IMPAIRED FUNCTION OF PREJUNCTIONAL ADENOSINE RECEPTORS ON PERIVASCULAR SYMPATHETIC NERVES IN SALT-SENSITIVE HYPERTENSION

By

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ABSTRACT

IMPAIRED PERIVASCULAR ADRENERGIC NEUROTRANSMISSION BY THE PREJUNCTIONAL ADENOSINE RECEPTORS IN DOCA-SALT HYPERTENSION

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Hypertension is a major public problem that will affect more than 1.6 billion people worldwide by 2025. Essential hypertension is associated with sympathetic nerve hyperactivity and 50% of the patients are salt sensitive. Deoxycorticosterone acetate (DOCA)-salt rat model is a low rennin form of hypertension with transient salt and water retention and is a model of salt-sensitive human hypertension. Norepinephrine (NE) and ATP are the major cotransmitters relased from perivascular sympathetic nerve varicosities supplying arteries and veins. NE and ATP regulate pheripheral arterial resistance and venous capacitance. Release of NE and ATP is precisely regulated by prejnctional autoreceptors. NE acts on the α_2-adrenergic receptors (α_2AR) to inhibit NE and ATP release and the α_2AR function is impaired in DOCA-salt rats. ATP is hydrolyzed to adenosine which activates prejunctional adenosine A_1 receptors (A_1Rs) and A_{2A}Rs to modulate NE and ATP release. This work determined role of prejunctional A_1Rs and A_{2A}Rs in adrenergic transmission. I also tested the hypothesis that prejunctional A_1R and A_{2A}R function is impaired

in sympathetic nerves supplying mesenteric arteries and veins of DOCA-salt rats. Electricall-evoked NE release and constriction of blood vessel were measured *in vitro* by using amperometry and video microscopy. Drug effects on the kinetics of NE oxidtion currents were measured. My results show that prejunctional A_1Rs couple to inhibition of NE release and A_1R function is impaired in periarterial nerves of DOCA-salt rats. I also showed that prejunctional A_{2A}Rs couple to stimulation of adenosine transporter in perivenous sympathetic nerves and this receptor function is also compromised in DOCA-salt hypertension. These data suggests that the perivascular sympathetic nerve varicosities are an important target for the pathophysiology of salt sensitive hypertension. Furthermore, sympathetic autoreceptor dysfunction is not specific to α_2-adrenergic receptors, but there is a more general disruption of prejunctional mechanisms controlling sympathetic neurotransmitter release. Finally, the data illustrate differential autoreceptor dysfunction in arteries and veins of DOCA-salt hypertension.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A_1R	A_1 adenosine receptors
$A_{2A}R$	A_{2A} adenosine receptors
$A_{2B}R$	A_{2B} adenosine receptors
A_3R	A_3 adenosine receptors
ACh	Acetylcholine
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CGS21680	2-*p*-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride hydrate
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CPA	N^6-cyclopentyl-adenosine
DA	Dopamine
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DMI	Desipramine
DOCA	Deoxycorticosterone acetate

DOPA	Dehydroxyphenylalanine
ENS	Enteric nervous system
GPCR	G protein coupled receptor
IMG	Inferior mesenteric ganglia
IP_3	Inositol 1,4,5-triphosphate
JNC7	Seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure
K_{ATP} channel	ATP-sensitive potassium channel
MA	Mesenteric artery
mAChR	Muscarinic acetylcholine receptor
MAO	Monoamine oxidase
MCFP	Mean circulatory filling pressure
mmHg	Millimeter mercury
MA	Mesenteric arteries
MV	Mesenteric vein
Na^+	Sodium ion
nAChR	Nicotinic acetylcholine receptor
NaCl	Sodium chloride
NE	Norepinephrine
NPY	Neuropeptide Y
OVLT	Organum vasculosum lamina terminalis
PLC	Phospholipase C

PNS	Peripheral nervous system
R	Resistance
SBP	Systolic blood pressure
SMG	Superior mesenteric ganglia
TH	Tyrosine hydroxylase
TPR	Total peripheral resistance
UK14304	5-Bromo-N-(2-imidazolin-2-yl)-6-quinoxalinamine,5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine, brimonidine
VMAT	Vesicular monoamine transporter
α_1-AR	Alpha1-adrenergic receptor
α_2-AR	Alpha2-adrenergic receptor
β-NAD	Beta-nicotinamide adenine phosphate

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview

Adenosine is an ATP precursor and metabolite that is ubiquitous and that produces a wide variety of physiological responses (Olah *et al.*, 2000; Tabrizchi *et al.*, 2001). In the cardiovascular system, adenosine plays an important role in controlling cardiac and vascular function (Srinivas *et al.*, 1998; Tabrizchi *et al.*, 2001). Considerable progress has been made in understanding adenosine receptor subtypes and their signaling mechanisms in the cardiovascular system. However, due to the breadth effects of adenosine, our understanding about adenosine receptor function in the sympathetic nerves supplying arteries and veins is less well developed, particularly in hypertension. This issue is important in MA and MV because they are densely innervated by sympathetic nerves (King *et al.*, 2007). Sympathetic nervous system control of the mesenteric circulation contributes significantly to blood pressure regulation by two mechanisms. Firstly, sympathetic nerves contribute to regulation of vascular resistance primarily in small MA (Fenger-Gron *et al.*, 1995; Kreulen, 2003; Rothe, 1983). Secondly, sympathetic nerves regulate capacitance of MV, which provide about 60-70% of venous return to the heart (Kreulen, 2003; Martin *et al.*, 1998; Pang, 2001). Understanding the contribution of adenosine and its receptors to control of the mesenteric circulation will help to understand the differential contributions of veins and arteries to blood pressure control and hypertension. This may also identify new drug targets for hypertension treatment.

1.2 The autonomic nervous system

Organization of the nervous system is illustrated in Figure 1. The nervous system is divided into the central nervous system (CNS) and peripheral nervous system (PNS). The CNS is composed of the brain and spinal cord. The PNS is further divided into the somatic and autonomic nervous systems (ANS). Somatic nervous system is divided into the sensory and motor systems, which are responsible for sensation and body movement respectively. Nearly all visceral reflexes are mediated by the autonomic nervous systems. The differences between the somatic and autonomic nervous systems are summarized in Table 1. The ANS is divided into three divisions: i) the sympathetic nervous system, ii) the parasympathetic nervous system, and iii) the enteric nervous system (ENS). The ENS resides within the gastrointestinal tract and it is a self-contained system as the disruption of connections between the CNS and the ENS does not alter substantially gastrointestinal function. The ENS is also supplied by sympathetic and parasympathetic nerves. Esophageal and stomach function are more dependent on sympathetic and parasympathetic innervation than the remaining portions of the gastrointestinal tract.

Figure 1. Shematic illustration of the organization of the nervous system.

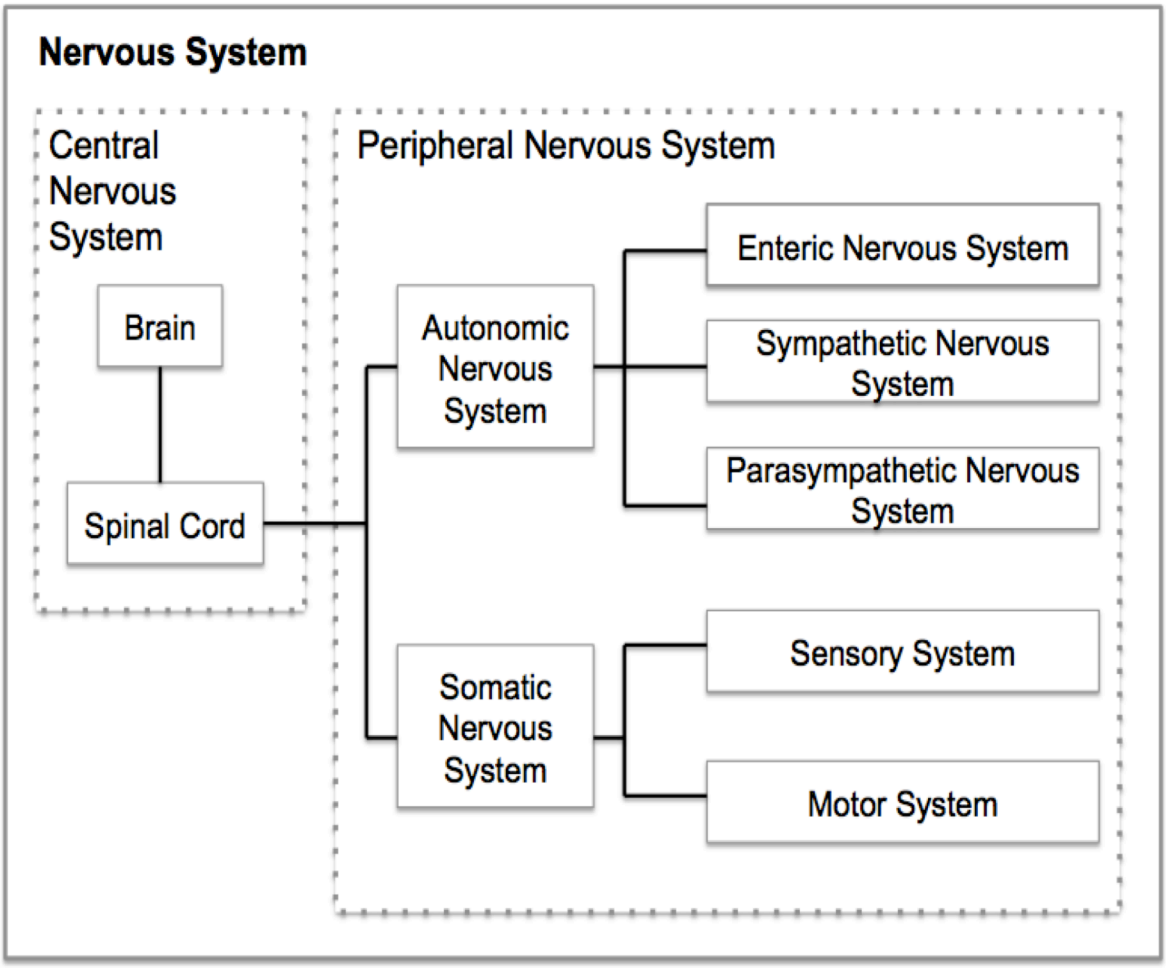


Table 1. Summary of differences between somatic and autonomic nervous system.

	Somatic Nervous System	Autonomic Nervous System
The number of neurons in efferent path	Single neuron from CNS to effector organs	Two-neuron chain from CNS to effector organs
Axon features	Heavily myelinated axon	Preganglionic neuron: Lightly myelinated axon Postganglionic neuron: Unmyelinated axon
Neurotransmitter/receptor at neuron-target synapse	ACh/nAChR	ACh/mAChR (Parasym) NE/ α or β-adrenoceptors (Sym)
Target tissue	Skeleton muscle	Smooth muscle Cardiac muscle Glands
Site of neurotransmitter release	Axon terminal	Varicosities and axon terminals
Effects on target tissue	Excitatory only: muscle contraction	Excitatory or inhibitory
Peripheral components found outside the CNS	Axon only	Preganglionic axons, ganglia, postganglionic neurons
Summary of function	Posture, movement, and sensation	Visceral functions (internal organ movement, and secretion, metabolism control)

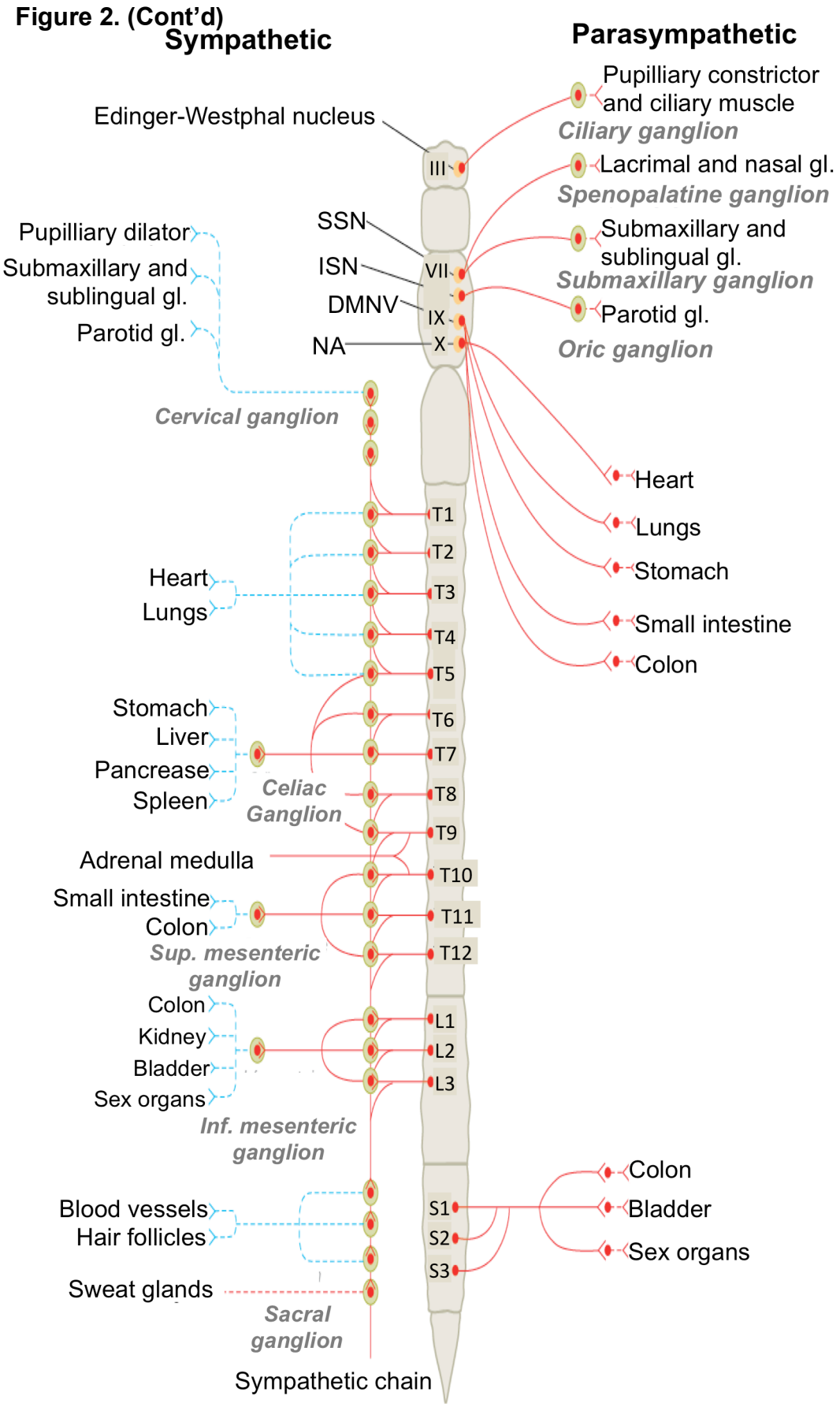
Figure 2 illustrates the anatomical features of the efferent paths of the sympathetic and parasympathetic nervous systems. The sympathetic neurotransmission consisted of a preganglionic cholinergic fiber, and a postganglionic adrenergic fiber. The preganglionic sympathetic axon leaves the spinal cord at thoracic and lumbar (l1-l2) level to innervate the cell body of the postganglionic sympathetic nerves at the peripheral ganglia of the sympathetic chains, which are alongside of the spinal cord. The postganglionic fibers from lower thoracic and lumbar regions pass through the sympathetic chain ganglia and form synapses onto the postganglionic neurons within the prevertebral glanglia including celiac ganglion, superior and inferior mesenteric ganglia. One preganglionic fiber could form synapse to numerous postganglionic neurons with approximately 1:10 ratio allowing well-established coordination of the sympathetic tone from different spinal levels. The postganglionic sympathetic axons (unmyelinated) leave the ganglia and projects onto their particular effector organs.

The parasympathetic preganglionic neurons reside within the brain stem and sacral spinal cord (Fig. 2). Unlike sympathetic chain ganglia that are close to the spinal cord, the parasympathetic ganglia lie close to or even reside in the effector target organs. Thus, the parasympathetic preganglionic axons are longer than that of sympathetic nervous system. The sympathetic and parasympathetic preganglionic axons release acetylcholine (ACh) onto the nicotinic-ACh receptor (nAChR) located on the postganglionic neurons. Once the postganglionic nerve is activated, it releases transmitter into effector organ to regulate the organ function. ACh is the major transmitter released from postganglionic parasympathetic nerves. Postganglionic

sympathetic nerve releases a variety of neurotransmitter including NE, ATP/ β-NAD and NPY onto the postjunctional receptors to regulate the activity of that particular target organ. The proportion of NE, ATP, β-NAD and NPY varies in different sympathetic varicosities. The sympathetic and parasympathetic nervous systems are tonically active exerting peripheral regulation under central control to maintain homeostasis including blood pressure, heart rate and water intake in response to environmental changes (Kandel *et al.*, 2000).

Figure 2 Anatomical features of sympathetic and parasympathetic divisions of the autonomic nervous system. ISN, Inferior salivatory nucleus; DMNV, Dorsal motor nucleus of vagus; gl, gland; NA, Nucleus ambiguus; SSN, Superior salivatory nucleus. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation. (Barrett *et al.*, 2012)**.**

Figure 2. (Cont'd)



1.3 Autonomic control of blood pressure

Arterial pressure drives blood flow through the entire systemic circulation. Arterial blood pressure is determined by cardiac output and total peripheral resistance (Martin *et al.*, 1998). Sympathetic nerve activation enhances heart rate and cardiac contractility leading to an increase in cardiac output and thereby increased arterial blood pressure. Conversely, parasympathetic nerves slow the heart and slightly affect peripheral resistance through release of the vasodilator nitric oxide. Parasympathetic nerves do not provide direct innervation to the vasculature. Under resting conditions, systemic arterioles are partially constricted by tonic sympathetic firing activity. Decreased sympathetic output leads to vasodilation (Mohrman *et al.*, 2010b). Besides, small arteries and arterioles primarily contribute to peripheral resistance. Thus, blood flow through an organ is regulated by adjustments in internal diameter of small arteries and arterioles. The sympathetic nervous system regulates vascular resistance and vascular capacitance by chemical synapse formation, which is described later in details in sympathetic neurotransmission section.

1.3.1 Sympathetic control of arterial resistance

Sympathetic nerves supply the adventitia of the arteries (Ekblad *et al.*, 1984). The unmyelinated periarterial postganglionic sympathetic nerve varicosities release NE and ATP/β-NAD that acts on the α_1-adrenergic and purinergic P2X receptors, respectively, on the arterial smooth muscle cells to mediate vascular contraction. Decreased arterial diameter enhances total peripheral resistance, hence decreases blood flow. Total peripheral resistance is a major contributor to arterial pressure. As the

systemic organs are arranged in parallel and the vessels in individual organ are connected in series, total peripheral resistance could be determined by this equation:

$$\frac{1}{TPR} = \frac{1}{R_{Organ_1}} + \ldots + \frac{1}{R_{Organ_n}}$$

$$R_{organ} = R_{arteries} + R_{arterioles} + R_{capillaries} + R_{venules} + R_{veins}$$

Where, TPR is total peripheral resistance, and R is resistance of each vascular bed as indicated.

The resistance of small arteries and arterioles is the key determinants for total peripheral resistance as the capillary and venous resistance is small in comparison to arterial and arteriolar resistance (Fig. 3).

1.3.2 Sympathetic control of venous capacitance

Veins are also very important for blood pressure control as 70% of the total blood volume resides in peripheral veins (Mohrman *et al.*, 2010c). The term “compliance” depicts how much the blood volume changes upon a change in pressure. The volume-pressure relationship in arteries is different from the venous compartment (Fig. 4). Veins have a relatively thin muscle layer and they are highly compliant. Therefore, venous pressures are relatively insensitive to normal volume changes. Arteries and arterioles have more muscular walls and are therefore less compliant than veins. This makes arterial pressure very sensitive to volume changes. It is important to note that small increase or decrease in venous compliance cause a significant amount of blood volume to shift to the heart increasing cardiac output and arterial blood volume.

Changes in cardiac output are transient however, and elevated arterial pressure is due largely to elevated blood volume in the low compliances arterial circulation. Thus, veins do make important contributions to normal regulation of blood pressure. Alterations in the factors that control venous compliance (sympathetic nerve activity for example) can then impact long-term blood pressure control and hypertension development (Fink *et al.*, 2000; Safar *et al.*, 1987; Simon *et al.*, 1975; Simon *et al.*, 1976).

Although the veins acquire sympathetic-independent myogenic tone, they also receive considerable sympathetic innervation to regulate venous capacitance. Sympathetic nerves distribute deeply into the border of the adventitia and media of the veins. Sympathetic nerves release NE onto venous smooth muscle to mediate venous contraction through activation of α_1-adrenergic receptors. The veins are more sensitive to neurogenic-(Nilsson *et al.*, 1985) ,and NE-induced contraction than the arteries (Perez-Rivera *et al.*, 2003).

Elevated sympathetic nerve activity associated with decreased peripheral venous capacitance has been established in animal models and hypertensive subjects. The importance of this phenomenon is that when peripheral veins contract, blood is shifted from peripheral venous pool and enters the central pool (Vena Cava). This increased central venous volume tends to increase cardiac filling, and mean circulatory filling pressure (MCFP) (Fink *et al.*, 2000; Perez-Rivera *et al.*, 2003); hence, increased stroke volume and cardiac output (Perez-Rivera *et al.*, 2003) via Starling's law of the heart. This is extremely importance as increased cardiac output raises arterial blood pressure.

1.3.3 Role of sympathetic supply on splanchnic circulation

As the splanchnic circulation receives high blood flow and contains approximately one third of total blood volume, splanchnic vascular adjustment importantly affects overall cardiovascular hemodynamics. The sympathetic nervous system plays an important role in controlling splanchnic vascular tone and the postganglionic sympathetic nerves densely innervate mesenteric arteries and veins. Maximal neurogenic vasoconstriction of splanchnic vasculature leads up to 80% reduction in blood flow through splanchnic region and causes a huge blood mobilization from peripheral mesenteric veins to the central vena cava. The mesenteric arteries and veins are the target of the present study as they imitate the most important peripheral resistance and capacitance vessels respectively.

1.3.4 Long-term regulation of blood pressure

There are a number of factors involved in long-term pressure regulation. Long-term pressure regulation is associated with blood volume control, and sodium handling, which affects plasma osmolality. Changes in mean arterial pressure can be accomplished by alteration of cardiac output and/or total peripheral resistance. Increased blood volume augments activity of sympathetic nerves supplying the heart, but not the kidney or lumbar regions. Increased plasma osmolality also influences long-term arterial pressure. Elevated osmolality by high-sodium intake is relevant to salt-sensitive hypertension. A small increase in plasma osmolality induced by high-salt intake enhances muscle sympathetic nerve activity and plasma NE, as osmoreceptors in hypothalamus (organum vasculosum of the lamina terminalis (OVLT), supraoptic

nuclei, and median preoptic nuclei), which are highly sensitive to plasma osmolality alterations. The supraoptic nuclei also synthesize vasopressin, which is then transported to posterior pituitary gland. The significance of vasopressin is described later in DOCA-salt rat model of hypertension. Moreover, high-salt intake is positively correlated with plasma Na^+ levels and sympathetic nerve activity, which may contribute to increased regional sympathetic activity in humans (Farquhar *et al.*, 2006) and animal models of salt sensitive hypertension (O'Donaughy *et al.*, 2006). It could be argued that increased plasma osmolality may not be sustained under normal physiological conditions because high plasma osmolality activates the thirst reflex causing increased fluid intake to restore osmolality. However, increased plasma osmolality may be a major importance under pathologic condition including salt-sensitive hypertension.

Figure 3. Differential attributions of peripheral blood volume, blood pressure, and vascular resistance in arteries, arterioles, capillaries, venules, and veins (Mohrman *et al.*, 2010a)

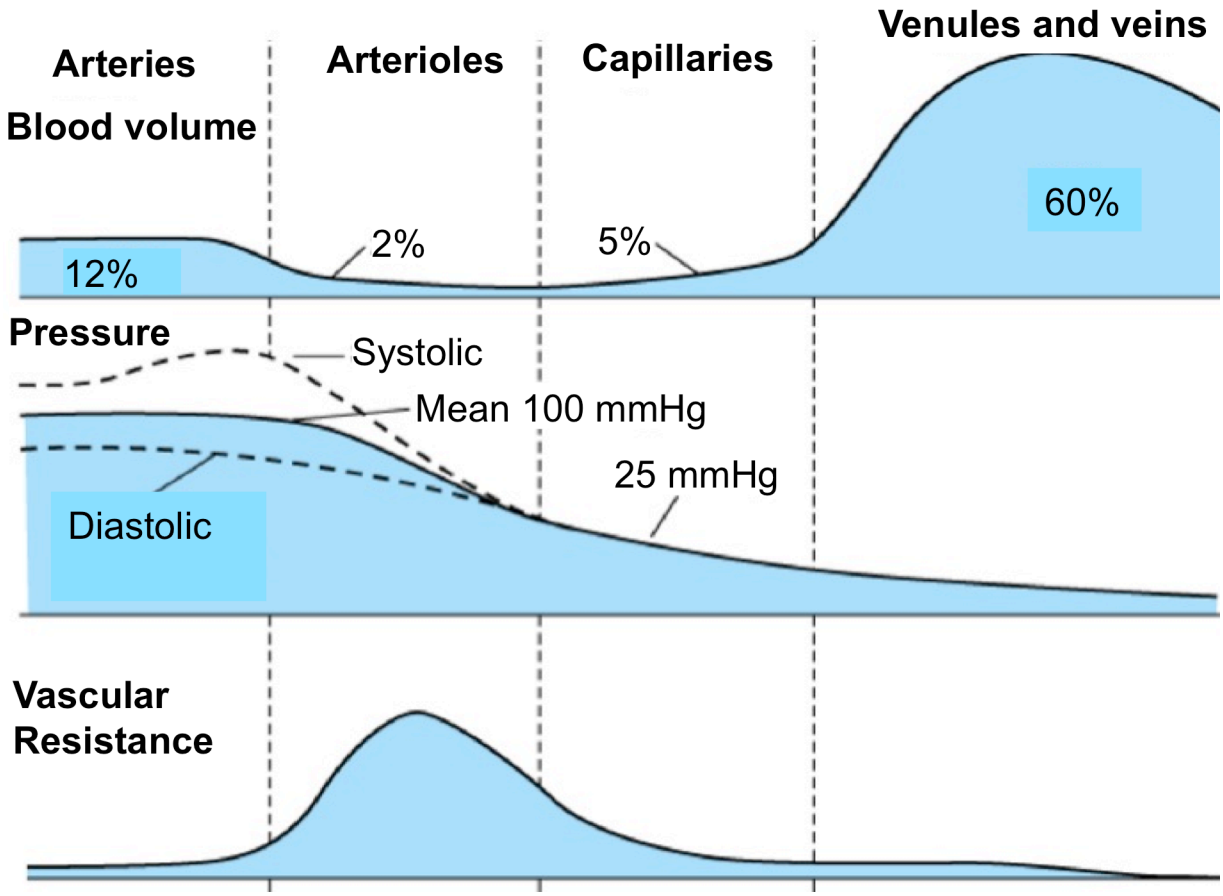
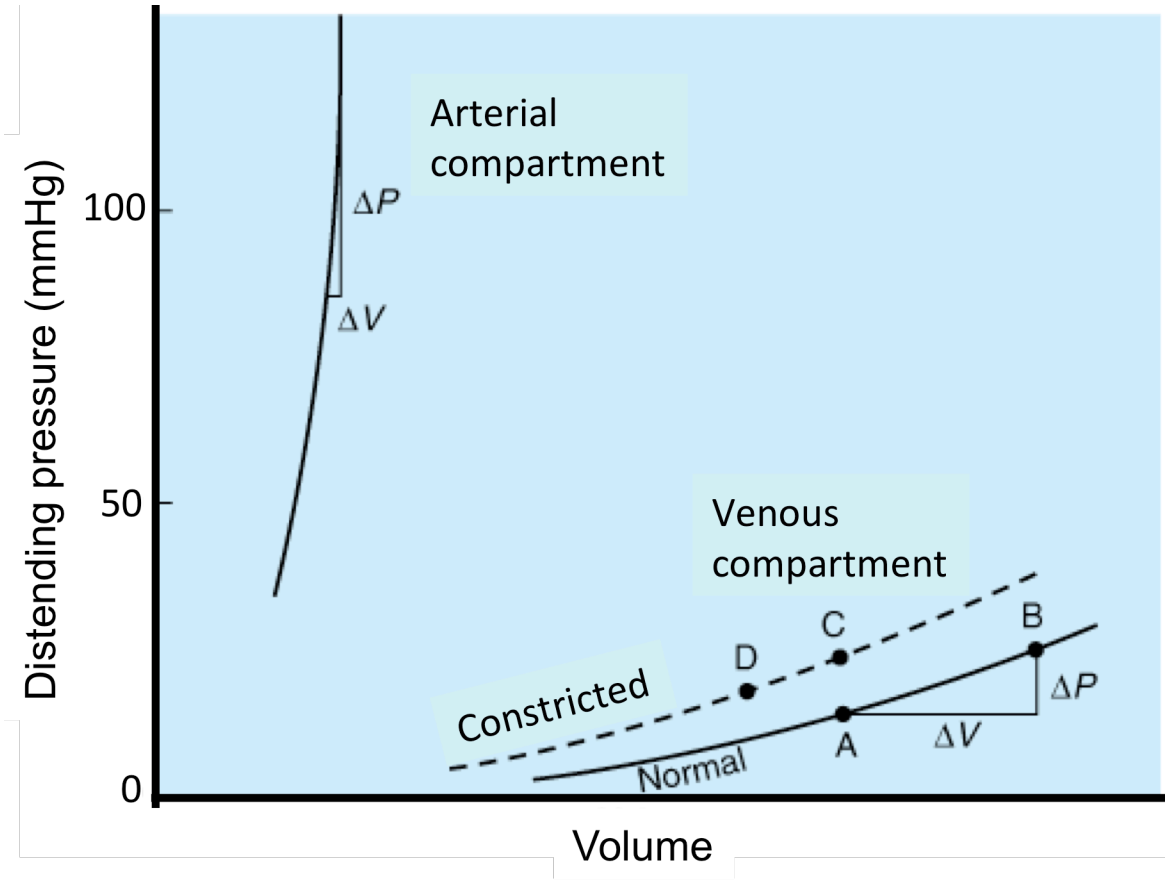


Figure 4. Volume-pressure relationships of arterial and venous compartments. ΔP, Pressure change; ΔV, volume change (Mohrman *et al.*, 2010a)

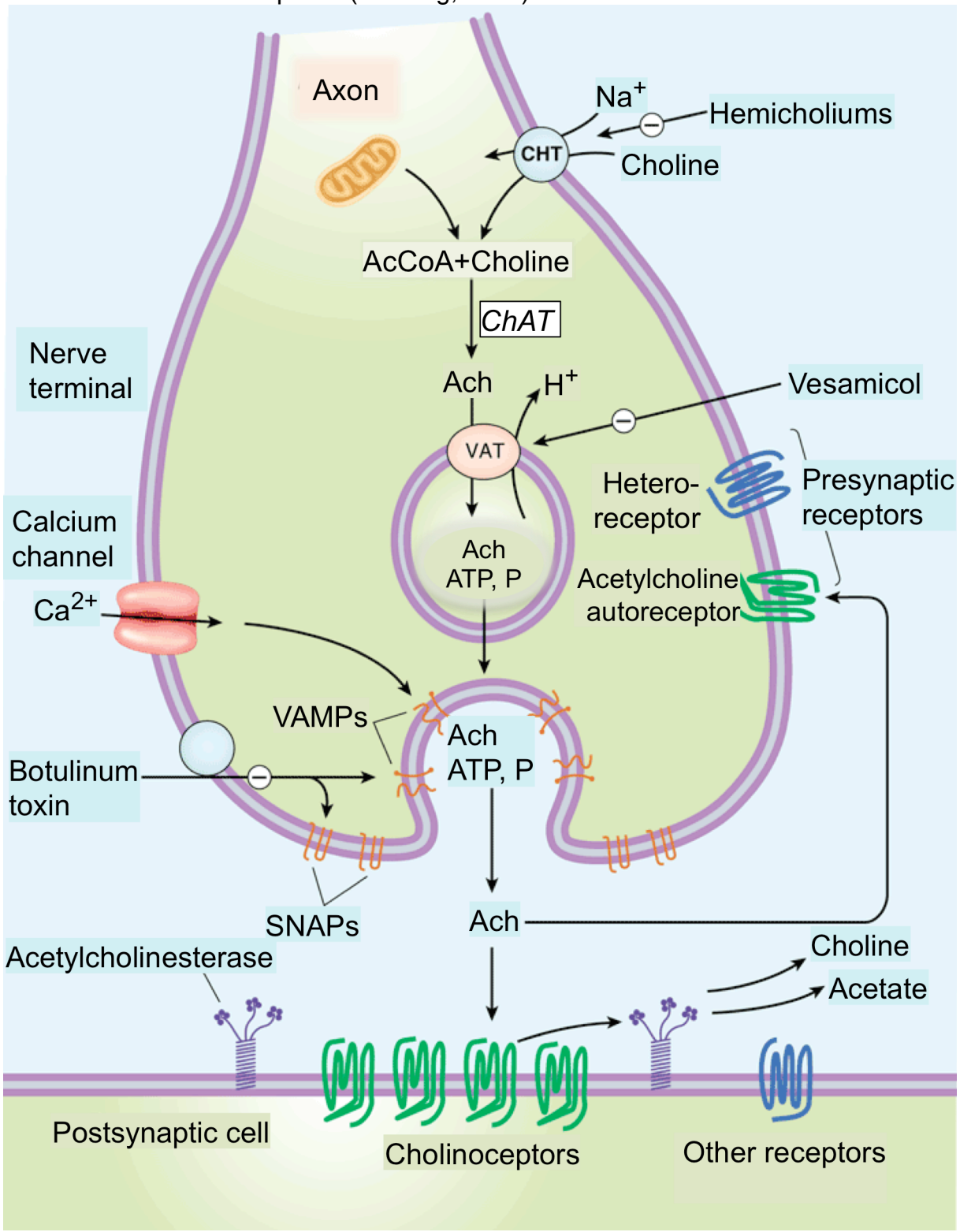


1.4 Sympathetic neurotransmission

1.4.1 Sympathetic preganglion neurotransmission

The axon terminal of the preganglionic sympathetic nerve contains acetylcholine (ACh). ACh is synthesized from acetyl-Coenzyme A (acetyl-CoA) and choline with enzyme choline acetyltransferase as the catalyst. The substrate choline is transported from extracellular fluid into the nerve terminal through the Na^+-dependent choline transporter. ACh is synthesized in the cytosol and is transported into the synaptic vesicles by the proton-driven vesicular-associated transporters (VAT). Once an action potential arrives, the voltage-gated Ca^{2+} channels are activated causing Ca^{2+} influx and exocytosis. Once released, ACh acts on the nicotinic cholinoceptors located on the cell body of postganglionic sympathetic nerves. One preganglionic neuron activates numerous of postganglionic neurons. The action of released ACh is shortly terminated by enzyme acetylcholinesterase into choline and acetate. Choline transporters capture choline back into the nerve terminal for further ACh synthesis (Fig. 5). Postganglionic sympathetic neurotransmission is described below.

