phox}$ and CGRP were localized in small mesenteric arteries removed from cap-tx animals using $p47^{phox}$ and CGRP antibodies. Low (fig. 22A) and high (fig. 22B) magnification images show peri-arterial nerve fibers co-labeled with anti-$p47^{phox}$ and anti-CGRP in control animals. The absence of anti-CGRP labeling in cap-tx rats indicates that treatment was effective in depleting sensory nerve fibers (fig. 22C). However, after depletion of sensory nerves $p47^{phox}$ immunostaining remains (fig. 22D). Figure 22E shows an overlay of these two images suggesting that $p47^{phox}$ exists in non-sensory peri-arterial nerve fibers.

Celiac ganglionectomy (CGx) reduces peri-arterial $p47^{phox}$ immunostaining.

Most sympathetic peri-arterial nerve fibers supplying mesenteric arteries originate in the celiac ganglia (CG) and afferent sensory nerves pass through the CG on their way to the dorsal root ganglia (Hsieh et al., 2000). However, there is also a population of perivascular nerve fibers that originate from nerve cell bodies in the myenteric plexus with the gut wall. These intestinofugal fibers project from the gut wall to the celiac ganglion and they contribute to regulation of gastrointestinal motor reflexes. Therefore, CGx was performed. CGx would not sever the axons of intestinofugal nerve fibers and this procedure would verify that nerve fibers described above were sympathetic or primary afferent nerve fibers originating in dorsal root ganglia. Two weeks following CGx, rats were sacrificed. Mesenteric vessels were removed, fixed and incubated with anti-tyrosine hydroxylase (TH) and anti-$p47^{phox}$. After CGx, few superficial $p47^{phox+}$ fibers remain (fig. 23A) and TH staining has been abolished (fig. 23B). This indicates that sympathetic nerve fibers have been successfully destroyed after CGx. It is possible that the superficially located $p47^{phox}$ staining nerve fibers which remain are either sensory fibers which were not abolished during the surgery, or intestinofugal fibers (Luckensmeyer and Keast, 1995).

In vitro apocynin treatment does not alter purinergic neurotransmission arteries from DOCA-salt rats.

Apocynin (0.1 mM) was added to the physiological buffer superfusing tissues *in vitro* to assess the role of ROS on impaired facilitation seen in DOCA-salt rats compared to normotensive controls (chapter 3). Figure 24A shows acute apocynin treatment was unable to increase % facilitation in DOCA-salt rats in response to 0.5 Hz nerve stimulation ($P > 0.05$). The effectiveness of antioxidant treatment on sympathetic nerves was assessed by measuring DHE intensity, a marker for O_2^-, in inferior mesenteric ganglia (IMG) *in vitro*. When DHE reacts with O_2^-, a planar molecule is formed. This intercalates with strands of DNA and fluoresces red. Because the perivascular nerve fibers are removed from their cell bodies, DHE fluorescence was assessed in the arterial SMCs (fig. 24B). No differences were seen between apocynin and control treated tissues. It is possible that the acute *in vitro* treatment protocol was inadequate to reduce O_2^- in the mesenteric arteries. It is also possible that elevated O_2^- in DOCA-salt rats produces long-lasting changes in the mechanisms controlling purinergic neurotransmission and acute apocynin treatment can not reduce these longer lasting changes. Therefore, an *in vivo* antioxidant treatment protocol was tested.

Chronic antioxidant treatment lowers blood pressure in DOCA-salt rats.

DOCA-salt rats were split into three groups: DOCA-salt (N=13), apocynin-treated (apocynin; N=8) and tempol-treated (tempol; N=9) (Fig. 25). Each week, fluid intake/24 hours and body weight were measured. There was a significant interaction between treatment and fluid intake over the course of the

study. However at each individual time point there was no difference in the fluid intake between treatment groups (interaction: $P < 0.05$; fig. 26A). As a measure of the health of the animal, body weights were assessed each week, and before being euthanized (fig. 26B) Body weight (g) was greater in apocynin and tempol treated rats compared to untreated DOCA-salt rats at the end of the study suggesting that antioxidant treatment improved the overall health of DOCA-salt rats.

Blood pressure was monitored continuously for 2 control (C) and 24 treatment (T) days (figs. 27A-C). Blood pressure was unchanged during the control period (fig. 27A-C; C1-2). Mean arterial pressure (MAP, fig. 27A), systolic blood pressure (SBP, fig. 27B) and diastolic blood pressure (DBP, fig. 27C) increased over experiment days (T1-24). SBP, DBP and MAP were lowered by apocynin and tempol. Heart rates (HR) declined throughout the experiment, but were not different between treatment groups (HR; 389 ± 6, 390 ± 8 and 377 ± 6 (day 3) to 346 ± 9, 353 ± 11 and 343 ± 6 (day 26) for control, apocynin and tempol groups respectively).

In vivo antioxidant treatment lowers O_2^- and sympathetic tone in DOCA-salt rats

Representative photomicrographs of DHE staining in IMG from control, apocynin- and tempol-treated rats show decreased O_2^- in treated animals compared to control (Fig. 28A). Average intensity of fluorescence was measured in individual neurons from each animal and this average value was used as a

measure of O_2^- in the inferior mesenteric ganglia (IMG) of each animal. N values refer to the number of rats from which DHE measurements were made. DHE staining was reduced by about 50% in tempol- and apocynin-treated compared to untreated DOCA-salt rats ($P < 0.05$).

It is well established that sympathetic drive to the vasculature is increased in DOCA-salt hypertension (de Champlain et al., 1987; Fink et al., 2000). Therefore, we used the fall in blood pressure caused by ganglion blockade with hexamethonium (1mg/kg, i.p., a nicotinic acetylcholine receptor antagonist) as a measure of sympathetic tone in treated and untreated DOCA-salt rats (fig. 28C) (Takata et al., 1988). Hexamethonium caused a fall in MAP, which peaked at 10 minutes after the injection, and returned to baseline values in about one hour. The peak drop in MAP was attenuated in apocynin and tempol-treated rats compared to that in untreated DOCA-salt rats after hexamethonium injection. A lower dose of apocynin (1 mM), also attenuated the peak drop in MAP caused by hexamethonium, but this was not significantly different from control. The drop in MAP in response to hexamethonium treatment was greater than the absolute difference in blood pressure on D23, so the difference in the MAP baseline does not account for the differences (D23 MAP = DOCA-salt 165 ± 7 mmHg; apocynin: 147 ± 8 mmHg and 162 ± 12 mmHg). These data suggest that sympathetic tone was reduced in apocynin and tempol treated DOCA-salt rats perhaps accounting in part for the blood pressure lowering effects of these drugs.

Chronic antioxidant treatment improves purinergic neurotransmission in mesenteric arteries from DOCA-salt rats.

Previous studies have shown an increased adrenergic, but decreased purinergic neurotransmission to mesenteric arteries in DOCA-salt hypertension (Luo et al., 2004). These studies tested the hypothesis that oxidative stress was responsible for this neurochemical change in sympathetic neurotransmission. Intracellular recordings from smooth muscle cells were used to detect EJPs and the EJP amplitudes were compared between arteries obtained from antioxidant treated and untreated DOCA-salt rats. Fig. 29 shows that EJP amplitudes means were not different between treatment groups and DOCA-salt control rats. However, EJP amplitudes from untreated DOCA-salt rats were skewed, while EJP amplitudes were normally distributed in arteries from antioxidant treated rats. Furthermore, the variance of EJP amplitudes was highest in arteries from untreated DOCA-salt rats compared to groups which received antioxidant treatment

Low (0.5 Hz) frequency trains of nerve stimulation cause facilitation of EJP amplitudes and EJPs are sustained throughout the train of stimulation. High frequency stimulation causes facilitation followed by rundown of EJP amplitudes in mesenteric arteries from DOCA-salt rats. Yohimbine was also used to block prejunctional α_2 autoreceptors. Representative tracings from the three treatment groups are shown in figure 30A and B. Individual EJPs from the 1st and 5th stimulus are shown before and after yohimbine are also shown. The 5th EJP tended to be bigger in the antioxidant treated groups compared to DOCA-salt

controls. An index of α_2AR autoreceptor function was calculated (fig. 30C). The ratio of facilitation before and after yohimbine was greater in apocynin-treated rats compared to DOCA-salt controls. No difference in the α_2AR autoreceptor index was observed between tempol-treated rats and untreated DOCA-salt rats.

When mesenteric arteries from DOCA-salt rats are stimulated with using a 10 Hz train, EJPs facilitate within 3-5 pulses, and then EJPs decline in amplitude (rundown)(see Chapter 3). Treatment of rats with apocynin, but not tempol, decreased EJP rundown (Fig. 31A). The peak amplitude of facilitation was determined before and after treatment with yohimbine (1 μM). Before yohimbine treatment there was no difference in peak amplitude (DOCA-salt: 12 ± 2 mV; apocynin: 15 ± 1 mV, tempol: 15 ± 1 mV), but after yohimbine EJPs facilitated to a greater extent in apocynin-treated rats compared to DOCA-salt control (DOCA-salt: 11 ± 2 mV; apocynin: 17 ± 1 mV; tempol 14 ± 1 mV; *$P < 0.05$).

Recovery of EJP amplitude was tested at 2 s after the end of the stimulus train be evoking a single EJP and comparing the amplitude of that test EJP to the amplitude of the first EJP in the stimulus train (Fig. 31C). EJP amplitude was fully recovered by 2 s in all groups.

Antioxidant treatment reduces NE release and restores α_2AR function in DOCA-salt rats.

NE release from perivascular nerves innervating the splanchnic circulation is increased in tissues from DOCA-salt rats. The increase in NE release is due in part to impairment of the α_2ARs (Westfall et al., 1987; Tsuda et al., 1989; Luo et

al., 2004). Previous studies using amperometric recordings confirmed that these changes occur at the mesenteric arterial neuroeffector junction in DOCA-salt rats (Park et al., 2008). Representative traces of NE current are shown for apocynin-treated, tempol-treated and DOCA-salt control groups (fig. 32A, B). Fig. 31C shows a frequency dependent increase in peak NE currents (60 pulses). Peak currents were smaller in arteries from apocynin- and tempol-treated rats ($P < 0.05$). To examine α_2AR function, idazoxan (1 μM) was superfused over tissues for 30 minutes and frequency response curves were repeated. Fig. 32D shows that idazoxan treatment increased the peak NE current in arteries from apocynin- and tempol treated but not in arteries from untreated DOCA-salt rats and there was no difference among peak current amplitudes across treatment groups ($P>0.05$). The rate of rise (slope) of the NE oxidation current is a measure of NE release rate, which is in part determined by α_2AR function. At 20 Hz, the rate of rise of the NE current was slower in arteries from apocynin- and tempol-treated rats that in arteries from untreated DOCA-salt rats (Fig. 32E). This difference was lost after blocking the α_2AR with idazoxan (1 μM; 32F). Together these data support a role for oxidative stress in the impairment of the α_2AR and subsequent increase in NE release and rate of release seen in DOCA-salt hypertension. Peak current at 10 Hz is normally distributed and variances are equal between the three groups examined (Fig. 33).

Table 3: Primary and secondary antibodies used for co-localization of NADPH oxidase subunits with sensory and sympathetic nerve fibers

Primary antibodies			
Antigen	**Host species**	**Dilution**	**Source**
TH^	Mouse	1:150	Calbiochem, La Jolla, CA
NPY	Goat	1:300	Santa Cruz Biotech., Inc., Santa Cruz, CA
CGRP	Sheep	1:1000	Abcam Inc., Cambridge, MA
p47phox	Rabbit	1:300	Mark T. Quinn, Montana St. Univ.
p22phox^	Mouse	1:1000	Mark T. Quinn, Montana St. Univ.
P22phox	Rabbit	1:300	Mark T. Quinn, Montana St. Univ.
Secondary antibodies			
Target species	**Host species**	**Dilution**	**Source**
Mouse	Donkey (FITC)	1:40	Jackson ImmunoResearch Lab., Inc., West Grove, PA
Rabbit	Donkey (Cy3)	1:200	Jackson ImmunoResearch Lab., Inc.
Goat	Donkey (FITC)	1:40	Jackson ImmunoResearch Lab., Inc.
Mouse	Donkey (Cy3)	1:200	Jackson ImmunoResearch Lab., Inc.

TH = tyrosine hydroxylase, NPY = Neuropeptide Y, CGRP = calcitonin gene-related peptide; ^ = monoclonal.

Fig. 17

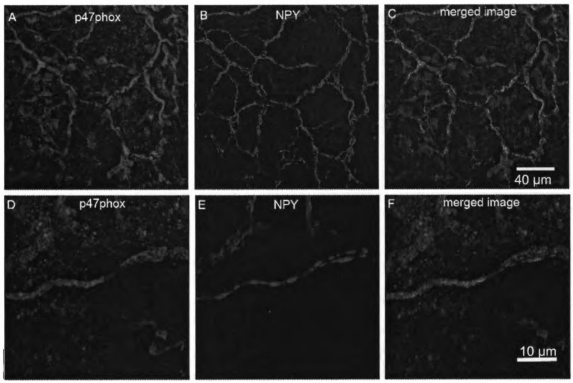


Fig. 17. Co-localization of immunoreactivity for $p47^{phox}$ and NPY in periarterial nerves. Periarterial nerve fibers were co-labeled with an antibody against NPY, a marker for sympathetic nerves, and $p47^{phox}$, a cytosolic subunit of NADPH oxidase. (A) $p47^{phox}$, (B) NPY, (C) merged image. Higher magnification images of $p47^{phox}$ (D), NPY (E) and the merged image (F) revealing close apposition of the two antigens in a subset of nerve fibers. Scale bar in (C) applies to (A,B) and scale bar in (F) applies to (D,E).