Figure 5. Schematic depiction of preganglionic sympathetic nerve transmission onto the postganglionic sympathetic nerve. AcCoA: Acetyl-CoA, ACh: Acetylcholine, ChAT: Choline acetyltransferase, CHT: Choline transporter, VAT: Vesicle-associated transporter (Katzung, 2012).



1.4.2 Sympathetic postganglion neurotransmission in focus on vascular system

Postganglionic sympathetic nerves densely innervate the vascular system to regulate vascular tone and maintain blood pressure. Postganglionic sympathetic axons are nonmyelinated with 0.25-0.5 μm in diameter and surrounded by Schwann cell sheaths. Each axon contains periodic swellings known as “varicosities”, which have 0.5-2 μm in diameter every 3-5μm interval. The varicose regions are Schwann-free with fenestration. Varicosities contain a large number of synaptic vesicles, which is believed to be the sites for transmitter storage. Perivascular sympathetic varicosities make very close contact with smooth muscle cells. The junction between varicosities and the smooth muscle cells is ranged between 0.1-10 μm. A nerve impulse depolarizes a series of varicosities and causes the release of transmitters (Brock *et al.*, 1993; Stjarne, 1989). There are three major transmitters that are costored and coreleased from periarterial sympathetic varicosities: NE, ATP, and NPY (Lamont *et al.*, 2006). In certain specific vasculatures, sympathetic nerve could release other transmitters. For instance, dopamine is released from sympathetic nerves supplying renal and hepatic blood vessels. Epinephrine is released from sympathetic nerves innervating amphibian heart. ACh is released from the nerve supplying small arteries in cat skeleton muscle (Tsuru *et al.*, 2002). Additionally, under continuous nerve stimulation, where enzyme dopamine *β*-hydroxylase become saturated, it causes accumulation of newly synthesized dopamine available for release from sympathetic nerves (Soares-da-Silva, 1988). Release of sympathetic transmitters is triggered by a transient influx of Ca^{2+} ion into nerve varicosity via voltage-sensitive Ca^{2+} channel. Conversely, an increase in intra-varicosity

Ca^{2+}concentration in from intraneuronal stores is likely to be ineffective for triggering transmitter release (Burgoyne *et al.*, 1995). Among these postganglionic sympathetic transmitters, NE is the principal neurotransmitter released upon sympathetic nerve stimulation.

1.4.3 Adrenergic neurotransmission

Synthesis of NE: The synthesis of NE is highly regulated and several enzymes are involved with this process (Fig. 6). NE and epinephrine are synthesized from tyrosine. Tyrosine is actively transported into the postganglionic sympathetic varicosity (Fig. 7). Tyrosine hydroxylase (TH) is the rate-limiting enzyme that catalyzes tyrosine hydroxylation to form dehydroxyphenylalanine (DOPA) using O_2 and the cofactor tetrahydrobioterin (BH_4). DOPA is decarboxylated by aromatic L-amino acid decarboxylase (DOPA decarboxylase) to produce dopamine. Dopamine is actively transported into synaptic vesicles via a vesicular monoamine transporter 2 (VMAT2). In the vesicle, NE is synthesized from dopamine by the membrane-associated enzyme dopamine β-hydroxylase. Most synthesized NE is leak from synaptic vesicular storage into the cytoplasm, but 90% of NE leakage is re-captured into synaptic vesicles by VMAT2. The other 10% of NE escapes the VMAT2 sequestration mechanism and is enzymatically degraded (Eisenhofer *et al.*, 2004) (see removal, turnover, and metabolism of NE). Additionally, in adrenal medulla approximately 85% of NE is converted to epinephrine (E) by enzyme phenylethanolamine-N-methyltransferase using S-adenosylmethionine as a methyl donor. NE production varies with neuronal activity. In 1912 Elliot was first observed that long train electrical stimulation of splanchnic nerves

did not cause NE depletion of adrenal NE content. Later in 1950s, Holland and Schumann proposed that nerve stimulation caused compensatory increases in TH activity and catecholamine production (Zigmond *et al.*, 1989). Generally, NE, epinephrine, dopamine, and DOPA (termed catechols) negatively regulate TH activity to stop further NE synthesis by reversibly competing with BH_4 binding to the catalytic site of TH (Dunkley *et al.*, 2004). However, numerous protein kinases and phosphatases modulate activity of TH. Phosphorylation augments enzyme affinity for the BH_4 cofactor, hence increased TH hydroxylation rate (Dunkley *et al.*, 2004) and diminishes the catechol negative feedback. It appears that TH activity is acutely increased by phosphorylation in response to elevated cAMP level and cellular depolarization (Zigmond *et al.*, 1989).

Figure 6. Biosynthesis process of catecholamines (Katzung, 2012)

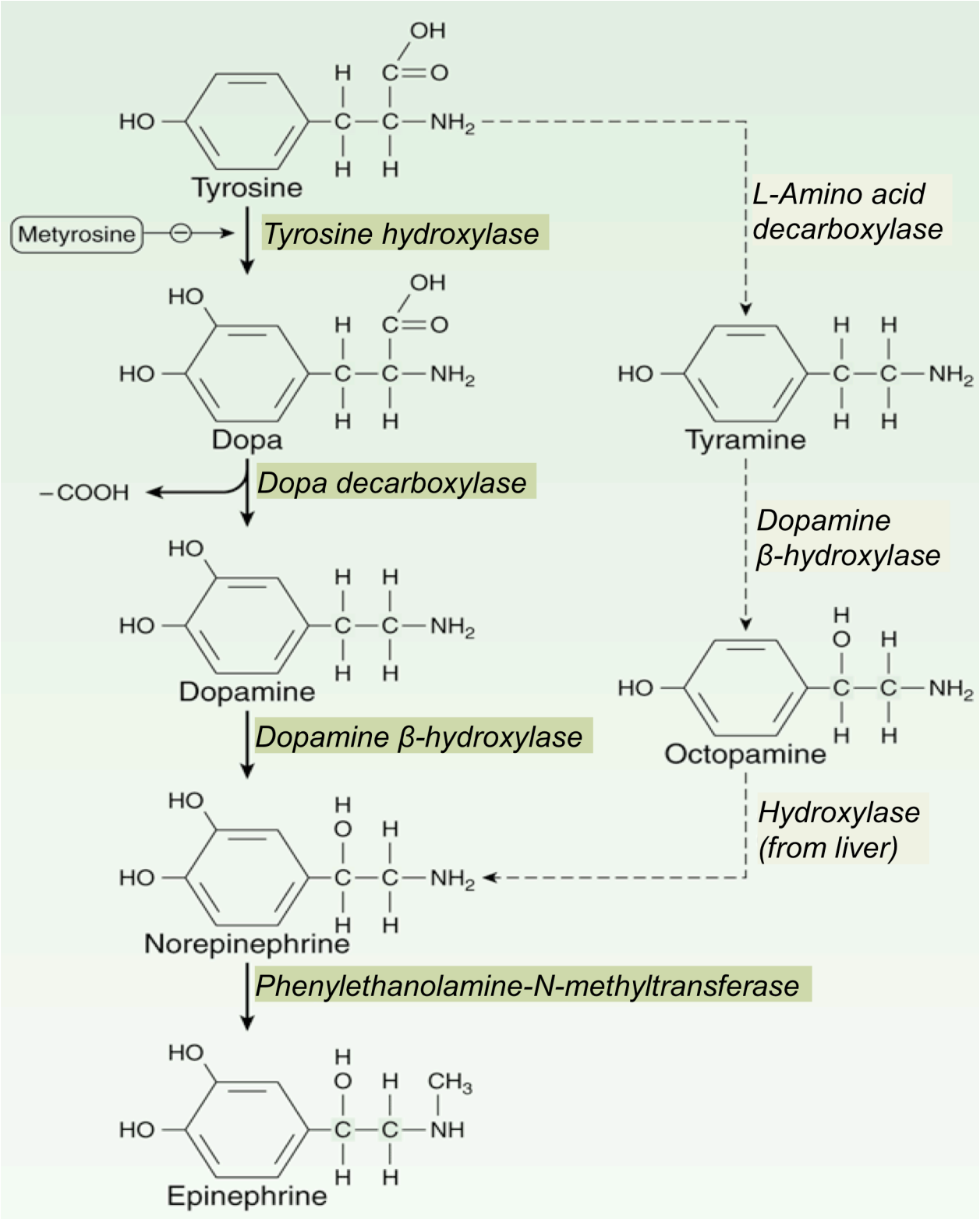
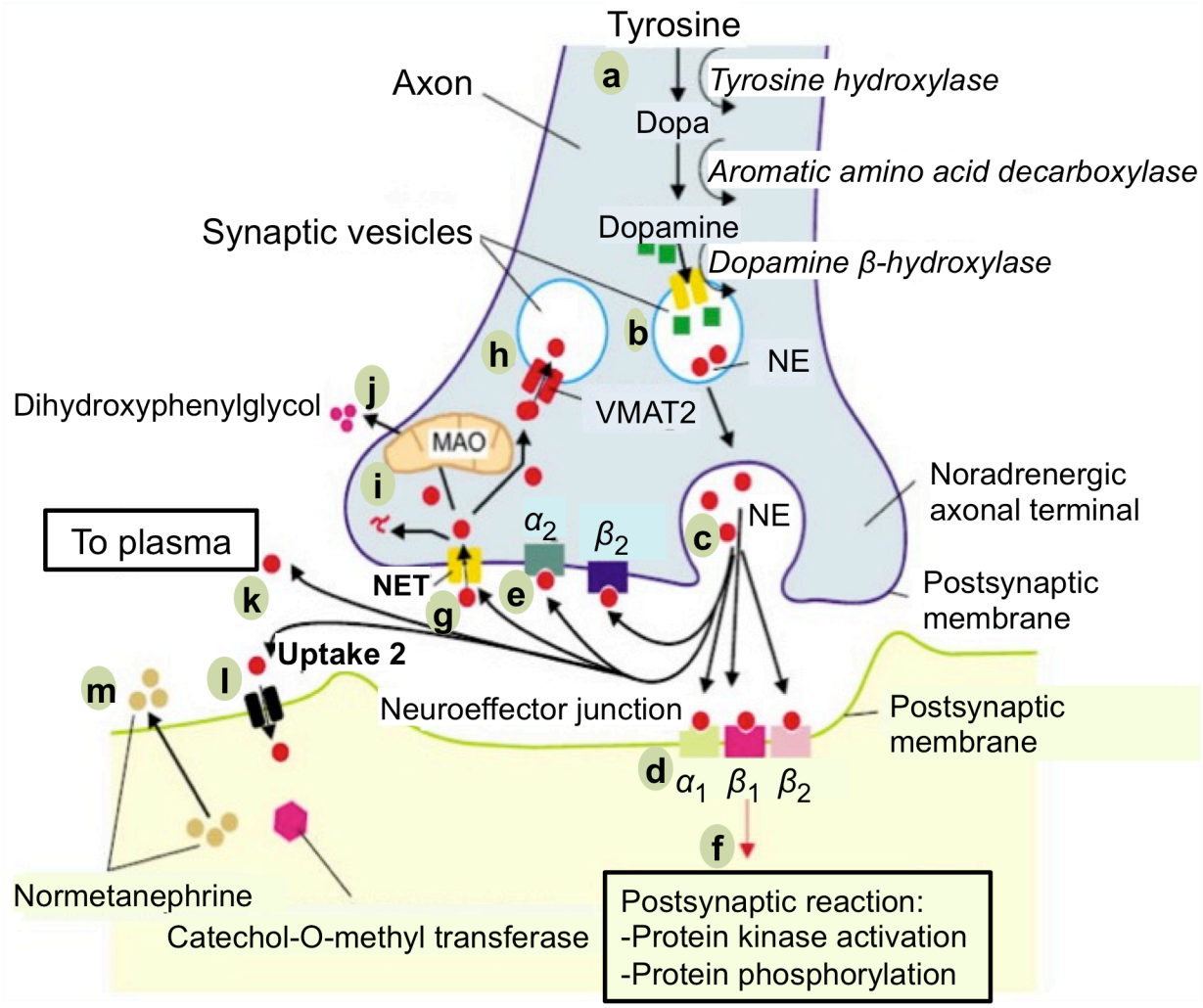


Figure 7. Summary depiction of adrenergic neurotransmission for vasculature. a) The essential amino acid tyrosine is captured into sympathetic axon for NE synthesis. To form NE, tyrosine is converted into dihydroxyphenylalanine (Dopa), and dopamine by tyrosine hydroxylase, and, aromatic amino acid decarboxylase respectively. b) Dopamine is next actively transported into a synaptic vesicle, where contains dopamine β-hydroxylase, and converted into NE and stored in the vesicle. c) Upon arrival of an action potential, vesicular exocytosis and NE release occurs. NE activates the postsynaptic receptors (α1, β1, β2) (d) leading to vascular contraction (f). e) NE also acts on the prejunctional receptors (α2, β2) to inhibit NE release. g) To terminate NE action, NE is removed from the neuroeffector junction by NET-mediated NE reuptake. h) After NE reentered into the axon terminal, NE is captured back into the vesicle via vesicular monoamine transporter 2 (VMAT2). i) Some NE escapes from VMAT reuptake and undergoes enzymatic metabolism by monoamine oxidase (MAO) within the nerve axon. j) degradation product dihydroxyphenylglycol is released into circulation. k) Partially, NE is uptake into postjunctional cell (uptake2) and degraded by catechol-O-methyl transferase into normetanephrine (Zhou, 2004).



Vesicular Storage of NE: In sympathetic axons, NE is stored in two types of synaptic vesicles: large, dense-core vesicles (Schwarzenbrunner *et al.*, 1990)(~ 75-200 nm in diameter)(Lagercrantz, 1976) and small, dense-core vesicles < 50 nm diameter (Fried *et al.*, 1985). In addition to NE, the large dense-core vesicles also contain a variety of neuropeptides, and ATP (Lagercrantz, 1976). It appears that different frequencies of sympathetic firing evoke different mixtures of neurotransmitter release. NE and ATP are release at low-frequency nerve stimulation, while the release of the other cotransmitter NPY peptide often occurs during period higher frequency of stimulation (Kennedy *et al.*, 1997). Furthermore, the transmitter release under high frequency of sympathetic nerve firing appears to be Ca^{2+} independent (Malpas, 2010). High frequency stimulation-induced multiple transmitter release causing activation of various receptors and second messengers. This results in a recruitment of Ca^{2+}-independent kinases and phosphorylation of transcription factors to modulate transmitter synthesis. Besides, there are other targets for Ca^{2+}-independent phosphorylation including cytosolic and vesicular proteins responsible for vesicle docking and fusion (Wakade *et al.*, 1995). In the adrenal medulla, NE and epinephrine are stored in chromaffin cells. Upon sympathetic nerve stimulation, chromaffin cells release ~80% epinephrine and 20% NE directly into the bloodstream.

Release of NE: Once an action potential arrives at the presynaptic nerve terminal, the voltage-gated Ca^{2+} channels located on the active zone change their conformation to the opening stage permitting Ca^{2+} influx. High concentration of Ca^{2+} in

active zone induces the fusion of synaptic vesicle membrane and the presynaptic neural membrane, hence exocytosis occurs. Impulse frequency could modulate the transmitter release. The docked small vesicles in active zones is more likely to exocytose during low frequency stimulation, while the high frequency burst causes the release of large vesicle from random sites.

Postjunctional action of NE at specific receptors: Once released, NE and co-transmitters diffuse across the neuroeffector junction and bind to their specific target receptors on the membrane of vascular smooth muscle (Fig. 7). NE activates the postjunctional α_1-adrenergic receptors which couple to stimulation of the G_q/PLC/IP_3 pathway and Ca^{2+}-induced Ca^{2+} release from endoplasmic reticulum stores to mediate smooth muscle contraction. Furthermore, activation of the postjunctional α_1-adrenergic receptors by NE also opens membrane pannexin-1 channels causing cellular ATP release to contribute to vascular contraction (see detail below). Prolonged exposure to NE causes desensitization in mesenteric veins, but not arteries. In addition to the postjunctional receptors, NE also acts on the prejunctional α_2-adrenergic receptors providing negative feedback to the sympathetic nerve terminal to stop further transmitter release. Agonists and antagonists for adrenergic receptors are list in table 2.

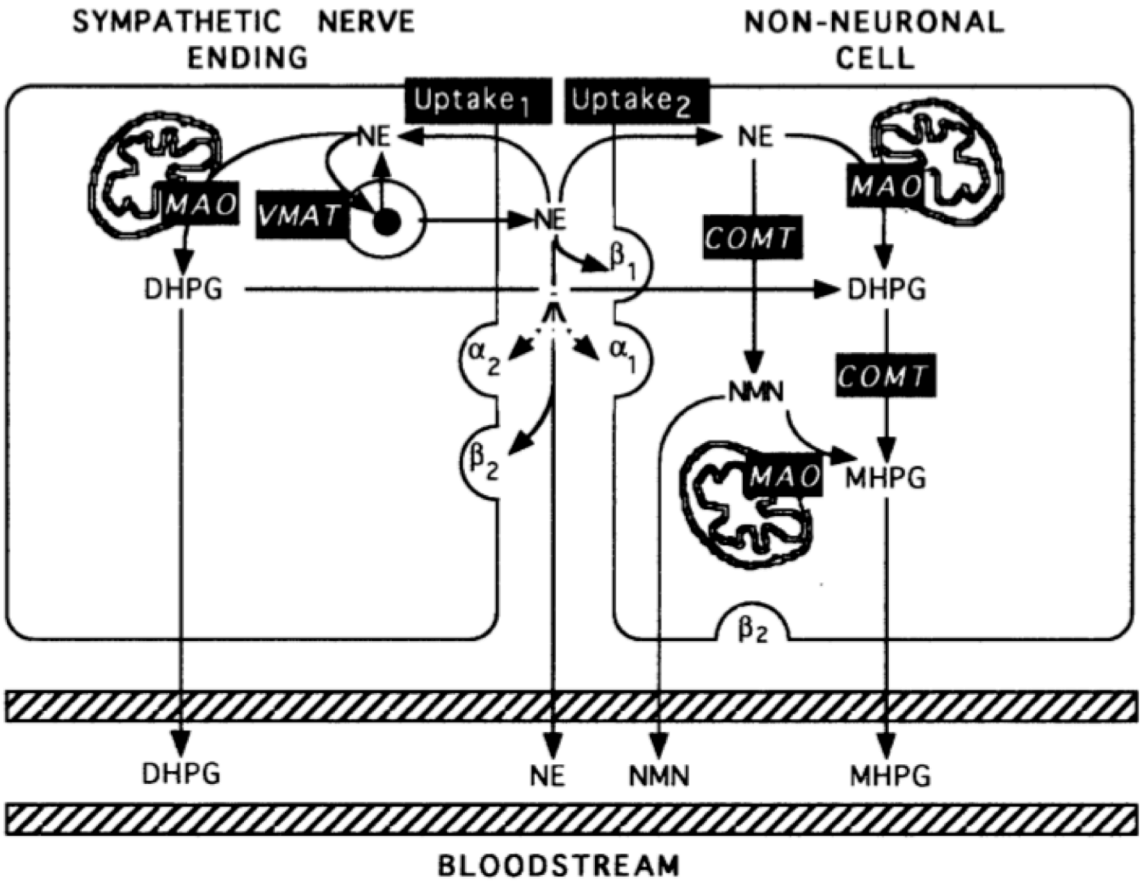
Table 2. Agonists and antagonist for α-adrenoceptors

	α_1-adrenoceptor	α_2-adrenoceptor
Location	Postjunctional site	Prejunctional site
Pharmacological action	Mediate vasoconstriction Opens pannexin1 channel	Inhibit transmitter release
Agonist	NE (non-selective) Phenylephrine	NE (non-selective) Clonidine UK14304
Antagonist	Prazosin	Idazoxan Yohimbine

Clearance and metabolism of NE: The action of NE is terminated mostly by the NE transporter (NET) reuptake (Fig. 7). Approximately 70% of released NE is recaptured back into the varicosities, and the remaining 30% is lost to extraneuronal transport and enzymatic metabolism (Eisenhofer *et al.*, 2004). Figure.8 shows two distinct NE reuptake systems: uptake1 and uptake2. Uptake1 transporters consist of 12-transmembrane domains and the reuptake mechanism is Na^+ and Cl^- dependent. Uptake1 transporters have high affinity, but low capacity to NE and are predominantly located on the nerve terminal. Uptake1 is the main mechanism responsible for remove NE from the neuroeffector junction and their action is blocked by cocaine or tricyclic antidepressant desipramine (DMI). Conversely, uptake2 transporters are considered as

non-neuronal transporters because they are located on the postjunctional site (vascular smooth muscle cells, for example). Uptake2 transporters are Na^+-and Cl^--independent and they have low affinity, but high capacity to NE. Besides, cocaine, and tricyclic antidepressants could not block the uptake2 system. Uptake 2 transporter could be blocked by the O-methylated metabolites of catecholamines (normethanephrine (NMN), and metanephrine) produced by COMT enzymes, and steroids (Eisenhofer, 2001). Once the uptake1 transporters transport NE into the nerve terminal, NE is mostly recaptured back into vesicles via vesicular monoamine transporter2 (VMAT2) and is available for another round of exocytosis. Sympathetic nerve terminal mitochondria contain monoamine oxidase (MAO), which metabolizes most NE not recaptured into synaptic vesicles. This intraneuronal metabolism pathway is also responsible for degradation of NE that leaks from synaptic vesicles. Some NE undergoes extraneuronal metabolism within the neuroeffector junction by catechol-O-methyltransferase (COMT) (Fig. 8).

Figure 8. Schematic sketch of NE reuptake and metabolism. Once an action potential arrives, the nerve releases NE onto the receptors on target cell. Then, NE is reuptake back into the nerve terminal via the uptake1 transporter, and is next reentered vesicular store via vesicular monoamine transporter2 (VMAT2). Escaped NE from transporter sequestration is metabolized by intraneuronal monoamine oxidase (MAO) and extraneuronal catechol-O-methyltransferase (COMT) (Eisenhofer, 2001).



1.4.4 Neuropeptide Y (NPY) neurotransmission

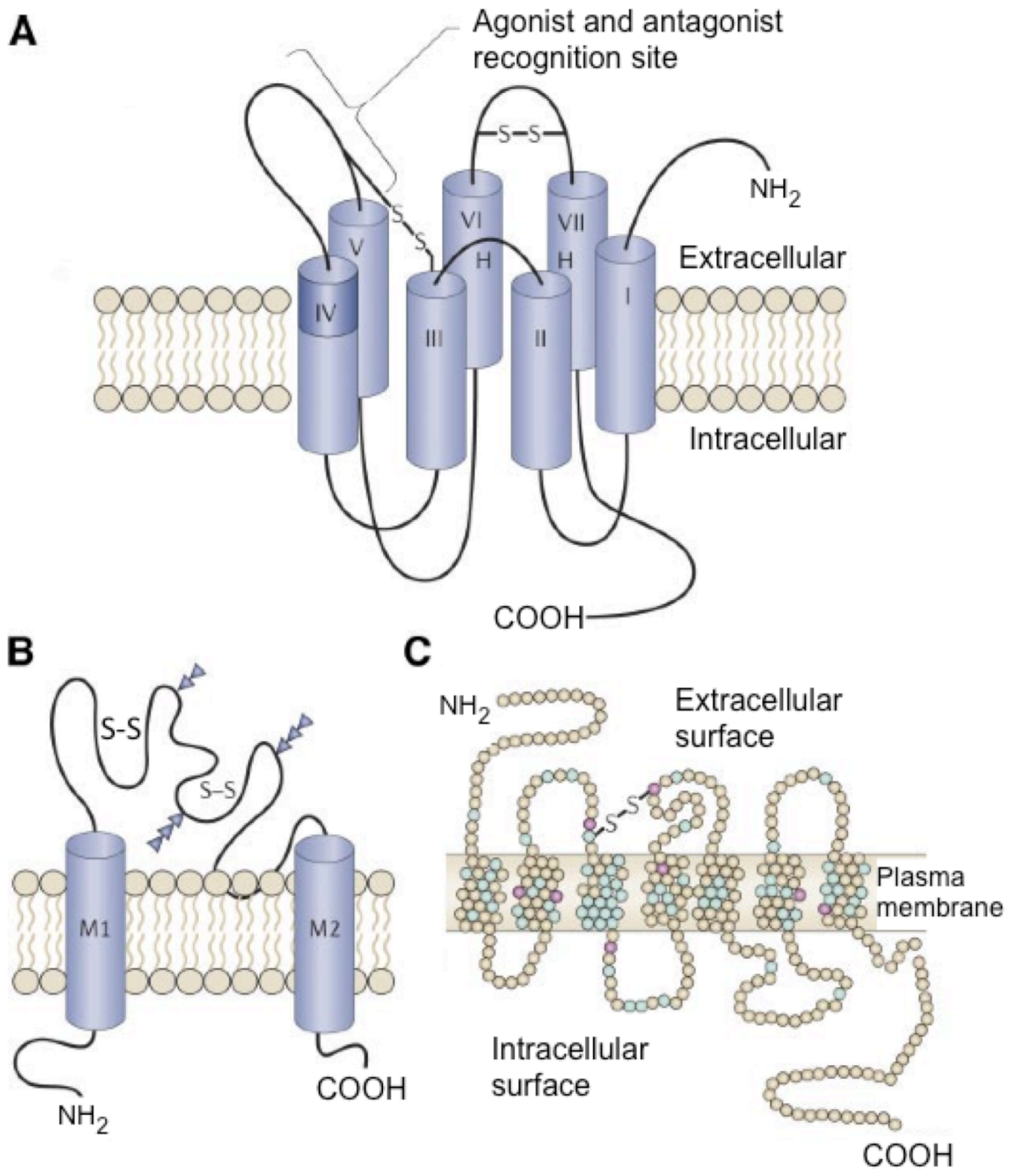
NPY is a small biologically active peptide (36 amino acids) found abundantly in the central and autonomic nervous systems (Reid, 2012). NPY is involved with regulation of numerous physiological processes, including thirst, appetite, blood pressure control, and energy metabolism. There are five subtypes of NPY receptors (Y1-Y5). All of them, except Y3, are GPCRs. Two subtypes of NPY receptors are important in the cardiovascular system: Y_1 and Y_2 receptors. In sympathetic neurotransmission, Y_1 receptors are primarily located on the postjunctional site to mediate vascular contraction and potentiated NE-induced vasoconstriction. Y_2 receptors express in both pre- and postjunctional sites. The prejunctional Y_2 receptors couple to inhibition of NE release. NPY is synthesized in the soma, packed into vesicles, and transported to nerve varicosities (Stjarne, 1989). NPY and NE are co-stored in large, dense core vesicles in sympathetic nerves supplying the rat vas deferens (Fried *et al.*, 1985). NPY is co-released with NE and ATP from sympathetic nerve endings and NPY is a potent vasoconstrictor in many vascular beds (Cowley-Jr *et al.*, 2008), including the mesenteric circulation of the rat (Westfall, et al.,). The constrictor effects of NPY are mediated by Y1 receptors as demonstrated in the guinea pig ear artery (Morris, 1999). Additionally, NPY potentiates the constrictor effects of NE and ATP via a postjunctional action in rat vas deferens, mesenteric artery (Donoso *et al.*, 1997a; Westfall *et al.*, 1995), and rabbit femoral arteries (Ekblad *et al.*, 1984). NPY is slowly cleared from the neuroeffector junction compared to other small molecule transmitters. This slow removal of NPY contributes to long duration of its effects. NPY probably removed by diffusion

and proteolysis by extracellular dipeptidyl peptidases 4 (DP4). DP4 is transmembrane glycoprotein consisted of 766 amino acid, which hydrolyzes post-proline bond of the N-terminus of NPY.

1.4.5 Purinergic neurotransmission

In sympathetic neurotransmission, ATP and its breakdown products act at pre- and postjunctional purinergic receptors. Purines refer to as adenosine, ADP and ATP as they contain adenine base; pyrimidines are UDP and UTP (Ralevic *et al.*, 1998). Both purines and pyrimidines mediate biological effect through purinergic receptors. Purinergic receptors are divided into two families: P1 and P2 receptors (P annotates purinergic or purine). P1 receptors are targets for adenosine, the final ATP degradation product. P1 receptors are metabotropic G-protein coupled receptors (Fig. 9). Adenosine receptors are further subdivided into A_1, A_{2A}, A_{2B}, and A_3 receptor subtypes. Details of adenosine receptors will be discussed later in adenosine signaling section. P2 receptors are targets for the nucleotides ATP, ADP, UTP, and UDP. There are 2 classes of P2 receptors: P2X receptors (ligand-gated ion channels) and P2Y receptors (G-protein coupled receptors) (Fig. 9). There are 7 P2X receptor subtypes ($P2X_{1-7}$) and 5 P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$). My work focused on ATP and adenosine as signaling molecules in peripheral sympathetic neurotransmission.

Figure 9. Schematic depiction of purinergic receptor structures. A) P1 metabotropic receptor (receptor for adenosine) is consisted of seven transmembrane domains. The second extracellular loop linked domain IV and V is critical for agonist/antagonist recognition. There are two subtypes of receptor for ATP: the ionotropic ligand-gated ion channel and metabotropic G protein-coupled receptor comprising two transmembrane domain (B), and the P2Y metabotropic receptor assembling seven transmembrane-spanning domains (C) (Burnstock, 2007).



1.4.5.1 ATP neurotransmission

The nucleotide adenosine 5'-triphosphate (ATP) is a key energy source in the cells. ATP is synthesized through mitochondrial electron transport chain and transported into the cytosolic compartment for further cellular energy utilization. ATP concentrations in the cytoplasm range between 2-5 milimolar (mM), and much higher in synaptic vesicles (up to 100 mM) (Burnstock, 2007). ATP and its degradation products (adenosine and ADP) are potent extracellular signaling molecules (Ralevic *et al.*, 1998). ATP is a critical co-transmitter in the central and peripheral nervous systems, and the contribution of ATP as an excitatory neurotransmistter varies across tissues. In chromaffin cells from adrenal medulla, ATP is co-released with epinephrine. In sympathetic nerves supplying vas deferens, ATP is an important co-transmitter with NE and NPY. It is revealed that ATP was co-stored with NE in large-dense core vesicles with the ratio of 7:1 in bovine splenic nerves (Lagercrantz, 1976). Likewise, ATP is also released from non-neuronal cells that are important in pain signaling pathways. ATP released from damaged cells triggers the naked endings of peripheral C-fiber through the activation of ionotropic P2X receptors. Also, healthy non-neuronal cells (can you list some examples?) release ATP in response to sheer stress, stretch, osmotic swelling, and hypoxia (Burnstock, 2007).

In the cardiovascular system, ATP signaling plays a crucial role during both normal physiological and pathological conditions including hypoxia-induced vasodilation, reactive hyperaemia, and hypertension. Periarterial sympathetic nerves supplying mesenteric artery co-release ATP and NE. ATP acts at ionotropic P2X1

ionotrop receptors on arterial smooth muscle cells to cause short latency (a few milliseconds) and short duration (200 milliseconds) excitatory junction potentials (EJPs). EJPs are brief depolarizations of smooth muscle cells that are caused by cation influx through P2X channels (Demel *et al.*, 2008b). EJPs are blocked by guanethidine or bretylium, drugs which also block NE release (Burnstock, 2007). These data support the conclusion that ATP and NE are co-stored in synaptic vesicles. Once the cotransmitter released, P2X activation by released ATP causes conformation change of the transmembrane channel leading to increased channel permeability to Na^+ and Ca^{2+}. Influx of cations causes cellular membrane depolarization and activation of voltage-gated L-type Ca^{2+} channels. Ca^{2+} entry through the P2X1 receptor and the L-type Ca^{2+} channel trigger and vascular contraction (Gitterman and Evans 2001).

Neurogenic arterial contraction is biphasic response including rapid-transient and slow-sustained phases. The rapid phase arterial contraction is caused by the action of neuronally released ATP on the postjunctional inotropic $P2X_1$ receptors. Meanwhile, NE-mediated contraction via the postjunctional α_1-adrenergic receptors is slower due to G-protein coupling and the involvement of second messengers providing sustained arterial contraction phase (Fig. 10). In P2X1-null mice, the rapid contraction phase is absent in neurogenic arterial contraction. Furthermore, prazosin (α_1-adrenoceptor antagonist) specifically attenuate the sustained contraction phase, without altering the transient rapid phase (Lamont *et al.*, 2006). Additionally, Park and coworkers showed that PPAD (P2X antagonist) blocked arterial neurogenic contraction without affecting

NE release suggesting that ATP is required for mediating neurogenic arterial contraction. It should be noted that Park *et al.* performed a brief train of stimulation for 6 seconds, therefore they can only detect change in contraction amplitude, but they could not distinguish the biphasic contraction response. Conversely, Lamont group used long train of stimulation for 1 minute permitting them to detect the changes in biphasic neurogenic contraction response.

1.4.5.2 α_1-adrenoceptor-evoked postjunctional ATP release

In 1970s Bunstock proposed that ATP is coreleased with NE from postganglionic sympathetic varicosities (Burnstock, 1976) and it was later revealed by Starke and coworkers that the majority of nerve-evoked ATP release is come from non-neuronal site, where 32% of electrically-evoke ATP release is come from nerve varicosities, but the majority of 68% of released ATP is come from non-neuronal site, which could probably be smooth muscle cells. Non-neuronal ATP release by activation of the postjunctional α_1-adrenoergic receptors is firstly reported in guinea-pig vas deferens (Kügelgen *et al.*, 1991). The fundamental mechanism of α_1-adrenoceptor-induced ATP release is involved with pannexin1 hemichannels (Dubyak, 2009).

1.4.5.3 Pannexin1-facilitated cellular purine release

Pannexin is a novel protein family discovered in the past decade. Pannexin is structurally similar to the invertebrate gap junction protein “innexin”, and mammalian gap junction protein “connexin”, but pannexin does not form gap junctions. Pannexin

contains several extracellular glycosylation sites and it is likely that these glycosylations are the key component that prevents pannexin from forming gap junctions with adjacent cells (MacVicar *et al.*, 2012). However, pannexin does form an unpaired hemichannel with extracellular space (Dubyak, 2009). Under basal physiologic condition, pannexin hemichannel remains in closing state, and changes in extra-or intra-cellular environment could induce pannexin protein into opening state leading to the release of intracellular signaling molecules onto extracellular compartment (D'hondt *et al.*, 2009). There are three isoforms of pannexin proteins: pannexin1, pannexin2 and pannexin3. Pannexin1 and 3 form hexameric channels, and pannexin2 forms either heptameric or octameric channel. Pannexin proteins are synthesized and assembled in the ER and Golgi; then, they are transported and anchor into the plasma membrane. Transmembrane pannexin protein serves as a channel for transport of molecules between the intra- and extracellular compartments. Unlike connexin, pannexin activation is Ca^{2+}-independent. Recent studies have provided a strong reason to assume that pannexin1 channel has permeability to ATP and contributes to cellular ATP release under physiological and pathological conditions. Pannexin1 protein is expressed on the membrane of vascular smooth muscle cells of middle cerebral artery (Burns *et al.*, 2012), thoracodorsal artery (Billaud *et al.*, 2011) and mesenteric artery (Galligan *et al.*, unpublished data). Activation of the α_1-adrenergic receptors located on smooth muscle leads to ATP release through pannexin1 channel and ATP-induced vascular contraction (Fig. 10-11). A panexin1 channel blocker and P2Y receptor antagonist prevented Pannexin-induced ATP release and ATP-induced contraction (Billaud *et al.*, 2011).