Fig. 18

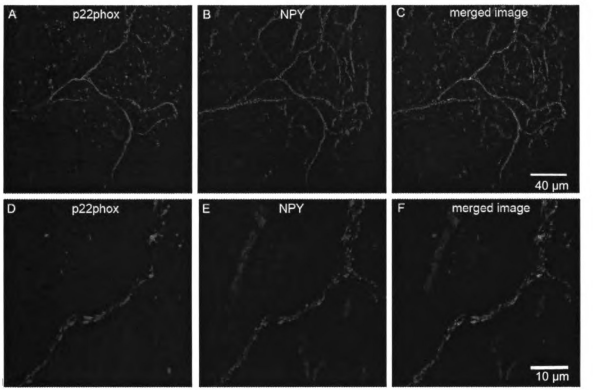


Fig. 18. Co-localization of immunoreactivity for $p22^{phox}$ and NPY in periarterial nerves. Periarterial nerve fibers were co-stained with neuropeptide Y (NPY), a marker for sympathetic nerves, and anti-$p22^{phox}$, a membrane-bound subunit of NADPH oxidase. (A) $p22^{phox}$, (B) NPY and (C) merged image. The bottom row shows higher magnification images of $p22^{phox}$ (D), NPY (E) and the merged image merged (F) revealing co-localization of the two antigens in a subset of nerve fibers. Scale bar in (C) applies to (A,B) and scale bar in (F) applies to (D,E).

Fig. 19

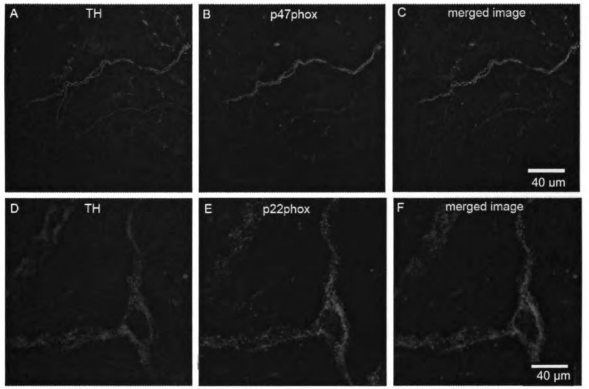


Fig. 19. Co-localization of immunoreactivity for $p22^{phox}$ or $p47^{phox}$ with TH in periarterial nerves. Periarterial nerve fibers were co-stained with tyrosine hydroxylase (TH), a marker for sympathetic nerves, and either anti-$p22^{phox}$, a membrane-bound subunit, or $p47^{phox}$, a cytosolic subunit, of NADPH oxidase. The top row shows high magnification images of (A) TH, (B) $p47^{phox}$ and (C) merged image revealing co-localization of the two antigens. The bottom row shows high magnification images of (A) TH, (B) $p22^{phox}$ and (C) a merged image of the co-localization of the two antigens. Scale bar in (C) applies to (A,B) and scale bar in (F) applies to (D,E).

Fig. 20

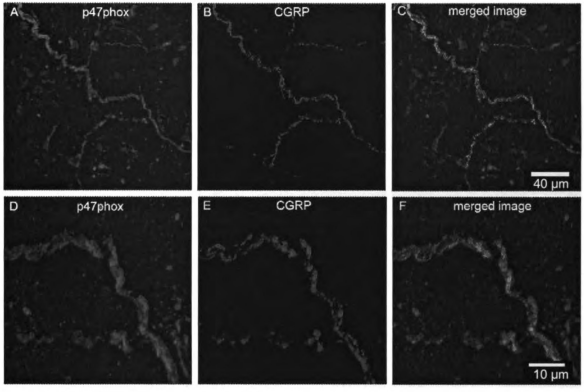


Fig. 20. Co-localization of immunoreactivity for $p47^{phox}$ with CGRP in periarterial nerves. Periarterial nerve fibers were costained with calcitonin gene-related peptide (CGRP), a marker for sensory nerves, and anti-$p47^{phox}$. The top row consists of representative pictures of (A) $p47^{phox}$ (B), CGRP and (C) merged image. The bottom row shows higher magnification images of $p47^{phox}$ (D), CGRP (E) and the merged image (F) revealing co-localization of the two antigens in a subset of nerve fibers. Scale bar in (C) applies to (A,B) and scale bar in (F) applies to (D,E).

Fig. 21

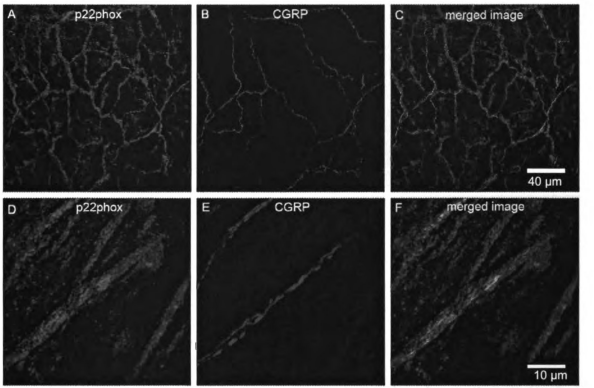


Fig. 21. Co-localization of immunoreactivity of $p22^{phox}$ with CGRP in periarterial nerves. Periarterial nerve fibers were co-stained with CGRP, a marker for sensory nerves, and anti-$p22^{phox}$. The top row consists of representative pictures of (A) $p22^{phox}$ (B), CGRP and (C) merged image. Notice the $p22^{phox}$ stained fibers which are not $CGRP^{+}$. The bottom row shows higher magnification images of $p47^{phox}$ (D), CGRP (E) and the merged image (F) revealing co-localization of the two antigens in a subset of nerve fibers. Scale bar in (C) applies to (A,B) and scale bar in (F) applies to (D,E).

Fig. 22

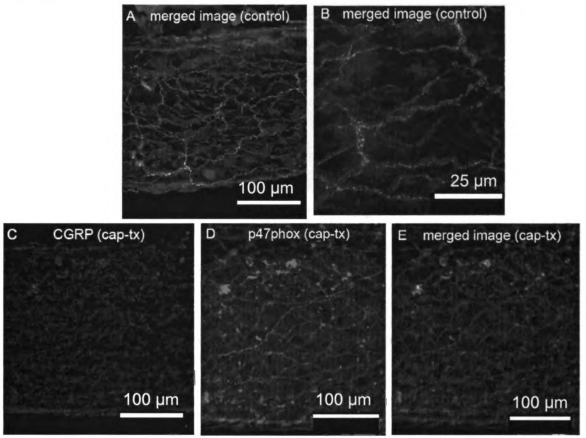


Fig. 22. Co-localization of immunoreactivity of $p47^{phox}$ with CGRP in control and capsaicin-treated rats. Chronic capsaicin treatment results in depletion of afferent nerve fibers (Ralevic et al., 1995). A low (A) and high (B) magnification image of $p47^{phox}$ and CGRP immunoreactivity in periarterial nerves from control rats. Some fibers are positive for both proteins, while others are $p47^{phox+}$ and $CGRP^{+}$. Representative images of $p47^{phox}$ (D) CGRP (C) and the merged image (E) from capsaicin-treated rats. In these vessels CGRP immunostaining is depleted (C), but $p47^{phox}$ remains (D and E). These images were taken with a conventional fluorescence microscope.

Fig. 23

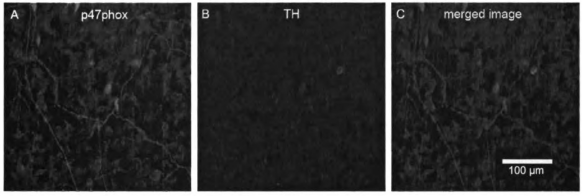


Fig. 23 Co-localization of immunoreactivity of p47phox with TH in celiac ganglionectomized (CGx) rats. CGx results in the depletion of afferent and efferent periarterial nerve fibers (King et al., 2007). (A) $p47^{phox}$ immunoreactivity is greatly reduced compared to the normally densely innervated mesenteric artery. (B) A representative image of mesenteric arteries incubated with TH antibody confirms sympathetic nerves are depleted in CGx-treated rats. (C) Overlay of the two images confirms that staining in previous images is specific for periarterial nerve fibers. These images were taken with a conventional fluorescence microscope. Scale bar in (C) applies to (A,B) also.

Fig. 24

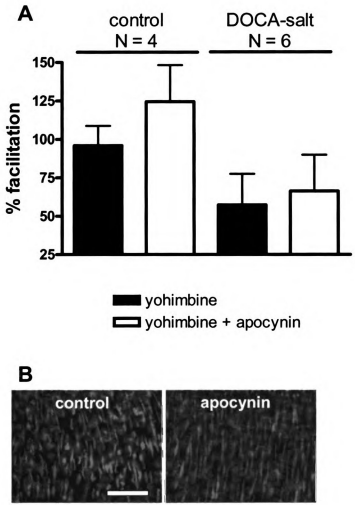


Fig. 24 Acute apocynin treatment did not improve facilitation of EJPs and did not alter vascular smooth muscle cell superoxide levels. (A) Facilitation of DOCA-salt and control animals in the presence of yohimbine (1 μM) before and after apocynin (0.1 mM) treatment. (B) Dihydroethidium staining of vascular smooth muscle cells was not different before and after apocynin treatment in control or DOCA-salt rats. Images taken with a conventional fluorescence microscope. Scale bar = 100 μm (scale bar applies to both figures).

Fig. 25

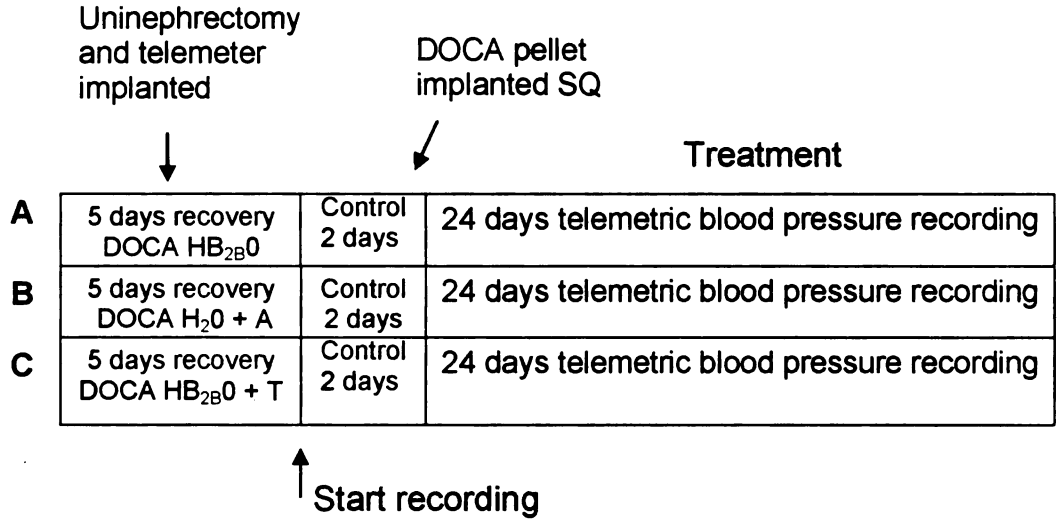


Fig. 25 Protocol for *in vivo* studies with chronic apocynin and tempol treatment. Male Sprague Dawley rats were given uninephrectomies and implanted with pressure transducers in their femoral arteries. They were started on either Na^+ alone (A) or Na^+ + either apocynin (B; 1 or 2 mM) or tempol (C; 1 mM). After five days recovery systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate were recorded for two control days (C). Animals were then implanted with DOCA-salt pellets, and recordings were continued for the next 24 days. A = apocynin; T = tempol

Fig. 26

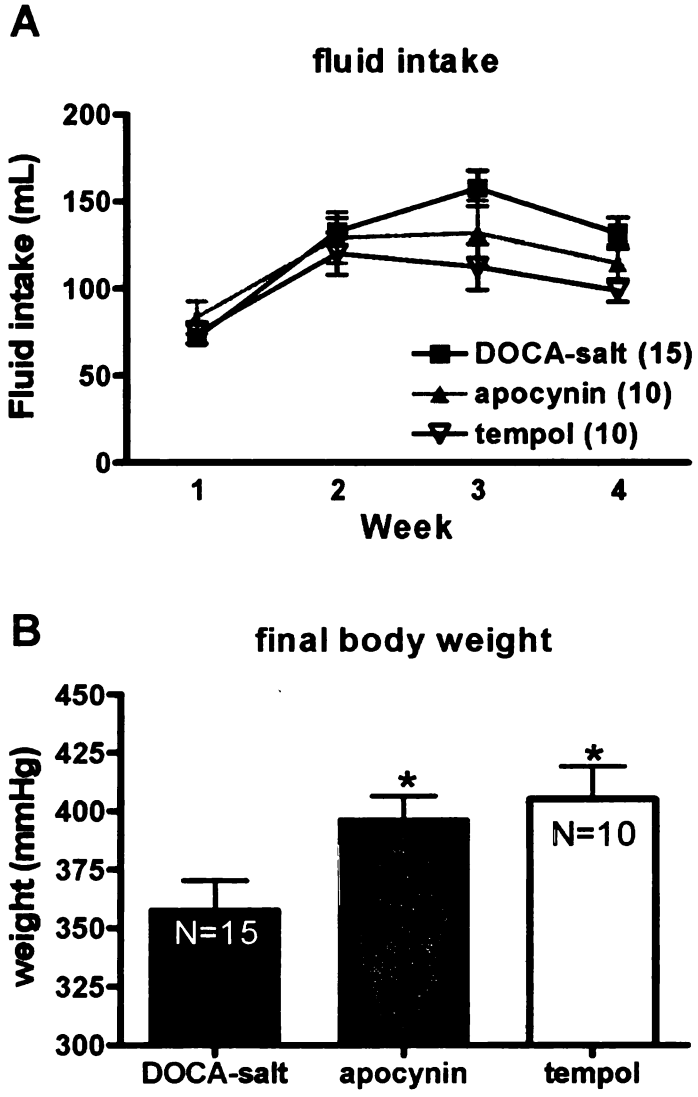


Fig. 26. Chronic anti-oxidant treatment affects on fluid intake and end body weight. (A) 24 h measurements of water intake were assessed one day of each week and averaged for each group. There was an interaction between treatment group and water intake over time (two-way ANOVA; * = $P < 0.05$), but there was no difference between groups at any one time point. (B) Body weights measured at the end of the study were significantly lower in the DOCA-salt control group (358 ±13, N = 14) compared to apocynin-treated (1 and 2 mM; 396 ± 10, N =14) and tempol-treated (405 ± 14, N = 10) groups (* = $P < 0.05$; one-way ANOVA).