1.4.5.4 Contribution of pannexin-1 to neurogenic vascular contraction

As shown previously that neuronal NE release could stimulate ATP release from vascular smooth muscle cells, the non-neuronal ATP could mediate synergistic arterial contraction along with NE. Postjunctional ATP release could activate neighboring P2X receptors and propagate neurogenic contractile response along the arteries (Fig. 10). However, venous contractile response is different from arterial contraction as perivenous varicosities contain different proportion of vasoactive transmitters. NE is the prominent transmitter to modulate venous contraction, but it is equivocal if ATP is coreleasd with NE from perivenous sympathetic varicosity. Role of pannexin channel in venous contraction response is undefined. Understanding the mechanism of pannexin-induced purine release in the vein would clarify the debating issue about source of purine release in perivenous nerves. A further extension question would be about pannexin channel selectivity. Does pannexin channel selectively transport ATP or it provides a general transport for all purines? It has been proposed that purine release is linked to metabolic demands via local blood flow regulation. For instance, ATP is release from skeleton muscle during contraction; adenosine is released in the central nervous system (Dale *et al.*, 2000; Frenguelli *et al.*, 2003) during hypoxia. Does pannexin hemichannel plays a key role in the release of these cellular purines?

Figure. 10 Contribution of pannexin1 in neurogenic arterial contraction. 1) ATP released from sympathetic varicosity activates P2X inotropic receptor (P2X) on vascular smooth muscle cell (VSMC) mediating rapid arterial contraction. 2-4) Released NE acts on α_1-adrenergic receptor (α_1) producing sustained arterial contraction by two major mechanisms: α_1-induced contraction (2) and α_1-stimulated pannexin1 (pan1) causing ATP release onto extracellular space (3). Non-neuronal ATP activates remote P2X receptors on neighboring muscle cell propagating further contraction (4). Biphasic neurogenic contractile response is from Lamont and co workers' work (Lamont *et al.*, 2006)

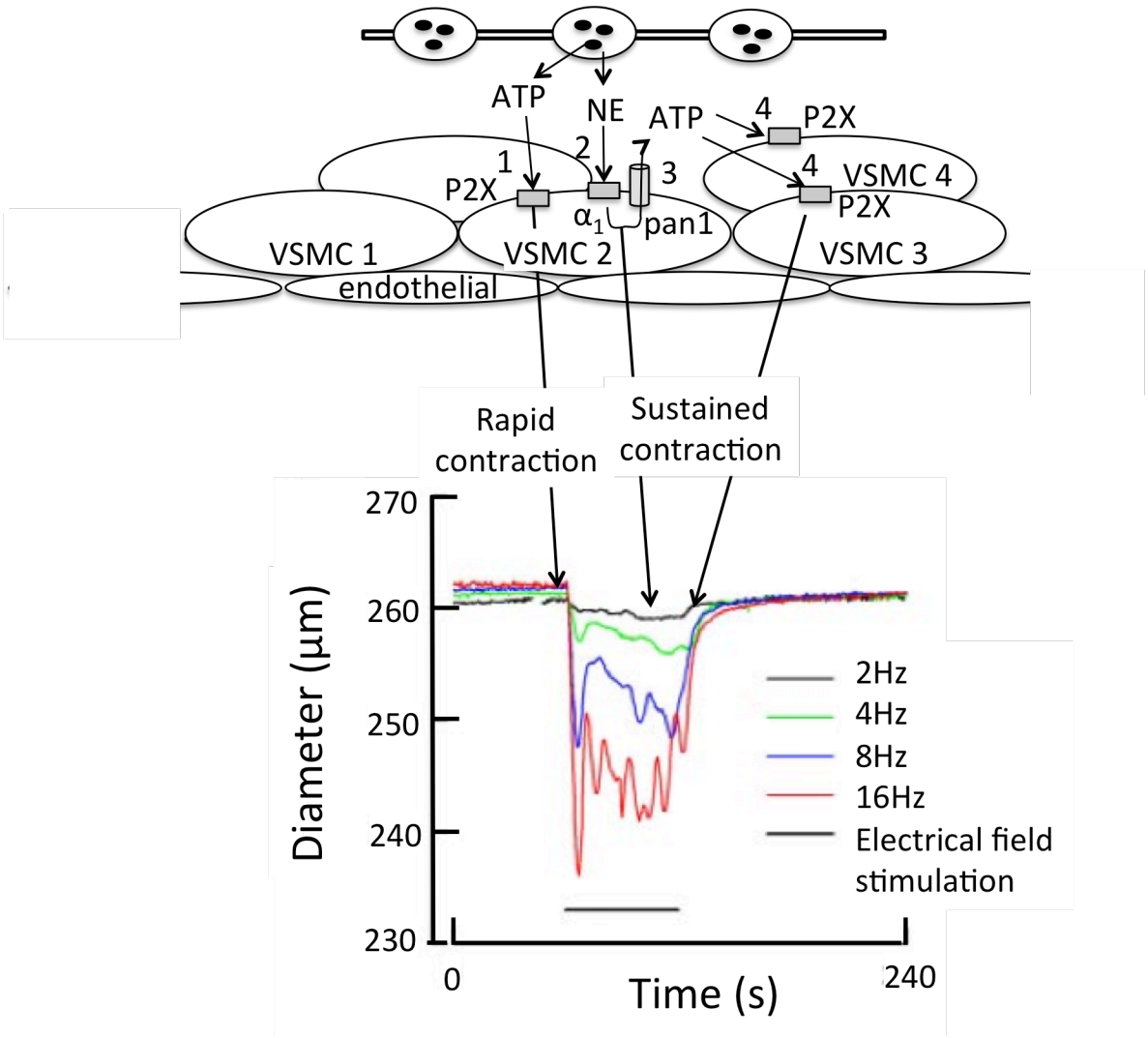


Figure 11. Proposed model of pannexin1-induced purine release in mesenteric artery and vein. A) Periarterial sympathetic nerve release ATP and NE. ATP binds to P2X receptor and NE acts on α_1-adrenergic receptor leading to vascular contraction. Activation of α_1-adrenergic receptor induced pannexin1 trafficking into plasma membrane. Active pannexin1 transports purines (ATP and adenosine) from inside the cell into the neurovascular junction to further modulate sympathetic neurotransmission and induce vascular contraction. B) Similar phenomenon occurs in sympathetic nerve supplying mesenteric vein. Unlike periarterial nerve, NE is the major neurotransmitter released from perivenous nerve. The source of purine is mainly from smooth muscle cells

A

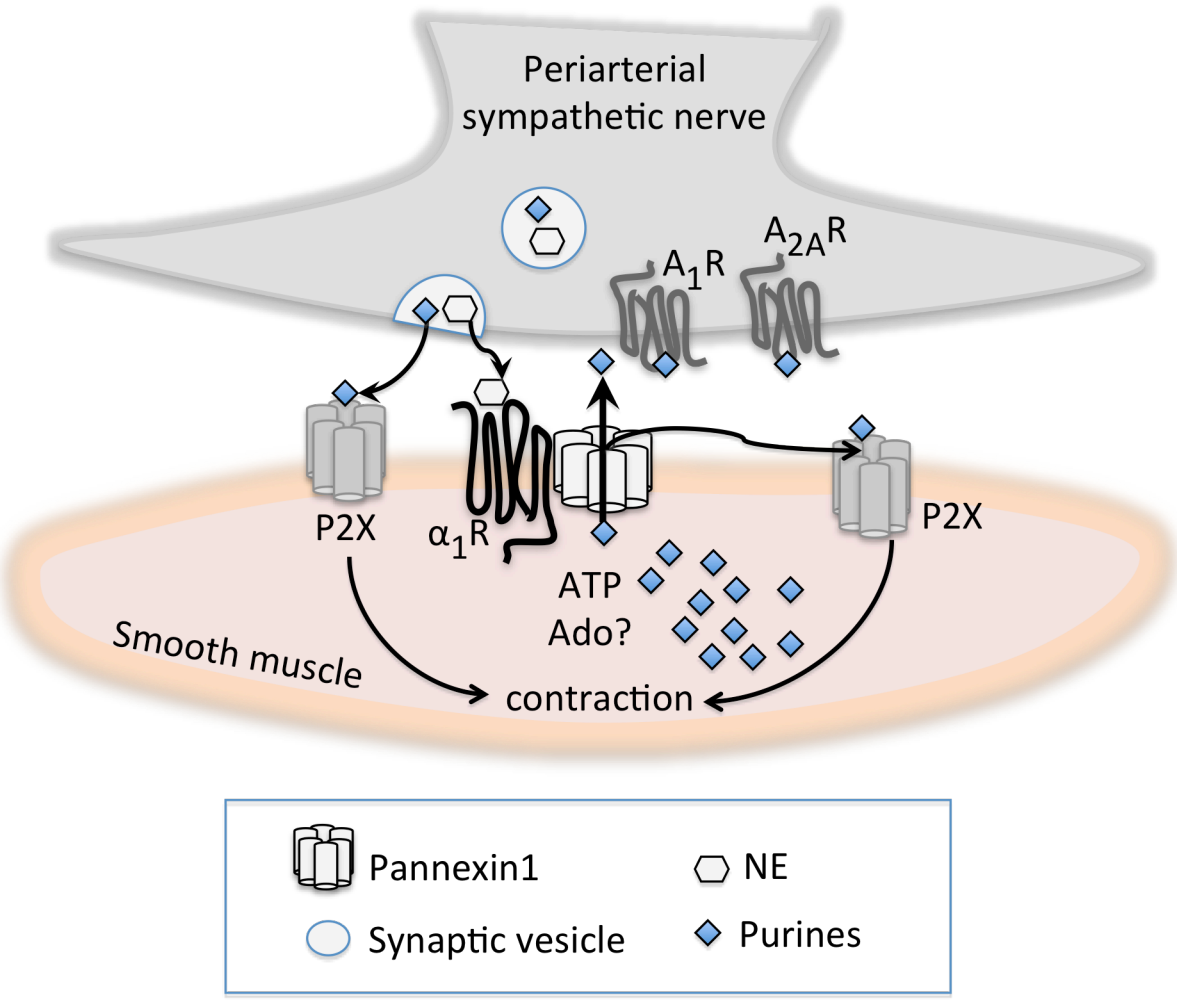
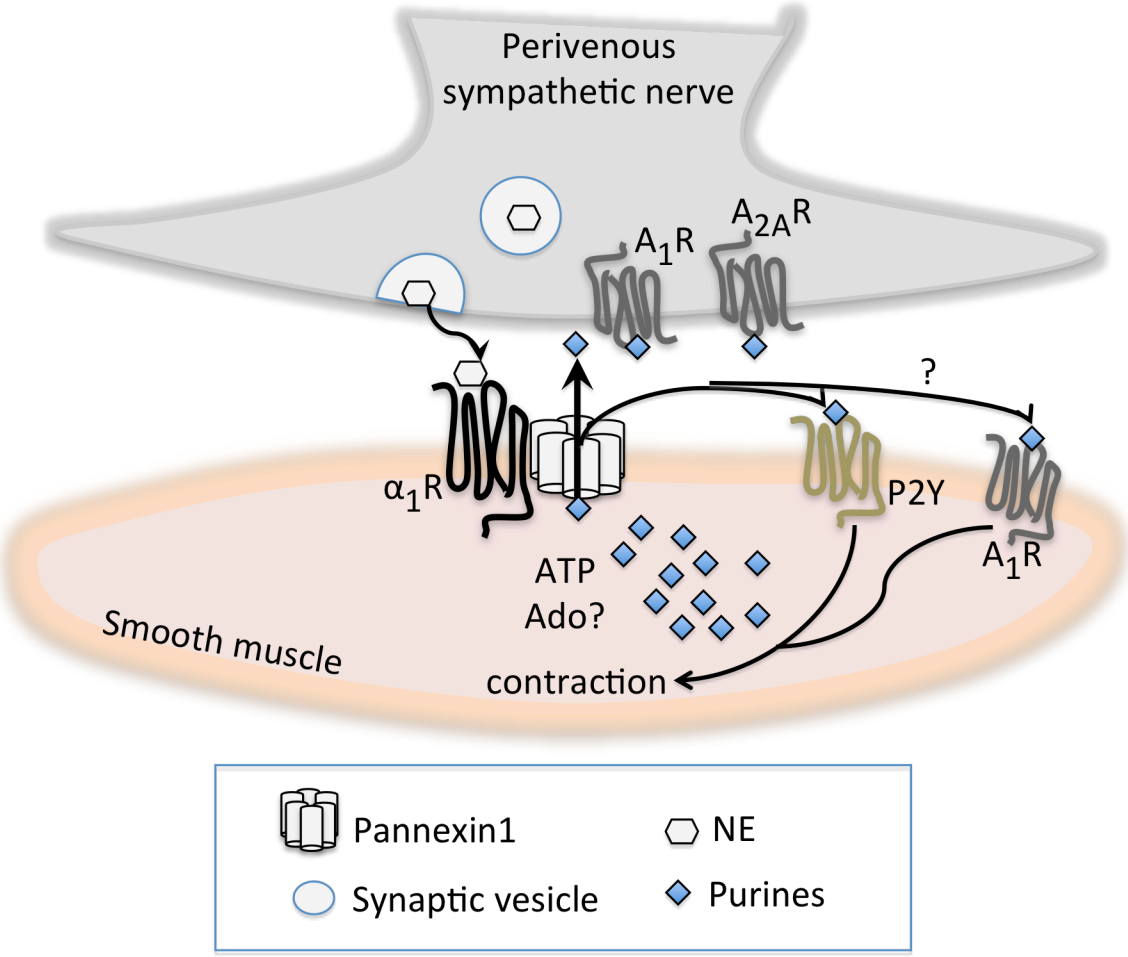


Figure 11. (Cont'd)

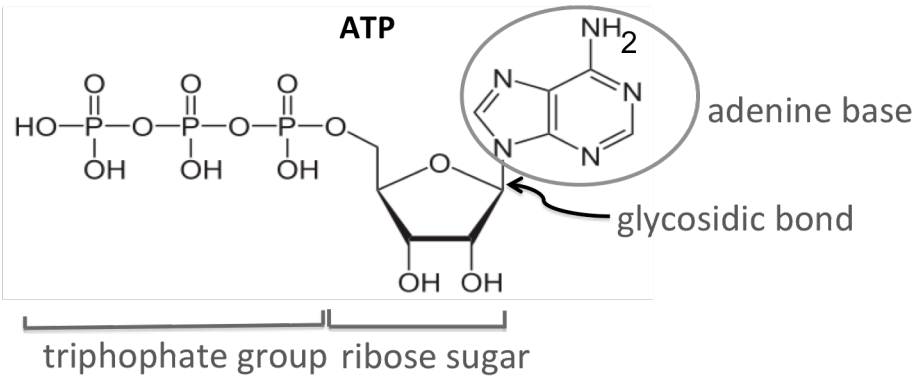
B

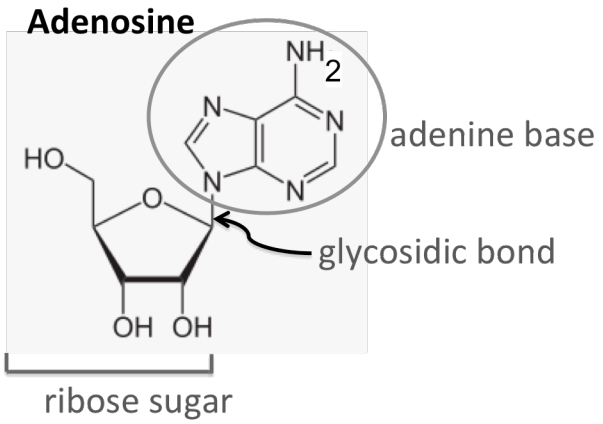


1.4.5.5 Beta-nicotinamide adenine dinucleotide (β-NAD) neurotransmission

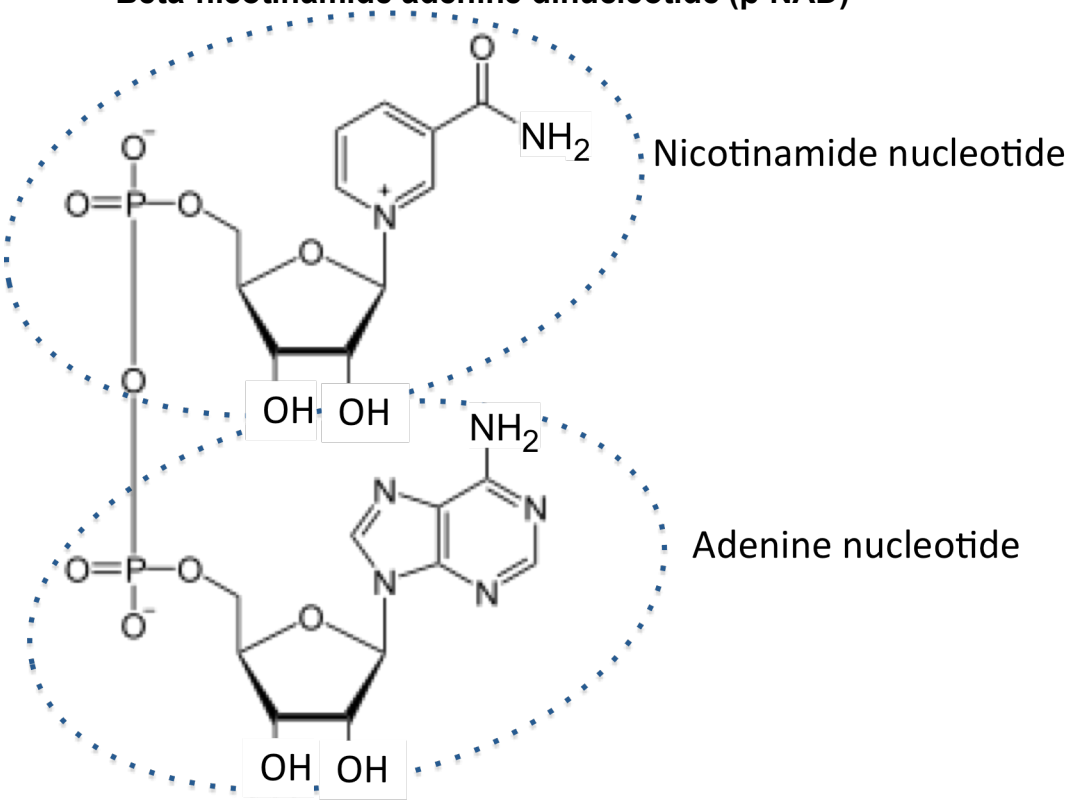
β-NAD is two adenine nicotinamide nucleotides bonded together through their phosphate groups (Fig. 12). β-NAD has been recently proposed as an extracellular signaling molecule and to be a novel neurotransmitter. In the gastrointestinal tract, β-NAD released by enteric nerves may provide inhibitory regulation of visceral muscle. This hypothesis is supported by data showing that the amount of β-NAD released by enteric nerve stimulation is 30-fold higher than the amount of ATP released from enteric nerves during the same stimulation period (Mutafova-Yambolieva *et al.*, 2007). Recent evidence suggests that sympathetic nerves also release β-NAD along with NE, and ATP (Smyth *et al.*, 2004; Smyth *et al.*, 2006) in canine mesenteric arteries. Similar to sympathetic NE and ATP release, the release of β-NAD is also dependent on the N-type Ca^{2+} channel as ω-conotoxin GVIA prevents β-NAD release (Smyth *et al.*, 2004). Further studies are needed to determine source(s), underlying mechanisms, and significance for health and disease of β-NAD signaling.

Figure 12. Purine chemical structures





Beta-nicotinamide adenine dinucleotide (β-NAD)



1.4.5.6 Adenosine neurotransmission

Adenosine is a purine nucleoside composed of adenine base and deoxyribose sugar (Fig. 12). Adenosine is produced intracellularly and extracellularly, but by different mechanisms (Latini and Pedata 2001). Intracellularly, adenosine is formed as AMP catabolism byproduct, which could be transported to extracellular space through the membrane via bidirectional nucleoside transporters. In the extracellular space, released nucleotides such as ATP are metabolized to adenosine by ecto-5'-nucleotidase (Cechova *et al.*, 2008).

Adenosine is released into extracellular space under physiologic and pathologic conditions. A large amount of adenosine is produced during hypoxia as cellular ATP consumption increases. When sympathetic nerves release ATP into the neuroeffector junction, ATP is rapidly degraded into ADP, AMP, and adenosine by membrane bound or soluble ecto-nucleotidases (Fig. 13). In the guinea-pig vas deferens, ATP released by sympathetic nerves is degraded by releasable nucleotidases (ATPDase and AMPase) as ATP metabolism profile is extremely low in the absence of nerve stimulation. Conversely, there is substantial ATP degradation during electrical nerve stimulation (Fig. 14) (Todorov *et al.*, 1997).

Figure 13. Schematic representation of NE and ATP cotransmission for perivascular sympathetic nerve terminal. NE and ATP nucleotidases are released from nerve terminal. NE acts on α_1-adrenoceptor, and ATP acts on P2X receptor in arteries or P2Y receptor in veins to elicit contraction. ATP undergoes sequentially metabolism to ADP, AMP, and adenosine (Ado) as the final degradation product via the action of releasable and extracellular membrane-bound nucleotidases. Releasable ATPDase exhibiting some similarity to ectonucleoside triphosphate/diphosphohydrolase (eNTPDase) converts ATP to ADP and ADP to AMP. Releasable AMPase exhibits similarity to ecto 5' nucleotidase (E5'N) in converting AMP to adenosine. Ado and NE act on P1 (A1-adenosine) receptor and α_2-adrenoceptor to inhibit further transmitter release.

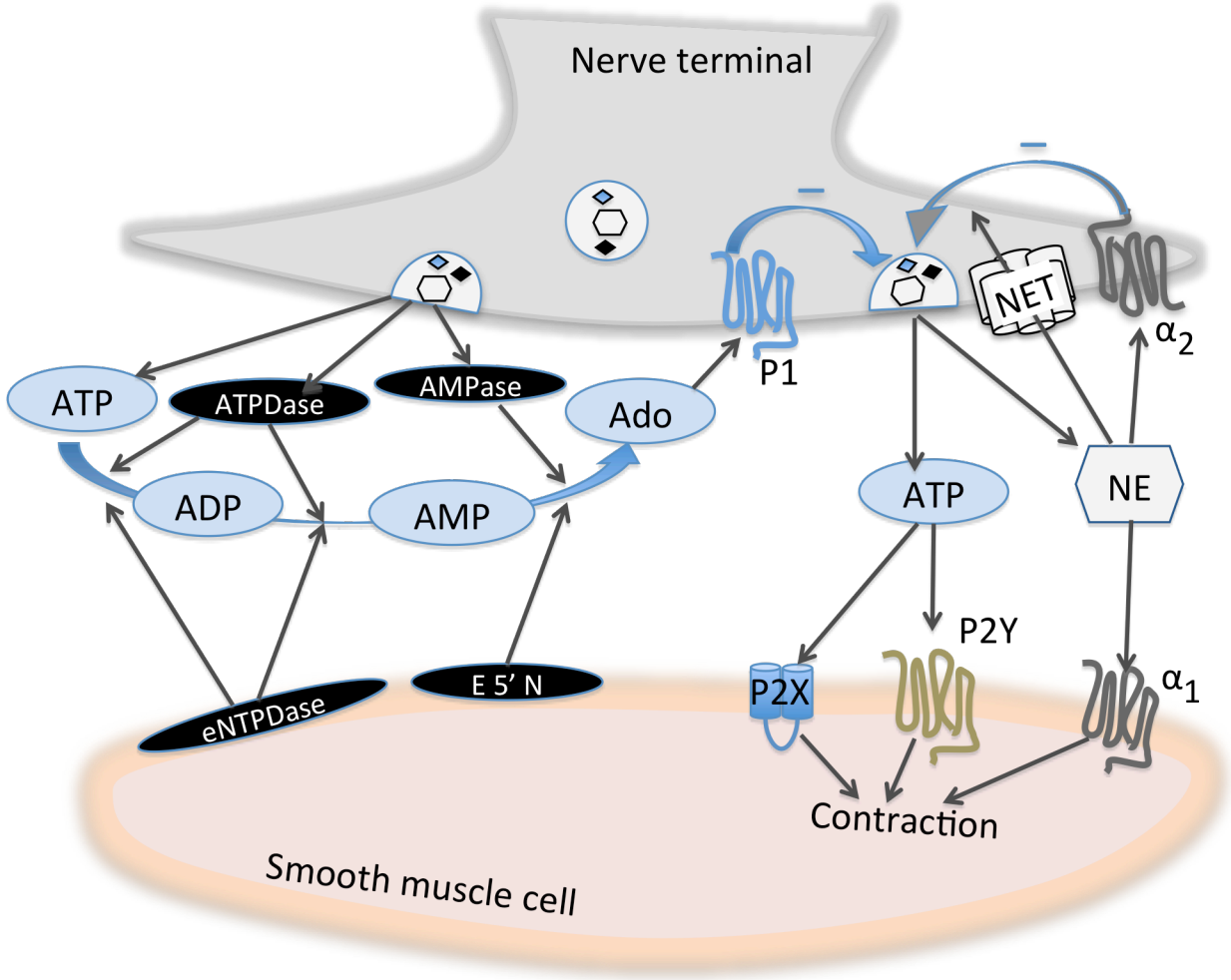
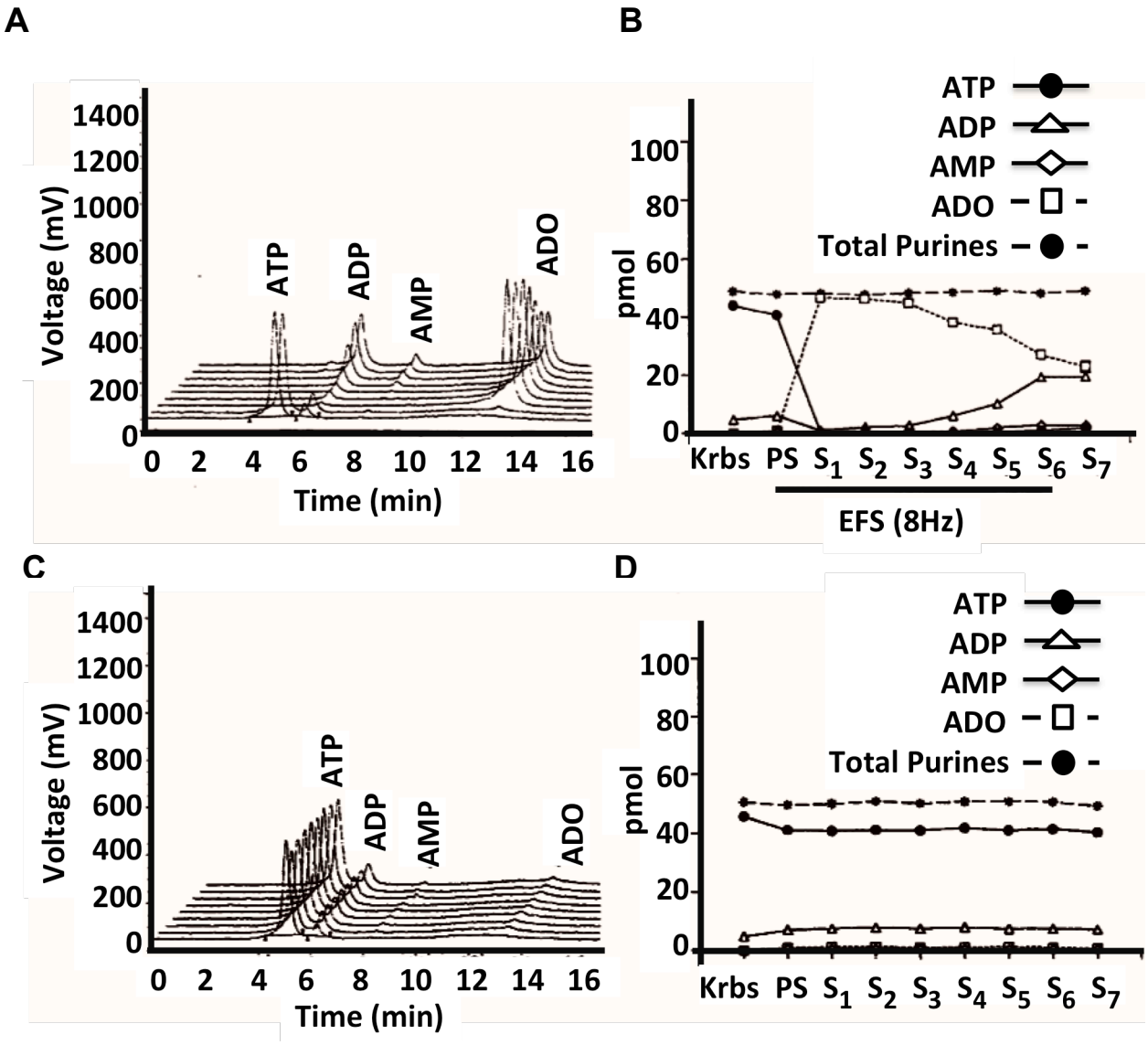


Figure 14. Neuronal ATP release and its degradation by guinea-pig vas deferens. To study neuronally released ATP breakdown, 9 consecutive superfusates were collected during nerve stimulations (8 Hz 6s) with 10-s interval. Krbs: control sample of Krebs solution, PS: 10s immediately before nerve stimulation, S1-S6: 6 consecutive nerve stimulation, S7: 10s after nerve stimulation. A) Original HPLC chromogograms for 9 consecutive superfusates with nerve stimulation arranging from front to back B) Converted mean data showing. C-D) Similar experiment to A and B were performed in the absence of nerve stimulation (Todorov *et al.*, 1997).

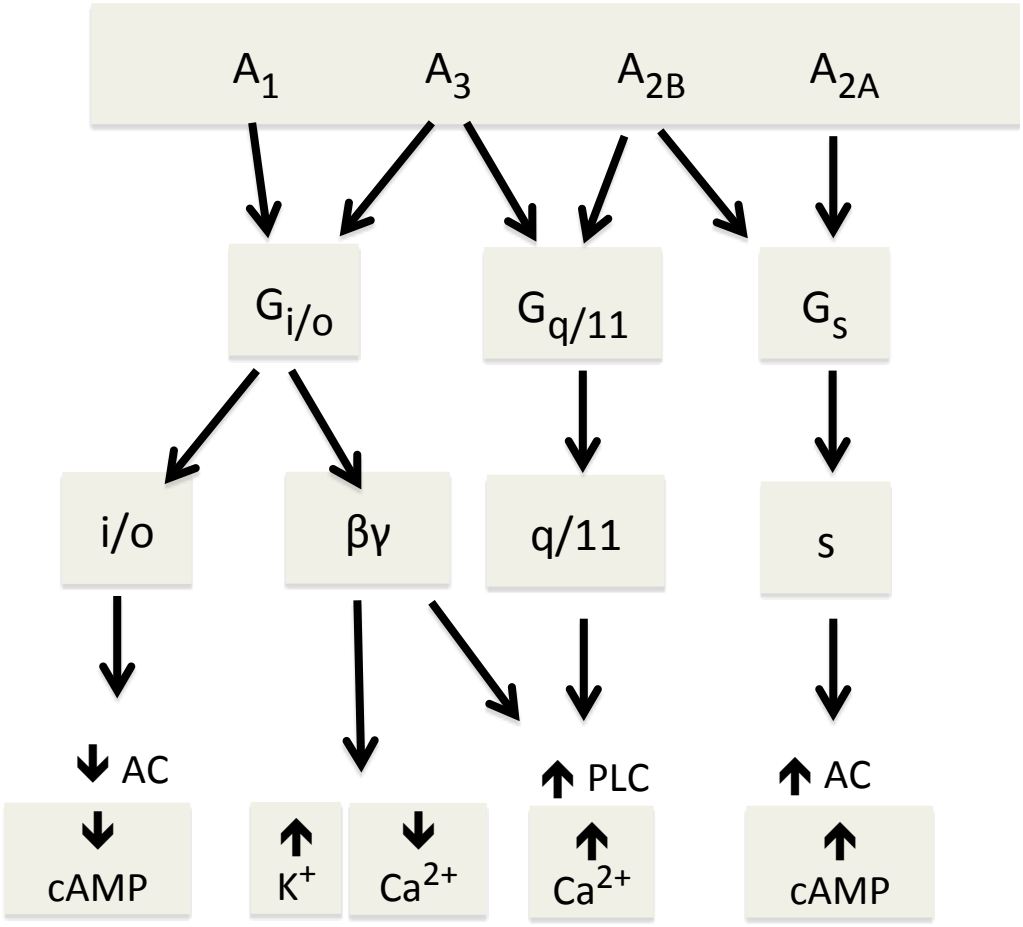


Adenosine receptor subtypes and signal transduction. Adenosine receptors are G protein-coupled receptors. There are four subtypes that are distinguished based on their ability to inhibit or stimulate adenylyl cyclase (AC) activity (Olah *et al.*, 2000) and different agonist and antagonist binding affinity (Alexander *et al.*, 1994; Ongini *et al.*, 1996). The four subtypes of adenosine receptors are the A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm *et al.*, 2001; Klotz, 2000; Linden, 2001; Tabrizchi *et al.*, 2001). Adenosine A_1 receptors (A_1Rs) couple to the inhibitory G proteins, G_i or G_o (Linden, 2001; Ralevic *et al.*, 1998) and receptor activation decreases cAMP due to adenylyl cyclase inhibition (Shen *et al.*, 1995; Tabrizchi *et al.*, 2001). A_1Rs also activate phospholipase C (PLC) leading to an increase in inositol 1,4,5-triphosphate (IP_3), diacylglycerol (DAG). IP_3 stimulates Ca^{2+} release from intracellular stores (eg. sarcroplasmic reticulum) (Ralevic *et al.*, 1998) causing contraction in airway smooth muscle (Abebe *et al.*, 1998). A_1R activation also opens K^+_{ATP} channels in cardiac muscle (Liang, 1996; Liang, 1998), coronary arteries (Davie *et al.*, 1999), and neurons (Andoh *et al.*, 2006). Activation of A_1R in the nervous system inhibits Ca^{2+} currents resulting in suppression of neurotransmitter release (Fig. 15) (Ralevic *et al.*, 1998; Storr *et al.*, 2002).

Adenosine A_{2A}Rs couple with Gs (Olah *et al.*, 2000; Ralevic *et al.*, 1998) to stimulate adenylyl cyclase and Ca^{2+} channels. A_{2A}Rs also activate G(olf) (first identified in olfactory epithelium) in striatum to activate type III adenylyl cyclase (Herve *et al.*, 1993;

Linden, 2001) (Fig. 15). A_{2B}Rs signal through G_s or G_q, which produce Ca^{2+} mobilization and MAPK activation (Fig. 15). Activation of A_3Rs signal through G_i and G_q proteins, which activate phospholipase C/D resulting in phosphorylation and desensitization of A_1Rs (Linden, 2001; Tabrizchi *et al.*, 2001).

Figure 15. Summary diagram of adenosine receptor subtype signaling pathways (Klotz, 2007)



Adenosine in the cardiovascular system. In the heart, pharmacological and genetic data reveal a cardioprotective effect of adenosine during hypoxia (Auchampach *et al.*, 1999; Lasley, 2005; Liang, 1996; Liang, 1998) via the activation of A_1Rs, A_{2A}Rs, A_{2B}Rs and A_3Rs on cardiac myocytes (Auchampach *et al.*, 1999; Eckle *et al.*, 2007; Lasley *et al.*, 2007). In coronary arteries, activation of A_{2A}Rs and A_{2B}Rs enhances nitric oxide production (Olanrewaju *et al.*, 2000) promoting vasodilation (Teng *et al.*, 2008) to increase coronary blood flow (Talukder *et al.*, 2003). This mechanism provides cardioprotection during ischemia. In the aorta, activation of A_{2A}Rs and A_{2B}Rs relaxes smooth muscle cells. In the renal vasculature, activation of A_1Rs via G_i coupling causes renal afferent arteriole contraction, while activation of A_2Rs via G_s coupling causes dilation of afferent arterioles (Welch, 2002). Endothelial cells also express A_{2A}R and A_{2B}R, and hypoxia modulates the expression of both receptor subtypes. Increased adenosine concentration during hypoxia reduces the high affinity A_{2A}R expression, but conversely increases mRNA expression of the low affinity A_{2B}R, which further promotes the release of an angiogenic factor, vascular endothelial growth factor (Feoktistov *et al.*, 2004).