Fig. 27

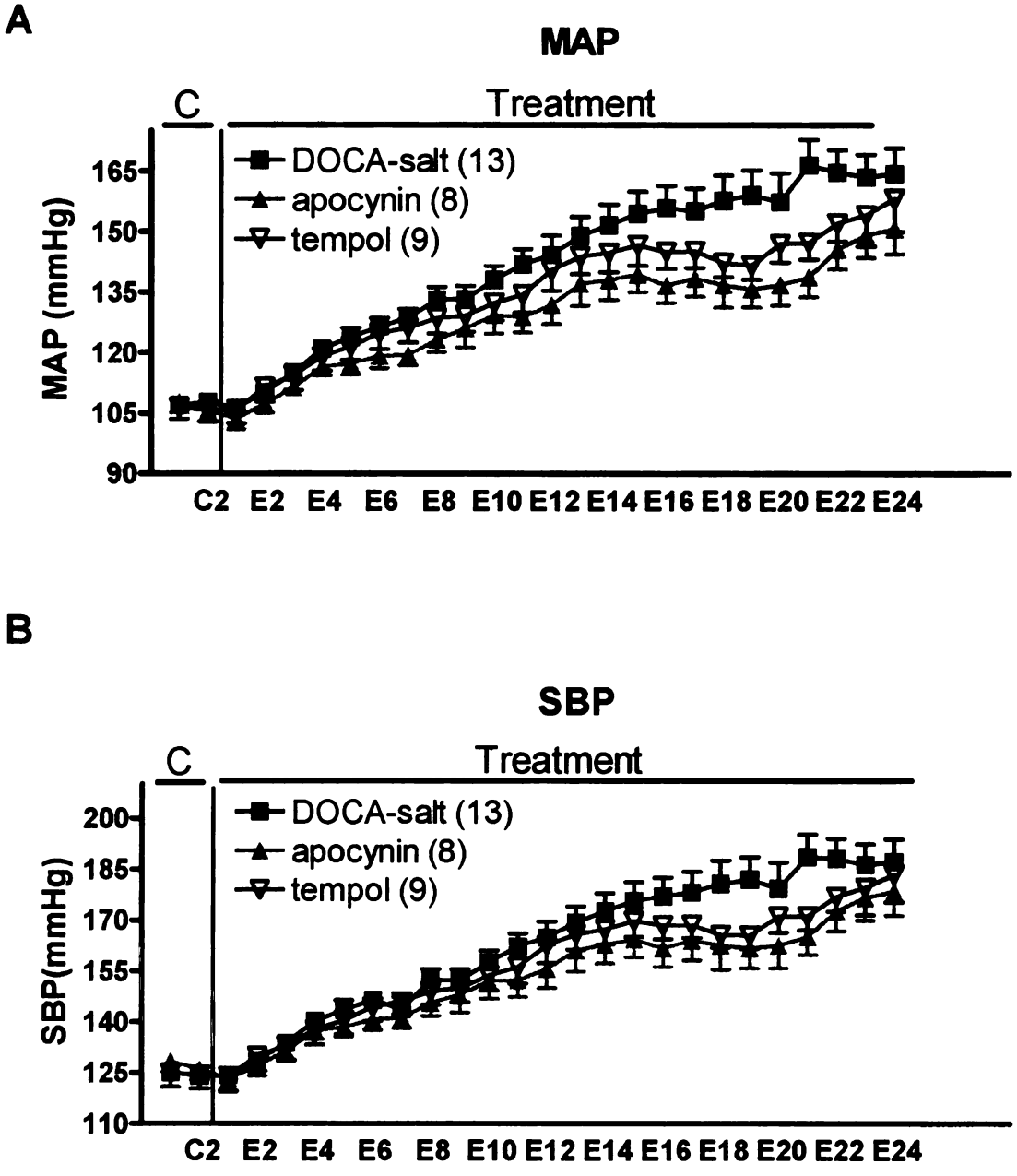

A
MAP
C
Treatment
DOCA-salt (13)
apocynin (8)
tempol (9)
MAP (mmHg)
165
150
135
120
105
90
C2 E2 E4 E6 E8 E10 E12 E14 E16 E18 E20 E22 E24
B
SBP
C
Treatment
DOCA-salt (13)
apocynin (8)
tempol (9)
SBP(mmHg)
200
185
170
155
140
125
110
C2 E2 E4 E6 E8 E10 E12 E14 E16 E18 E20 E22 E24

Fig. 27 continued

C

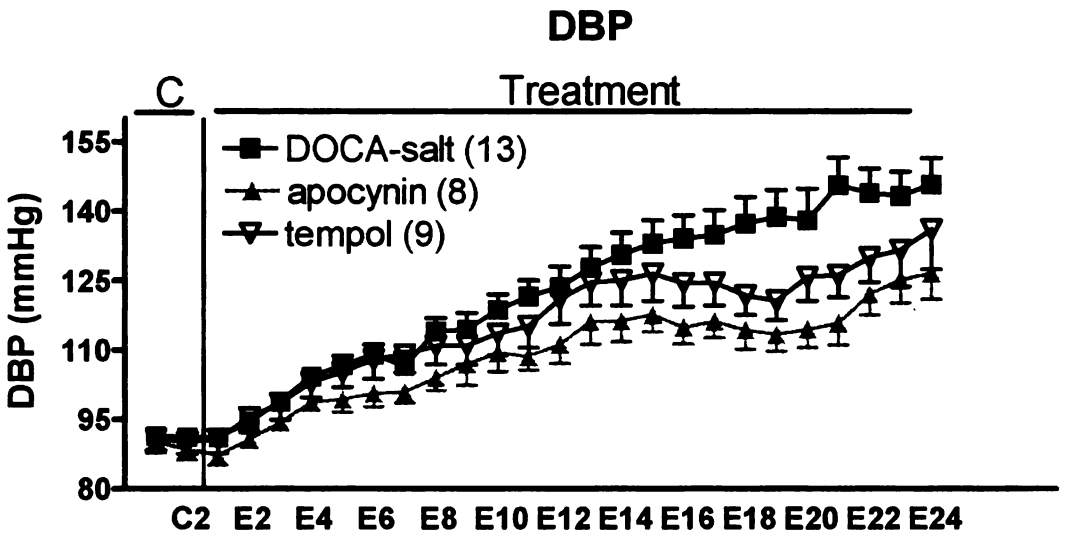


Fig. 27. Chronic Tempol and apocynin treatment lowers blood pressure in DOCA-salt rats. Mean arterial pressure (MAP; A), systolic blood pressure (SBP; B) and diastolic blood pressure (DBP; C) all decreased in response to chronic treatment with apocynin (gray triangle, N = 8) and tempol (open triangle, N = 9) compared to DOCA-salt controls (solid square, N = 13; two-way ANOVA, interaction $P < 0.05$). The most significant drop was in DBP.

Fig. 28

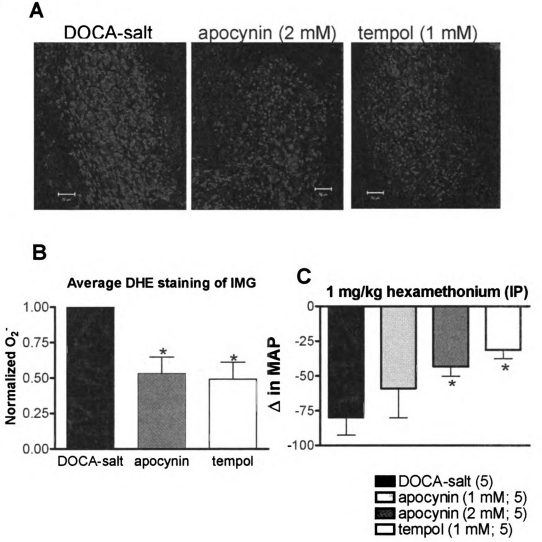


Fig. 28. Chronic anti-oxidant treatment lowers superoxide (O_2^-) and sympathetic neurotransmission in DOCA-salt rats. (A) Representative images of dihydroethidium (DHE) stained inferior mesenteric ganglia (IMG) show a reduction in O_2^- in neuron cell bodies with apocynin and tempol treatment. Scale bar = 50 μM. (B) Staining was quantified by selecting individual soma (7-31) and measuring the fluorescence intensity. Average intensities from each day were averaged (N = number of ganglia; * = $P < 0.05$; 47 ± 11% and 51 ± 11% reduced, respectively; one-way ANOVA). (C) Response to a one-time injection of hexamethonium (1mg/kg, i.p.) on the 23rd experimental day. Tempol (1 mM) and apocynin (2 mM) significantly lowered the response to hemamethonium compared to DOCA-salt control rats Apocynin (1mM) showed a trend for a decreased drop in MAP, but this was not significant compared to control (Δ MAP = DOCA-salt: 80 ±13mmHg; apocynin (1 mM): 59 ± 21 mmHg; apocynin (2mM): 43 ± 7 mmHg; tempol: 31 ± 6 mmHg, *$P < 0.05$). Scale bar = 50 μm.

Fig. 29

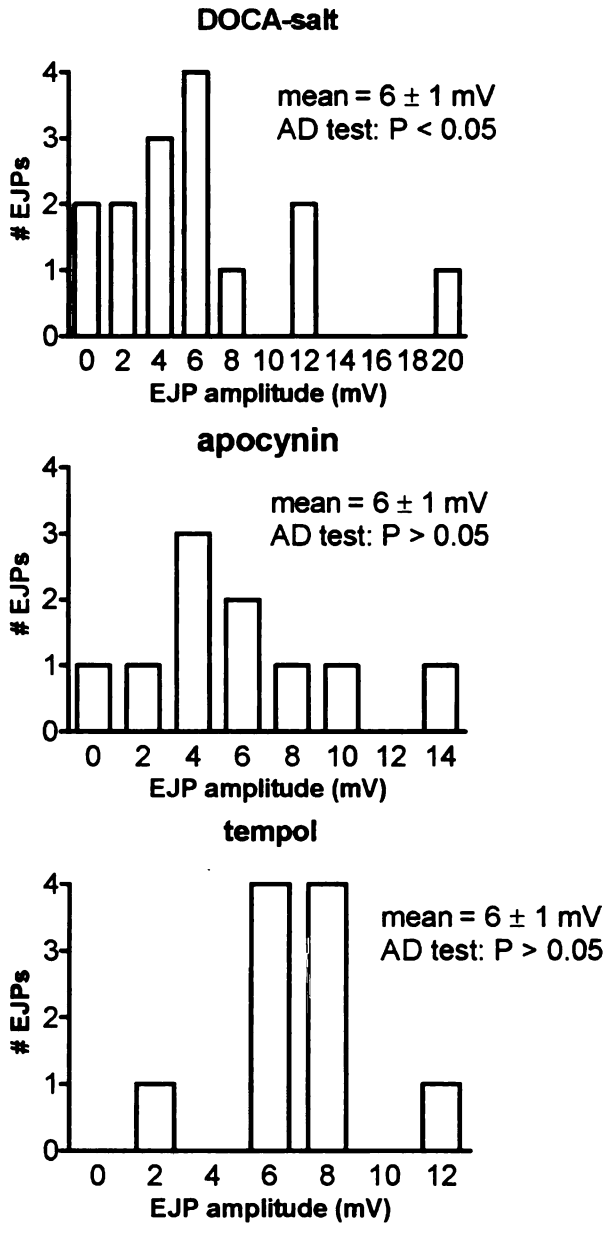


Fig. 29. EJP amplitude distribution patterns change with chronic antioxidant treatment. EJPs were elicited with peri-arterial nerve stimulation (50-120V). The stimulus was increased until maximal responses were reached, and that voltage was used to elicit EJPs. EJP amplitudes were not different between the three groups (DOCA: 6.0 ± 1.4 mV, apocynin: 5.8 ± 1.3 mV; tempol: 7.0 ± 0.8 mV). EJP amplitudes from DOCA-salt rats were not normally distributed (A^2 = 0.74, $P < 0.05$; Anderson-Darling Normality Test), and the variance was greatest in this group compared to the antioxidant treated groups (variance = 29, N = 15). EJP distribution in mesenteric arteries from apocynin and tempol treated animals were normally distributed (apocynin: $A^2 = 0.47$, $P > 0.05$; tempol: $A^2 = 0.31$, $P > 0.05$) and the variances were decreased (variance = 17 and 6 respectively, N = 10). Testing for equal variances resulted in an F-test statistic which was significantly different between DOCA-salt and tempol treated rats ($F = 4.53$, $P < 0.05$), but not DOCA-salt and apocynin-treated rats ($F = 1.67$, $P > 0.05$). AD = Anderson-Darling Normality Test result.