The adenosine-induced effects are concentration dependent as low adenosine concentrations (micromolar range) activate A_1Rs, while higher concentrations activate A_1Rs and A_2Rs. In MA, adenosine produces both relaxation and constriction.

Vasodilation of MA by adenosine is primarily mediated by A_2Rs on smooth muscle cells, which link to K_{ATP} channels (Tabrizchi *et al.*, 2001). Another possibility is that activation of A_2Rs located on endothelial cells is associated with nitric oxide release. Activation of A_3Rs reduces blood pressure in rats, but there is no evidence to indicate that A_3R activation could elicit vasodilation (Tabrizchi *et al.*, 2001). Furthermore, postganglionic sympathetic nerves innervating mesenteric blood vessels express A_1Rs, which modulate sympathetic neurotransmission (Illes *et al.*, 1988; Ralevic, 2000). Stimulation of adenosine receptors relaxes the portal vein of rats and saphenous and hind limb vein of dogs (Ralevic, 2000). Adenosine action on vascular tissues is especially important when oxygen demand is high and supply is low, as occurs in ischemia. In this situation, adenosine release is increased dramatically from cells and this can have a profound impact on blood vessel tone and blood flow (Tabrizchi *et al.*, 2001). Although there is growing evidence revealing adenosine receptors subtypes and their functions in different blood vessels, characterization of these processes in MA and MV are not clearly described (Tabrizchi *et al.*, 2001) particularly in hypertension. Therefore, further clarification of the role of adenosine in the mesenteric circulation is needed.

Adenosine in sympathetic neurotransmission The sympathetic nervous system regulates blood pressure by regulating cardiac function and tone of resistance arteries and capacitance veins, particularly in the mesenteric circulation (King *et al.*, 2007). Sympathetic nerves supply blood vessels at the adventitial-medial surface for arteries and they distribute deeper into the media for veins (Birch *et al.*, 2008). Sympathetic

release of NE and ATP is precisely regulated by the prejunctional α_2-autoreceptors and A_1Rs (Demel *et al.*, 2008a; Illes *et al.*, 1988; Ralevic, 2000). NE acts at prejunctional α_2-autoreceptors to inhibit co-transmitter release from sympathetic nerves. On the other hand, ATP is quickly terminated following its release by enzymatic degradation to ADP, AMP, and adenosine as a final product (Todorov *et al.*, 1997; Todorov *et al.*, 1999). Adenosine binds to prejunctional A_1Rs to inhibit transmitter release. Evidence showed that chronic inhibition of a non-selective adenosine receptor antagonist, 1,3-dipropyl-8-sulphophenylxanthine (DPSPX), increased blood pressure and purinergic and adrenergic neurotransmission (Guimaraes *et al.*, 2003). This indicates that adenosine regulates blood pressure partly by acting at prejunctional adenosine receptors.

Adenosine transporters Regarding sympathetic neurotransmission, adenosine could be formed by hydrolysis of released ATP or directly released from the postjunctional site. Under physiological condition, the major source of adenosine would be ATP degradation by ectoendonuclease in the neuroeffector junction. Conversely, during hypoxia, adenosine is probably released from the cells to modulate cell-surviving mechanisms (Frenguelli *et al.*, 2003; Görlach, 2005).

1.5 Modulation of sympathetic neurotransmitter release by prejunctional autoreceptors

The release of sympathetic neurotransmitter is precisely regulated by various endogenous substances acting on a variety of prejunctional autoreceptors, which link to complex second messenger pathways (Brock *et al.*, 1993). The autoreceptors are local machineries expressed on sympathetic varicosities to modulate the release of transmitters. Autoreceptors could facilitate/inhibit transmitter release or alter the transmitter synthesis (Brock, 1995). For instance, NE action on the prejunctional α_2-AR inhibits further NE release, while circulating epinephrine action on the prejunctional β_2-AR facilitates the release of NE (Lennarstjarne *et al.*, 1975).

To study the function of the prejunctional autoreceptors, one may demonstrate that activation of the autoreceptor by adding selective exogenous agonist showing greater modulation of the release. Conversely, specific antagonist should alter transmitter release oppositely from that receptors being activated by the endogenous/exogenous agonist (Brock, 1995). For example, activation of prejunctional α_2-AR by exogenous agonist UK-14304 or NE provides a negative feedback to sympathetic nerves resulting in a reduction of transmitter release and neurogenic contractile response. Conversely, blockade of α_2-AR by its selective antagonist yohimbine causes an increase in NE release (Park *et al.*, 2010) from periarterial sympathetic nerves. However, failure of an antagonist to alter transmitter may because the autoreceptors are not operative under experimental condition (Brock, 1995).

More interestingly, modulation of sympathetic transmitter release exhibits sophisticated cross-inhibitory regulation. Activation of prejunctional α_2-AR inhibits both NE release (Park *et al.*, 2010) and ATP-induced excitatory junction potential (EJP) (Demel *et al.*, 2008b). Similarly, prejunctional adenosine A_1R activation attenuates NE release (see chapter 3) and ATP-induced EJP response (Demel *et al.*, 2008b).

Voltage-gated Ca^{2+} channels are the key component of neurotransmitter release. Increased intra-terminal Ca^{2+} concentration via voltage-gated Ca^{2+} channel is the key trigger of transmitter release, while Ca^{2+}-induced Ca^{2+} release from intracellular stores seems to be ineffective mechanism for transmitter release (Burgoyne *et al.*, 1995). Therefore, voltage-gated Ca^{2+} channels are biological target for autoreceptors in modulating transmitter release. Here, a specific example of how the prejunctional α_2-adrenergic modulates sympathetic transmitter release is described.

1.5.1 The α_2-adrenergic autoreceptors

The prejunctional α_2-adrenergic autoreceptors provide inhibitory modulation of transmitter release in both central and peripheral nerves. The prejunctional $\alpha_{2A/D}$-AR is the major autoreceptor providing negative feedback to stop sympathetic transmission (Guimaraes *et al.*, 2001). The α_{2A} subtype is cloned from human and pocrine tissue, which is slightly different from its homologous receptors cloned from rat, mouse, and guinea pig. The homologous of α_{2A} subtype is named α_{2D} and these two receptors are

simply referred to as $\alpha_{2A/D}$-AR (Guimaraes *et al.*, 2001). Furthermore, α_{2C}-AR also serves as an additional mechanism providing inhibitory modulation of transmitter release with different time course of expression from $\alpha_{2A/D}$-AR. The $\alpha_{2A/D}$-AR is operative immediately at birth, while the α_{2C}-AR is become operative later in mice. The α_{2C}-AR is a high affinity receptor; hence it regulates basal NE release. Conversely, the low affinity $\alpha_{2A/D}$-AR regulates transmitter release at higher sympathetic tone (Guimaraes *et al.*, 2001; Philipp *et al.*, 2002). Additionally, α_{2C}-AR function is more prominent in peripheral, while $\alpha_{2A/D}$-AR is likely to be important in central nervous system (Guimaraes *et al.*, 2001).

The $\alpha_{2A/D}$ and α_{2C}-AR inhibit transmitter release by modulating voltage-gated Ca^{2+} channels. The α_2-AR inhibits L-type Ca2+ channels in rat retina (Dong *et al.*, 2007), and N-type channels in sympathetic nerves (Schwartz, 1997). In sympathetic neurons, only $\alpha_{2A/D}$ subtype, but not α_{2C} contributes to inhibition of transmitter release. As α_2-AR is coupled to inhibitory G-protein (G_i), it was first proposed that α_2-AR might inhibit transmitter release by inhibiting adenylate cyclase activity. However, several studies found that neither forskolin nor phosphodiesterase inhibitors alter the effect of α_2-AR-induced inhibition of NE release in various tissues leading to a conclusion that cAMP does not a link in presynaptic α_2-AR (Starke, 1987).

Meanwhile, G protein *βγ* subunits inhibit voltage-gated Ca^{2+} channels resulting in inhibition of transmitter release, and this Ca^{2+}channel inhibition is relieved by protein kinase C (PKC) (Herlitze *et al.*, 2001; Swartz, 1993), which generally coupled to various prejunctional facilitatory autoreceptors including angiotensin AT_1 receptors (Talaia *et al.*, 2006), and prejunctional adenosine A_{2B} receptors (Talaia *et al.*, 2005). Phosphorylation of the voltage-gated Ca^{2+} channels by PKC removes tonic G protein-mediated inhibition of the Ca^{2+} channels.

The α_2-AR expression is not restricted to the prejunctional site as they also express on vascular smooth muscle and endothelial cells. The $\alpha_{2A/D}$, α_{2B}, and α_{2C} subtypes express on vascular smooth muscle cells mediating vasoconstriction (Kanagy, 2005), while α_2-AR expressed by vascular endothelial cells couples to NO release and vasodilation in isolated coronary, renal and mesenteric arteries (Guimaraes *et al.*, 2001). As the α_2-ARs express in both pre-and post-junctional sites, it is equivocal to determine if the receptor agonist/antagonist effects on neurogenic contraction response is caused by pre- or post-junctional receptor activation. Continuous amperometry with microelectrode permit one to study sympathetic transmission by directly measuring transmitter release as an oxidation current proportional to concentration of transmitter release (See chapter2). This electrochemical technique provides unequivocal evidence of the autoreceptor function on transmitter release regardless of interference from the drug interacting at postjunctional α_2-ARs.

1.6 Differential sympathetic regulation of arteries and veins

As arteries and veins obtain different hemodynamic functions, they receive differential sympathetic nerve innervation. Nonmyelinated sympathetic nerves form plexuses at border of tunica adventitia and media of arteries, while penetrate further down into media of veins suggesting closer contact between nerve varicosities and venous smooth muscle cells (Fig. 16A, C) than that in arterial bed (Fig. 16 B, D). Furthermore, glycosidic acid-induced fluorescent catecholamine data revealed that the veins have less sympathetic nerve fibers than the arteries with different nerve arrangement. The veins receive mostly circular nerve arrangement; while arteries receive both circular and longitudinal sympathetic innervation (Park *et al.*, 2007). This differential nerve organization contributes to a unique “upstream” and/or “lateral” regulation of transmitter release and clearance at the neuroeffector junction (Stjarne, 1989) in periarterial, but not perivenous nerves (Fig. 17). Stärke and Stjärne proposed that transmitter release could be regulated by the autoreceptors expressed by remote varicosities (Stjarne, 1989). A train stimuli causes transmitter release from varicosities, accumulated, and diffusing to neighboring varicosities causing further activation of autoreceptors and transporters. The early evidence of upstream/lateral regulation was reported by Stjärne and Stjärne. They found that inhibition of ATP release from varicosities inside the patch was caused by activation of α_2-autoreceptors outside the patch by exogenous agonist clonidine. It should be noted that α_2-autoreceptors inside the patch were initially blocked by yohimbine (α_2 antagonist) to prevent the receptor activation. Hence, the inhibition of ATP release observed inside the patch was caused

by the activation of neighboring α_2-autoreceptors outside the patch (Stjärne *et al.*, 1988). Furthermore, Park and coworkers also revealed differential kinetics of NE release and clearance of periarterial and perivenous sympathetic nerves. Periarterial is capable to inhibit NE release and remove NE more efficiently than that in perivenous nerves apparently at low frequency of nerve stimulation. This may because low frequency stimulation permits sufficient time for released transmitter to accumulate and diffuse to surrounding autoreceptors. They also revealed differential rising phase of NE oxidation current evoked by electrical nerve stimulation. Arterial NE current exhibited biphasic rise phase: initial rapid rise following by gradual rise. They proposed that the second phase (gradual rise) was influenced by the activation of prejunctional autoreceptors to inhibit NE release and this gradual rise was absent in venous NE current (Park *et al.*, 2010). Furthermore, activation of α_2-AR by exogenous agonist UK-14304 causes a dramatic increase in NE release in periarterial nerves, while induces slightly increase in NE release in perivascular nerves. Additionally, α_2-AR blockade by exogenous yohimbine increases NE release in periarterial, but not perivenous nerves (Park *et al.*, 2007). Meanwhile, lateral NE clearance is also established predominantly in periarterial nerves. Cocaine, NE transporter blocker causes a remarkable increase in NE release in periarterial, but not perivenous nerves (Park *et al.*, 2007). Therefore, it is logical to imply that upstream/lateral regulation provides higher efficiency to periarterial nerve to control release and removal of NE from neurovascular junction, which may account for smaller basal NE oxidation current measured by amperometry of periarterial nerves compared to perivenous nerves (Park *et al.*, 2010; Park *et al.*, 2007). Lack of

lateral regulation in perivenous nerves may be due to less sympathetic supply or/and less amount of transmitter release from varicosities to accumulate and diffuse to autoreceptors and/or transporters located on surrounding varicosities.

Furthermore, arteries and veins may contain different transmitters in sympathetic varicosities and this issue is still controversial. In rat mesenteric bed, NE is likely to be a major transmitter released from perivenous nerves, while periarterial varicosities contain cotransmitter cocktail: ATP, NPY, and NE (Talaia *et al.*, 2011). Donoso and coworkers demonstrated that blockade of NPY Y_1, P2 purinergic, or α1-adrenergic receptors by specific exogenous antagonist reduces neurogenic vasomotor response and combination of antagonists for these three receptor completely abolish neurogenic response in rat mesenteric arteries (Donoso *et al.*, 1997b). This is consistent with Park and coworkers' work reporting that blockade of α_1-adrenergic receptors by prazosin partially inhibited neurogenic arterial contraction, while prazosin abolished neurogenic venous contraction (Park *et al.*, 2010) suggesting that NE is only major neurotransmitter released from perivenous sympathetic nerves. However, NPY and ATP seem to be cotransmitters along with NE in guinea pig mesenteric veins. Smyth *et al* showed colocalization of NPY and tyrosine hydroxylase-containing nerve fibers in both guinea-pig mesenteric arteries and veins. Additionally, NPY Y1 antagonist abolishes neurogenic venous contraction in NE-depleted veins. Moreover, blockade of purinergic receptors by PPAD and sumarin attenuates neurogenic contraction in NE-depleted veins (Smyth *et al.*, 2000). These findings suggested that perhaps, perivenous sympathetic nerves also release ATP, but in only a small fraction and it does not

necessarily to plays an important role at the postjunctional site. Conversely, this small fraction of released ATP only acts prejunctionally to modulate cotransmitter release.

Figure. 16 Differential sympathetic nerve distribution of arteries and veins. Sympathetic nerves supply arteries at border of adventitia and media (A), and penetrate deeper into tunica media of veins (B). Arteries are more densely innervated with sympathetic nerves (C) compared to veins (D). T, tunica; VSMC, vascular smooth muscle cell.

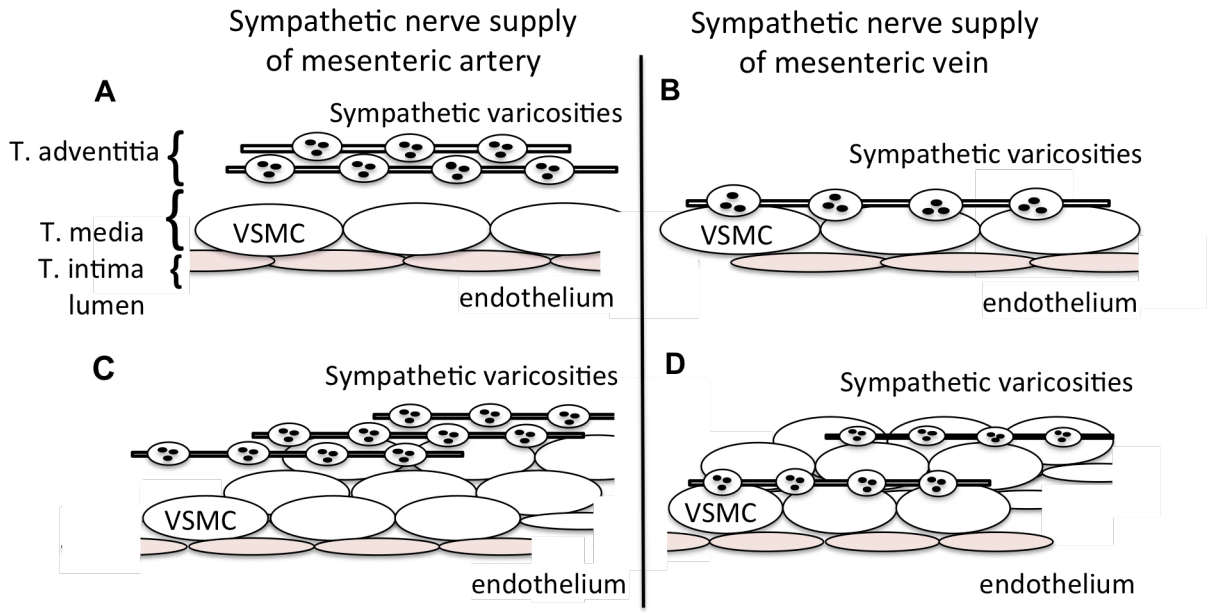
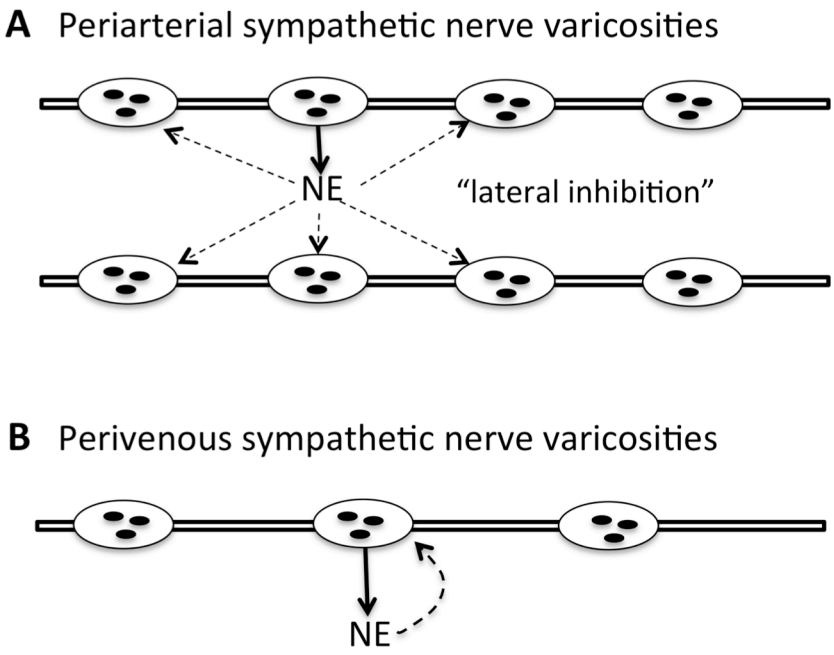


Figure. 17 Diagram illustrating lateral regulation for NE release and clearance of periarterial sympathetic nerves. The lateral regulation hypothesis proposed that NE released from one varicosity could diffuse to activate on remote varicosities to turn on autoinhibition negative feedback. Also, released NE from one site is removed by the NE transporters located on neighboring varicosities. This lateral inhibition is exclusive for periarterial (A), but not perivenous nerves (B).



1.7 Hypertension

Hypertension is an important public health problem. About 1 billion people worldwide had hypertension in 2000. This represents 30% of the global population which will be increased to 1.56 billion by 2025 (Patricia M Kearney, 2005). Hypertension causes approximately 7.1 million deaths worldwide each year (Chobanian *et al.*, 2003). Hypertension is the key risk factor driving increased cardiovascular disease. The World Health Organization estimates that 62% of cerebrovascular disease and 49% of ischemia heart disease occurs in hypertensive patients with suboptimal blood pressure control (systolic blood pressure >115 mmHg)(Rodgers, 2002). Data from animal models of hypertension and from human studies have clearly shown that increased sympathetic activation contributes to hypertension development (Anderson *et al.*, 1989; Schlaich *et al.*, 2004).

The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC7, (Chobanian *et al.*, 2003)) has defined essential hypertension as systolic/diastolic blood pressure over 140/90 mmHg. More importantly, elevated blood pressure is related to increased cardiovascular mortality risk. Every increment of 20/10mmHg systolic/diastolic pressure from the optimal blood pressure115/75 mmHg increases risks for ischemia heart disease and stroke by two and three fold, respectively (Fig. 18, 19). The stages for high blood pressure have been classified by JNC7 shown in table 3.

Table 3. JNC7 Blood Pressure Classification. SBP, systolic blood pressure; DBP, diastolic blood pressure (Chobanian *et al.*, 2003)

SBP/DBP	Condition
< 120/80	Normal
120-139/80-89	Prehypertension
≥ 140/90	Hypertension
140-159/90-99	Hypertension stage 1
>160/100	Hypertension stage 2

Figure 18. Ischemic heart disease (IHD) mortality rate in each decade of age vs usual systolic. (A) and diastolic (B) blood pressure reported in the JNC 7. (Chobanian *et al*., 2003)

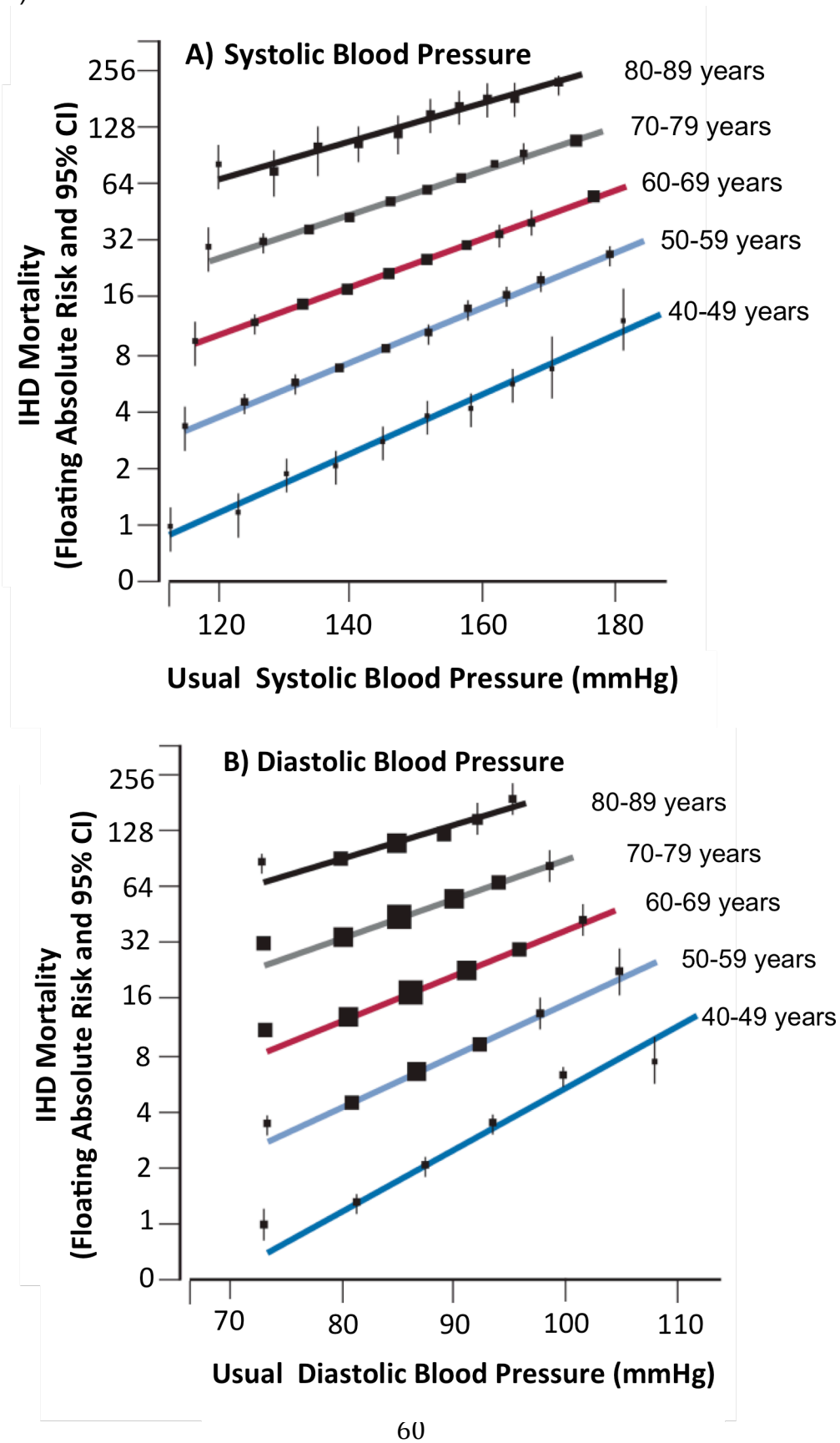
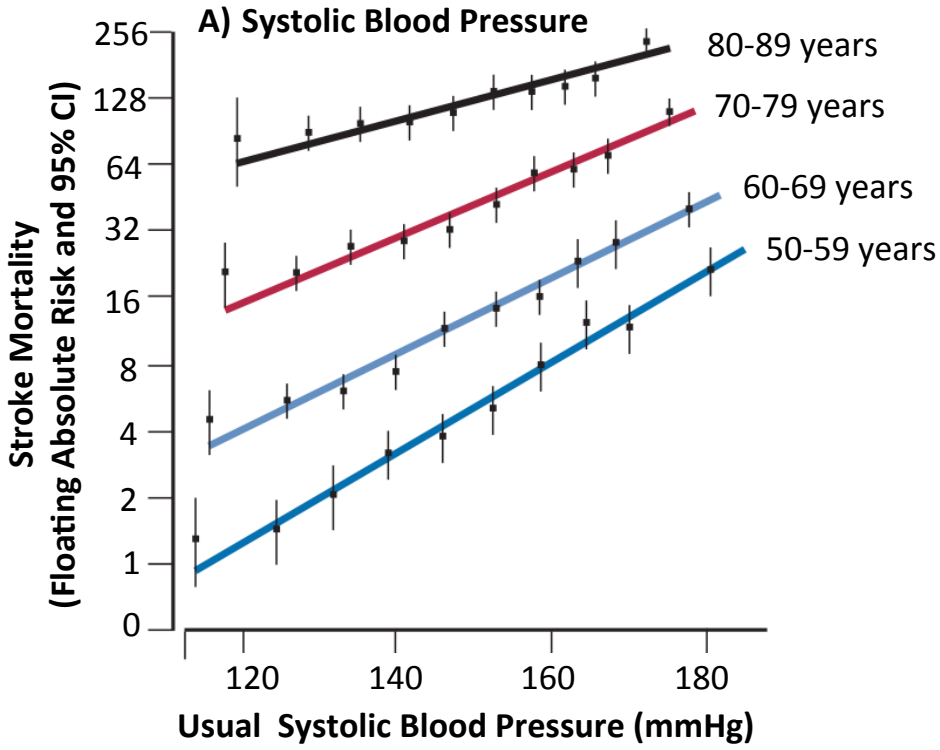
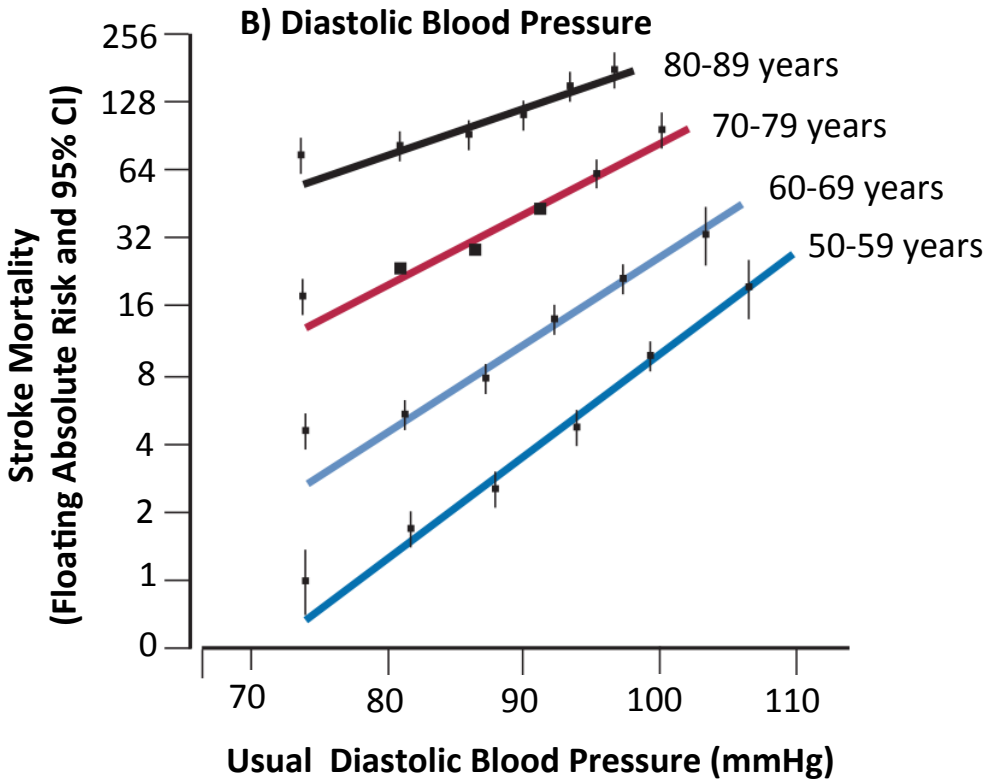


Figure 19. Stroke mortality rate in each decade of age *vs* usual systolic (A) and diastolic (B) blood pressure. (Chobanian *et al.*, 2003)





1.7.1 Sympathetic overactivity in hypertension

Chronic elevated sympathetic nerve activity is associated a variety of cardiovascular diseases including obesity, hypertension, sleep apnea (high prevalence in hypertension and stroke patients), and mental stress. A large-scale study has showed a significant correlation between work-place mental stress and hypertension development (Malpas, 2010). Regarding essential hypertension, it appears that sympathetic nerve activity is elevated in both animal models and human.

1.7.2 Autoreceptors in hypertension

Data from animal models of hypertension and from human studies have clearly shown that increased sympathetic activation contributes to hypertension development (Anderson *et al.*, 1989; Schlaich *et al.*, 2004). Nevertheless, the mechanisms for sympathetic activation occurring in essential hypertension have not been conclusively determined (Mancia *et al.*, 1999). One possibility is that activation of the sympathetic nervous system depends on the function of autoreceptors located on sympathetic nerve terminals (Demel *et al.*, 2008a; Min Luo, 2004). Increasing evidence suggests that impairment of α_2 autoreceptors, which provide negative feedback for sympathetic neuromodulation, contributes to development of hypertension (Bobalova *et al.*, 2001; Demel *et al.*, 2008a; Luo *et al.*, 2003; Min Luo, 2004). However, it is not clear if impairment of A_1 autoreceptor function is also involved in hypertension.

1.7.3 DOCA-salt rat model of hypertension

Administration of the mineralocorticoid deoxycorticosterone acetate (DOCA) in combination with a high salt diet and unilateral nephrectomy is used to induce hypertension in experimental animals (Pintoa *et al.*, 1998; Schenk *et al.*, 1992). This model is considered as a low rennin form of hypertension with salt and water retention, which is relevant to salt-sensitive human hypertension (Luo *et al.*, 2003; Schenk *et al.*, 1992). Although the DOCA-salt rat model of hypertension has been established for decades, one may question what mechanism leads to hypertension in this animal model. Uninephrectomy decreases renal Na^+ excretion. Chronic high-salt administration induces a sustained increase in plasma NaCl and osmolality, which activates neurons in the (O'Donaughy *et al.*, 2006) organum vasculosum lamina terminalis (OVLT) and hypothalamus (Guyenet, 2006). That particular central activation drives sympathoexcitation outflow. Moreover, negative alteration of renal Na^+ excretion allows DOCA-induced Na^+-retention leading to expansion of extracellular fluid and blood volume (Haack *et al.*, 1977). However, volume expansion in DOCA-salt hypertension occurs during the first few weeks then returns to basal volume due to compensatory renal mechanisms. Yamamoto(Yamamoto *et al.*, 1983) and Fink (Fink *et al.*, 2000) found moderately increased blood volume in DOCA-salt hypertension during the early phase of DOCA-salt hypertension. O'Donaughy also proposed that mineralocorticoid; DOCA amplifies Na^+ signal to central network leading to augmented sympathoexcitation. In the absence of DOCA administration, the animals did not develop increased lumbar sympathetic nerve activity or elevated blood pressure under

unilateral nephrectomy and high-salt diet (O'Donaughy *et al.*, 2006). Moreover, evidence suggested that vasopressin was elevated during increased plasma osmolality. Vasopressin (arginine vasopressin, AVP) is an antidiuretic polypeptide hormone produced by magnocellular secretory cells in hypothalamus, and stored and released from the posterior pituitary gland. AVP acts on the distal convoluted tubules and the medullary collecting duct to decrease water excretion. AVP could increase vascular resistance, as it is a potent vasoconstrictor. AVP is released in response to decreased blood volume or increased plasma osmolality. As small as 1% increase in plasma osmolality above the threshold could be detected by the osmoreceptor neurons in the hypothalamus causing AVP release. Selective inhibition of V1 vasopressin receptors significantly decreased plasma NaCl concentration, blood pressure, and lumbar sympathetic nerve activity (O'Donaughy *et al.*, 2006). Crofton and co-workers also reported an increase in plasma vasopressin and urine vasopressin excretion in DOCA-salt rats early after DOCA-salt administration, which matched the timeline of elevated blood pressure of the animals (Crofton *et al.*, 1979). Furthermore, administration of inactive synthetic vasopressin analogs: [l-deaminopenicillamine, 4- valine, 8-D-arginine] vasopressin or [l-(β-mercapto-β, β- cyclopentamethylenepropionic acid), 4-valine, 8-D-arginine]vasopressin caused dramatically decreased in blood pressure in established DOCA-salt hypertension (Crofton *et al.*, 1979). It should be noted that the animals used in Crofton experiments were Long-Evans strain and these rats were received 30mg/kg body weight DOCA and 1%NaCl drinking water. Moreover, blood pressure of DOCA-salt rats with genetic vasopressin deficiency were significantly lower than of those

DOCA-salt with normal synthesizing vasopressin (Kunes *et al.*, 1989). Thus, vasopressin may partly account for development of DOCA-salt hypertension.

Additionally, endothelin-1 (ET-1) level and mRNA expression are increased in DOCA-salt arteries (Day *et al.*, 1995; Larivière *et al.*, 1993; Watts *et al.*, 2002). Endothelin (ET) is a local vasoconstrictor peptide released from vascular endothelial cells. There are three subtypes of ETs: ET-1, ET-2, and ET-3. Of these, ET-1 is the most potent vasoconstrictor (Larivière *et al.*, 1993); hence it is logically implied that ET-1 is involved in pathophysiologic mechanism of hypertension (Deng *et al.*, 1992). Two endothelin receptor subtypes have been cloned: ET_A and ET_B receptors. Similar to NE-induced response, the veins (rat vena cava) are more sensitive to ET-1 induced contraction that arteries (rat aorta) and ET-1 induced contraction mechanism may be impaired in DOCA-salt hypertension (Watts *et al.*, 2002). Chronic administration of ET_A antagonist could lower blood pressure of DOCA-salt hypertensive rats (Callera *et al.*, 2006; Matsumura *et al.*, 1995). Interestingly, elevation of ET-1 contributes to increased superoxide level in DOCA-salt rats (Callera *et al.*, 2006; Li *et al.*, 2003). Li and coworkers revealed that ET-1 and superoxide levels were elevated in DOCA-salt hypertension. Furthermore, ET-1 causes an increase in superoxide production in concentration (0.01-1 nM)-dependent manner in DOCA-salt carotid arteries and this effect is prevented by ET_A antagonist (Li *et al.*, 2003). More interestingly, Yu *et al* reported synergistic effect of a non-selective ET antagonist (bosentan) and vasopressin V_1 antagonist on a reduction of total peripheral resistance and blood pressure of DOCA-salt hypertensive rats compared to single administration of bosentan. Vasopressin

antagonist would only reduce blood pressure when an ET-1 antagonist was also administered (Yu *et al.*, 2001). These findings suggest that development of DOCA-salt hypertension is multifactorial associating with ET and vasopressin hormonal alterations.

DOCA-salt–induced hypertension may involve in both brain-initiated sympathoexcitation and renal fluid retention. While Xu and co-workers found unaltered renal sympathetic nerve activity between DOCA-salt hypertensive and control rats (Xu *et al.*, 2002), Takeda and Bunag directly illustrated elevated splanchnic sympathetic nerve activity in DOCA-salt hypertensive rats in basal and hypothalamic stimulation response (Takeda *et al.*, 1980). Although this study was done in anesthetized rats, it is valuable finding to support the concept that increased sympathetic nerve activity, particularly splanchnic sympathetic nerves, is associated with hypertension development in DOCA-salt rats. Furthermore, De Champlain and Kopin groups reported elevated plasma catecholamine level as an indirect indicator for augmented sympathetic activity, in DOCA-salt hypertensive rats (Bouvier *et al.*, 1986; Reid *et al.*, 1975). Taken these data together, it is likely that there is a selective increase in splanchnic sympathetic nerve activity provided by the celiac ganglion in DOCA-salt hypertensive rats. The unchanged renal sympathetic nerve activity is because the celiac ganglion provides very slightly input to the kidney (Fig.1). On the other hand, it is possible that the alteration of sympathetic nerve activity may occur independently from the central network. For instance, the alteration of peripheral sympathetic transmission may occur due to disruption of the prejunctional control mechanism leading to increased transmitter released onto the postjunctional site. Previous students in our lab have shown that the disruption of adrenergic autoreceptor on postganglionic sympathetic

nerves supplying splanchnic circulation cause an increase in transmitter (ATP and NE) release. The present study focused on the postganglionic sympathetic neurotransmission at the level of the prejunctional purinoautoreceptor alterations leading to changes in transmitter release and clearance in both control and hypertensive animals.

In addition to DOCA-salt rat model of hypertension, recently Kandlikar and coworkers have developed a mild DOCA-salt hypertensive rat. In this model the animals are received low dose of DOCA implantation (50 *vs* 200 mg/kg) without uninephrectomy (Kandlikar *et al.*, 2011; Luo *et al.*, 2004). The animal blood pressure is elevated gradually and the established arterial pressure is increased approximately 10-20 mmHg. They proposed that the small gradual increase in blood pressure permits one to study the casual mechanisms driving hypertension (Kandlikar *et al.*, 2011).

1.7.4 Amperometric measurement of norepinephrine

Amperometry with microelectrodes is an electrochemical technique adapted from voltammetry which is well characterized in chemistry laboratories for almost one century (Evanko, 2005). Amperometry was first introduced for ion chromatography and later it was implicated for detection of electroactive neurotransmitters including catecholamines (dopamine, norepinephrine, epinephrine) and ATP (Llaudet *et al.*, 2005), which can be oxidized at the electrode surface at a given potential. The major breakthrough was when Adams and co-workers implanted carbon fiber microelectrode into rat. Catecholamines are oxidized to quinone and release electrons, which are transferred to the electrode surface and detected as an oxidation current (Mundorf *et al.*, 2002). An

amperometric cell includes a carbon fiber microelectrode (working electrode), a platinum counter (auxiliary) electrode, and silver-silver chloride (Ag/AgCl) reference electrode. The direction of the electron flow depends on the properties of the neurotransmitter and can be controlled by potential applied to the working electrode. Generally, the potential of the working electrode is held sufficiently positive so that all released transmitters are oxidized. This will cause an accumulation of an oxidation current in response to a brief train of stimuli. The working electrode for transmitter detection is usually constructed from carbon fiber or diamond (Park *et al.*, 2010; Park *et al.*, 2007; Park *et al.*, 2005). A reference electrode provides a fixed potential against which potential applied to the working electrode is measured and controlled. Platinum counter electrode is used to complete amperometric circuitry. Amperometric measurement produces transient electro-oxidation current that is proportional to the concentration of the neurotransmitter oxidized per unit time. Area under the curve is used to calculate the amount of released transmitter. The shape of oxidation current contains information about transmitter release, autoinhibition, and transmitter reuptake.

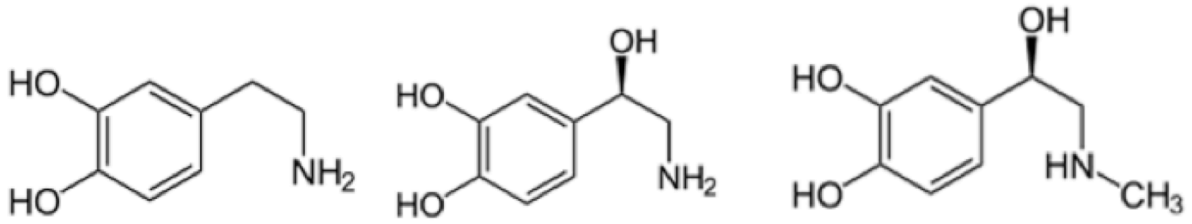
1.7.5 Contribution of other catecholamines in amperometric detection of NE

As NE share similar structure with dopamine and epinephrine (Fig. 20), one may question that at the given potential 0.6 Volts, would the amperometric oxidation current results from the mixture of these three catecholamines? Although NE, dopamine and epinephrine become oxidizable at 0.6 Volts potential, epinephrine is exclusively released from adrenal medulla during stress response. Perivascular sympathetic nerves

do not contain enzyme phenylethanolamine *N*-methyltransferase to convert NE into epinephrine.

However, dopamine could be released from sympathetic nerves supplying canine mesenteric blood vessels (Soares-da-Silva, 1988). Silva showed that prolonged sympathetic nerve depolarization with KCl causes dopamine release along with NE. Sustained nerve stimulation causes dopamine *β*-hydroxylase become rate-limiting resulting in accumulation of dopamine available for release. Once release, dopamine could act on the prejunctional dopamine receptors providing the negative feedback to shut down sympathetic transmission. In my experiment, I used brief electrical-train stimuli (60 stimuli at 10 Hz frequency, 0.5 ms pulse duration). At this stimulation parameter, it would be hardly dopamine release.

Figure 20. Catecholamine structures



Dopamine Norepinephrine Epinephrine

1.8 Perspectives

Although there have been substantial advances in anti-hypertensive medication development and use, many patients do not respond well to these drugs or resist their use because of troubling side effects. Continued research directed at identifying new drug targets is needed. Clarifying the mechanism that leads to enhanced neurogenic vasoconstriction may open a door for a new avenue of anti-hypertensive drug development.

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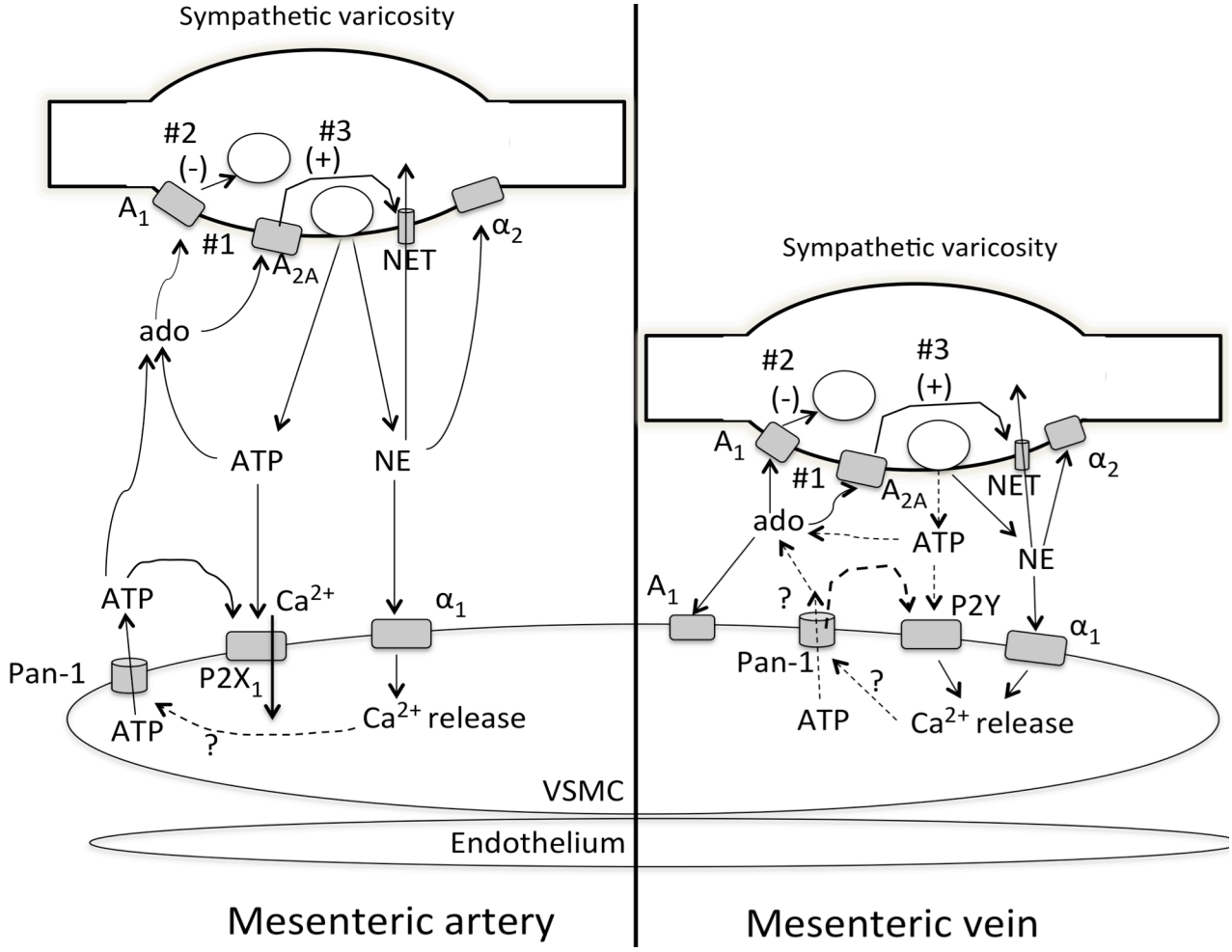
CHAPTER 2

RESEARCH SPECIFIC AIMS AND GENERAL METHODS

2.1 Overall research goal

Adenosine, a final ATP metabolite, is a signaling molecule in the cardiovascular system. Adenosine receptors are G-protein coupled receptors and there are four subtypes: A_1, A_{2A}, A_{2B}, and A_3 but receptors controlling arterial and venous tone, particularly in the mesentery, have not been identified. This is important because small mesenteric arteries (MA) and veins (MV) contribute to the resistance and capacitance functions of the peripheral circulation and identification of the adenosine receptor subtypes in this vascular bed is still unclear (Tabrizchi *et al.*, 2001). Thus, my first goal is to identify the expression of adenosine receptors expressed by the sympathetic nerves supplying MA and MV. The second goal is to determine the function of prejunctional adenosine A_1 and A_{2A} receptors in modulating adrenergic neurotransmission. The last part of my research goal was to test the hypothesis that adenosine receptor dysfunction contributes to increased NE release from sympathetic nerves supplying MA and MV in deoxycorticosterone acetate (DOCA)-salt hypertensive rats. The overall goal is described in a summary diagram (Fig. 21).

Figure 21. Overall goals. Aim 1, Characterize adenosine receptors expressed by sympathetic nerves supplying MA and MV (#1). Aim 2, Determine the function of the prejunctional A1 receptors (A_1) in modulating NE release; test if A_1R dysfunction contributes to elevated NE release in DOCA-salt hypertension (#2). Aim 3, Determine the role of prejunctional Adenosine $_{2A}$ receptors (A_{2A}) in sympathetic transmission and tested if A_{2A} receptor dysfunction contributes to elevated NE availability in DOCA-salt hypertension. α_1, α_1-adrenergic receptor; α_2, α_2-adrenergic receptor, ado, adenosine; NET, NE transporter; Pan1, pannexin-1; VSMC vascular smooth muscle cells. Solid lines indicate established mechanism. Dashed lines indicate equivocal mechanisms.



2.2 Specific Aims

Specific aim #1 Test the hypothesis that A_1 and A_{2A} adenosine receptors localize to sympathetic nerves innervating MA and MV (prejunctional site), while A_1Rs are localized to venous but not arterial smooth muscle cells (postjunctional site).

Specific aim 1a: Localize all adenosine receptor subtypes on the sympathetic nerves supplying mesenteric arteries and veins. Immunohistochemistry (IHC) for all adenosine subtypes (A_1, A_{2A}, A_{2B}, and A_3) and tyrosine hydroxylase, a marker for sympathetic nerve will be used to determine the co-localization of the prejunctional adenosine receptors on perivascular sympathetic nerves. The expression of the prejunctional adenosine receptors are not determined by PCR or western analysis, as these two techniques could not distinguish the pre-and postjunctional adenosine receptors. My strategy is to combine the IHC data with functional studies to reveal the role of adenosine in modulating sympathetic transmission.

Specific aim 1b: Identify postjunctional adenosine receptors mediating MV constriction *in vitro* by using agonists and antagonists for adenosine receptor subtypes.

Specific aim #2 Examine the inhibitory modulation by the prejunctional A_1R on sympathetic NE release and test the hypothesis that A_1R dysfunction causes an increase in NE release in DOCA-salt hypertension.

Specific aim 2a: Establish concentration response curves (CRCs) for adenosine receptor agonists for inhibition of neurogenic constrictions of MA and MV *in vitro*. Adenosine receptor antagonists, and adenosine transporter blocker are used for further receptor characterization.

Specific aim 2b: Measure NE release and vasoconstriction *in vitro* using continuous amperometry with carbon-fiber microelectrode and video microscopy, respectively. Adenosine receptors on sympathetic nerves will be studied in isolated MA and MV *in vitro*. Adenosine receptor antagonist and adenosine transporter blocking agents are used to reveal role of endogenous adenosine.

Specific aim #3 Determine role of the prejunctional A_{2A} receptor in adrenergic transmission and test if the $A_{2A}R$ impaired function contributes to DOCA-salt hypertension.

Specific aim 3a: test the hypothesis that activation of $A_{2A}R$ decreases NE availability in the neuroeffector junction by increasing the activity of the NE transporter.

Specific aim 3b: Test the hypothesis that $A_{2A}R$ receptor dysfunction causes an increase in neurogenic NE availability in the neuroeffector junction of DOCA-salt hypertension.

Measurement of NE release and vasoconstriction *in vitro* are accomplished using amperometry and video microscopy respectively. $A_{2A}R$ agonists, and antagonists are used reveal the role of the $A_{2A}R$ in modulating adrenergic transmission and its alteration in DOCA-salt hypertensive rats.

2.3 Research design and methods

2.3.1 Specific aim 1 Establish the expression of adenosine receptor subtypes on sympathetic nerves innervating MA and MV (prejunctional) using immunohistochemistry (IHC).

Rationale I use IHC to determine receptor localization by observing the co-localization of adenosine receptors and marker proteins for sympathetic nerves (TH). IHC data reveal the distribution of adenosine receptors in mesenteric blood vessels and their sympathetic nerve supply. The IHC preliminary results provide background for further functional studies.

2.3.2 Immunohistochemistry protocol

Prejunctional receptor staining: MA and MV from DOCA-salt and sham rat were excised, cleaned of access fat and connective tissue in ice-cold 0.1M PBS. Vessels are perfused with cold saline to remove blood from the lumen. Tissues

were cut in1 cm lengths and fixed in Zamboni fixative for 2 hours on ice. Then, the tissues were washed 3x with 0.1M PBS and then incubated in 4% mixture of goat-sheep blocking serum for 1 hour. Then, the vessels were incubated with antibodies against particular adenosine receptor and TH to show localization of adenosine receptor and sympathetic nerve fibers, respectively. To control for non-specific binding, control vessels were incubated with no primary antibodies. To prevent contamination, the IHC for particular adenosine receptor subtypes were performed at different day. Next, the vessels were incubated with the secondary antibodies-conjugated to fluorescent markers. Specimens were viewed under a confocal microscope.

2.3.3 Specific aim 2 Determine if activation of the prejunctional A_1 receptors inhibits NE release and test the hypothesis that A_1R dysfunction causes an increase in NE release in DOCA-salt MA and MV. The pharmacological properties of adenosine receptors on sympathetic nerves will be studied in isolated MA and MV maintained *in vitro*. Neurogenic vasoconstriction and real time NE release are measured by using video microscopy and amperometry respectively.

Rationale Although measuring nerve-mediated vasoconstriction *in vitro* is an indirect method to measure changes in NE release, this method is still useful for electro-inactive compounds (adenosine), which cannot be measured by amperometry (see below). Another benefit of this method is to screen the

pharmacology responses before doing amperometry, which is more challenging than measuring blood vessel diameter alone.

2.3.4 Protocol for measurement of vasoconstriction *in vitro*

Tissue preparation: Rats were killed using a lethal pentobarbital injection (100 mg ip). The entire mesentery was removed and tertiary MA and MV (120-300 µm) are dissected out and transferred to a small recording chamber. The tissue was pinned flat with 50 µm diameter stainless steel pins. MA and MV were carefully cleaned from adipose and connective tissues under dissecting microscope. The Tissue was superfused continuously at flow rate of 3 ml/min with warmed (36°C), oxygenated (95% O_2, 5% CO_2) Krebs' solution containing mM: 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 $NaHPO_4$, and 11 dextrose. The output of black and white video camera attached to the microscope was fed to a PCVision Plus frame-grabber board (Imagine Technology, Woburn, MA) mounted in a personal computer. The video images were recorded and analyzed using Diamtrak® software, which tracks the distance between the outer edges of the vessel in the observation field. The sampling rate was 10 Hz and changes in blood vessel diameter of 0.5 µm were resolved. The tissue was allowed to equilibrate for 30 minutes before beginning experiment. During this time, MA and MV relax to stable resting diameter.

Drug application: Agonists are applied using a system three-way stopcock so that superfusing Krebs' solution can be changed to one containing a known concentration of drug. It took 4 minutes for drugs to reach the tissue. Vessels

were first tested with maximum concentration of NE (10 μM for MA, 1 μM for MV) 3 times to verify stable responses. Then, agonist CRCs were constructed using cumulative addition for agonists that do not cause desensitization (e.g. NE, PE). For agonists that cause desensitization, a control experiment was done to determine recovery time (e.g. 15 minutes for α,β-me-ATP, or 45 min for CPA) and the CRCs were constructed using noncumulative addition of each drug concentration. Drugs were applied for 10 minutes and washed for optimum interval between applications of each agonist concentration with normal Krebs' solution. Antagonists were applied for 20 minutes prior to testing their effect on agonist or nerve-mediated responses in the continued presence of antagonist.

Transmural stimulation of perivascular nerves: This protocol was for measurement of contractile response mediated by nerve stimulation. Two wire electrodes were placed parallel to the longitudinal axis of the vessel under the Krebs' solution level, but not touching the tissue. Parameters for nerve stimulation were 30 stimuli, stimulus duration of 0.5 ms, frequency 0.2-30 Hz and voltage of 60 V. The neurogenic origin of constrictions caused by electrical stimulation was verified in each preparation by initially demonstrating that constriction caused by 20-Hz stimulus was completely blocked by the Na^+ channel blocker, tetrodotoxin (TTX, 0.3 μM). Preparations in which the 20-Hz constriction was not blocked by TTX were discarded. Peak constriction was measured using Diamtrak® software.

Table 4. Agonists and antagonists for rat adenosine receptors

Subtypes	Selective Agonist	Selective Antagonist
A_1	CPA, CCPA, R-PIA	DPCPX
A_{2A}	CGS 21680	SCH58261
A_{2B}	-	-
A_3	IB-MECA	BWA 1433, MRS 1523
A_{2A}/A_{2B}	NECA	8-*p*-sulfophenyltheophilline, ZM 241385

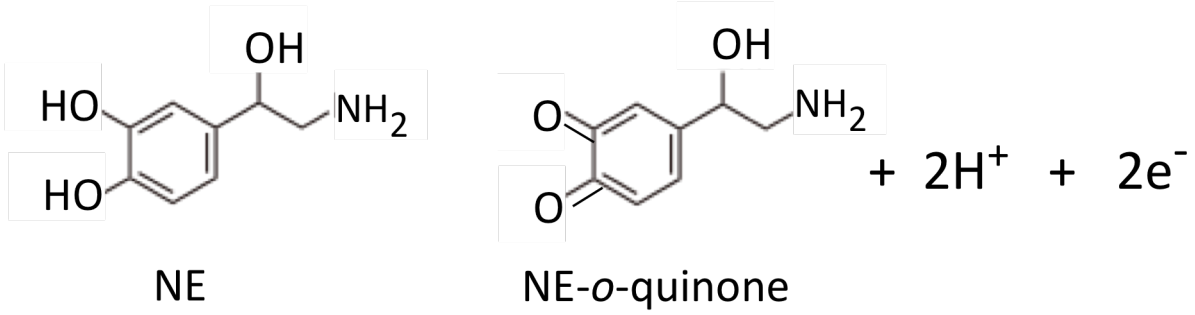
Data analysis: The frequency response curves (0.2-30 Hz) and CRCs were created and half maximal effective concentrations (EC_{50}) were determined using a non-linear fitting routine and a logistic equation (Origin 8.0 software). Comparisons of tissues from control and DOCA-salt rats will be made. Statistical differences will be assessed using Student's t-test and analysis of variance with $P<0.05$ indicating significance differences among treatment groups.

2.3.5 Specific aim 3 Determine the function of the prejunctional A_{2A} receptors on sympathetic neurotransmission and test the hypothesis that dysfunction of prejunctional A_{2A}Rs contributes to increased NE release from sympathetic nerves in DOCA-salt hypertensive rats. Electrically-evoke NE release is measured by using NE amperometry. Kinetics of NE release and clearance are calculated.

Rationale Continuous amperometry is used to detect catecholamine release from nerve endings in the central and peripheral nervous system (Brock *et al.*,

1997; Brock *et al.*, 2000; Brock *et al.*, 2004; Dugast *et al.*, 2002; Dunn *et al.*, 1999; Gonon *et al.*, 1993; Park *et al.*, 2007; Yavich *et al.*, 2005). This technique takes advantage of the electroactive property of catecholamines, like NE. Amperometry detects small quantities of NE by measuring the oxidation current (Teschemacher, 2005) produced by the redox reaction (Fig. 22). This technique provides real-time information on the local concentration of NE near the neuroeffector junction in the vasculature. HPLC and radioligand detection are also used to measure NE release in response to nerve stimulation. However, these methods cannot measure NE release in real time and they require long periods of nerve stimulation to evoke sufficient NE release for detection. Our laboratory has successfully detected NE released from sympathetic nerve stimulation in rat mesenteric blood vessels using amperometry (Dong *et al.*, 2009; Park *et al.*, 2007; Park *et al.*, 2006).

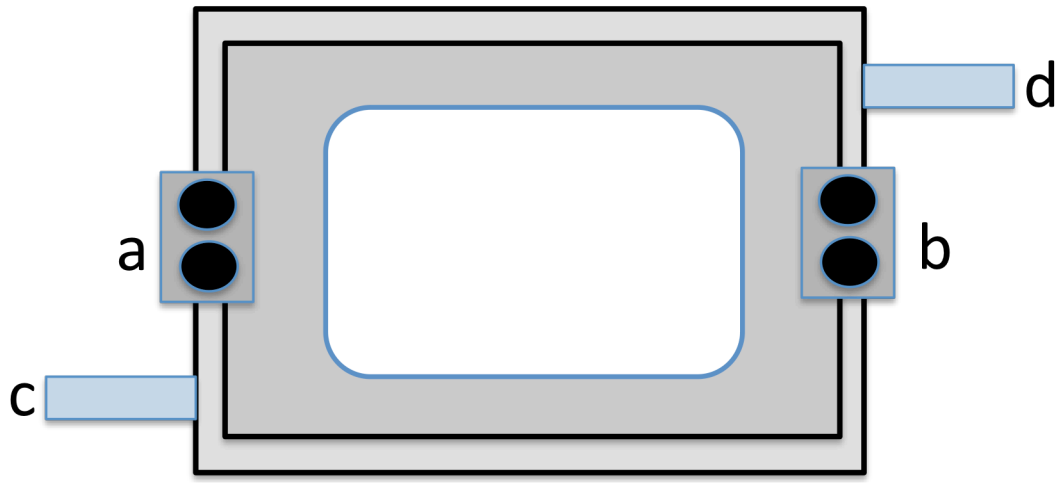
Figure 22. Redox reaction of norepinephrine.



2.3.6 Protocol for amperometric measurement of NE

Preparation of recording chamber: A plastic chamber was designed for holding two electrodes (reference and counter electrodes) on each side of the chamber. A recording chamber was prepared by gluing a coverslip to the bottom of a customized plastic chamber (Fig.23) using superglue and let dry. The next day, the bottom of chamber was filled with Sylgard, vacuums the bubbles for 5 minutes, and let dry for 2-3 days at room temperature. Sylgard is non-toxic and broken coverslip is easily to replace.

Figure 23. Recording chamber for amperometry. The chamber includes two holders for reference and counter electrodes (a,b), inlet at lower level (c), and outlet at higher level (d). The bottom of the chamber is made from clear glass cover slip and topped with clear silicone Sylgard®.



Carbon fiber microelectrode preparation: The electrode was produced as previously described (Dong, 2009; Park *et al.*, 2007). Briefly, the carbon fiber was insert into a propylene pipet tip (Dot Scientific, Michigan, #PF2411). Then, the electrode tip was carefully melted by the heated-puller machine (Sutter Instrument, Model P-30, CA, USA), and gently pulled by a flat-end forceps to create a slight angle (Fig. 24a) permitting a horizontal contact to the vessel. The carbon fiber tip was then cut to approximately 700μm length under a microscopy by using a glass scale. Each strip on the scale indicates 300 μm in width). Two ends of the silver wire were peeled off insulation (Fig. 24b,d). One end of the wire was connected to the carbon fiber by silver-epoxy glue (Fig. 24b, Fig. 25) and the other end was left for a connection with the potentiostat. The open gap between the pipet and silver wire is sealed with clear epoxy glue (Fig. 24c, Fig. 25).

Figure 24. Carbon fiber microelectrode. a) Carbon-fiber electrode tip is sealed with slight angle and cut into 700µm, b) Carbon fiber is connected to the silver wire by silver epoxy, c) Electrode gap is sealed with epoxy glue, d) the other end of the electrode was blared of insulation for potentiostat connection.

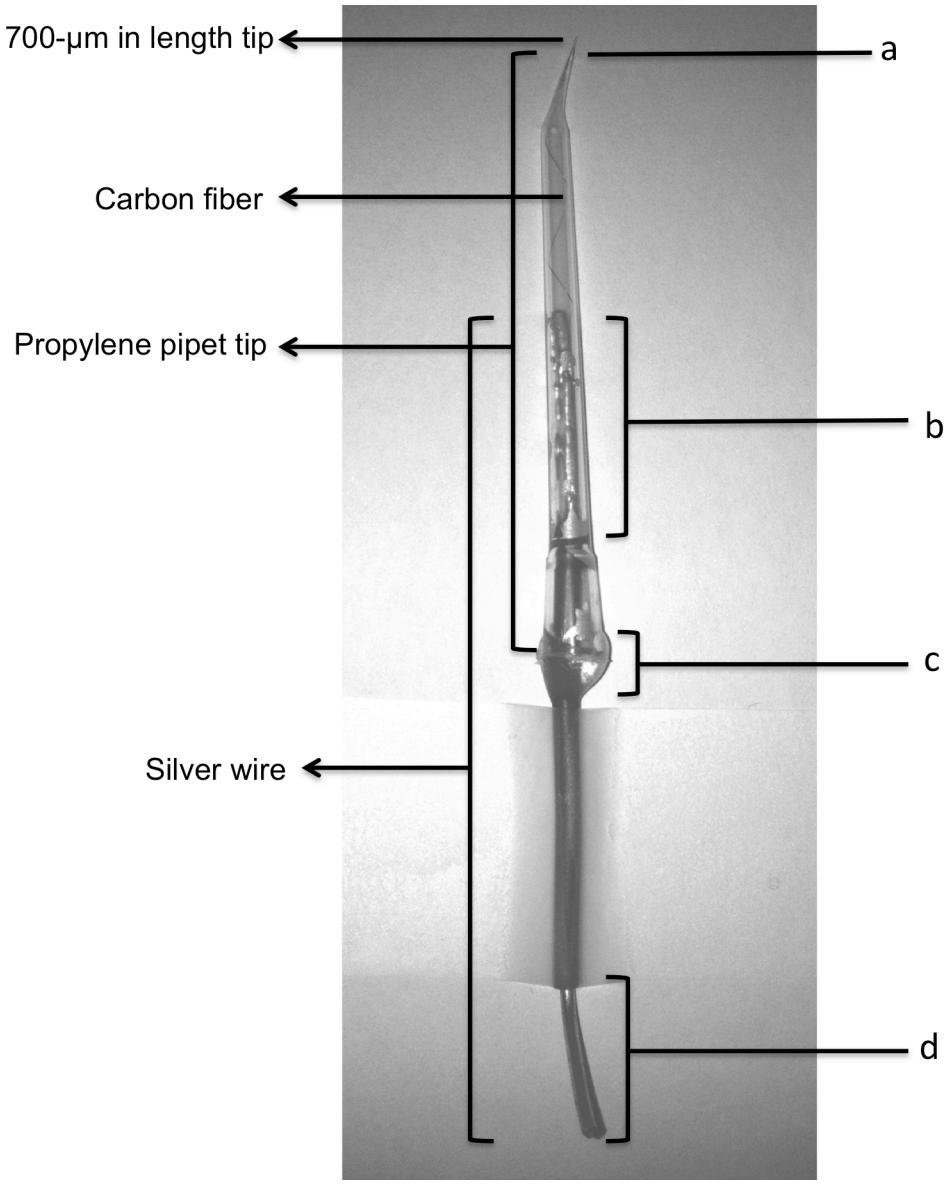
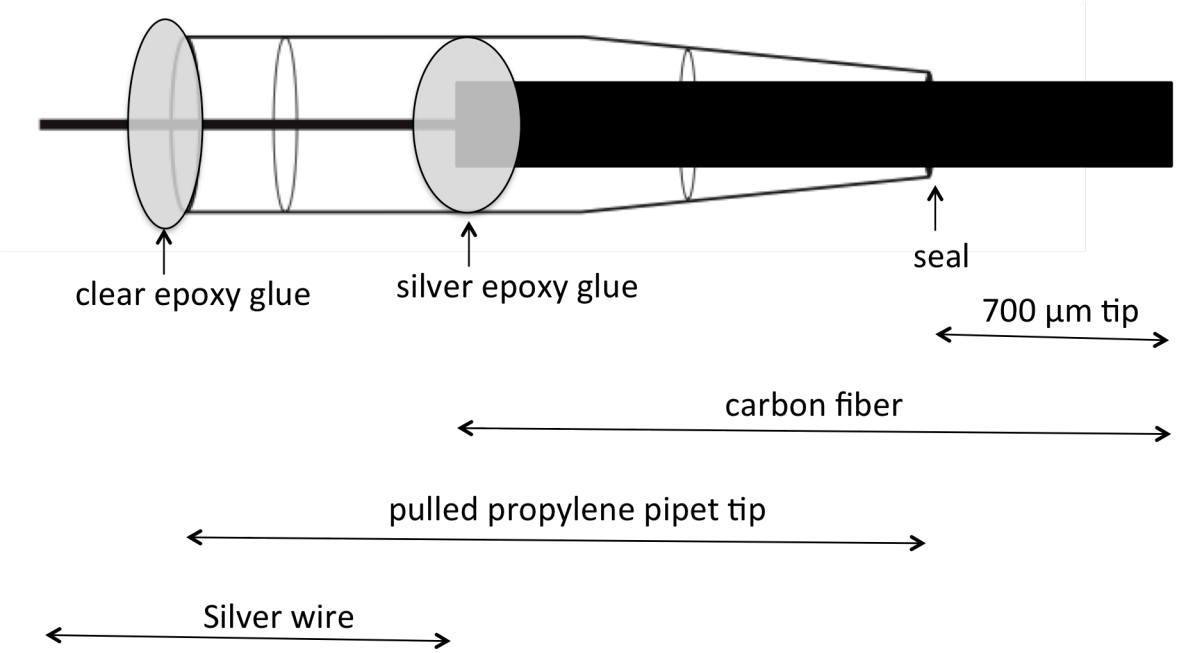


Figure. 25 Schematic diagram of the tip of a carbon-fiber microelectrode. All items are not drawn to scale. Modified from (Mundorf *et al.*, 2002).