Fig. 30 EJPs from rats treated with apocynin facilitate to a greater extent than DOCA-salt control rats in response to low frequency nerve stimulation. Trains of nerve stimulation (0.5 Hz, 10 s) were used to elicit EJPs. (A) Representative traces show EJPs recorded from apocynin-treated and DOCA-salt controls before (*left*) and after (*right*) yohimbine (1 μM). 1st and 5th EJPs from each group are shown individually before and after yohimbine to show the increased facilitation in apocynin-treated rats, but not DOCA-salt controls after yohimbine. (B) Representative traces show EJPs recorded from tempol-treated and DOCA-salt controls before (left) and after (right) yohimbine (1 μM). 1st and 5th EJPs from each group are shown individually before and after yohimbine. However, the difference between tempol-treated and DOCA-salt rats was not significant. Before addition of yohimbine EJPs from DOCA-salt control rats, apocynin-treated and tempol-treated DOCA-salt rats facilitate equally well (38 ± 14%, 34 ± 5%, 45 ± 13 mV, respectively; $P > 0.05$) while after blocking the prejunctional α_2AR facilitation is greater in antioxidant treated rats compared to DOCA-salt controls (86 ± 16%; 120 ± 13%; 122 ± 7%, respectively). (C) To determine the function of the α_2AR in facilitation for each group, an α_2AR autoreceptor function index was calculated. The ratio was significantly greater in the apocynin-treated group (* = $P < 0.05$; students t-test, DOCA-salt vs. apocynin).

Fig. 30

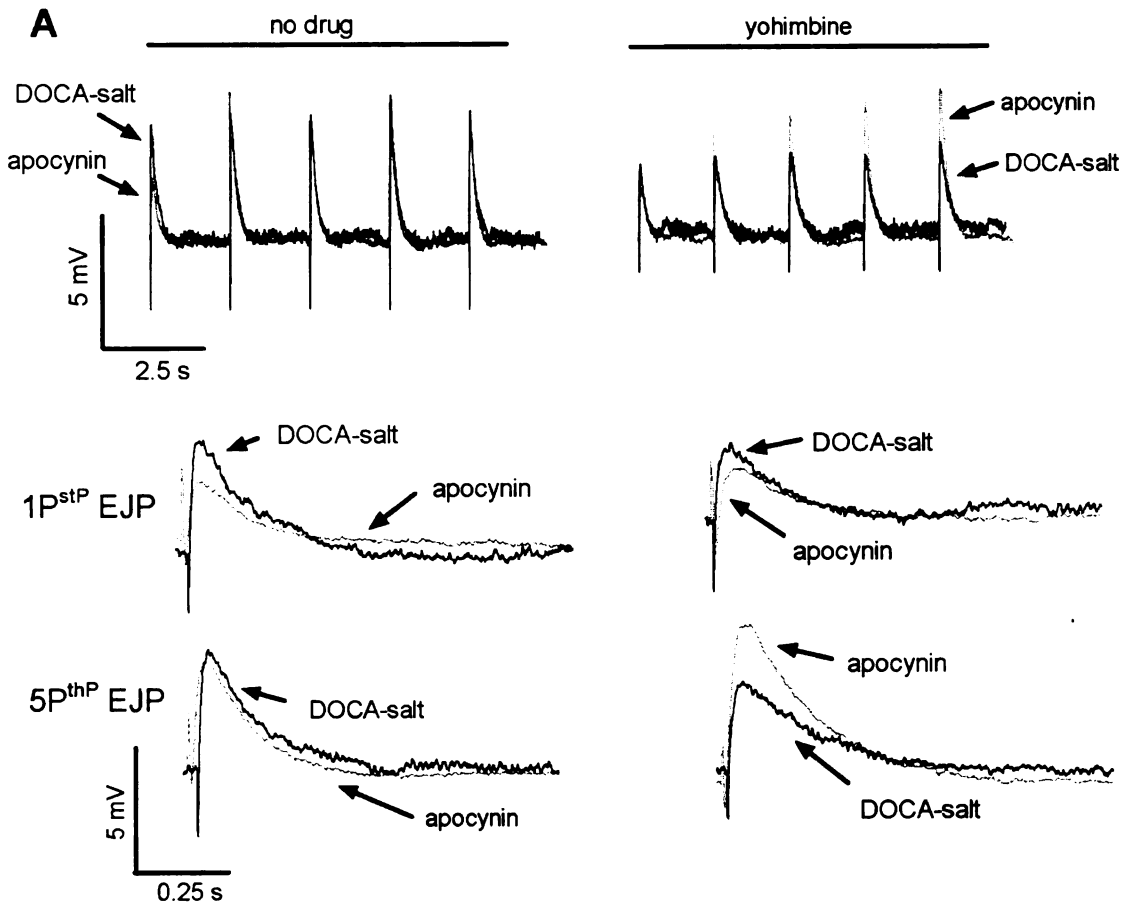
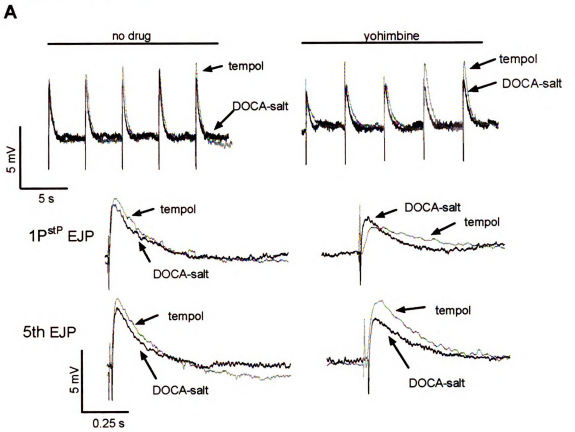

A
no drug
yohimbine
DOCA-salt
apocynin
5 mV
2.5 s
apocynin
DOCA-salt
DOCA-salt
apocynin
1P^stP EJP
DOCA-salt
apocynin
DOCA-salt
apocynin
5P^thP EJP
DOCA-salt
apocynin
5 mV
0.25 s
apocynin
DOCA-salt

Fig. 30 continued



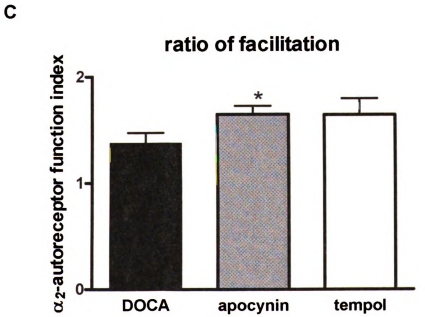
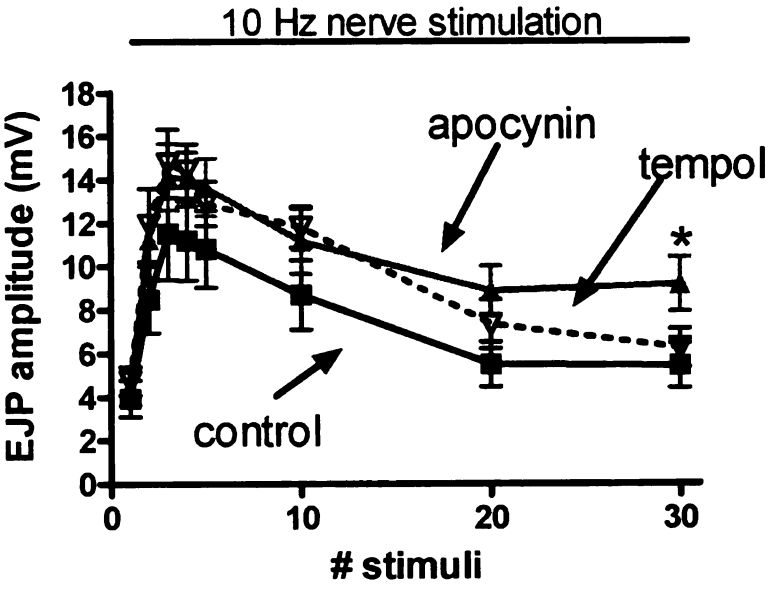
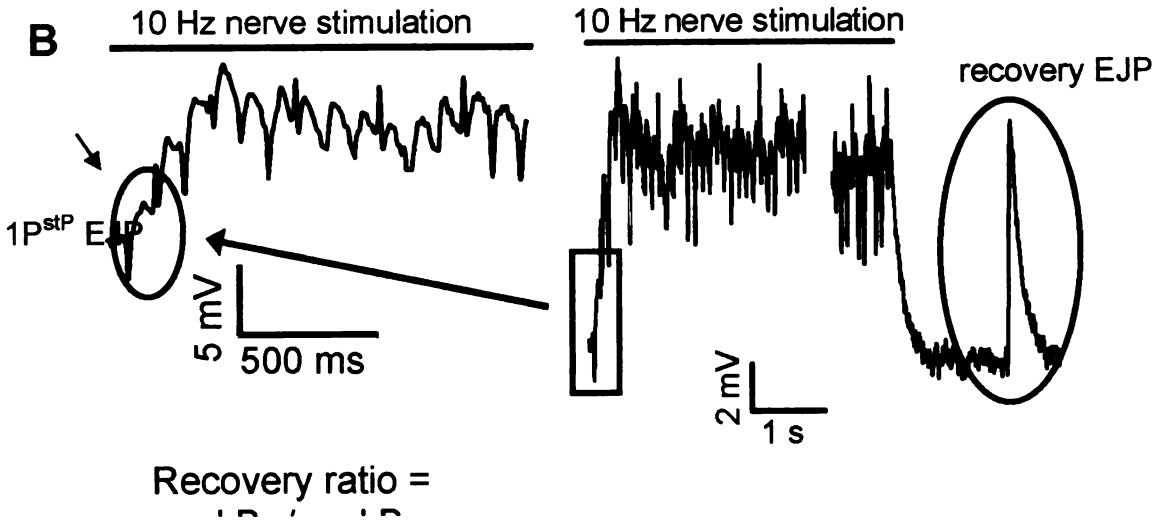


Fig. 31

A



B



Recovery ratio =

Fig. 31 continued

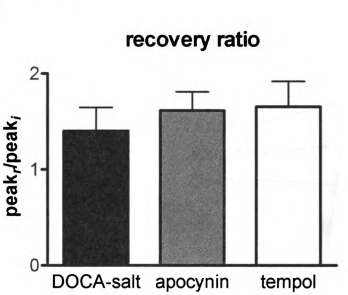


Fig. 31 Rundown was decreased and facilitation increased in arteries from apocynin, but not tempol, treated rats compared DOCA-salt controls. High frequency trains of nerve stimulation were used to examine facilitation and rundown in the three treatment groups. Peak amplitude, and EJP amplitude at 3 s was determined. (A) EJP amplitude was significantly greater in apocynin-treated, but not tempol-treated, rats compared to DOCA-salt controls at 3 (* = $P < 0.05$; one-way ANOVA). (B) A single stimulation after the end of the train was used to determine if the EJPs were fully recovered after rundown. (C) EJPs were recovered to the same extent 2 s after the end of the train.

Fig. 32

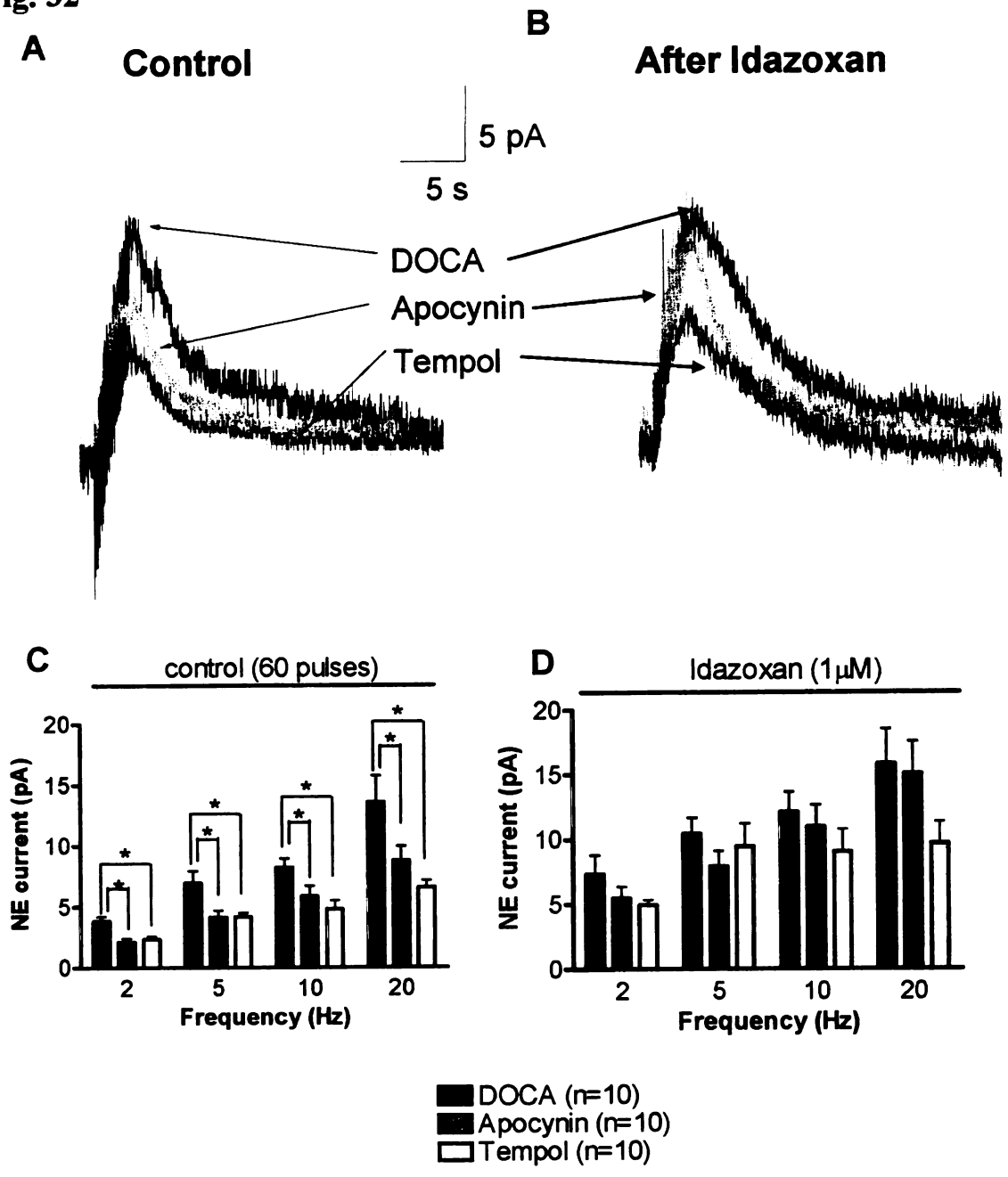

A
Control
B
After Idazoxan
5 pA
5 s
DOCA
Apocynin
Tempol
C
control (60 pulses)
NE current (pA)
Frequency (Hz)
D
Idazoxan (1μM)
NE current (pA)
Frequency (Hz)
DOCA (n=10)
Apocynin (n=10)
Tempol (n=10)