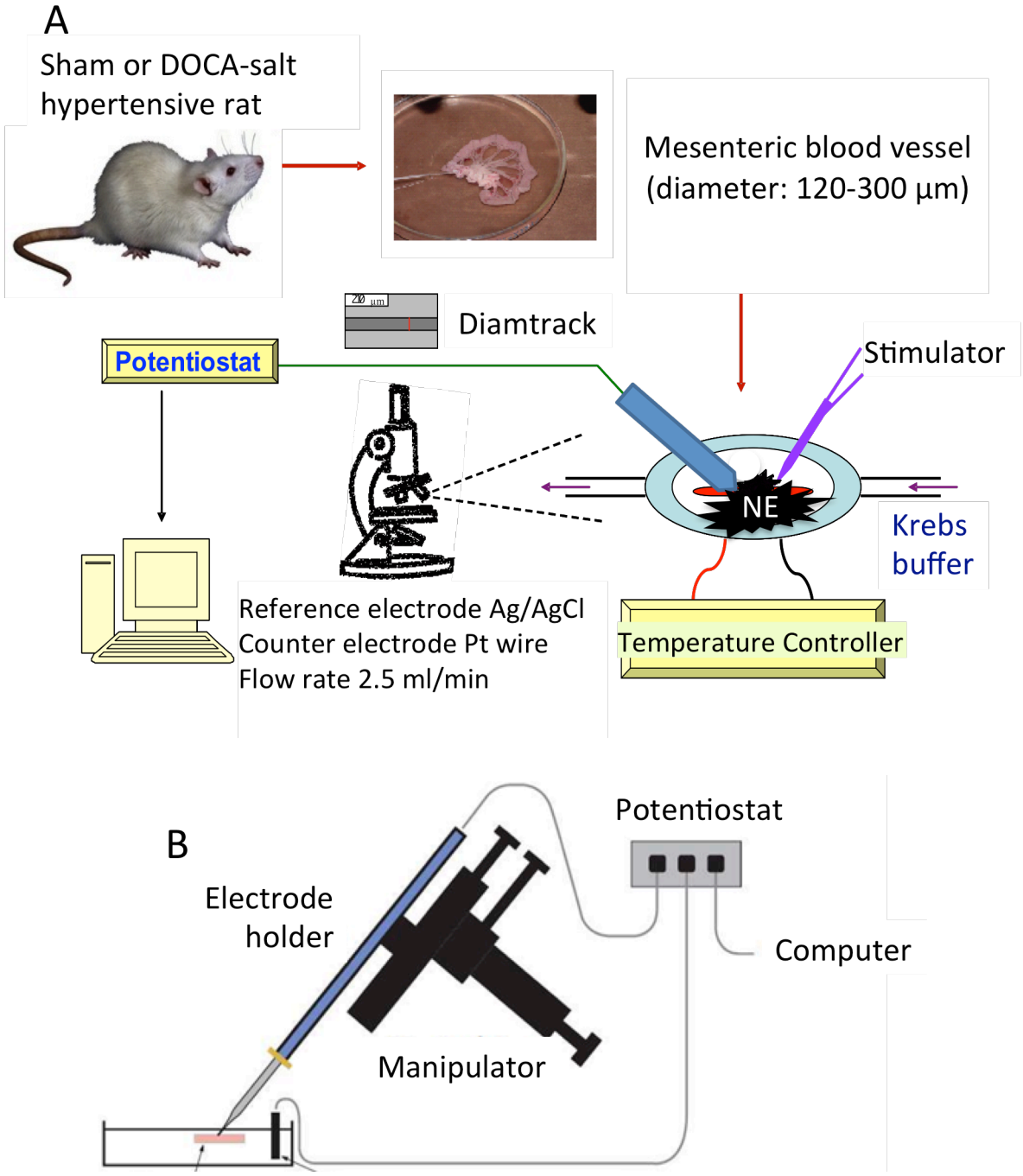


Tissue preparation: Tertiary MA and MV were isolated, cleaned of adipose and connective tissue, placed into a small silicone chamber, and perfused with warmed (36°C) oxygenate (95% O_2, 5% CO_2) Krebs' solution at flow rate 2.5 ml/min (Fig. 26A).

Amperometric detection: Firstly, a platinum wire counter electrode and a commercial "no leak" Ag-AgCl (3 M KCl, model EE009, Cypress System Inc., USA) reference electrode were mounted in the chamber to complete the electrochemical circuit. Carbon fibers working electrode was briefly dipped into distilled isopropyl alcohol (Ranganathan *et al.*, 1999) and affixed to a micromanipulator (MP-1, Narishige USA). The working electrode was connected to the electrode holder and mounted into the micromanipulator. The electrode was lower just above the surface of the blood vessel by using micromanipulator. All electrodes were then connected to potentiostat. The microelectrode was then adjusted the position parallel to the blood vessel so that NE flux from nearby release site was sensed by the electrode surface. The microelectrode was pressed gently against the vessel to enable the microelectrode to main contact with the vessel during stimulation-evoked constrictions (Fig. 27A,B). Amperometric measurements were made with an Omni 90 analog potentiostat (Cypress Systems Inc.), a mini digi analog-to-digital converter and a computer running Axon 9.0 (Axon Instruments, Union City, CA, USA). Data were obtained at 100 Hz sampling rate. An applied potential of 400 mV was used to detect NE current because this is approximate voltage for NE to be oxidized at mass-transfer limited rate (Park *et al.*, 2007). Current recording was low pass filtered at

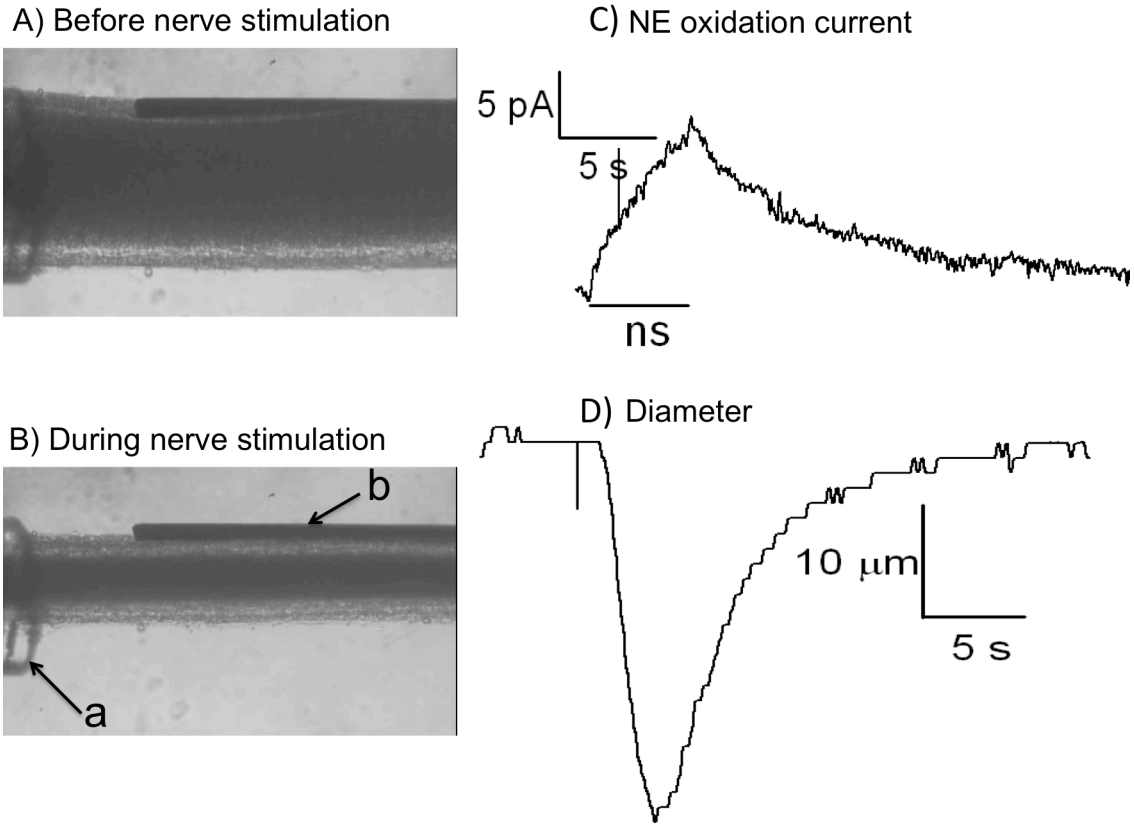
a time constant of 200 ms (5 Hz) before being digitized using A/D converter at a sampling rate of 100Hz. Data were stored on the computer for further analysis (Fig. 26).

Figure 26. Instrumental setup for amperometric NE detection. A, overall amperometry setup. B, Magnification part of carbon-fiber electrode positioned on the vessel and connected to the potentiostat (Dong, 2009).



Focal nerve stimulation: Short trains of electrical stimulation will be applied to trigger NE release using bipolar focal stimulation electrode positioned along the surface of the vessel. The electrode will be placed at a distance of 200 μm from the carbon fiber to minimize the stimulus artifact in the current recording (Fig. 29 A,B). Trains of 60 stimuli (0.3 ms pulse width, 60 V) at frequency of 0.5 to 20Hz will be used to stimulate transmitter release. ***Drug Application:*** Drugs (Table 5) will be added to the Krebs' buffer and applied for 20 min before assessing their effects on nerve-mediated responses.

Figure 27. Amperometric detection of NE release by a mesenteric vein. A, The vessel is gently cleaned off fat and connective tissue permitting electrode contact. A bipolar stimulating electrode (a) and a carbon fiber microelectrode (b) are positioned on the vessel surface. Nerve stimulation (ns) is applied to evoke NE release and contraction. C-D, NE oxidation current (C) and vasoconstriction (D) are detected in real-time. pA, pico ampere; s, second; μm, micrometer.



2.3.7 How to minimize unstable baseline?

It is necessary to acquire stable baseline before beginning any amperometric experiment. Having stable baseline allow one to measure current amplitude and kinetics of the oxidation current accurately. Here are some suggestions when experiencing unstable baseline.

1. Check if the tubing is cracked especially in the pump connection areas. Cracked tubing causes unstable superfusing rate resulting in unstable baseline.
2. Check the solution volume in the recording chamber. Low level of solution in the chamber causes noise. I found that the solution should cover approximately one third of reference electrode to stabilize amperometric baseline.
3. Slow down the flow rate. Too fast flow rate causes noises, but too slow flow rate kills the tissue. My superfusing flow rate was set at 2.5 ml/minute.
4. Carbon-fiber electrodes that are “leaky” with poor seal around the carbon fiber can cause unstable baseline and alter the oxidation current characteristics. Switching to a new carbon electrode may be needed.

2.3.8 When is carbon fiber electrode no longer useful? Ionic composition in Krebs solution appears to deteriorate the surface of carbon fiber microelectrode. The oxidation current is found to decay over time after used for several days. The

microelectrode may require reconditioning with purified isopropyl alcohol (distilled and kept with carbon powder to precipitate impurities) to improve microelectrode sensitivity. The control peak amplitude of NE oxidation current obtained from sham arteries and veins are 12.7±4.8 and 14.7±6.0 pA (n=53 and 48, respectively) and 13.3±3.8 and 17.3±4.6 pA from DOCA arteries and veins (n=19 and 20, respectively). Data are mean ± S.D. This electrically-evoked NE releases was performed under a brief train of 60 stimuli at stimulation parameter of 10 Hz frequency, 0.5 ms pulse duration, and 60-80 V intensity. These oxidation currents were measured by using carbon fiber electrode with 700± 150 μm in length, which equals to 2.5 blocks of glass scale (1block = 300 μm). Control peak current amplitude below the average range in part indicates loss of electrode sensitivity and the carbon electrode should be discarded. It should be noted that there are several factors that can determine the amplitude of oxidation current. Healthy isolated blood vessel is the key factor of getting a pleasurable oxidation current. A healthy vessel can be prepared by gently and fast cleaning of connective tissue and quickly incubating the tissue with oxygenated warm Krebs. Furthermore, short tip of carbon electrode detect smaller amount of released NE resulting in low oxidation current; hence small current may result from the tip of electrode is far below 700 μm. Moreover, the animal itself could be a problem. Some very hypertensive DOCA-salt blood vessels exhibit low current or probably no response to nerve stimulation as the nerves are dramatically damaged. Thus, to verify if the low oxidation current is caused by the less-sensitive electrode or an unhealthy vessel, one may have to compared the current response with the

contractile response. The contractile response for sham arteries and veins are 20.9±5.4 and 33.6±8.2% (n=40 and 34, respectively) and for DOCA arteries and veins are 19.8±7.1 and 27.0±9.4% (n=15 and 14, respectively). The low oxidation current, but high contraction response may be another key indicator for discarding a carbon-fiber electrode.

2.3.9 General methods

Animals: All animal procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University. Upon arrival at the animal care facility, rats are acclimatized for 2-3 days in clear plastic boxes with wood chip bedding in temperature and humidity-controlled rooms with a 12:12 light-dark cycle. Rats are fed with pelleted rat chow (Harlan/Tekled 8640 Rodent Diet) and *ad libitum* water.

DOCA-salt hypertensive rats: Male Sprague-Dawley rats (250-275g) were used for all of the studies. DOCA-salt and control (sham) rats were prepared as previously published procedure (Luo *et al.*, 2003; Watts *et al.*, 2002). Briefly, DOCA-salts rats received unilateral nephrectomy, Deoxycorticosterone acetate (DOCA) subcutaneous implantation, and 1%NaCl+0.2%KCl drinking water. Sham rats only undergo unilateral nephrectomy and received distilled drinking water. After 4 weeks of recovery, the animal's mean arterial blood pressure was measured by tail-cuff method. The blood pressure above 150 mmHg is considered hypertensive.

Data analysis: Data were reported as mean ± SE with 'n' values indicating the number of rats from which the data will be obtained. Concentration-response data were fitted using non-linear regression (Origin 8.0 or Graphpad Prism) in order to determine EC_{50} values. Statistical differences between treatment groups were determined using one- or two-way ANOVA, and Student's t-test for paired or unpaired data, as appropriate. Differences were considered significance when $P<0.05$.

2.4 Ethical aspects of the proposed research

To study the expression and functional properties of adenosine receptor in normotensive and hypertensive rats, the use of animal subjects is necessary for this project. Every attempt was made to reduce animal use and expense in accordance with AAALAC accreditation.

2.4.1 Vertebrate animal subjects

Male Sprague Dawley rats (8-9 weeks, 250-275 g) were used in this study. Rats were obtained from Charles River Laboratories, Inc.

Number of rats used in specific aim #1: 10 rats (5 sham, 5 DOCA) for immunohistochemistry (IHC). Tissue from 1 rat was used for staining all adenosine receptor subtypes from artery and vein. This will include both positive and negative controls. Experiments were done in triplicates.

Number of rats used in specific aim #2: approximately 100 rats for contractility and amperometry studies (50 sham, 50 DOCA)

Number of rats used in specific aim #3: approximately 100 rats for amperometry studies (50 sham, 50 DOCA)

It is important to note that I work as part of a larger group of investigators who study DOCA-salt hypertension at Michigan State University. It is a policy of our group that all animals are shared amongst the group. As I only used some of the mesenteric blood vessels from each rat, I shared these animals with 4 other students. The numbers of rats indicated above were not for my studies only but these animals supported 3 other ongoing projects as well. This arrangement further reduces the number of rats used in our research group.

Justification of animal use: The justification for the choice of using rats is that great amount of studies have been performed in rat. This provides a logical basis for prediction and interpretation of data. I reduced the number of rats used whenever it was possible. Moreover, I shared the subjects with our collaborators who also study hypertension using the same model to minimize the animal used.

2.4.2 Veterinary Care

Animal husbandry was provided by University Laboratory Animal Resources (ULAR) staff under the guidance of supervisors who are certified as Animal Technologists by the American Health Association for Laboratory Animal Science (AALS). Animals were cared for and checked on everyday by members of Dr. Galligan's laboratory. ULAR staff provided veterinary care. The animal care and use program conforms to the standards in The Guild for the Care and Use of Laboratory Animals DHEW pub. No. (NIH 78-23, Revised 1996). This includes

periodic surveillance of animal facilities, review of all funded projects for human use of animals, and the appropriate use of surgical anesthesia, analgesics, and tranquilizers.

2.4.3 Euthanasia

Euthanasia was performed by giving a high dose of pentobarbital (50 mg/kg, intraperitonial injection). This protocol is consistent with the recommendation of the panel on euthanasia of the American Veterinary Medical Association.

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CHAPTER 3

IMPAIRED FUNCTION OF PREJUNCTIONAL ADENOSINE A_1 RECEPTORS EXPRESSED BY PERIVASCULAR SYMPATHETIC NERVES IN DOCA-SALT HYPERTENSION

3.1 Abstract

Increased sympathetic nervous system activity contributes to DOCA-salt hypertension in rats. ATP and norepinephrine (NE) are co-released from perivascular sympathetic nerves. NE acts at prejunctional α_2-adrenergic receptors (α_2-AR) to inhibit NE release and α_2-AR function is impaired in DOCA-salt rats. Adenosine, an enzymatic ATP degradation product, acts at prejunctional A_1 adenosine receptors (A_1R) to inhibit NE release. We tested the hypothesis that prejunctional A_1R function is impaired in sympathetic nerves supplying mesenteric arteries and veins of DOCA-salt rats. Electrically-evoked NE release and constrictions of blood vessels were measured *in vitro* using amperometry and video microscopy. Adenosine and N^6-cyclopentyl-adenosine (CPA, A_1R agonist) constricted mesenteric veins but not arteries. Adenosine and CPA (0.001-10 μM) inhibited neurogenic constrictions and NE release in mesenteric arteries and veins. DOCA-salt arteries were resistant to adenosine and CPA-mediated inhibition of NE release and constriction. The A_{2A} adenosine receptor agonist, CGS21680 (0.01-0.1 μM) did not alter NE oxidation currents. We conclude that there are prejunctional A_1Rs in arteries and both pre and postjunctional A_1Rs in veins hence adenosine selectively constricts the veins. Prejunctional A_1R function is impaired in arteries, but not veins, from DOCA-salt rats. Sympathetic autoreceptor dysfunction is not specific to α_2-adrenergic receptors, but there is a more general disruption of prejunctional mechanisms controlling sympathetic neurotransmitter release.

3.2 Introduction

Adenosine is an ATP precursor and metabolite and an intercellular signaling molecule (Mark and Olah, 2001; Tabrizchi and Bedi, 2001). In the cardiovascular system, adenosine plays an important role in controlling cardiac output and vascular function (Tabrizchi and Bedi 2001; Shryock and Belardinelli, 1997). Considerable progress has been made in understanding adenosine receptor subtypes and their signaling mechanisms in the cardiovascular system. However, due to the breadth effects of adenosine, our understanding about adenosine receptor function in the sympathetic nerves supplying arteries and especially veins is less well developed, particularly in hypertension. This issue is important in mesenteric arteries and veins because they are densely innervated by sympathetic nerves (King et al., 2007). Sympathetic nerves supply blood vessels at the adventitial-medial border in arteries and they distribute more deeply into the media for veins (Pang, 2001; Birch et al., 2008). Sympathetic nervous system control of the mesenteric circulation contributes significantly to blood pressure regulation by two mechanisms. Firstly, sympathetic nerves regulate resistance in small mesenteric arteries (Kreulen, 2003; Rothe 1983; Fenger-Gron et al., 1995). Secondly, sympathetic nerves regulate capacitance of mesenteric veins which hold a large fraction of total blood volume and which provide 60-70% of venous return to the heart (Martin et al., 1998; Pang, 2001). Venous constriction shifts blood volume from the high compliance capacitance veins to the low compliance resistance arteries causing an increase in arterial pressure (Fink, 2009).

Adenosine receptors are G-protein coupled receptors (Mark and Olah, 2001; Morato et al., 2008). There are four types of adenosine receptor (A_1 A_{2A}, A_{2B}, and

A_3)(Tabrizchi and Bedi, 2001; Fredholm et al., 2001) that are distinguished based on their ability to inhibit or stimulate adenylyl cyclase and by different agonist and antagonist binding profiles (Mark, and Olah, 2001; Stephen et al., 1994; Fredholm et al., 2003). We focused on adenosine A_1 receptors (A_1Rs) which couple to inhibition of norepinephrine release. A_1Rs couple to the inhibitory G protein, G_i, leading to inhibition of adenylyl cyclase (Tabrizchi and Bedi, 2001; Linden, 2001; Ralevic and Burnstock, 1998). Activation of A_1Rs in the nervous system inhibits Ca^{2+} currents resulting in suppression of neurotransmitter release (Ralevic and Burnstock, 1998). Sympathetic nerves supplying mesenteric arteries release norepinephrine, ATP and/or β-nicotinamide adenine dinucleotide (β–NAD)(Burnstock, 2004; Smyth et al; 2004). Norepinephrine (NE), ATP and β–NAD can act on postjunctional α_1 adrenergic receptors and $P2X_1$ purinoceptors respectively to cause arterial smooth muscle contraction (Mutafova-Yambolieva et al., 2000; Smyth et al., 2004; Demel and Galligan, 2008). NE and ATP/β–NAD release is regulated by prejunctional α_2-receptors (α_2ARs) and A_1Rs (Demel and Galligan, 2008; Illes et al., 1988; Ralevic, 2000; Rongen et al., 1996). The action of ATP is quickly terminated by enzymatic degradation to ADP, AMP, and adenosine as a final product (Todorov et al., 1997; 1999; Westfall et al., 2002). Adenosine binds to prejunctional A_1Rs to inhibit NE and ATP/β–NAD release. Chronic treatment of rats with a non-selective adenosine receptor antagonist, 1,3-dipropyl-8-sulphophenylxanthine (DPSPX), increased blood pressure and purinergic and adrenergic neurotransmission (Guinarães et al., 2003). This indicates that adenosine regulates blood pressure partly by acting at prejunctional adenosine receptors. Data from animal models and from human studies showed that increased sympathetic

activation contributes to hypertension (Schlaich et al., 2004; Anderson et al., 1989). Previous work has shown that augmented sympathetic activity in DOCA-salt hypertensive rats is due partly to disruption of prejunctional α_2AR function (deChamplain et al., 1987; Luo et al., 2004). However, it is not known if impairment of A_1R function also occurs in hypertension. Thus, the purpose of this study was to determine if the disruption of prejunctional autoreceptors is specific to α_2ARs or if there is a more general disruption of prejunctional autoreceptor function in hypertension. Furthermore, we tested the hypothesis that dysfunction of prejunctional A_1Rs contributes to increased norepinephrine release from sympathetic nerves in DOCA-salt hypertensive rats. This model is a low rennin form of salt-sensitive hypertension that is driven by increased sympathetic nerve activity (Schenk and McNeil, 1992). Finally, there have been a number of studies of adenosine receptor function on periarterial sympathetic nerves but interactions of adenosine with sympathetic nerves supplying mesenteric veins in normotensive or hypertensive animals have not been studied.

3.3 Materials and Methods

Preparation of DOCA-salt hypertensive rats

Animals use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University. Adult male Sprague-Dawley rats (250-275g) were obtained from Charles River Laboratories (Portage, MI) and they were acclimated 2-3 days before entry into experimental protocols. Rat chow (Harlan/Teklad 8640 Rodent Diet) and tap water were provided *ad libitum*. Rats were housed in temperature and humidity controlled room with 12:12 hour dark-light cycle.

The surgical procedures and drug treatment protocols for producing sham control and DOCA-salt hypertensive rats have been described in detail previously (Luo *et al.*, 2004). After recovery from the surgical procedures, rats were housed under standard conditions for 4 weeks. DOCA-implanted rats received standard pelleted rat chow and drinking solution containing 1%NaCl+0.2%KCl in distilled water, while sham rats receive standard pelleted rat chow and tap water. Blood pressure was measured using tail-cuff plesmythography 3-5 days prior to experimentation. Rats with mean arterial pressure $\geq$150 mmHg were considered hypertensive.

Tissue preparation for in vitro studies

4 weeks after DOCA-salt or sham surgery, rats were euthanized with a lethal injection of pentobarbital (100 mg/kg i.p.). The mesentery was removed and transferred to a silicone elastomer-lined Petri dish with Krebs solution (mol/L): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 $NaHPO_4$, and 11, glucose. Sections of tertiary mesenteric arteries (145-250μm outside diameter) and veins (190-390μm outside diameter) were dissected out and transferred to a recording chamber. The tissue was

pinned flat with 50 μm-diameter stainless steel pins. Mesenteric arteries and veins were cleaned of adipose and connective tissue under a dissecting microscope. The chamber was mounted on the stage of an inverted microscope and tissues were superfused continuously at flow rate of 3 ml/min with warmed (36° C), oxygenated (95% O_2, 5% CO_2) Krebs solution. Tissues were allowed to equilibrate for 30 minutes before beginning experiments. Video images were obtained using a black-and-white video camera (Hitachi model KP-111; Yokohama, Japan) connected to the microscope and fed to a Picolo frame grabber board (Euresys Inc., Itasca, IL) mounted in a personal computer. Video images were analyzed using Diamtrak® edge tracking software (Adelaide, Australia). Diameter changes of 1 μm can be resolved.

Drug-induced constrictions

Drugs were applied using a 3-way stopcock system so that the superfusing Krebs solution could be changed to one containing a known drug concentration. Blood vessels were initially constricted with 3 consecutive applications of NE (10μM for arteries and 1μM for veins) at 15-minute intervals to verify viability and response stability. Responses greater than 20% of initial diameter in arteries and 30% in veins were considered acceptable and only those blood vessels were used for further experiments. Adenosine (0.001-100μM) and N^6-cyclopentyl-adenosine (CPA, 0.01-10μM) were added non-cumulatively. Successive concentrations were applied at 45minute intervals. Antagonists were applied for 30 minutes before testing the effect of agonists. Increasing concentrations of the A_1R antagonist, 1,3-dipropl-8-cyplopentylxanthine (DPCPX) were applied cumulatively with a 15 minute incubation

period for each concentration. Dipyridamole dose response curves were obtained non-accumulatively with 15-minute wash between concentrations.

Transmural stimulation of perivascular nerves

Perivascular sympathetic nerves were stimulated using Ag/AgCl wire electrodes placed parallel to the length of the blood vessel. Parameters for nerve stimulation were: 30 stimuli (0.5ms duration) at 10Hz, 60-80V. Pilot experiments and previously published work (Park et al., 2007, Park et al., 2010) showed that these parameters produced a maximum constriction. Constrictions caused by electrical stimulation were blocked by the Na^+ channel blocker, tetrodotoxin (0.3μM).

Amperometric detection of norepinephrine (NE)

Construction of carbon fiber microelectrodes was described in detail previously (Park *et al.*, 2007). The microelectrode was positioned parallel to the blood vessel so that NE flux from nearby release site will be sensed by the electrode surface. The microelectrode was pressed gently against the vessel to enable the microelectrode to maintain contact with the vessel during stimulation-evoked constrictions. A platinum wire counter electrode and a commercial “no leak” Ag-AgCl (3M KCl, model EE009, Cypress System Inc., USA) reference electrode also were mounted in the chamber to complete the electrochemical cell. Continuous amperometric measurements were made with an Omni 90 analog potentiostat (Cypress Systems Inc.), a Minidigi analog-to-digital converter and a computer running Axoscope 9.0 (Axon Instruments, Union City, CA, USA). Data were obtained at a 100 Hz sampling rate. An applied potential of 600 mV was used to detect NE currents because this is the oxidation potential for NE at a mass-transfer limited rate (Park *et al.*, 2007). Currents were low pass filtered with at a time

constant of 200 ms. Data were stored on the computer hard drive for further analysis. A bipolar focal stimulation electrode positioned along the surface of the vessel was used to excite perivascular nerves. The electrode was placed at a distance of 200 μm from the carbon fiber microelectrode to minimize the stimulus artifact in the current recording.

Immunohistochemistry.

MA and MV were excised, cleaned of excess fat and connective tissue. Vessels were perfused with saline to remove blood from the lumen. Tissues were cut into 1 cm lengths and fixed in Zamboni fixative overnight at 4 °C. The next day, the tissues were washed 3 times with 0.1 M PBS and then incubated in 4% goat serum in PBS for 1 h. Then, the vessels were incubated overnight with primary antibodies against particular adenosine receptor subtypes (Table 5) and tyrosine hydroxylase (TH) (mouse monoclonal anti-TH antibody, 1:200 in PBS, Millipore). To control for non-specific binding, control vessels were incubated without primary antibodies. After overnight incubation, unbound primary antibody was rinsed with 3 washes with PBS and the blood vessels were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:40) and Cy^3-conjugated goat anti-rabbit IgG (1:50, Jackson ImmunoResearch Laboratories, Inc.) to visualize TH and A_1R staining respectively. After 1 hour incubation, unbound secondary antibody was rinsed away with 3 washes with PBS. Specimens were mounted on microscope slides in buffered glycerol (pH, 8.6) and were viewed under using a fluorescence microscope. Images are acquired and analyzed using MetaMorph software.

Table 5. Sources of primary and secondary antibodies and working dilutions for adenosine receptor staining.

Primary antibodies

Antigen	Source	Host Species	Dilution
A_1R	Santa Cruz	Rabbit (polyclonal)	1:200
	Sigma	Rabbit (polyclonal)	1:100
$A_{2A}R$	Sigma	Rabbit (polyclonal)	1:100
A_{2B} R	Santa Cruz	Rabbit (polyclonal)	1:200
A_3 R	Sigma	Rabbit (polyclonal)	1:100
TH	Millipore	Mouse (monoclonal)	1:100

Secondary antibodies

Antigen	Source	Host species	Dilution	Fluorophore
Rabbit IgG	Jackson immunolaboratory	Goat	1:400	Cy3
Mouse IgG	Jackson immunolaboratory	Sheep	1:50	FITC

Drugs. All drugs were obtained from Sigma Aldrich, USA. All drugs, except DPCPX, were dissolved in deionized water as a concentrated stock solution kept at 0°C. DPCPX and was made as stock solution in ethanol. Control experiments with the highest concentration of ethanol (0.01% vol/vol) did not affect neurogenic or agonist-induced blood vessel contraction (data not shown).

Statistics. Data are reported as mean ± S.E., N values are the number of animals. Differences between groups were assessed using Student's t-test. Differences in agonist concentration response curves were assessed first by 2-way ANOVA and Bonferoni's test for multiple comparisions (GraphPad Prism 5.0, LaJolla, CA).

Adenosine and CPA half maximal inhibitory concentrations (IC_{50}) and maximum effect values (E_{max}) were determined from individual concentration response curves using a non-linear fitting routine with a logistic equation (Origin 8.0, Northampton, MA). Mean IC_{50} and E_{max} values were compared using Student's t-test. IC_{50} values were expressed as the negative log of the drug concentration that causes 50% of the maximum inhibitory response (pIC_{50}). $P<0.05$ was considered significant.

3.4 Results

3.4.1 Immunohistochemical localization of A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R on sympathetic nerves supplying MA and MV in sham and DOCA-salt hypertension.

Antibodies raised against A_1Rs and tyrosine hydroxylase (TH), a marker for sympathetic nerves, were used to show that immunoreactivity for A_1Rs and TH is co-expressed in sympathetic nerves innervating sham mesenteric arteries and veins. There was no obvious difference for A_1R expression on TH-containing structure between sham and DOCA-salt arteries and veins (Figure 28).

Additionally, immunostaining for the other three adenosine receptor subtypes was also performed by using their particular specific antibodies shown in table 5. Figure 29-30 shows co-expression of TH and $A_{2A}R$ or $A_{2B}R$ subtypes, respectively in arteries and veins of sham and DOCA-salt hypertensive rats. There was also no obvious difference in $A_{2A}R$ or $A_{2B}R$ expression on TH-containing fibers between sham and DOCA-salt arteries and veins. However, A_3R are likely expressed by non-neuronal structures, most likely fibroblasts (Fig. 31 A, D, and G) and/or vascular smooth muscle cells (Fig. 31 C, F).

Figure 28. Localization of A_1Rs on periarterial and perivenous sympathetic nerves of sham and DOCA hypertensive rats. Sympathetic nerves were labeled with an anti-TH antibody shown in green labeling. A_1Rs were detected by anti-A_1R antibody shown in red labeling. Overlay of photomicrographs shows the co-localization of TH and A_1Rs (yellow labeling). The images were obtained by confocal microscopy. Bars indicate magnification. MA, mesenteric artery; MV, mesenteric vein.

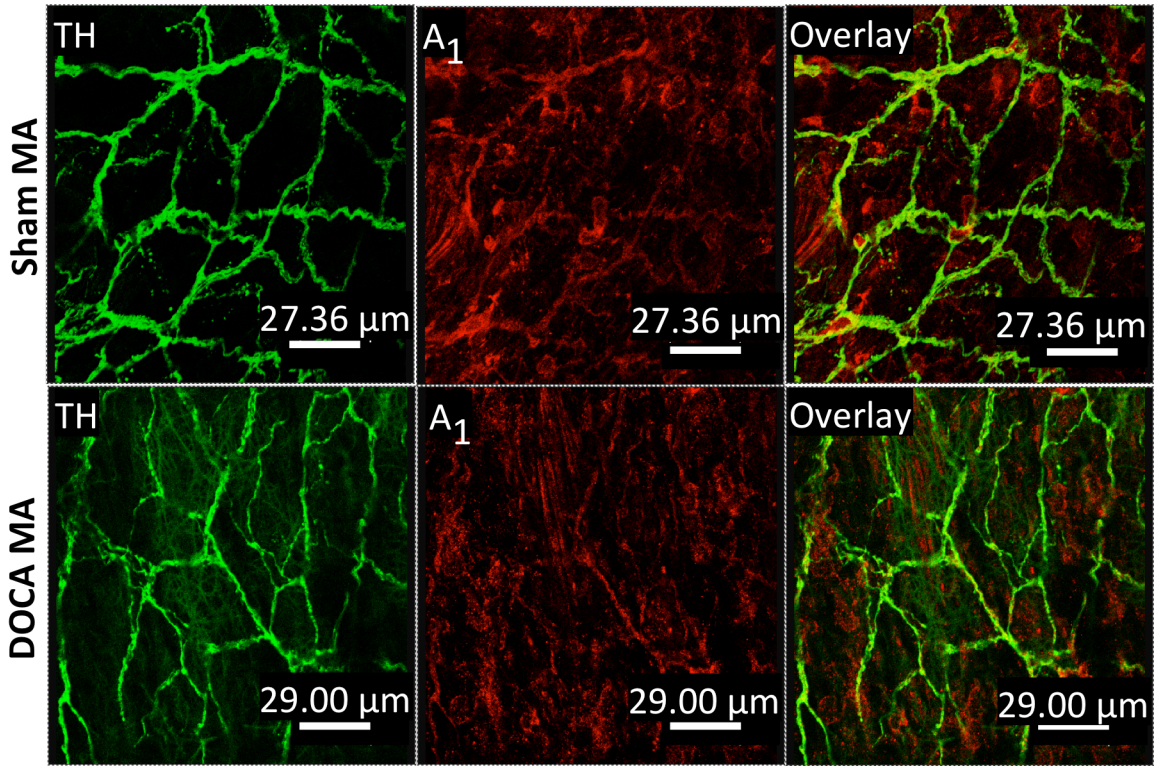


Figure 28. (Cont'd)

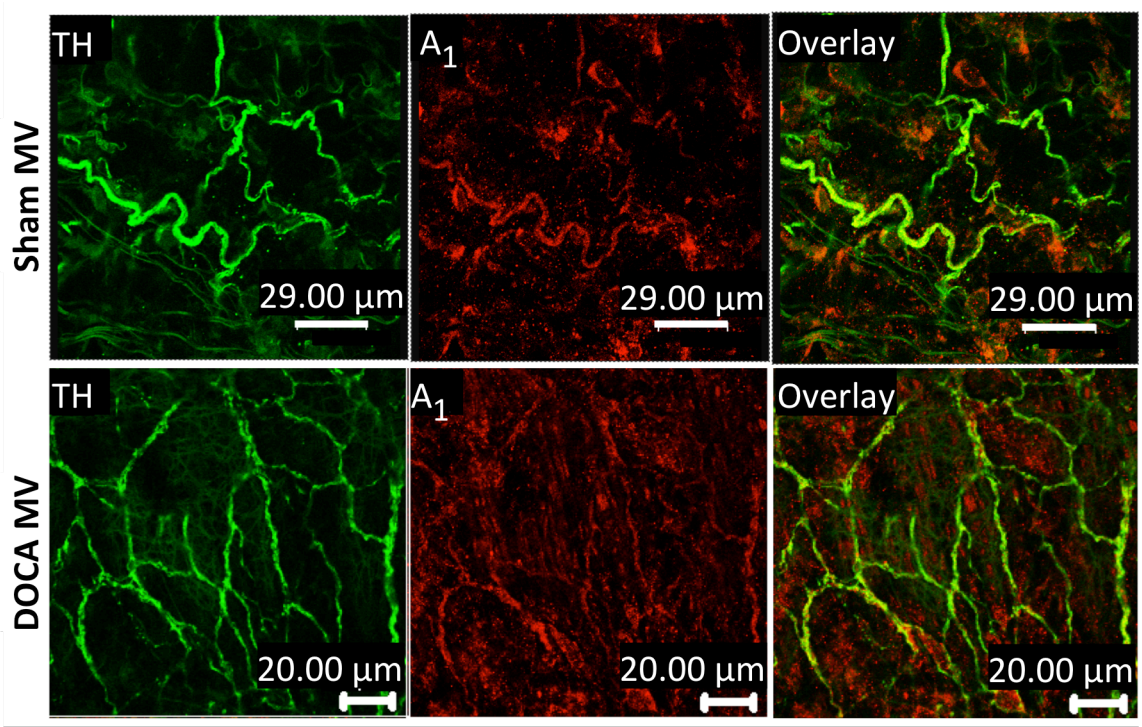
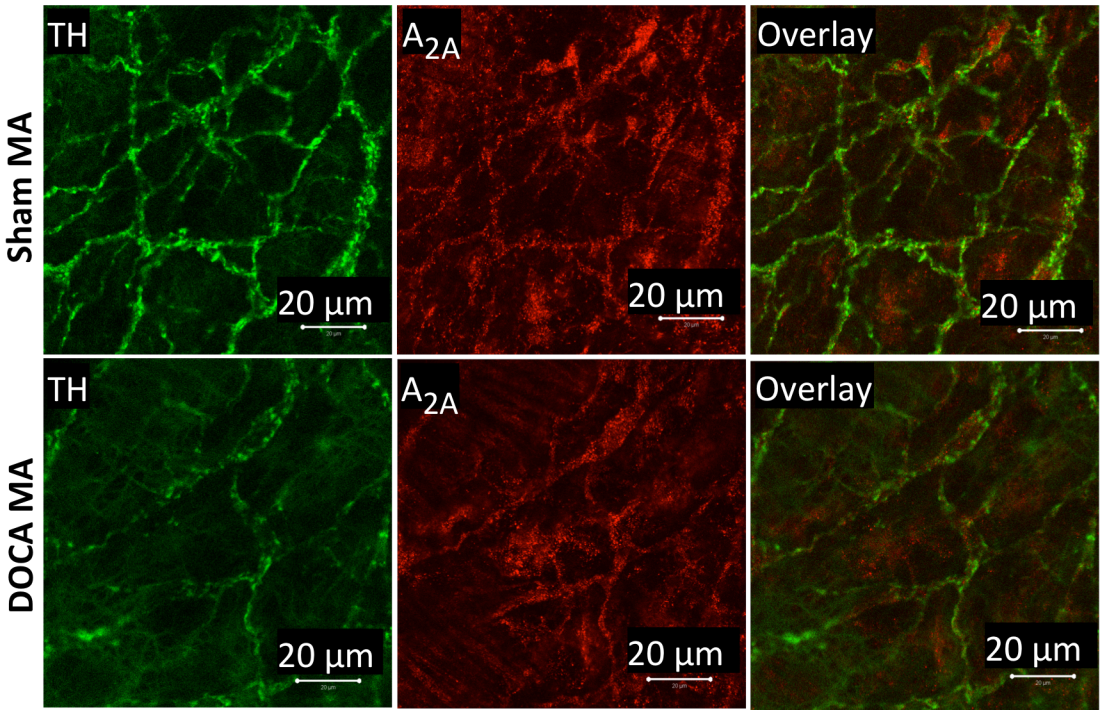


Figure 29. Localization of A_{2A}Rs on periarterial and perivenous sympathetic nerves of sham and DOCA hypertensive rats. Sympathetic nerves were labeled with an anti-TH antibody shown in green labeling. A_{2A}Rs were detected by anti-A_{2A}R antibody shown in red labeling. Overlay of photomicrographs shows the co-localization of TH and A_{2A}Rs (yellow labeling). The images were obtained by confocal microscopy. Bars indicate magnification. MA, mesenteric artery; MV, mesenteric vein.



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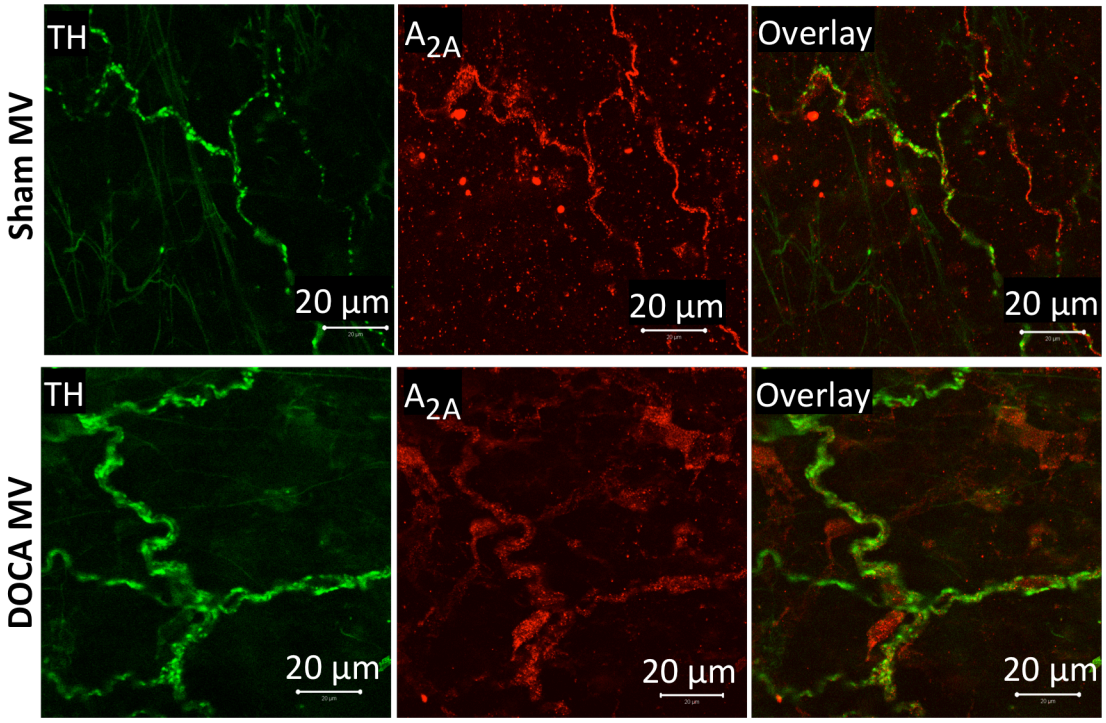


Figure 30. Localization of A_{2B}Rs on periarterial and perivenous sympathetic nerves of sham and DOCA hypertensive rats. Sympathetic nerves were labeled with an anti-TH antibody shown in green labeling. A_{2B}Rs were detected by anti-A_{2B}R antibody shown in red labeling. Overlay of photomicrographs shows the co-localization of TH and A_{2B}Rs (yellow labeling). The images were obtained by confocal microscopy. Bars indicate magnification. MA, mesenteric artery; MV, mesenteric vein.

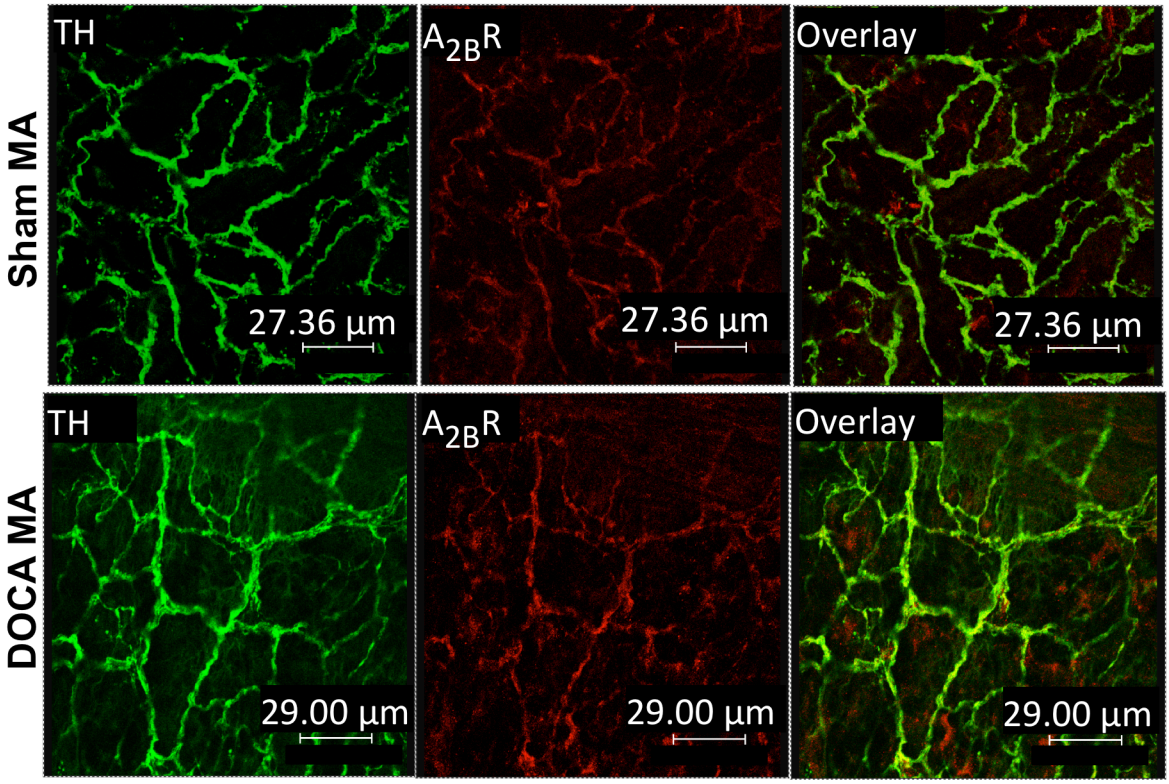


Figure 30. (Cont'd)

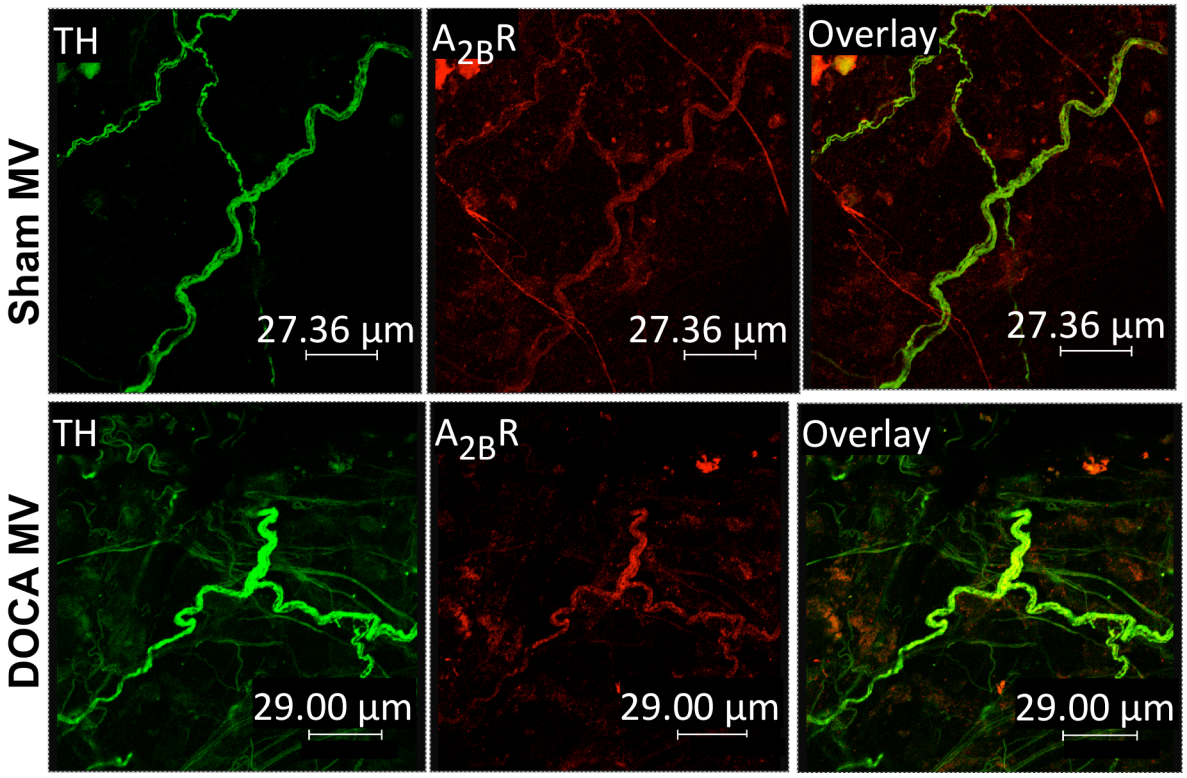
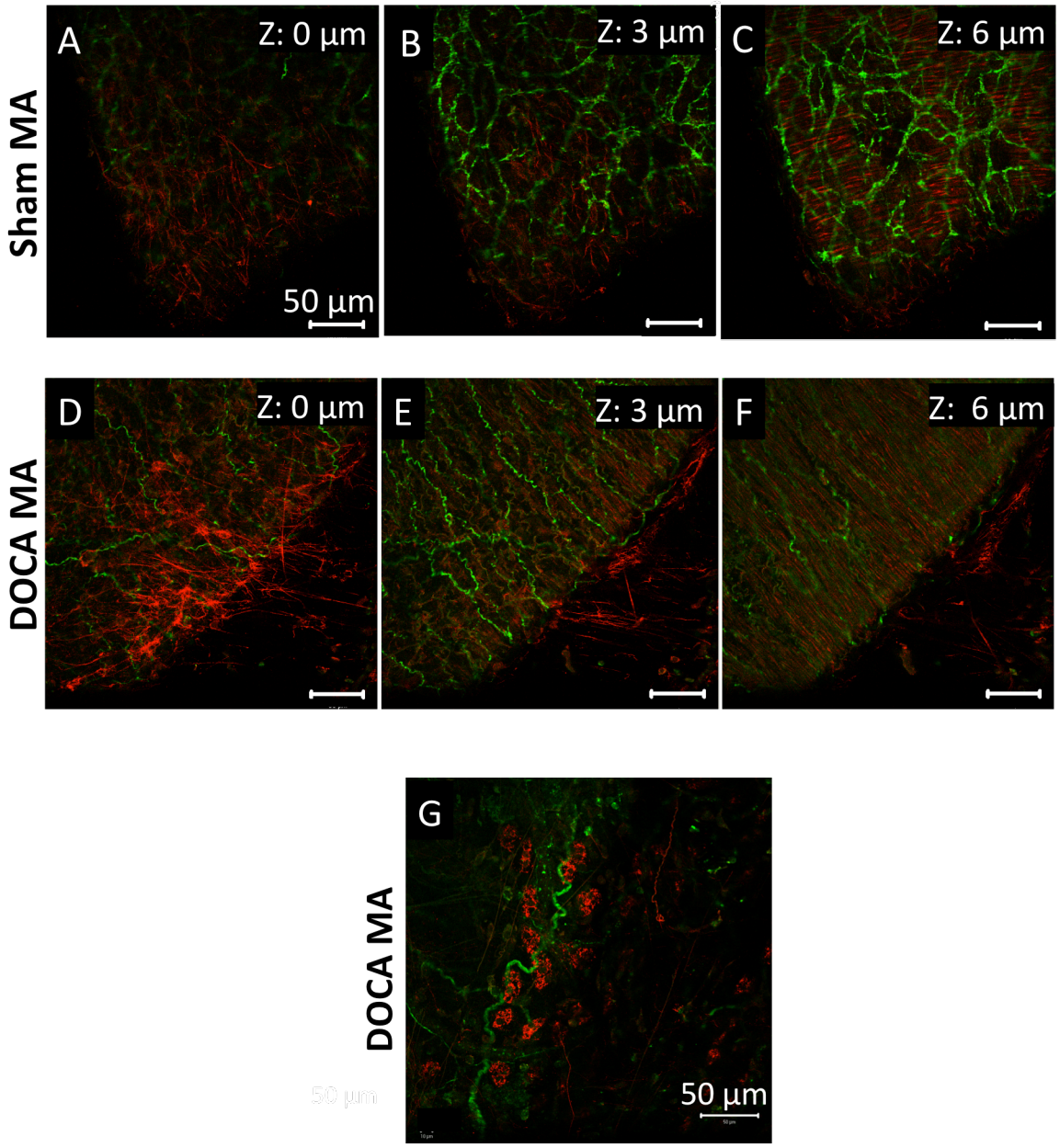


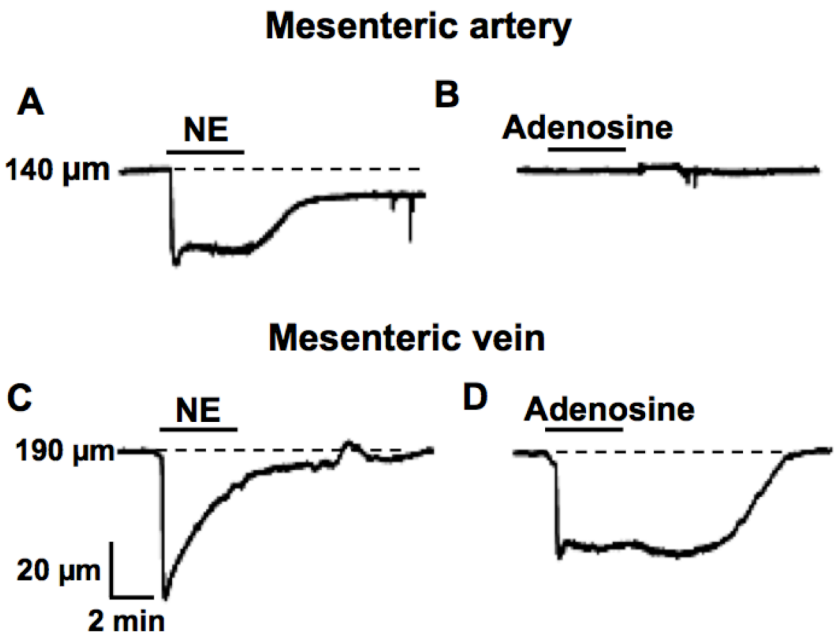
Figure 31. A_3Rs does not express on periarterial and perivenous sympathetic nerves. Sympathetic nerves were labeled with an anti-TH antibody shown in green. A_3Rs were detected by anti-A_3R antibody shown in red. Overlay of photomicrographs shows A_3R staining outside the TH-containing nerves (A-G). A-F, Series of overlay images of A_3R and TH-containing nerves from adventitia down to smooth muscle layer. Z indicates z-axis distance from adventitia. The images were obtained by confocal microscopy. Bars indicate magnification. MA, mesenteric artery; MV, mesenteric vein.



3.4.2 Adenosine and CPA constricted mesenteric vein but not artery.

Adenosine and, selective A_1 adenosine receptor, CPA were tested for their constrictor effects in mesenteric arteries and veins. NE produced a sustained constriction of arteries while neither adenosine nor CPA constricted arteries (Fig. 32A, B). In sham arteries adenosine (10μM) and CPA (1μM) caused changes in diameter of only 0.4±0.3% (n=6) and 0.8±0.5% (n=8), respectively. Adenosine and CPA caused sustained constrictions of veins while NE produced a transient constriction of veins (Fig. 32C, D). In sham veins, adenosine and CPA caused decreases in diameter of 19±2% (n=5) and 28±3% (n=3), respectively. Similar data were obtained in DOCA-salt mesenteric arteries and veins.

Figure 32. Representative traces of norepinephrine (NE) and adenosine induced-constrictions of sham mesenteric arteries and veins. A, NE-induced constriction of arteries was smaller than veins (C) in peak amplitude but the constriction was sustained throughout the period of NE application. Venous constriction showed rapid desensitization to NE (C). D, Adenosine constricted mesenteric veins but not arteries (B). Agonists were applied during the period indicated by the bar. Y-axis is vessel diameter (μm)

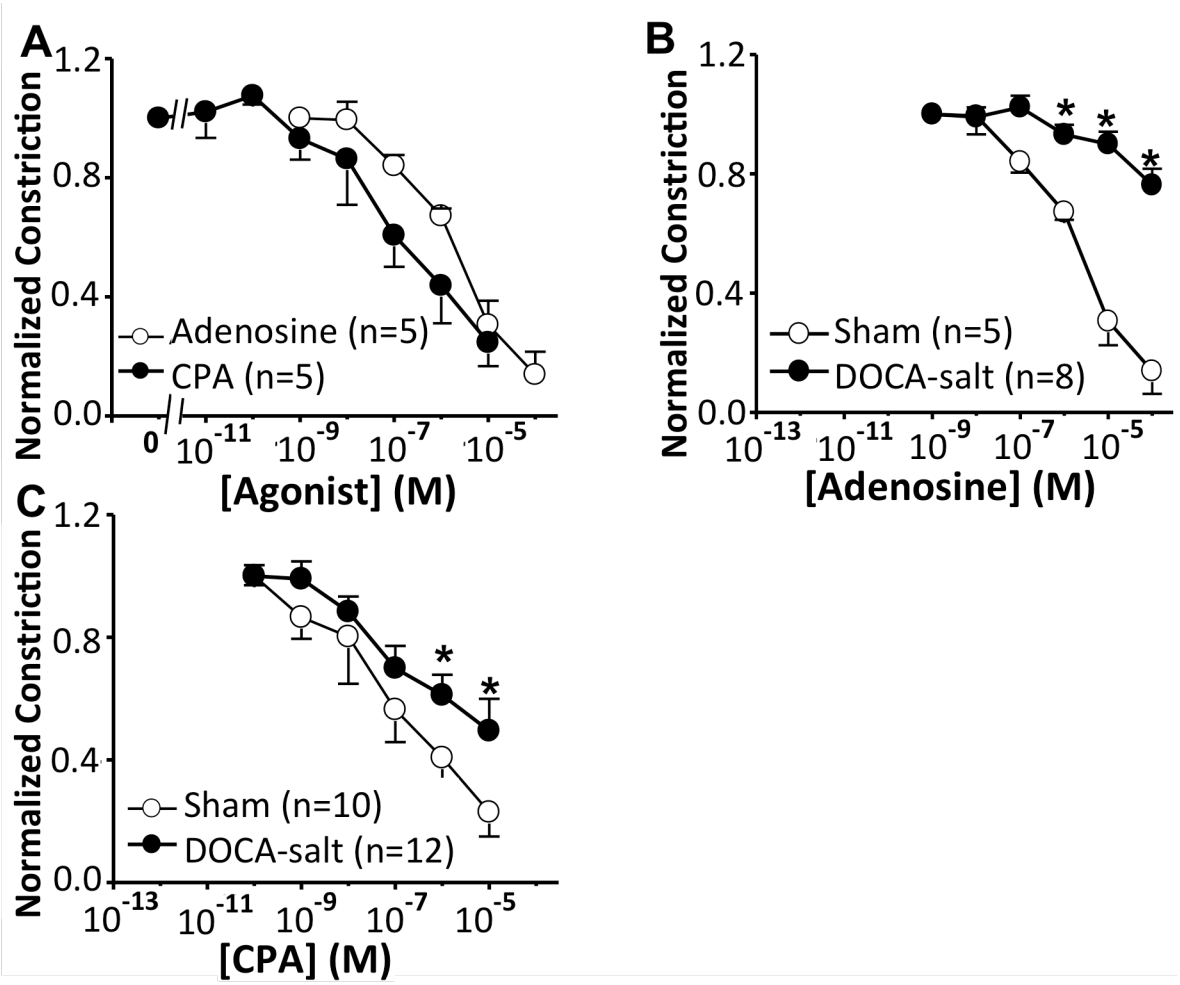


3.4.3 The prejunctional A_1Rs inhibited neurogenic constriction of mesenteric arteries.

The actions of adenosine and CPA on neurogenic constrictions in mesenteric veins were not studied because these agonists constricted veins directly. However, adenosine and CPA both caused concentration-dependent inhibition of neurogenic constriction in arteries (Fig. 33A). The concentration response curve for CPA was left-shift compared to that for adenosine. The pIC_{50} values for CPA and adenosine were 7.0±0.4 and 5.6±0.2, respectively ($P<0.05$).

We next compared adenosine and CPA concentration-response curves for inhibition of neurogenic constrictions in arteries from sham and DOCA-salt hypertensive rats. Adenosine and CPA concentration response curves were both right shifted in DOCA-salt arteries compared to those obtained in sham arteries (Fig. 33B,C). The rightward shift was greater for adenosine compared to that for CPA.

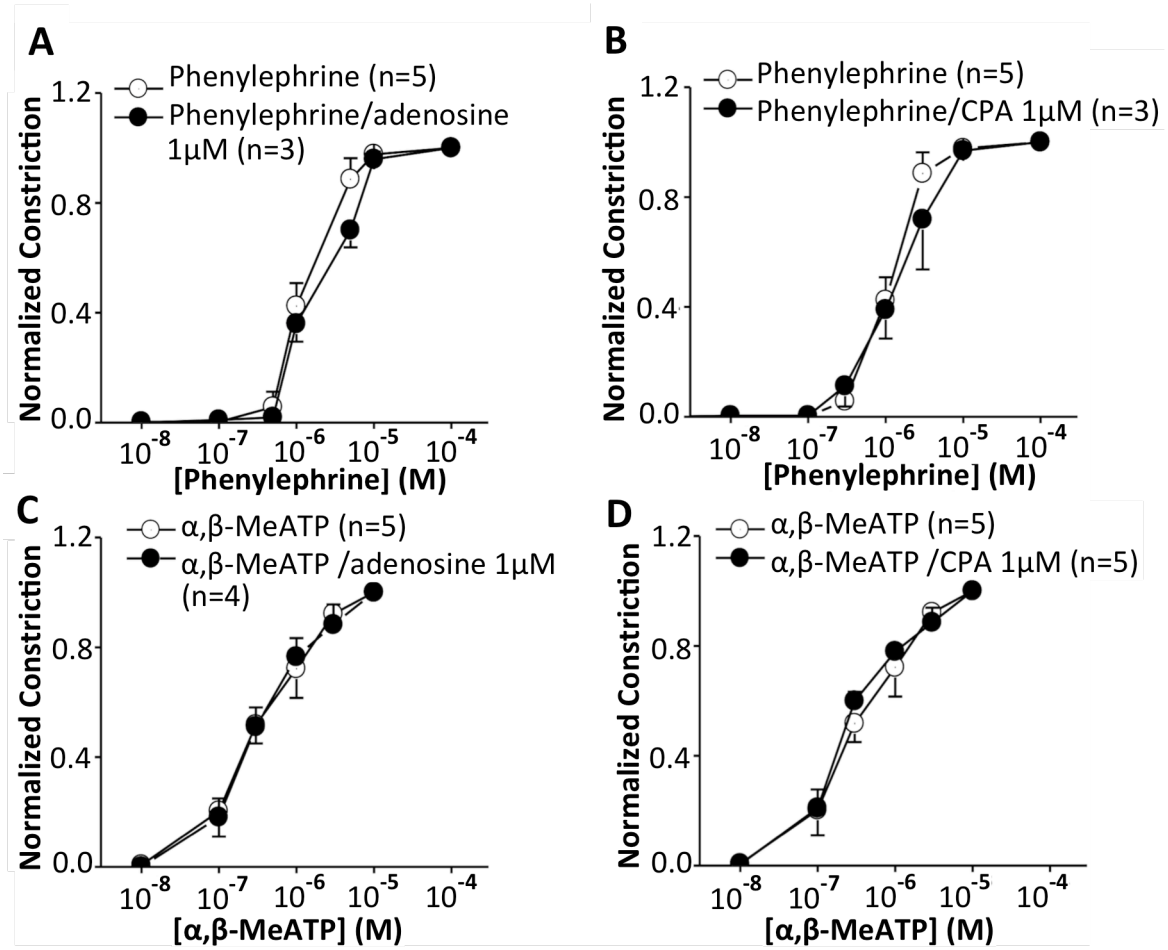
Figure 33. Adenosine and CPA concentration-response curves (CRCs) for inhibition of neurogenic constrictions of sham, but not DOCA-salt mesenteric arteries. A, Adenosine and CPA inhibited neurogenic constriction in sham arteries. The CPA curve was significantly left-shifted compared to the adenosine CRC ($p<0.05$). B-C, Adenosine and CPA-induced inhibition of neurogenic constriction were reduced in DOCA-salt arteries compared to sham arteries. Data are mean ± S.E. Data were analyzed using a 2-way ANOVA and Bonferoni's post-hoc test. *indicates significantly different from sham arteries.



3.4.4 Adenosine and CPA did not affect mesenteric artery reactivity

Control experiments were performed to determine whether the inhibition of neurogenic constriction produced by adenosine or CPA was due to pre- or postjunctional effects. We determined the influence of exogenous adenosine and CPA on two postjunctional receptors, the α_1 adrenergic and the $P2X_1$ receptors. The selective α_1-adrenergic receptor agonist, phenylephrine, and the $P2X_1$ receptor agonist, α,β-methylene ATP both caused concentration dependent constrictions of mesenteric arteries. Neither adenosine (1 μM) nor CPA (1 μM) altered phenylephrine or α,β-methylene ATP-induced constrictions (Fig. 34A-D). This indicates that inhibition of the neurogenic constriction by adenosine and CPA was mediated prejunctionally.

Figure 34. CPA and adenosine do not inhibit constrictions caused by phenylephrine (α_1-adrenergic receptor agonist) and α,β-MeATP (P2X receptor agonists). Phenylephrine CRCs were not altered by adenosine (A) or CPA (B) in sham MA. α,β-MeATP CRCs were not altered by adenosine (C) or by CPA (D) in sham MA. Data are mean ± S.E., n values represent the number of animals for each experiment.

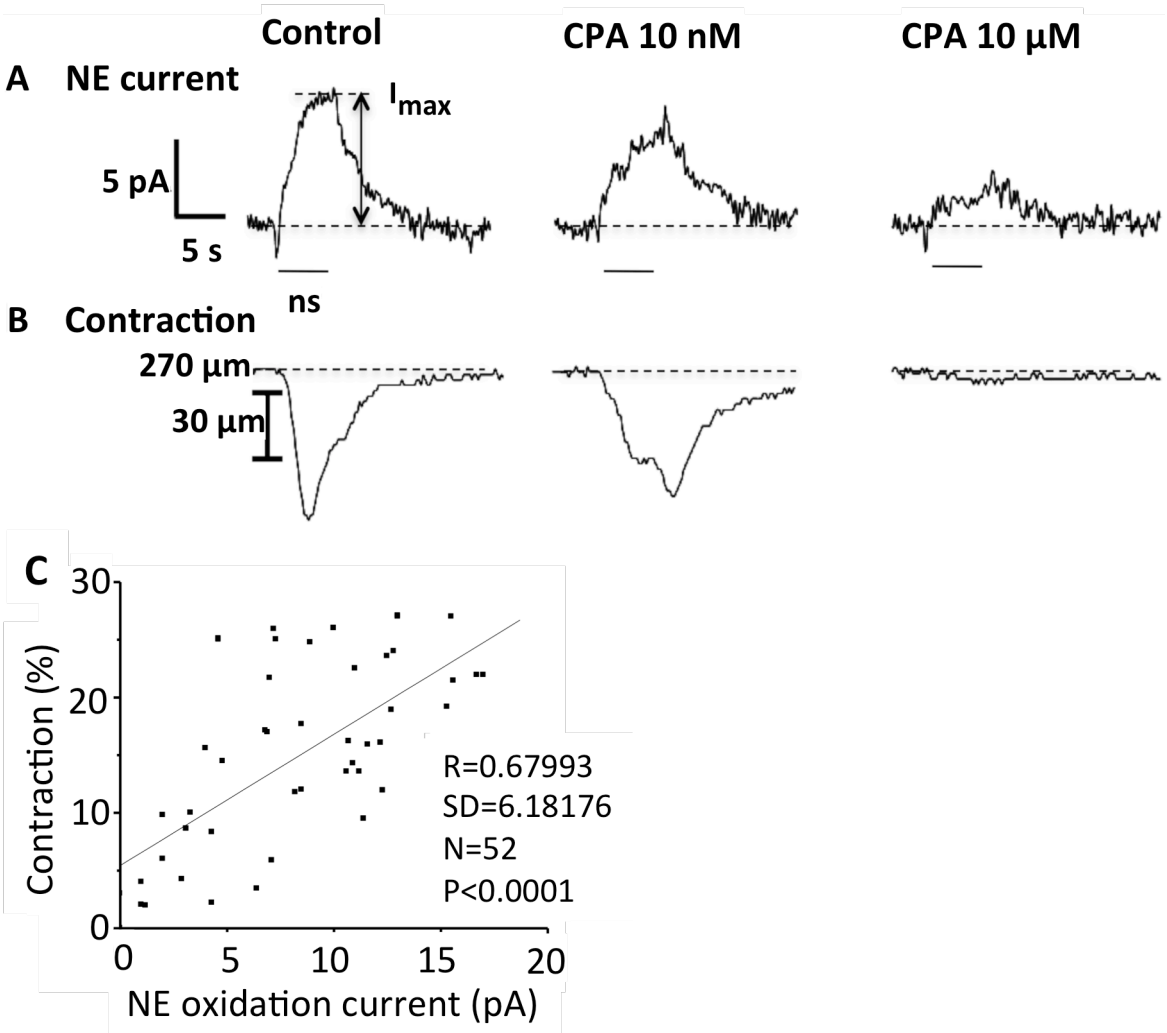


3.4.5 A_1Rs but not A_{2A}Rs mediated inhibition of NE release

Neurogenic constriction was used as an indirect measure of NE release, which could be performed only in arteries because adenosine and CPA both constricted veins directly. In the next experiments, we made more direct measures of NE release using continuous amperometry with carbon-fiber microelectrode. This allowed measurement of NE release in real time near the surface of both arteries and veins. CPA was used in these studies as it is A_1R selective and actions at other adenosine receptors would not complicate our data interpretation. In addition, adenosine could not be used in these studies as it interacted with the carbon fiber electrode making the electrode baseline current unstable.

Short trains of stimulation evoked an oxidation current whose time course tracked the time course of the neurogenic constriction in mesenteric arteries (Fig. 35A,B). Besides, the data exhibit positive correlations between NE oxidation currents and neurogenic arterial contractions (Fig. 35C).