Fig. 32 continued

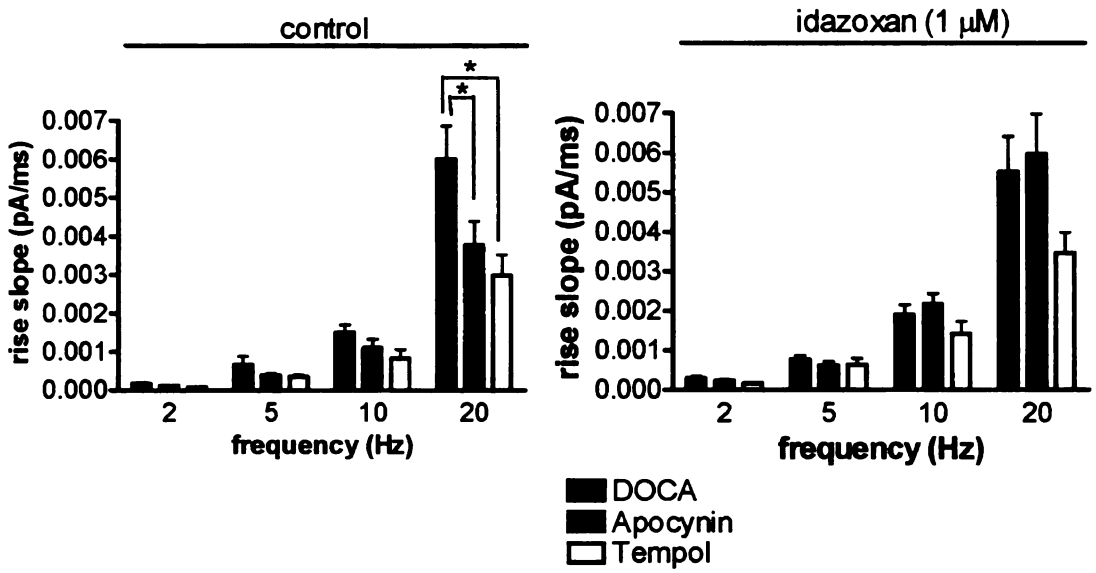


Fig. 32 Chronic antioxidant treatment reduced NE oxidation current and improved α_2AR function compared to DOCA-salt controls. NE oxidation current was assessed at 2, 5, 10 and 20 Hz nerve stimulation (60 pulses). Representative traces of NE oxidation current from each of the treatment groups (20 Hz) before (A) and after (B) treatment with idazoxan (1 μM). (C) Under control conditions peak oxidation current was significantly lower in apocynin- and tempol-treated rats compared to DOCA-salt control at all frequencies (*P < 0.05 compared to DOCA-salt). (D) After application of idazoxan, differences in oxidation current in response to nerve stimulation were abolished (P > 0.05). The rise slope (from baseline to peak current) was also assessed in control (E) and idazoxan treated tissues at 2,5,10 and 20 Hz (60 pulses). At 20 Hz, there was a significant decrease in rise slope in apocynin- and tempol- treated rats (* P < 0.05 vs. DOCA-salt) which was abolished in idazoxan treated tissues in response to the same frequencies of nerve stimulation (F; P > 0.05 vs. DOCA-salt).

Fig. 33

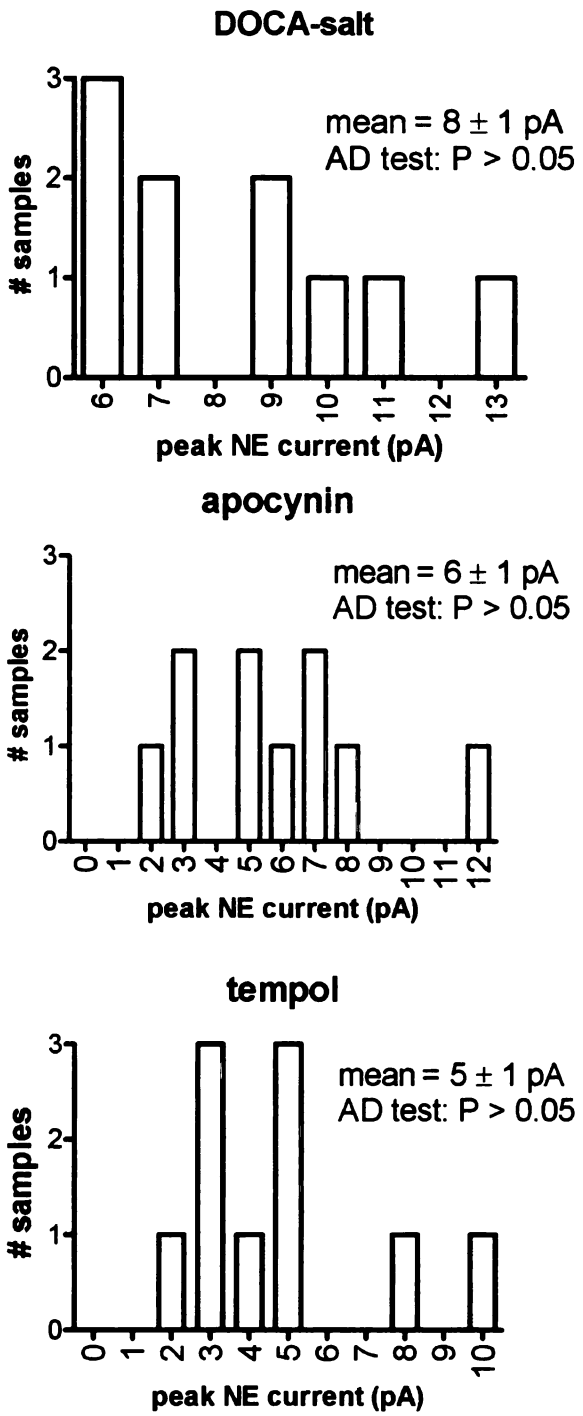


Fig. 33 NE peak current is normally distributed and variances are equal between the three treatment groups. Distribution patterns of NE peak current in response to 10 Hz nerve stimulation was analyzed for DOCA-salt ($A^2 = 0.26$), apocynin- ($A^2 = 0.25$) and tempol-treated ($A^2 = 0.62$) rats. Mean current amplitudes were lower in antioxidant treated rats compared to untreated DOCA-salt rats. Distribution patterns and variances were not different between groups.

Discussion

In this study I showed that NADPH oxidase subunits are expressed in sympathetic and sensory nerve fibers innervating mesenteric arteries. Previous studies showed the presence of NADPH oxidase subunits in central and peripheral nervous tissue (Tammariello et al., 2000; Glass et al., 2006; Cao et al., 2007), but this is the first study to systematically evaluate the NADPH oxidase expression in perivascular nerve fibers. This result is important because O_2^- is a short-lived molecule but its production in sympathetic nerve endings would allow O_2^- induced modulation of sympathetic neuroeffector transmission. The expression of NADPH oxidase in sympathetic and sensory nerves supplying blood vessels is also important because in brain cardiovascular centers, O_2^- changes firing rates and ion channel function (Sun et al., 2005). It is very likely that the functional properties of peripheral nerves can also be altered by O_2^- , which will directly affect neurotransmission and vascular tone. This may have important implications in blood pressure regulation and hypertension where NADPH oxidase activity is increased (Beswick et al., 2001a; Dai et al., 2004; Dai et al., 2006; Cao et al., 2007).

In an attempt to identify if NADPH oxidase subunits were localized to specific subsets of perivascular nerves (sensory or sympathetic nerve fibers) two surgical manipulations were made. NAPDH oxidase subunits were localized in arteries from rats treated neonatally with capsaicin. This treatment destroys perivascular sensory nerves (Nagy et al., 1981). The prominent meshwork that

remained allowed me to conclude that sensory nerves are not the only location of NADPH oxidase. The complementary experiment would be to deplete sympathetic nerves following *in vivo* treatment with the sympathetic neurotoxin, 6-hydroxydopamine (6-OHDA). The primary concern is that 6-OHDA, which is taken up by norepinephrine transporter (NET), uses NADPH oxidase pathways as one of three neurotoxic pathways used to kill sympathetic nerve fibers (Rodriguez-Pallares et al., 2007), which we have shown to be in both sensory and sympathetic nerve fibers. NET, the primary mediator of 6-OHDA into sympathetic nerves, is also found on sensory nerve fibers (Zheng et al., 2000). Therefore specificity is a concern. In order to avoid the complications associated with using neurotoxins, CGx was used to remove all perivascular nerve fibers which would either originate in the celiac ganglion (sympathetic nerves) or would pass through the celiac ganglion enroute to other target tissues (sensory nerves originating from dorsal root ganglia).

CGx did not completely remove $p47^{phox}$ immunostaining. Also, $p22^{phox}$ was not found in all NPY containing fibers. These data support two conclusions 1) There are subpopulations of sympathetic and sensory nerves which do not show $p22^{phox}$ immunostaining and 2) there are $p47^{phox}$ containing nerves which contain neither NPY^{+} nor $CGRP^{+}$. The first conclusion raises the possibility that nerve fibers may contain different subunit compositions. $P47^{phox}$ was consistently found in all nerve fibers, but there may be a membrane-bound subunit other than $p22^{phox}$ in nerve fibers. It may also be possible that $p22^{phox}$ found in nerve fibers contains a polymorphic site which eludes antibody detection (Zalba et al., 2005).

The 2nd conclusion suggests that there are a set of nerve fibers located in paravascular nerve fibers, which do not originate in the celiac ganglia, such as the intestinofugal fibers. Intestinofugal fibers are afferent nerve fibers which originate in the myenteric plexus of the enteric nervous system and terminate near nerve cell bodies in the celiac ganglia (Costa and Furness, 1983; Gibbins et al., 2003). However the $p47^{phox+}$ nerve fibers remaining after CGx could also be due to fibers which were not abolished during ganglionectomy (Furness et al., 2001).

Recently, non-vascular NADPH oxidase and its role in blood pressure regulation have been recognized. Zimmerman et al. showed *Rac-1* dependent NADPH oxidase was required for the brain to elicit Ang II-dependent increases in blood pressure (Zimmerman et al., 2004). This was followed by a number of studies focusing on the localization and regulation of NADPH oxidase in different cardiovascular-regulating nuclei in the brain (Kim et al., 2005; Glass et al., 2006). Meanwhile, several laboratories suggested that NADPH oxidase in the peripheral neurons also contributes to cardiovascular regulation. For example, the regulation of NADPH oxidase-derived O_2^- in prevertebral sympathetic ganglia and primary sensory ganglia were shown to be associated with DOCA-salt hypertension (Dai et al., 2004; Dai et al., 2006; Cao et al., 2007).

Therefore we were interested in knowing whether increased O_2^- production in hypertension could directly affect the function of sympathetic nerve terminals. Increased O_2^- production contributes to increased blood pressure and associated end-organ damage in DOCA-salt hypertensive rats (Somers et al., 2000; Beswick et al., 2001a; Li et al., 2003; Luo et al., 2003). Furthermore,

chronic apocynin or tempol treatment lowers blood pressure in rodent models of hypertension (Schnackenberg and Wilcox, 1999; Beswick et al., 2001a; Beswick et al., 2001b). Finally, NADPH oxidase subunit mRNA is increased in central autonomic nuclei in hypertension (Ye et al., 2006).

However, the role of oxidative stress in changes seen in sympathetic neuroeffector transmission has not been studied. The goal of these studies was to assess the role of oxidative stress on impaired neurotransmission to resistance arteries in DOCA-salt hypertension. Equipped with the evidence that NADPH oxidase localizes to mesenteric periarterial sympathetic nerves, I hypothesized that treatment with apocynin, an NADPH oxidase inhibitor or tempol, an SOD mimetic, would improve sympathetic nervous system function in DOCA-salt rats.

Treatment of arteries with apocynin *in vitro* did not improve facilitation in DOCA-salt rats compared to sham controls. Although treatment with apocynin had no effect in this acute setting, I showed that chronic antioxidant treatment had beneficial effects on the overall health and on sympathetic nerve function in DOCA-salt treated rats. After a 4-week treatment protocol NE release was decreased and ATP handling improved in sympathetic nerve endings. Findings of lower O_2^- in peripheral sympathetic ganglia and decreased responses to hexamethonium suggest that oxidative stress plays a role in the impairment of sympathetic nerve function. Sympathetic nerve dysfunction is known to contribute to DOCA-salt hypertension (Bouvier and de Champlain, 1985; Luo et al., 2003).

The effects of apocynin and tempol on blood pressure were not apparent until after 2-3 weeks of treatment and the greatest differences in blood pressure between treatment groups and control were seen during the third week of treatment. By the end of the study, antioxidant treatment prevented the weight loss seen in DOCA-salt controls. This suggests that ROS generation may be secondary to increased pressure on the vascular wall, and after changes to nerve fibers and the blood vessel wall have occurred. It also supports a role for ROS in mediating end-organ damage. Apocynin also attenuated blood pressure and prevented structural changes and end organ damage in Ang II-infused mice, whereas in the same study hydralazine treatment lowered blood pressure without protection of end-organ damage (Virdis et al., 2004). This suggests that the inhibition of NADPH oxidase and reduction in oxidative stress is particularly important in the health of these animals.

The novel finding in the studies presented here is the beneficial effects of antioxidant treatment on sympathetic nerve function, which occurred in a variety of ways. Previously in the literature ROS has been shown to act via postjunctional mechanisms in DOCA-salt hypertension (Touyz, 2004; Touyz and Schiffrin, 2004; Virdis et al., 2004). However, these data suggest that ROS may have prejunctional targets as well. One component of synaptic regulation altered in DOCA-salt hypertension is the regulation of NE release by the α_2AR (Bouvier and de Champlain, 1985; Moreau et al., 1995; Knapp and Klann, 2000; Luo et al., 2004). These results of these studies show that NE current decreased in the antioxidant treatment groups compared to control, but that in the presence of

idazoxan the differences in peak NE oxidation current were abolished. Similarly, a decrease in the rise slope in apocynin and tempol treated animals at 20 Hz was abolished after idozoxan treatment. Together, these data reaffirm that increased NE release in DOCA-salt hypertension from sympathetic nerve terminals is due in part to impairment of α_2AR function. The role of oxidative stress in its impairment is a novel finding, but may be targeted by ROS due to structural and physiological properties. The α_2AR contains two cysteine residues on the first and second extracellular loops, which are connected by a disulfide bond and are thought to play a role in ligand binding (Strosberg, 1993). Cysteine residues contain thiol groups, which are one of many targets of ROS (Knapp and Klann, 2000). Alterations in these residues could effect the folding and function of the receptor. It's also possible that ROS target Gi/Go proteins, downstream from the receptor, causing impaired signaling (Nishida et al., 2000)

ATP is also released from sympathetic nerve terminals in response to nerve stimulation, where it is the primary neurotransmitter in physiological conditions, but is decreased in DOCA-salt hypertension (Luo et al., 2004). Previously we found that EJPs, an indirect measure of ATP release, display decreased amplitude and increased variability in DOCA-salt rats compared to normotensive controls. The ability of EJPs to facilitate can give us insight the handling and release of ATP-containing synaptic vesicles. In DOCA-salt hypertension facilitation is decreased and ATP is depleted faster in response to high frequency trains of nerve stimulation. Together this suggests impaired handling and vesicular release of ATP. In these studies, treatment with apocynin

improved purinergic neurotransmission. The effects of tempol were less consistent. Purinergic neurotransmission in apocynin-treated vs. control rats was improved in a number of ways including decreased rundown, and increased facilitation, especially in the presence of yohimbine. Tempol had its greatest effect on EJP amplitude, where variance was decreased compared to DOCA-salt control rats.

The α_2AR plays a role in regulating ATP release through pertussis toxin-sensitive mechanisms, similar to its role in NE regulation (Todorov et al., 1999; Bobalova and Mutafova-Yambolieva, 2001) and as mentioned above, its function is impaired in DOCA-salt hypertension (Tsuda et al., 1989; Luo et al., 2004). The decreased facilitation in response to 10 Hz nerve stimulation appear to be α_2-AR dependent, as they were only significantly different from control after treatment with yohimbine. This is consistent with the ability of yohimbine to increase facilitation in arteries from apocynin-treated rats, but not in arteries from untreated DOCA-salt rats. This is also consistent with previous findings where yohimbine increased facilitation in normotensive rats to a greater extent than DOCA-salt rats (Chapter 3). In addition, the difference in facilitation before and after yohimbine treatment in response to low frequency stimulation resulted in a greater difference in arteries from apocynin-treated rats compared to arteries from untreated DOCA-salt controls. Together this suggests that the α_2AR's regulation of ATP is also impaired, as yohimbine had an effect on facilitation only in the apocynin-treated but not the DOCA-salt control rats.

In addition, there is a non-α_2AR component of impaired purinergic neurotransmission as indicated by increased rundown at high frequency and impaired facilitation at low frequency stimulation, which may be a direct effect of oxidative stress on ATP availability. Alterations in purinergic neurotransmission can also be due to impaired ATP handling, defined as either decreased ATP storage into vesicles or decreased vesicle mobilization, between which I did not discriminate. Mitochondria are known to be targets of ROS, which are rich in sympathetic nerve endings. In addition there may be other vesicle regulating proteins besides the α_2AR in the sympathetic nerve terminal which are sensitive to oxidative stress. For example, SNAP25, a vesicular release protein is also a target of ROS in the CNS (Giniatullin et al., 2006) and may be a target in the periphery as well.

The differential results of *in vivo* tempol and apocynin treatment on purinergic neurotransmission may be due to the different mechanisms of action of these two antioxidants. Apocynin is an inhibitor of NADPH oxidase, which is known to be upregulated in sympathetic ganglia of DOCA-salt hypertensive rats (Cao et al., 2007). However, the mechanism of action of apocynin has recently been disputed. Apocynin inhibited O_2^- in human embryonic kidney (HEK)-293 cells in the absence of NADPH oxidase subunits. It also was found to act as an antioxidant in endothelial and vascular SMCs, independent of NADPH oxidase (Heumuller et al., 2007). Although apocynin has been shown to inhibit NADPH oxidase-dependent generation of O_2^- (Dai et al., 2006), it is not known if this alternative antioxidant mechanism for apocynin is active in sympathetic neurons.

In addition, NADPH oxidase is only one potential sources of O_2^-. Tempol, an SOD mimetic is not specific, but rather it should decrease O_2^- regardless of its' enzymatic origin. This is beneficial due to the numerous generators of free radicals such as mitochondrial-generated ROS at the sympathetic nerve terminal (Szeto, 2006; Puddu et al., 2007). Xanthine oxidase can also produce O_2^-. However as a result of tempol's action on O_2^-another free radical, H_2O_2, is formed. H_2O_2 is not only more stable, and longer-lived, but also depresses neurotransmission in the CNS (Avshalumov et al., 2000) and NMJ (Giniatullin et al., 2005). It is possible that H_2O_2 contributes to changes in neurotransmitter release form sympathetic nerve terminals, although this has not been studied. Although tempol and apocynin had different effects on purinergic neurotransmission they had a similar effect on O_2^- in neurons in the sympathetic ganglia. A recent study has shown that apocynin has radical scavenging effects independent to NADPH oxidase (Heumuller et al., 2007), which could account for these results. Tempol also directly inhibits sympathetic nerve activity *in vivo*, independent of its SOD-mimetic actions (Xu et al., 2004).

It is possible that long-term inhibition of sympathetic nerve activity could also have beneficial effects. Perhaps high levels of sympathetic activity generate O_2^- or other metabolites which might be deleterious to sympathetic nerve endings. Blood pressure is reduced more in apocynin-treated compared to tempol rats, and O_2^- production secondary in increased blood pressure may be a reason for some changes at the neuroeffector junction.

The sympathetic nervous system plays an important role in regulating vascular tone of the splanchnic circulation. The results of this study show that oxidative stress contributes to the increase in blood pressure in DOCA-salt hypertension by altering neurotransmitter release properties at sympathetic nerve terminals. The localization of NADPH oxidase subunits to periarterial nerves provides a novel mechanism for O_2^- production in the nerve terminal where it can interact with vesicular release processes. While ROS have an important role as signaling molecules at physiological levels, increases in ROS affects the function of proteins, such as the α_2AR which are irreversible with acute antioxidant treatment. These alterations at the nerve terminal affect both adrenergic and purinergic neurotransmission in DOCA-salt hypertension. Inhibiting NADPH oxidase with apocynin, restores purinergic neurotransmission while the SOD mimetic tempol, does not. This suggests that H_2O_2 may regulate purinergic neurotransmission, with increases in H_2O_2 contributing to the depletion of ATP stores in the nerve terminal. The restoration of adrenergic neurotransmission by both apocynin and tempol suggests that the two neurotransmitters are regulated differently. The increase in NE release, without an increase in ATP release may contribute to an impaired regulation of vascular tone and hypertension.

Perspectives

The abundance of recent literature regarding oxidative stress and hypertension reveals the importance of understanding these processes and perhaps targeting them for treatment of high blood pressure. While antioxidants are

beneficial in animal models of hypertension and cardiovascular disease, clinical trials in human hypertensive subjects have been disappointing. These data show for the first time that NADPH oxidase subunits are found in sympathetic and sensory nerve endings which densely innervate the splanchnic circulation and that NADPH oxidase derived free radicals impairs the sympathetic nerve terminal resulting in increased NE release and impaired regulation of ATP-containing vesicles.

Until now, the relationship between purines and increased ROS found in hypertension has not been established in the peripheral nervous system. However, the development and maintenance of hypertension and the resulting end-organ damage has all been linked to oxidative stress. These data provide a novel site of ROS regulation, and a target for antioxidant therapy in hypertension.

CHAPTER 6

GENERAL CONCLUSIONS AND PERSPECTIVES

Purinergic neurotransmission from mesenteric sympathetic nerves is now widely accepted, but details regarding ATP storage, release and regulation are still under investigation. Few studies have focused on purinergic neurotransmission from mesenteric perivascular nerves, which have particular importance in blood pressure regulation. Impairment in sympathetic nerve function underlies several types of hypertension including DOCA-salt hypertension. Understanding alterations in sympathetic neurotransmission will help us to understand and treat pathological changes in diseases such as hypertension. The few studies that have looked at purinergic neurotransmission in hypertension were conducted in spontaneously hypertensive rats, a genetic model of hypertension, where the RAS may play role (Brock and Van Helden, 1995; Guitart et al., 2002). The experiments described here focus on alterations in purinergic neurotransmission in the DOCA-salt model of hypertension, a salt-dependent model of hypertension, where renin levels are low (Gavras et al., 1975).

The results of these studies extend our knowledge of purinergic neurotransmission from sympathetic nerves, the role of purinergic transmission in blood pressure regulation, and the role of ROS in altering ATP and NE release from sympathetic nerves *in vitro* and *in vivo*. The results of these studies are novel and have particular importance in those patients with salt-sensitive hypertension including the elderly and African-American population.

There are four major conclusions which can be drawn from the results of these studies collectively.

1. There is impaired regulation of the purinergic component of neurotransmission from sympathetic periarterial nerves in DOCA-salt rats.

2. Sympathetic and sensory nerves contain NADPH oxidase subunits which, if active, would generate ROS in nerve fibers and endings. This provides a rationale for NADPH oxidase-derived O_2^- interactions with synaptic vesicles and release proteins, contributing to altered sympathetic neurotransmission and increased blood pressure.

3. ROS effects on sympathetic nerves contribute to high blood pressure in DOCA-salt hypertensive rats.

4. Results from these studies provide support for separate ATP- and NE-containing vesicles, but doesn't preclude the possibility that there is also a subset of vesicles co-storing ATP with other sympathetic neurotransmitters.

Increased EJP variability and rundown in DOCA-salt arteries suggests changes in ATP handling and storage in nerve endings.

NE, ATP and NPY are all released as neurotransmitters from peri-arterial sympathetic nerves in the rat mesentery (Donoso et al., 1997; Huidobro-Toro and Donoso, 2004). Stimulating perivascular nerves results in a biphasic contraction, where the first part is transient, and predominately mediated by ATP, while the second part is a longer-lasting response due to NE binding to α_1-ARs. In the literature, the relative contribution of ATP vs. NE to neurogenic constriction varies depending on tissue-type, age and disease state of animal. In the

mesenteric perivascular nerves, ATP is the predominant neurotransmitter under physiological conditions (Luo et al., 2004; Rummery et al., 2007).

In hypertension, several changes at the neuroeffector junction occur which alter adrenergic neurotransmission. A few key findings include impairment of α_2ARs (Luo et al., 2004), increased NE release (Ekas et al., 1983; Esler et al., 1986; Masuyama et al., 1986) and increased kinetics of NE release (Miranda-Ferreira et al., 2008). Together, the increase in adrenergic drive results in increased arterial tone, and increased blood pressure. However alterations in purinergic neurotransmission in hypertension are not known. ATP released from sympathetic perivascular nerves plays a role as a neurotransmitter acting at P2X receptors. ATP and its metabolite adenosine are neuromodulators at the sympathetic nerve terminal. Therefore understanding the regulation of ATP as a neurotransmitter in physiological and pathophysiological conditions is needed.

Several changes in purinergic neurotransmission in hypertension were revealed in these studies, which, taken together, give insight into changes in the nerve terminal. First, I found increased variability of EJPs in DOCA-salt rats which on average had lower amplitude than controls. Second, I saw that EJPs rundown faster in DOCA-salt arteries. It was important to design a series of experiments to differentiate pre- from postjunctional changes because in DOCA-salt hypertension as post-junctional changes could also affect EJP rundown and variability. Some of the results include: 1) EC_{50}s and maximum constriction in response to exogenous ATP were not different between DOCA-salt and control arteries and 2) The rate of desensitization of P2X receptors in response to

exogenous α,β methylene ATP application was slower in DOCA-salt rats compared to controls, suggesting that desensitization of the P2X receptors was not responsible for the observed rundown of EJPs in response to 10 Hz trains of nerve stimulation.

Another postjunctional change which may account for some of the variability in EJP amplitude distribution is changes in the properties of SMCs. SMCs are coupled through an electrical syncytium making it more difficult to control local from distant neurotransmitter release sites during intracellular recordings. Although RMP was depolarized in hypertensive rats, variability of RMP, a measure of SMC coupling (Young et al., 2007) did not change in DOCA-salt hypertension. In addition, adding hyperpolarizing current to SMCs from DOCA-salt rats did not change EJP amplitude even slightly. So, although changes do occur to SMCs in hypertension, the results of these studies suggest that the alterations in purinergic neurotransmission are due primarily to changes in ATP handling in sympathetic nerve terminals.

In order to come to the conclusion that changes in purinergic neurotransmission are likely to originate at the sympathetic nerve terminal, I used pharmacological techniques to differentiate between ATP depletion, increased inhibition of release, altered Ca^{2+} handling, etc. There are many factors which contribute to the probability and amount of neurotransmitter released. As Bennett concluded in his review of autonomic neuromuscular transmission, "...transmission from individual varicosities involves the release of variable size packages of transmitter onto different size receptor patches, with different

varicosities possessing markedly different probabilities for secretion of a transmitter package (Bennett, 1996). In DOCA-salt hypertension, the increased variability may due to changes in any of these factors.

I was able to make several conclusions about changes in the pre-junction due to the following results: 1) N-type calcium channel function was unchanged; 2) Ca^{2+} sensitivity was unchanged; 3) and α_2AR receptor function is impaired in DOCA-salt rats. However I also learned that α_2AR only regulates the first few EJPs in a high frequency train. Therefore α_2AR dysfunction is not responsible for increased rundown for example. Other prejunctional receptors which regulate neurotransmitter release via PTX-sensitive Gi/Go proteins are also not responsible for the changes in purinergic neurotransmission.

The results of these studies show the following: 1) increased EJP recovery time (τ) after trains of stimulation in DOCA-salt rats and 2) decreased facilitation remains in the presence of α_2AR inhibition in DOCA-salt hypertension. There are several possible mechanisms that could contribute to these changes. One possibility is that there is decreased ATP bioavailability in the nerve terminal. Other possible mechanisms include changes in the ability of ATP to be stored in vesicles or for vesicles to be mobilized and docked in DOCA-salt rat sympathetic nerve endings. There is precedent for decreased ATP bioavailability in hypertension and in synapses where there is increased ROS (Postnov et al., 2007). ATP is depleted in nerve endings which show characteristics of increased Ca^{2+} handling and vesicular turnover, such as the case in hypertension (Postnov et al., 2007). For example, ATP is decreased and ADP increased in SHR rats compared

to WKY controls (Pisarenko et al., 2000). Mitochondria, targets for ROS, are densely distributed throughout the nerve terminal, which, if damaged, could result in a significant decrease in ATP production. For example decreased ATP content in patients with hypertension secondary to thyrotoxicosis is a result of uncoupling of mitochondrial oxidative phosphorylation (Silvestri et al., 2005). Also mitochondria from brains of SHRs have a decreased rate of ATP synthesis (Doroshchuk et al., 2004). Increased rundown and impaired facilitation and smaller EJPs could all be explained by decreased ATP availability. However, I would expect EJPs to also be inhibited faster in DOCA-salt rats compared to controls with lower Ca^{2+} in the extracellular buffer. However this was not the case. This conclusion also does not explain the increased variability in EJP amplitudes. This idea could be strengthened by measuring ATP/ADP ratios in the nerve endings, but the small size of the varicosities makes this technically challenging. Assessing mitochondrial shape, size and number using fluorescent or TEM techniques may provide insight into this hypothesis. Measuring ATP in the sympathetic ganglia may be a way to assess overall availability in the nerve.

The other possibilities, that ATP storage into vesicles is impaired, or vesicle mobilization is impaired can be grouped together as a hypothesis of impaired ATP handling. Facilitation at the neuroeffector junction occurs due to increased calcium in nerve terminals in response to trains of stimulation (Brain and Bennett, 1997), but is impaired in DOCA-salt arteries. These data in conjunction with increased τ suggests that calcium influx may be compromised in these nerve terminals, but my results suggest that this isn't the case. I attempted

to disrupt uptake of ATP into vesicles to assess if there were changes in ATP storage in nerve terminals from DOCA-salt rats. NE is taken up into vesicles via a vesicular monoamine transporter, but until recently it was not known how ATP was transported into vesicles for release. Bafilomycin A1, a vATPase inhibitor was used to decrease the H^+ gradient which neurotransmitters use to load into vesicles. However, I obtained inconclusive results, as it did not lower EJPs in control or DOCA-salt arteries after up to 2 h of bafilomycin treatment. Guanethidine, a known inhibitor of NE secretion, decreased EJPs to a similar extent in control and DOCA-salt arteries, although Hill-slope was diminished (Chapter 4). Guanethidine is transported into the terminal via the norepinephrine transporter. Therefore a change in Hill-slope is difficult to interpret due to alterations in NET function in hypertension (Esler, 1993), as decreased cooperativity of ATP inhibition could be due to decreased NET function. Recently a vesicular nucleotide transporter (SLC17A9) has been identified and should help reveal the molecular mechanism of ATP vesicular storage and secretion from sympathetic nerves (Sawada et al., 2008). Future studies targeting this transporter pharmacologically or with knock-out studies will also provide insight into the importance of purinergic neurotransmission in hypertension.

Vesicular mobilization is also hard to study at the sympathetic neuroeffector junction due to the small nerve terminals. Transmission electron microscopy would help to gain insight into the sympathetic nerve terminal and patterns of vesicle distribution (Luff et al., 1987; Glass et al., 2006). Photomicrographs would be able to show decreased number of vesicles or altered

arrangement of vesicles in the nerve terminal, although identifying purinergic vs. adrenergic vesicles is not possible. Photomicrographs could also identify changes in mitochondrial number and ultrastructure, distances from the postjunction and density of nerve terminals.

Role of ROS in purinergic neurotransmission

Whether it is ATP storage or vesicular mobilization, it appears that ROS play a role in altering their function. ROS are increased in the neuroeffector junction of DOCA-salt hypertensive rats where they modulate neurotransmission (Kolo et al., 2004b, 2004a). However a number of questions remain including, what are the most sensitive targets of ROS in the nervous system, and how do they modulate neurotransmission in physiological and pathophysiological conditions? O_2^- and H_2O_2 are oxidants which can modify cysteine, methionine or tyrosine groups on proteins. The α_2AR contains cysteine residues which share disulfide bonds. Another vesicular release proteins, SNAP25 is particularly sensitive to ROS (Giniatullin et al., 2006). In these studies, I attempted to make a connection between the increase in ROS and impairment of the α_2AR, in particular, in DOCA-salt hypertension. I hypothesized that increased ROS targets proteins in sympathetic nerve endings resulting in impaired neurotransmission and increased blood pressure.

It was unclear how ROS, thought to be generated in SMCs and endothelial cells could impair proteins and vesicle release processes in the sympathetic nerve terminal. ROS are generated as a byproduct of oxidative phosphorylation in the

mitochondria, which are abundant in the nerve terminal. In addition the presence of O_2^- generating enzymes, such as NADPH oxidase in the nerve terminal provides another source of ROS. The co-localization of NADPH oxidase subunits to periarterial nerve fibers and endings provides a realistic possibility for the interaction of ROS with vesicle release apparatus in sympathetic nerve endings. The presence of NADPH oxidase subunits in sympathetic and sensory nerve findings is novel, and further studies of its function in nerve fibers will provide information regarding the origin of ROS found at the NEJ.

The relationship between O_2^- and neurotransmission may also involve an intermediate signaling molecule such as H_2O_2. O_2^- is spontaneously, or with the help of SOD, converted to H_2O_2 almost instantaneously *in vivo*. H_2O_2 is a more stable free radical which can cross plasma membranes and modulate synaptic transmission. H_2O_2 acting at presynaptic targets is not novel. H_2O_2 affects neurotransmission in several CNS brain regions including dopamine (DA) release in dorsal striatum, substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA), (Avshalumov et al., 2000; Chen et al., 2002) and glutamate-generated populations spikes in hippocampal slices (Avshalumov et al., 2000). H_2O_2 has also been shown to alter neurotransmission in NMJs (Giniatullin et al., 2005; Giniatullin et al., 2006), producing a depressant action on synaptic transmission via direct impairment of SNAP25, a protein important for vesicular release (Giniatullin et al., 2006). It is possible that H_2O_2, or another metabolite of O_2^- is the actual effector molecule in sympathetic neuroeffector junction as well.

If H_2O_2 is the effector molecule, then this could explain the differential effects seen with tempol and apocynin *in vivo*. Tempol, an SOD mimetic, decreases O_2^-, but generates H_2O_2, a molecule which according to studies in other synapses, may be just as detrimental to nerve endings. On the other hand, apocynin inhibits O_2- generation, albeit through only one of several mechanisms. Therefore the superior effects of this drug on several of the endpoints assessed in purinergic neurotransmission may be due to decreased generation of ROS and implicates NADPH oxidase as a major ROS contributor in the nerve ending as it is in vascular cells in hypertension (Lassegue and Clempus, 2003).

NAPDH oxidase must be stimulated for cytosolic components to be translocated to the membrane to form a functional complex. In vascular cells this occurs in response to humoral (Ang II) and physical factors (stretch, strain) (Lassegue and Clempus, 2003). There are several generators of ROS in the nerve terminal, but the predominant source is not known. In order for NADPH oxidase to be functional and contribute to ROS production, membrane-bound subunits must be phosphorylated and cytosolic subunits translocated to the membrane. Research by Glass et al. have shown that NADPH oxidase subunits in cardiovascular nuclei in the brainstem can be translocated in response to Ang-II or phenylephrine-induced hypertension (Glass et al., 2007). Phenylephrine, an αAR agonist, may mimic increased adrenergic neurotransmission as seen in DOCA-salt hypertension and stimulate NADPH oxidase subunit translocation.

P2 receptor signaling uses ROS to regulate neurotransmitter release. P2 receptor activation increases O_2^- via glial NADPH oxidase in the CNS

(Parvathenani et al., 2003). Also in the frog NMJ, ATP has an inhibitory effect on ACh release via ROS production (Giniatullin et al., 2005). This possibility may deserve more attention at the sympathetic nerve terminal as P2 receptors found there regulate neurotransmitter release in adrenergic nerves of rat prostate (Morikawa et al., 2007). P2X receptors may not only be generators, but targets of ROS. Because of the similarities between ACh release at NMJs and ATP release from sympathetic nerves, comparisons between the two can be made (Zimmermann, 2008). Damage to the nicotinic acetylcholine receptor (nAChR) due to ROS in post-ganglionic nerves results in a use-dependent, long-lasting rundown of ACh-evoked currents in cultured sympathetic neurons. These effects were found to be specific for nAChRs on neurons, but not muscle resulting in impaired signaling (Campanucci et al., 2008). It is possible, that prejunctional P2X receptors on sympathetic nerve terminals may also be sensitive to ROS.

It is likely that O_2^- in sympathetic nerve endings is also produced from non-NADPH oxidase mechanisms, with a likely candidate being the electron transport chain in mitochondria. This pathway provides a direct connection between ROS and ATP production, strengthening the relationship between the two. Increased neurotransmitter release and vesicle recycling, seen in hypertension, requires more energy (ATP). O_2^-, a byproduct of oxidative phosphorylation, will therefore also increase. Mitochondria, which are densely distributed in nerve endings, are particularly vulnerable to oxidative stress and ATP levels decrease as a result (Ballinger et al., 2000). Hazel Szeto points out that "because dysfunctional mitochondria will produce more ROS, a feed-forward

loop is set up whereby ROS-mediated oxidative damage to mitochondria favors ROS generation, resulting in a vicious cycle" (Szeto, 2006). This suggests that in addition to NADPH oxidase derived ROS, mitochondrial-derived oxidative stress might also damage nerve fibers and therefore serve as a potential target for anti-oxidant therapy and neuroprotection of sympathetic nerves.

Acute treatment with apocynin did not improve purinergic neurotransmission in DOCA-salt rats. Chronic treatment with apocynin, begun before rats were hypertensive, protected sympathetic nerves from changes to the neuroeffector junction which occurred in DOCA-salt control rats. These data suggest that once ROS have increased above a certain threshold oxidative stress causes permanent damage to proteins and lipids among other irreversible changes such as vascular remodeling and deposition of extracellular matrix proteins (Landmesser and Harrison, 2001). Therefore regardless of the source of ROS in hypertension, antioxidant therapy would be most effective if given early, and administered chronically.

I've shown that changes in sympathetic nerve terminals play an important role in blood pressure regulation and dysfunction in sympathetic nerves due to oxidative stress results in high blood pressure. However the mechanisms for these alterations remain elusive. Unfortunately, efficacious and selective anti-oxidants are lacking. The choices available are short-lived and have various off-target effects. For example, apocynin, once thought to be a selective NADPH-oxidase inhibitor, has been shown to be a general ROS scavenger (Heumuller et al., 2007), and tempol has been shown to have direct sympathomimetic effects as

a BK channel agonist (Xu et al., 2004). It might be beneficial then, to investigate the role of ROS on nerve endings and blood pressure regulation using genetic models such as $gp91^{phox}$ knockout mice which were used to study the role of ROS on neurogenic vasodilation (Starr et al., 2008). Challenging the $gp91^{phox}$ knockout with a uninephrectomy and mineralocorticoid activation would result in attenuated blood pressures and unaltered neurotransmitter release compared to DOCA-salt controls if NADPH oxidase-derived ROS are involved in DOCA-salt hypertension. This model and similar models with genetic manipulations of NADPH oxidase subunits could provide more information regarding the role of NADPH oxidase-derived ROS on sympathetic neurotransmission and hypertension.

In summary, nerves use ROS as signaling molecules and neuromodulators. However too many ROS can lead to oxidative stress. Cysteine residues, and downstream signaling molecules, such as Gi/Go proteins (Nishida et al., 2000), provide targets for oxidative stress. I have shown that impaired purinergic neurotransmission is associated with increased blood pressure. Treatment with antioxidants restored blood pressure, lowered sympathetic nerve activity and restored neurotransmission at sympathetic nerve terminals. These data show the importance of ROS as a signaling molecule and a neuromodulator in both physiological and pathophysiological conditions.

Mechanisms and differential storage, regulation and release of ATP and NE

NE and ATP are both released from sympathetic nerve terminals onto mesenteric arteries. In chapter 5 two complimentary techniques were used to detect the release of ATP and NE from nerve terminals in DOCA-salt rats with and without treatment with antioxidants. Intracellular recordings of EJPs in combination with electrochemical detection of NE from a few sympathetic nerve endings allows for a novel look at the regulation and release patterns of cotransmission. The results reveal differential release patterns of the two neurotransmitters. For example the distribution pattern of EJP amplitudes is different between control and DOCA-salt arteries (Chapter 4) and changes with antioxidant treatment (Chapter 5). However NE peak current distribution pattern is normally distributed in all treatment groups. This suggests that changes in the NEJ of DOCA-salt hypertensive rats differentially affect regulation of ATP and NE release. This is also seen in the differential effects of tempol and apocynin on EJP amplitudes and NE current, as tempol restores NE release, but does not have an effect on ATP release. Interestingly, in the somatic NMJ, ATP (but not adenosine) uses H_2O_2 to inhibit ACh release (Giniatullin and Sokolova, 1998), and in the CNS H_2O_2 modulates dopamine release in the substantia nigra (Chen et al., 2002). Perhaps H_2O_2 has a preferential effect on regulation of NE release, while O_2^- inhibits ATP release. Thereby using tempol as an antioxidant increases H_2O_2 ($\rightarrow$ decreased NE release) and decreases O_2^- ($\rightarrow$ increased ATP release).

ROS signaling is one possible mechanism for differential regulation of NE and ATP. There are several other explanations including: 1) different ratios of NE and ATP in the nerve terminal 2) Different distances of NE and ATP-

containing vesicles from calcium channels 3) different isoforms of synaptic proteins or Ca^{2+} channel subtypes in sympathetic nerve endings. A review of neurotransmitter specific exocytosis mechanisms by Langley and Grant lists almost a dozen proteins involved in exocytosis which vary between different cell types and possibly between the particular NT being released (Langley and Grant, 1997). Examples include Ca^{2+} channel subtype, synapsin, synaptotagmin isoforms, SNAP-25, etc. Exploring protein differences within a single synapse will shed light onto the mechanisms for differential regulation of co-transmitters at the neuroeffector junction.

Limitations of experiments

Most of what we know about vesicle pools, facilitation, rundown, and synaptic transmission we know from calyx-like nerve endings in the CNS or large neuromuscular junctions, where the presynaptic nerve endings are large enough for direct intracellular or whole-cell patch recordings. However, the sympathetic nerve terminal is extremely small, and therefore techniques which are available to study neurotransmission in larger synapses can't be used. Autonomic nerves innervating the vas deferens are also small, but due to the dense innervation, lose patch pipettes have been used to measure excitatory junctional currents (EJCs) (Brock and Cunnane, 1988). Recently, the use of orthograde transport of calcium indicators from the sympathetic ganglia to the nerve terminal have been used in conjunction with EJC measurements to study neurotransmission from single varicosities (Brain and Bennett, 1997). Mesenteric arteries are not as densely

innervated as isolated vas deferens, and this may be why these techniques have not been used in this preparation. However the use of calcium imaging allows for the identification of nerve terminals, and may make it possible to study sympathetic terminals of perivascular nerves using lose patch pipettes.

Amperometry is another technique which provides precise spatial and temporal measurements of vesicular release (Bruns, 2004). It has been applied to many other systems *in vitro*, but only recently has been used to study the vascular neuroeffector junction. Thus far only NE has been studied from perivascular nerves using amperometry, but in combination with intracellular recordings of EJPs, data about both transmitters can be made simultaneously on an impulse by impulse basis (Dunn et al., 1999). Adenosine is also electroactive and amperometric techniques coupled to carbon-fiber or diamond microelectrodes may also help us understand neuroeffector transmission. Using these techniques will provide more information on neurotransmitter regulation and release and how this is altered in hypertension.

Importance of purinergic neurotransmission in vivo

It has been firmly established that ATP acts as a cotransmitter at a large number of synapses and junctions (Sneddon and Burnstock, 1984; Sneddon and Westfall, 1984; Msghina et al., 1992; Burnstock, 1995; Bobalova and Mutafova-Yambolieva, 2001; Westfall et al., 2002; Conceicao et al., 2005), including myenteric neurons, where ATP has been identified as a fast synaptic transmitter in a physiologically identified pathway (Galligan and North, 2004). However, the

physiological role of purinergic neurotransmission has not been fully elucidated in the cardiovascular system. There are several hypothetical roles for purinergic neurotransmission in blood pressure regulation. I will address and provide evidence for possible physiological roles for ATP as a co-transmitter at the vascular neuroeffector junction.

ATP is a short acting molecule, and therefore one of its most important roles may be in mediating immediate, transient modifications of vessel tone, opposed to the longer-lasting effects of NE (Tarasova et al., 1998; Golubinskaya et al., 1999) In this regard it plays an important role in renal hemodynamics and the renal microcirculation (Inscho, 2001), and impaired P2X receptor signaling is associated with Ang II-induced hypertension in rats (Zhao et al., 2005). ATP also plays an important role as a neuromodulator at sympathetic nerve endings (Boehm, 1999) and possibly communicates with afferent sensory nerves as well. ATP and its metabolite adenosine feedback to sympathetic nerve endings to increase or decrease ATP, respectively. This modulatory action is important in blood pressure regulation as exemplified by treatment of rats with DPSPX, a non-selective antagonist of adenosine receptors. Treatment with DSPX causes a hypertensive state which lasts for at least one month after the administration of the drug had been stopped (Guimaraes et al., 2003). This suggests that a decrease in ATP release in DOCA-salt hypertension could cause increased NE release from sympathetic nerves. Thirdly, when ATP is released it can modify other effector cells, besides SMC, such as neighboring sensory nerves which contain P2Y receptors (Burnstock, 2004). This type of communication is called cross-talk and

is possible due to the close proximity of sensory and sympathetic fibers, and the nature of non-synaptic NEJs shown in TEM photomicrographs (Burnstock, 2008).

Finally, I have only looked at arteries in these studies, and the effects of ATP acting at veins are still controversial. Although venous constriction can be blocked almost completely with prazosin, an α_1AR antagonist, there are functional P2Y receptors located on mesenteric veins, which are important *in vivo*. The role of these receptors has not been completely characterized, but may produce either constriction (Galligan et al., 2001) or relaxation (Calvert et al., 2008). Dysfunction of P2Y receptors on veins, resulting in decreased relaxation for example, may have dramatic impact on blood pressure.

As ATP is likely to play a role in some or all of these physiological mechanisms, its role in the development and maintenance of hypertension is not known, and binding to P2X receptors may not be the only important mechanism in blood pressure regulation. The *in vivo* effect of altered purinergic neurotransmission from sympathetic nerve terminals in hypertension should be explored further.

Possible applications of these results in the treatment of essential hypertension

The results of these studies show that 1) purinergic neurotransmission is altered in DOCA-salt hypertension and 2) Some of these dysfunctions can be restored with the antioxidant treatment. Modification of purinergic neurotransmission by targeting P2 or P1 receptors for the treatment of high blood pressure is a novel idea, and one that would be particularly useful if alterations in

ATP release are a cause, rather than an effect of high blood pressure. There are several types of purinergic receptors which may be activated by the release of ATP from the nerve terminal (Burnstock, 2008), which increases the number of receptors as pharmacological targets. ATP activation of $P2X_1$ receptors on arterial SMCs is one example. Targeting $P2X_4$ receptors on endothelial cells provides another example as blood pressure was higher and remodeling lower in $P2X_4$ knock-out mice (Yamamoto et al., 2006).

There are other possible pathways involving ATP which are possible targets for treatment of hypertension. One of the largest questions remaining is the mechanism for ATP storage into synaptic vesicles. ATP is a negatively charged molecule, precluding it from diffusing across lipid membranes. It has been suggested that ATP sequestration from the cytoplasm is carrier-mediated, similar to carrier-mediated transport systems in rat brain synaptosomes (reviewed by Sperlagh and Vizi). Recently a vesicular nucleotide transporter (SLC17A9) has been identified opening the door for future studies which may elucidate mechanisms of ATP storage and secretion from nerves (Sawada et al., 2008) as potential therapies for high blood pressure.

Despite the extensive amount of money and research put into high blood pressure research over the last few decades, effective therapies for essential hypertension remain elusive. This may be due to the fact that we still don't know what is initiating these changes to begin with; why they occur; and what sets the "set-point?" Recent findings have opened the door for new avenues of research. I have found one area of research particularly interesting. Yuvenalii Postnov

investigates the correlation between energy conversion, ATP production and hypertension. He states, "Attenuated intracellular ATP content results in the long-term maintenance of elevated BP by increased sympathetic outflow, whereas augmented ROS production following mitochondrial dysfunction lowers the capacity of the NO-dependent vascular relaxation." (Postnov et al., 2007). This novel work shifts the role of ATP depletion to a cause and not just a result, of hypertension, and provides an integrated signaling cascade between ROS, ATP, NE and downstream effects of vascular tone. I think further studies of the altered energy balance of sympathetic nerves in chronic diseases such as hypertension will reveal an important role for ATP as a neurotransmitter.

In conclusion, the results of these studies show that purinergic signaling is impaired in DOCA-salt hypertension. The increased variability of EJP amplitudes, with normal responses to Ca^{2+}, suggest that ATP storage and/or mobilization of vesicles to the membrane is impaired, and that increased ROS in perivascular nerves plays a role in these changes. The differences in variability are seen only in measurements of purinergic neurotransmission suggesting that there are subsets of vesicles in the nerve terminal which contain primarily NE and some which contain primarily ATP, and these two subsets are differentially regulated. Treatment with apocynin and tempol changed the distribution of EJP amplitudes suggesting that ROS plays a role in the storage of transmitters or mobilization of vesicles. Finally, treatment with antioxidants decreased blood pressure and restored sympathetic neurotransmission in DOCA-salt hypertensive rats.

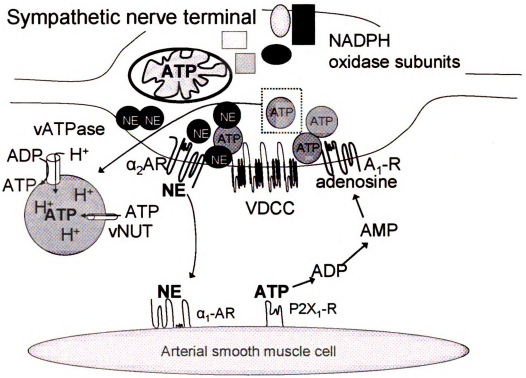


Fig. 34 Mesenteric neuroeffector junction in control rats. NE and ATP are released from sympathetic nerve terminals in response to nerve stimulation and depolarization of the nerve terminal. Activation of voltage dependent calcium channels provides sufficient Ca^{2+} influx for vesicle fusion with the membrane and subsequent neurotransmitter release into the extracellular space. NE binds to α_1ARs post-junctionally resulting in intracellular Ca^{2+} release, while ATP binds to ligand-gated $P2X_1$Rs post-junctionally resulting in Ca^{2+} influx and subsequent constriction of the smooth muscle cell (SMC). In control animals purinergic neurotransmission dominates. Both neurotransmitters feed back to prejunctional autoreceptors which decrease the further release of NE and ATP through Ca^{2+} channel blockade. vATPase on the vesicle membrane creates a H^+ gradient which transporters use to fill vesicles with NE and ATP. ATP is thought to move into the membrane via the newly discovered vesicular nucleotide transporter (vNUT). Although reactive oxygen species are produced as a byproduct of oxidative phosphoylation in the mitochondria, endogenous antioxidants keep levels low. NADPH oxidase subunits are present, but activity of the enzyme is not known, and assumed to be relatively low. Cross talk between receptors on the nerve ending results in a tight regulation of NE and ATP release.

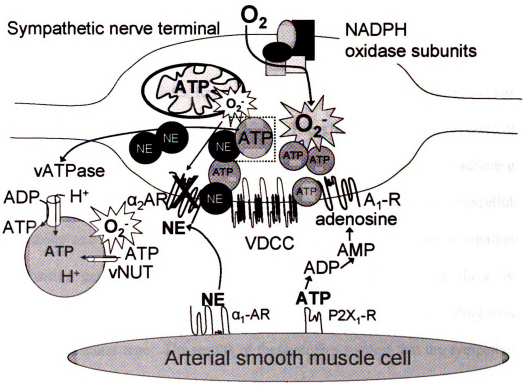


Fig. 35 Mesenteric neuroeffector junction in DOCA-salt rats. In DOCA-salt hypertension several changes occur to the sympathetic nerve ending which affects neurotransmitter release. Voltage dependent calcium channels remain functional, providing sufficient Ca^{2+} influx for vesicle fusion with the membrane and subsequent neurotransmitter release into the extracellular space. However, there is a shift towards increased NE release, but decreased ATP release which is likely to play an important role in increased vascular tone in hypertension, as the transient control provided by ATP is lost. Furthermore there is impaired handling of ATP due either to altered storage of ATP into vesicles or impaired mobilization of vesicles to the membrane which results in increased variability of EJPs. Increased ROS in the nerve terminal may target either vATPase or vNUT to cause alterions in the storage of ATP into synaptic vesicles. Negative feedback is also impaired, due to ROS targeting the α_2AR, affecting both NE and ATP release. NAPDH oxidase subunits are present and active; as NADPH oxidase inhibitor, apocynin, restores function at the sympathetic nerve ending and lowers blood pressure. The increase in ROS in hypertension is likely to play a role in the changes seen in DOCA-salt hypertension.

Perspectives

The last two decades have solidified ATP as a co-transmitter in many synapses including the sympathetic NEJ. The present work has revealed the importance of understanding changes in purinergic as well as noradrenergic components of neurotransmission in hypertension. The use of intracellular recordings and amperometry to detect ATP and NE from a few sympathetic varicosities gave me insight into the differential regulation of these two neurotransmitters, and revealed the complexity of neurochemical signaling which maintains vascular tone. The results of these studies suggest that the sympathetic nerve terminal is dynamic and alterations in neurotransmission are one of many factors contributing to salt-sensitive hypertension. Continuing advancements in the ability to measure from a few, or even one varicosity from perivascular nerve endings will advance our knowledge of neurotransmitter storage, regulation and release from these small nerve terminals and how they are altered in hypertension.

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