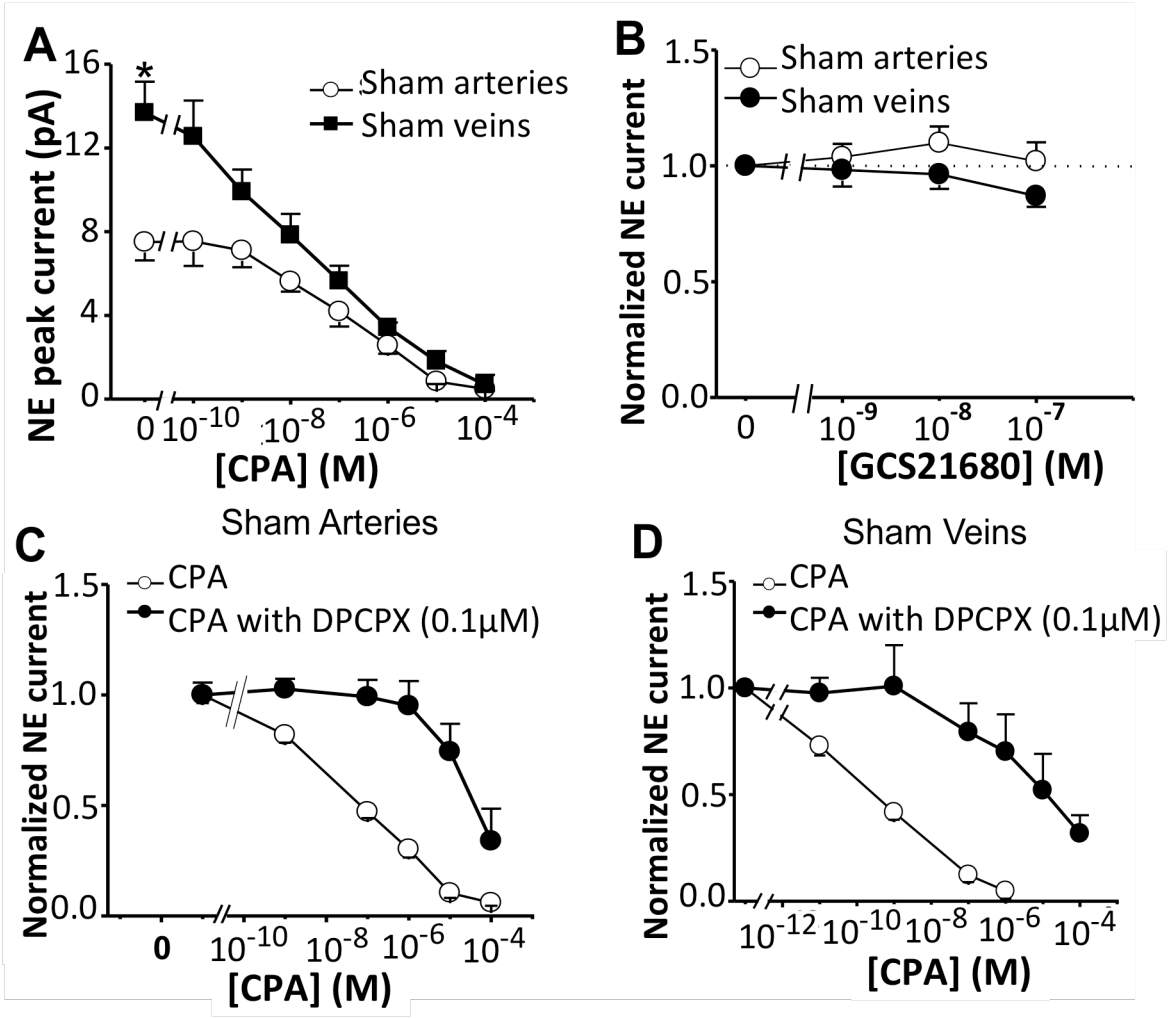
Figure 35. Representative traces of CPA-mediated inhibition of NE oxidation currents and constrictions in sham MA. Electrically-evoke NE release (60 stimuli, 10 Hz frequency, 0.5 ms pulse duration, 60-80 V intensity) was detected by amperometry with a carbon fiber microelectrode. Peri-arterial sympathetic nerves were stimulated and oxidation currents (A) and constrictions (B) were inhibited by CPA**.** C, Positive correlation between NE oxidation current and neurogenic contraction. Duration of nerve stimulation (ns) is indicated by the bars. I_{max} indicates amplitude of NE oxidation current. n indicates the number of animals. R, relative correlation; s, second; SD, standard deviation.



CPA produced a concentration dependent inhibition of the oxidation current and the neurogenic constriction (Fig. 35A,B 36A). Similar data were obtained in mesenteric veins (where only oxidation current was monitored due to direct constrictor effect of CPA). Peak NE currents in sham veins were greater than sham arteries by 36% (13.7±1.5 *vs*. 7.5±0.9 pA, $P<0.05$)(Fig. 36A). Furthermore, DPCPX (0.1μM), a selective A_1R antagonist, caused a rightward shift in the CPA concentration response curve in sham arteries and veins (Fig. 36C, 36D). The pIC_{50} values for CPA in sham arteries in the absence and presence of DPCPX were 6.6±0.2 and 4.7±0.2 respectively ($P<0.05$). The pIC_{50} values for CPA in sham veins in the absence and presence of DPCPX were 9.6±0.2 and 5.2±0.4 respectively ($P<0.05$).

Amperometric measurement of NE release after brief electrical stimulation (60 stimuli at 10Hz) showed that the $A_{2A}R$ agonist CGS21680 in a range of concentrations selective for the $A_{2A}R$ (1-100nM) did not alter significantly NE oxidation currents (Fig. 36B).

Figure 36. CPA, but not CGS21680 inhibit norepinephrine (NE) oxidation currents in sham mesenteric arteries and veins. A, Nerve stimulation was used to evoke NE oxidation currents, which were inhibited by CPA in sham arteries and veins. * Indicates that baseline oxidation currents were significantly larger in veins compared to arteries. B, Increasing concentrations of A_{2A} adenosine receptor agonist CGS 21680 did not significantly change amplitude of NE oxidation currents in sham mesenteric arteries or veins. CPA concentration response curves for inhibition of NE oxidation currents in arteries were right shifted by the A_1R antagonist DPCPX in arteries (C) and veins (D). Data are mean ± S.E.



3.4.6 Impaired function of prejunctional A_1Rs in DOCA-salt mesenteric artery but not vein

The data presented above indicate that adenosine and CPA act pre-junctionally to inhibit NE oxidation currents and neurogenic constrictions. The next study determined if this mechanism was impaired in DOCA-salt hypertension. It was found that adenosine- and CPA-induced inhibition of neurogenic constriction was reduced in DOCA-salt artery (Fig. 33B, C). CPA produced a concentration-dependent inhibition of NE oxidation currents in arteries and veins but DOCA-salt arteries were less sensitive to the inhibitory effects of CPA effect compared to sham arteries (Fig. 37A, $P<0.05$). In DOCA-salt arteries, the maximum inhibition of NE release produced by CPA (100 μM) was reduced and the pIC_{50} value was decreased compared to sham artery values (Table 6). The effects of CPA on NE oxidation currents were not different in sham and DOCA-salt veins (Fig. 37B, Table 6).

Figure 37. Impaired A_1R function in DOCA-salt mesenteric arteries but not veins. A, A 10 Hz stimulus train was used to evoke NE oxidation currents. CPA was then added in increasing concentrations. The CPA CRC was right shifted in DOCA-salt arteries compared to sham arteries. B, There were no differences in CPA CRCs between sham and DOCA-salt veins. Data are mean ± S.E. *Indicates significantly different from sham arteries. #Indicates significantly different from baseline values.

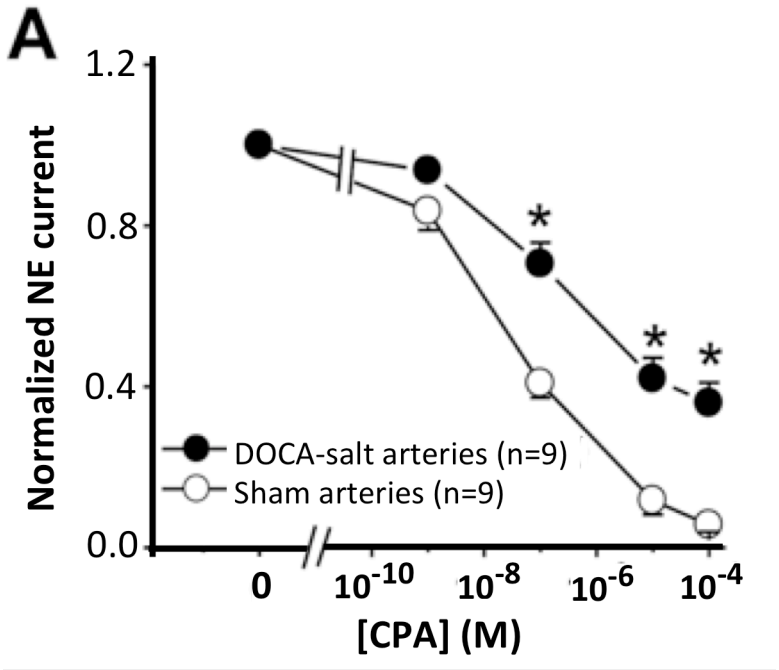


Figure 37. Cont'd

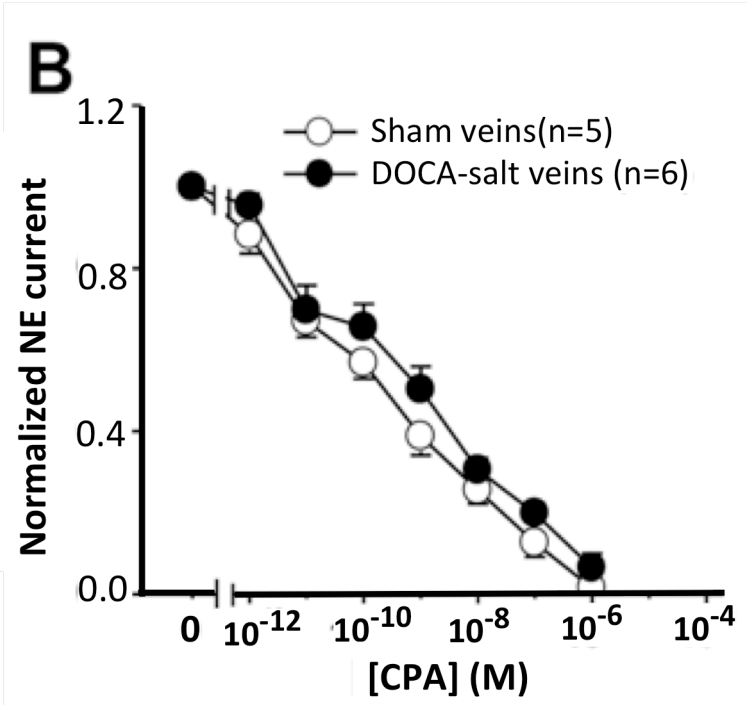


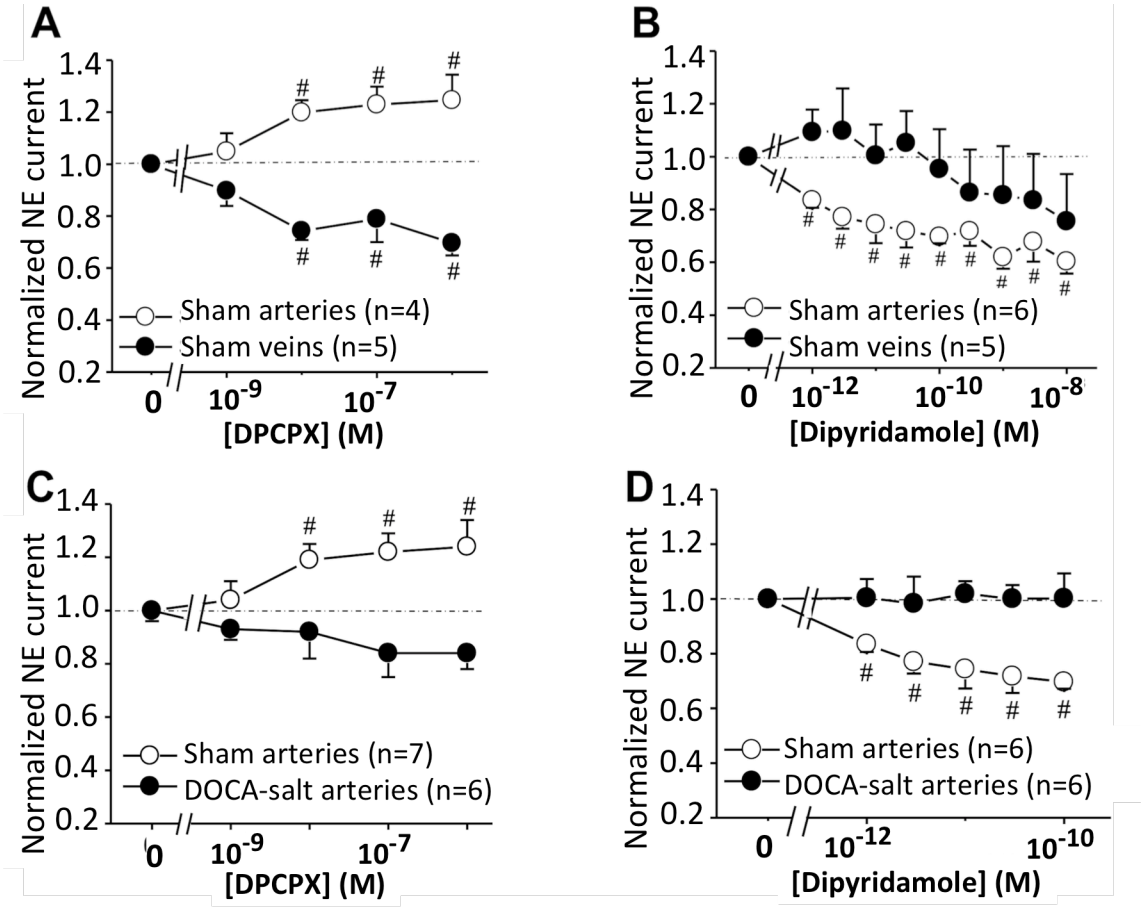
Table 6. Analysis of concentration response curves for the effects of the A_1R agonist, CPA, on norepinephrine oxidation currents recorded from the surface of mesenteric arteries and veins from sham and DOCA-salt rats. pIC_{50} values and maximum inhibition were determined from nonlinear curve fits of concentration response curves from individual preparations. Mean values were then compared using Student's t-test.

	N	pIC_{50}	% Max Inhibition
Mesenteric arteries			
Sham	9	7.6 ± 0.2	94 ± 2.0
DOCA-salt	9	6.4 ± 0.3*	64 ± 4.2*
Mesenteric veins			
Sham	5	8.5 ± 0.5	95 ± 3.6
DOCA-salt	6	8.6 ± 0.2	99 ± 1.4

* Indicates significantly different from sham values.

3.4.7 Role of endogenous adenosine in modulation of adrenergic transmission. We next determined the role of endogenous adenosine as a neuromodulator of sympathetic neurotransmission in mesenteric arteries and veins. DPCPX, a selective A_1R antagonist, was used to block A_1R autoinhibition. DPCPX caused an increase in NE current in concentration-dependent manner in arteries. Conversely, DPCPX caused a reduction of NE current in concentration-dependent manner in veins (Fig. 38A). Furthermore, dipyridamole, an adenosine transporter blocker, was used to produce an accumulation of endogenous adenosine providing a greater A_1R activation. Dipyridamole produced a decrease in NE current in concentration-dependent manner is arteries, but not veins (Fig. 38B). DPCPX and dipyridamole did not change NE oxidation currents in DOCA-salt arteries (Fig. 38C-D).

Figure 38. Role of endogenous adenosine in adrenergic transmission of sham and DOCA-salt rats. Dose-response curves of DPCPX (A_1R antagonist) and dipyridamole (adenosine transporter blocker) were performed under a 10 Hz stimulus train to evoke NE oxidation currents. A, DPCPX increased NE current in concentration-dependent manner in sham mesenteric arteries, nut not veins. B, dipyridamole caused a reduction of NE current in sham mesenteric arteries, but not veins. DPCPX (C) and dipyridamole (D) did not alter the amplitude of NE oxidation currents in DOCA-salt arteries. Data are mean ± S.E. #Indicates significantly different from baseline values. n values indicate the number of animals in the experiment.



3.5 Discussion

3.5.1 Adenosine A_1, A_{2A}, A_{2B}, but not A_3 receptors localize on periarterial and perivenous sympathetic nerves

My data show that there are three subtypes of adenosine receptors (A_1, A_{2A}, and A_{2B}, but not A_3) located on the sympathetic nerve fibers supplying mesenteric arteries and veins. My research focuses on A_1 and A_{2A} subtypes as they are high affinity adenosine receptors (Olah *et al.*, 1995). Thus, these two prejunctional receptors are more relevant to physiologic sympathetic neurotransmission where adenosine concentrations in the neurovascular junction are ranged in the nanomolar range. The low affinity $A_{2B}R$ may play a pivotal role under pathologic conditions where adenosine concentrations may reach the micromolar range (Ralevic *et al.*, 1998).

3.5.2 Adenosine constricts mesenteric veins but not arteries via A_1R activation

Our data show that adenosine can modulate venous tone by directly constricting venous smooth muscle and by inhibiting NE release from perivenous sympathetic nerves. Adenosine does not constrict mesenteric arteries and acts only to inhibit NE release from periarterial sympathetic nerves. Adenosine-induced constriction of mesenteric veins was mimicked by the A_1R agonist, CPA and adenosine produced a sustained constriction indicating that the A_1R mechanism is desensitization resistant. It is not surprising that adenosine or CPA did not constrict mesenteric arteries as previous work has shown that adenosine dilates mesenteric arterioles (Mian *et al.*, 1995) and this

effect is mediated by A_2R in the rat stomach (Nagata *et al.*, 1996). In rabbit mesenteric arteries, adenosine induced vasodilation is mediated also by the $A_{2A}R$ subtype (de Brito *et al.*, 2002). We did not detect a vasodilation in our studies because we did not pre-constrict the blood vessels. A novel finding in our study is the A_1R mediated constriction of mesenteric vein in light of previous studies showing that adenosine dilated rat mesenteric vein *in situ* (Mian *et al.*, 1995). In these studies, the veins were blood perfused while our veins were maintained in a physiological buffer solution *in vitro*. Methodological differences might contribute to these differences in results. As the focus of our study was adenosine modulation of sympathetic neuroeffector transmission we did not pursue this issue.

3.5.3 A_1Rs couple to inhibition of NE release

Adenosine, a degradation product of ATP metabolism in the neuroeffector junction (Tabrizchi *et al.*, 2001; Todorov *et al.*, 1997; Westfall *et al.*, 2002) modulates sympathetic neurotransmission in the mesenteric circulation (Diniz *et al.*, 2004; Donoso *et al.*, 2006; Illes *et al.*, 1988; Ralevic, 1995), in the rat tail artery (Bucher *et al.*, 1992; Diniz *et al.*, 2004), rat caudal arteries (Shinozuka *et al.*, 1988), and in human forearm blood vessels (Rongen *et al.*, 1996). Adenosine-induced inhibition of sympathetic neuroeffector transmission is mediated by prejunctional A_1Rs (Diniz *et al.*, 2004; Ralevic, 1995; Ralevic, 2000) and/or A_{2A}Rs (Donoso *et al.*, 2006). These previous studies showed that A_{2A}Rs could either couple to increased or decreased NE release

from perivascular sympathetic nerves (Diniz *et al.*, 2004; Donoso *et al.*, 2006). They used long trains of electrical stimulation and NE was measured in the bath solution collected by overflow and high performance liquid chromatography. We used amperometry to measure NE release in real time near a few release sites on a single blood vessel with brief trains of electrical stimulation that more closely mimic sympathetic nerve activity *in vivo*. Under these conditions, we found that the $A_{2A}R$ agonist CGS21680 in a range of concentrations selective for the $A_{2A}R$ (1-100nM) did not alter significantly NE oxidation currents.

Our immunohistochemical data show that A_1Rs are localized to perivascular sympathetic nerves supplying rat mesenteric arteries and veins. Sympathetic nerves were identified by localizing tyrosine hydroxylase (TH) immunoreactivity in nerve fibers. TH is the rate-limiting enzyme in NE synthesis. The immunohistochemical data are consistent with our conclusion that A_1Rs modulate NE release in the mesenteric circulation. This differs from the conclusion of Donoso and co-workers (Donoso *et al.*, 2006) who concluded that A_1Rs do not couple to inhibition of NE release in the rat mesentery. This conclusion is based partly on RT-PCR studies, which did not detect A_1R transcripts in the mesentery. However, mRNA encoding the A_1R protein would be found in highest concentrations in the nerve cell body of sympathetic neurons in prevertebral ganglia. It is not surprising that they failed to detect the A_1R mRNA in the mesentery where sympathetic nerve terminals are located. Immunohistochemical methods detect the A_1R protein, which was found in periarterial and perivenous nerve

fibers. These methodological differences would account in part for differences in conclusions about the role of the A_1R in perivascular sympathetic nerves. The result also demonstrates that there are TH negative nerve fibers expressing A_1R immunoreactivity. It is possible that these A_1Rs are localized to periarterial primary afferent nerve fibers (Burnstock *et al.*, 1996). We did not measure mRNA or protein levels of prejunctional A_1R in mesenteric blood vessels as A_1Rs are expressed by sympathetic nerves fibers, and by vascular smooth muscle in mesenteric veins. Hence, measurements of A_1R expression in whole blood vessel wall protein extracts would not distinguish the amount of A_1Rs in the nerve fibers vs. smooth muscle or other cell types such as fibroblasts (Gines *et al.*, 2000) in the blood vessel wall.

As adenosine and CPA constricted the vein we could not study neurogenic venous constrictions in the presence of these agonists. However, we were able to study A_1R modulation of arterial constrictions. Neurogenic constriction also permits comparisons between adenosine and CPA for inhibition of this response. Adenosine could not be used in our amperometry studies as it interacted with the microelectrodes causing an unstable baseline. Neurogenic constrictions of rat mesenteric arteries are mediated by NE acting at α_1-adrenergic receptors and ATP acting at P2X1 receptors (Dunn *et al.*, 1999). We confirmed that A_1R stimulation does not directly affect arterial smooth muscle reactivity by showing that constrictions caused by phenylephrine or the P2X1 receptor agonist, α,β-methylene ATP, were unaffected by adenosine and CPA. However, adenosine and CPA produced concentration dependent decreases in nerve-

mediated constrictions supporting the existing data that A_1R activation couples to inhibition of neurotransmitter release.

Our data show that inhibition of neurogenic constriction by adenosine is more sensitive to impairment in DOCA-salt hypertension compared to CPA. This may be due to different potency profile of adenosine analogues acting at A_1Rs. Additionally, adenosine as a non-selective AR agonist may also act at other AR subtypes that couple to inhibition of neurotransmitter release. A previous study revealed that NE overflow of periarterial nerves was decreased by activation of A_3 adenosine receptors (A_3Rs) (Donoso *et al.*, 2006). It is possible that A_3R function is also impaired in DOCA-salt hypertension. Further studies are needed to clarify this issue.

Measurement of neurogenic constrictions is an indirect measure of neurotransmitter release and it does not discriminate between effects mediated by NE or ATP release. Continuous amperometry with carbon fiber microelectrodes detects NE release from sympathetic nerves (Dunn *et al.*, 1999; Park *et al.*, 2007). We used amperometry to directly assess adenosine receptor modulation of NE release from sympathetic nerves. Our amperometry data confirm previous work showing that A_1Rs mediate prejunctional inhibition of sympathetic neuroeffector transmission in the mesentery but we expand on these findings in two ways. Firstly, previous work used increases in vascular perfusion pressures, arterial contractions or overflow of ^{3}H-NE following long trains of electrical stimulation to assess A_1R-mediated modulation of NE release. We used microelectrodes to measure NE release from a small number of

nerve fibers on the blood vessel surface. This technique allows measurements in real time and also permits use of short trains of stimulation that more closely mimic sympathetic nerve activity occurring *in vivo*. Secondly, we show that endogenous adenosine can inhibit NE release. This conclusion is based on the finding that the A_1R selective antagonist, DPCPX, increased NE oxidation currents while the adenosine uptake inhibitor, dipyridamole, decreased NE oxidation currents in mesenteric arteries from normotensive rats. It is important to note that enhancement of NE release by endogenously released adenosine could be due only to blockade of inhibitory A_1Rs or to a combination of inhibition of A_1Rs which reveals an action of adenosine at facilitatory A_{2A} or A_{2B} receptors on sympathetic nerve terminals (Diniz *et al.*, 2004; Fresco *et al.*, 2002). Additional studies are needed to address this issue.

However, previous work has shown that prejunctional $A_{2A}Rs$ could couple to increased or decreased NE release from perivascular sympathetic nerves (Diniz *et al.*, 2004; Donoso *et al.*, 2006). These investigators used long trains of electrical stimulation and NE was measured in the bath solution collected by overflow and high performance liquid chromatography. We used amperometry to measure NE release in real time near a few release sites on a single blood vessel with brief trains of electrical stimulation that more closely mimic sympathetic nerve activity *in vivo*. Under these conditions, we found that the $A_{2A}R$ agonist CGS21680 in a range of concentrations selective for the $A_{2A}R$ did not alter significantly NE oxidation currents. The function of

facilitatory A_{2A} receptors may be most prominent when A_1Rs are blocked. Additional studies are needed to address these differences in results and their interpretation.

3.5.4 Impaired prejunctional A_1R function in DOCA-salt MA

Sympathetic nerves regulate blood pressure and increased sympathetic nerve activity contributes to increased blood pressure in DOCA-salt hypertension (deChamplain *et al.*, 1987). Increased sympathetic vasoconstrictor tone in DOCA-salt hypertension is not due to vascular hyperreactivity to NE or ATP released by sympathetic nerves (Luo *et al.*, 2003) as might occur in angiotensin-induced hypertension(Griendling *et al.*, 1997). One possibility is that augmented sympathetic tone is due to loss of inhibitory α_2-AR function on sympathetic nerve terminals (deChamplain *et al.*, 1987; Demel *et al.*, 2008; Luo *et al.*, 2004). Prior to this study, it was not clear if A_1R function is also impaired in DOCA-salt hypertension. Here, we assessed whether dysfunction of the prejunctional autoreceptors is specific to the α_2-AR or if there is a more general disruption of prejunctional mechanisms controlling neurotransmitter release from periarterial sympathetic nerves. We found that the CPA concentration response curve for inhibition of neurogenic constrictions of mesenteric arteries and for inhibition of NE release from periarterial nerves are both right-shifted in DOCA-salt hypertension suggesting reduced A_1R function. Impaired A_1R function is not specific for the exogenously applied agonist, but it also affects the inhibitory actions of endogenously released adenosine. This conclusion is based on data showing that

increases in NE oxidation currents caused by DPCPX (A_1R antagonist) and reductions in NE oxidation currents caused by dipyridamole (adenosine uptake inhibitor) were also reduced in DOCA-salt arteries. We also found that concentration response curves for inhibition of neurogenic constrictions by exogenous adenosine and CPA were right shifted in MA from DOCA-salt hypertensive rats. These data are similar to those obtained using α_2-AR agonists to inhibit neurogenic constrictions (Luo *et al.*, 2004; Park *et al.*, 2010). These results also indicate that prejunctional α_2-ARs are not selectively affected in DOCA-salt hypertension, but there is a more general disruption of prejunctional mechanisms controlling neurotransmitter release form periarterial sympathetic nerves. Indeed, there is evidence that α_2-ARs and A_1Rs interact to modulate NE released from sympathetic nerves supplying the rat tail artery (Bucher *et al.*, 1992). It is also important to note that A_1R modulation of NE release from perivenous sympathetic nerves was not altered in DOCA-salt hypertension. Similar results have been obtained when α_2-AR function was studied in perivenous sympathetic nerves from DOCA-salt hypertensive rats (Park *et al.*, 2010). The mechanisms responsible for decreased function of prejunctional autoreceptors in arteries are unclear. It is possible that arterial autoreceptor impairment is due to receptor downregulation. Western blot analysis or radioligand binding studies would be useful for these measurements. However, neuronal protein in the arterial wall is a small fraction of total protein present. Furthermore, A_1Rs are both expressed by nerve fibers and other cell types (smooth muscle, fibroblasts (Bruns, 1979; Gines *et al.*, 2000)) in the arterial wall.

These limitations would make it difficult to determine if there are neuronal specific changes in receptor expression in arteries in DOCA-salt hypertension.

3.6 Perspective.

Our data support previous findings that A_1Rs localized to periarterial nerves couple to inhibition of NE release. We also show that the disruption of autoreceptor function on periarterial sympathetic nerves is not specific for the α_2-adrenergic receptors as A_1 autoreceptors on periarterial nerves of DOCA-salt hypertensive rats are also impaired. We also show for the first time that A_1Rs also couple to inhibition of NE release in perivenous sympathetic nerves. An important difference between mesenteric arteries and veins is that A_1R and α_2-AR function is disrupted in periarterial but not perivenous sympathetic nerves in DOCA-salt hypertension. α_2-AR function is at least partly restored in DOCA-salt hypertensive rats that had been treated with apocynin an NADPH oxidase inhibitor (Demel *et al.*, 2010). NADPH oxidase produces O_2^- in the vasculature(Szasz *et al.*, 2007) and this enzyme is also localized to perivascular sympathetic nerves (Cao *et al.*, 2009). Increased O_2^- levels in arteries is associated with higher arterial pressures and elevated O_2^- could disrupt signaling mechanisms linking α_2-AR and A_1R to inhibition of NE release. Venous pressure is not increased in DOCA-salt hypertension (Ralevic, 2000) so levels of O_2^- would not be elevated in veins

and this might spare autoreceptor function. There are also likely to be differences in arteries and veins in the enzyme systems that produce and degrade reactive oxygen species (Szasz *et al.*, 2007). This difference could also contribute to maintained autoreceptor function in perivenous sympathetic nerves in DOCA-salt hypertension.

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CHAPTER 4

DOCA-SALT HYPERTENSION FAILED TO ENHANCE SYMPATHETIC NE CLEARANCE THROUGH PREJUNCTIONAL A_{2A} ADENOSINE RECEPTOR ACTIVATION

4.1 Abstract

Norepinephrine (NE) and ATP are released from perivascular sympathetic nerves to control arterial resistance and venous capacitance. NE is cleared from the neuroeffector junction via the NE transporter (NET). ATP is hydrolyzed to produce adenosine, which activates prejunctional A_1-adenosine receptors (A_1Rs) to inhibit NE release. A_{2A}-adenosine receptors (A_{2A}Rs) are also located on perivascular nerves and these receptors facilitate sympathetic transmission. In the central nervous system A_{2A}Rs also stimulate neurotransmitter re-uptake. This effect has not been investigated in peripheral nerves. We tested the hypothesis that A_{2A}Rs enhance NE clearance at the sympathetic neuroeffector junction by increasing NET activity, and this response is impaired in DOCA-salt hypertensive rats. Electrically evoked NE release was measured *in vitro* in real time using amperometry. Drug effects on the kinetics of NE oxidation currents were measured. Activation of A_{2A}Rs by CGS21680 (1-10nM, A_{2A}R agonist) enhanced NE uptake via NET in control mesenteric veins, but not arteries, an A_{2A}R antagonist (SCH58261 400nM), and NET blocker (cocaine 10μM) prevented this effect. Forskolin (1 μM, adenylyl cyclase activator) mimicked CGS21680 effects and H-89 (1 μM, protein kinase A inhibitor) blocked CGS21680-mediated effects. In DOCA-salt veins, CGS21680 failed to promote NE clearance, but forskolin restored the response. Impaired A_{2A}R function to promote NE clearance in perivascular veins accounts for

increased NE availability in mesenteric veins of DOCA-salt hypertension. DOCA-salt hypertension impairs this pathway by inhibiting $A_{2A}R$ activation of adenylyl cyclase.

4.2 Introduction

Hypertension (HT), a major risk factor for stroke, cardiac and renal injury and HT affects 1 billion people worldwide (Chobanian *et al.*, 2003). HT is associated with elevated sympathetic nerve activity and circulating catecholamines (Bouvier *et al.*, 1986; deChamplain *et al.*, 1976). Mesenteric arteries and veins are highly innervated by sympathetic nerves which supply the adventitia-media border of arteries and tunica media of veins (Birch *et al.*, 2008). Periarterial sympathetic nerves control vascular resistance by releasing NE and ATP as co-transmitters (Astrand *et al.*, 1989; Bobalova *et al.*, 2001b). Once released, NE and ATP act at the postjunctional α_1-adernergic and $P2X_1$ receptors respectively to cause constriction. The actions of NE and ATP are terminated by two mechanisms. Firstly, NE is quickly taken up into nerve terminals via the high affinity NE transporter (NET) while ATP is degraded by extracellular 5'ectonucleotidase enzyme into ADP, AMP and adenosine as a final breakdown product. Secondly, NE and adenosine activate prejunctional α_2-adrenergic and A_1-adenosine autoreceptors, respectively to stop further transmitter release. Deoxycorticosterone acetate (DOCA)-salt hypertension is a salt sensitive, renin independent model of hypertension (Schenk *et al.*, 1992). Periarterial and perivenous sympathetic nerves in DOCA-salt hypertensive rats release more NE (Luo *et al.*, 2004) causing increased vascular tone. Increased NE release in DOCA-salt mesenteric arteries is partly due to impaired sympathetic autoreceptor function (deChamplain, 1990; Luo *et al.*, 2004). However, autoreceptor function is preserved in mesenteric veins (Park *et al.*, 2010). Luo and co-workers also reported downregulation of the

postjunctional α_1-adrenoceptor resulting in decreased sensitivity to NE and phenylephrine (an α_1-receptor agonist) in mesenteric veins of DOCA-salt hypertensive rats (Luo *et al.*, 2003). Since the number of adrenergic receptors is in part regulated by catecholamines (Michel *et al.*, 1990), it is possible that the receptor downregulation is caused by elevated NE availability (deChamplain *et al.*, 1976). It is unclear what drives elevated NE availability in DOCA-salt veins.

Adenosine acts on four different G-protein coupled receptor subtypes: A_1, A_{2A}, A_{2B}, and A_3-adenosine receptors. A_1 and A_{2A} subtypes are high affinity receptors that desensitize quickly (Olah *et al.*, 1995). In the nervous system, activation of A_{2A}Rs requires higher adenosine concentrations of adenosine compared to those required to activate A_1R (Ralevic *et al.*, 1998). Low affinity A_{2B}Rs require sustained, high concentrations of adenosine for activation suggesting that A_{2B}Rs are activated only under pathophysiological conditions where adenosine levels are high (ischemia for example) (Eckle *et al.*, 2008; Koeppen *et al.*, 2011; Ralevic *et al.*, 1998).

A_{2A}Rs are also located on sympathetic nerves supplying mesenteric arteries and veins. Neuronal A_1Rs couple to Ca^{2+} channel inhibition (Ralevic *et al.*, 1998) resulting in inhibition of transmitter release. A_{2A}Rs couple to the stimulator G-protein G_s to activate adenylyl cyclase and Ca^{2+}channels leading to facilitation of neurotransmitter release. Recent studies have revealed a novel mechanism of A_{2A}R in modulating

neurotransmission where A_{2A}Rs modulate GABA and glutamate uptake via and action on GABA and glutamate transporters, respectively (Cristóvão-Ferreira *et al.*, 2011; 2009; Gonzalez *et al.*, 2006), (Matos *et al.*, 2012). However, A_{2A}R-modulated transporter function has not been investigated in peripheral nerves. Thus, the first aim of this study was to test the hypothesis that prejunctional A_{2A}Rs couple to enhanced NET function to remove NE from the sympathetic neuroeffector junction in mesenteric blood vessels. The second aim was to determine whether this mechanism was impaired in DOCA-salt hypertensive rats.

4.3 Materials and Methods

4.3.1 Animal. All animal use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University. Male Sprague-Dawley rats (250-275g) were obtained from Charles River Laboratories (Portage, MI). Upon arrival, rats were placed in temperature and humidity controlled room with 12:12 hour light-dark cycle. They were provided with Rat chow (Harlan/Teklad 8640 Rodent Diet) and tap water were provided *ad libitum*. The animals were acclimated 2-3 days before entry into experimental protocols.

4.3.2 DOCA-salt hypertensive rats. The surgical procedures and drug treatment protocols for producing sham control and DOCA-salt hypertensive rats have been described in detail previously (Luo *et al.*, 2003). After surgery, rats were housed under standard conditions for 4 weeks for recovery. DOCA-implanted rats received standard

pelleted rat chow and drinking solution containing 1%NaCl+0.2%KCl in distilled water. Sham rats received standard pelleted rat chow and distilled water. Blood pressure was measured using tail-cuff plesmythography 3-5 days prior to experimentation. Rats with mean arterial pressure $\geq$150 mmHg were considered hypertensive.

4.3.3 Tissue preparation for in vitro studies. 4-weeks post surgery, blood pressure had risen substantially in DOCA-salt but not in sham control rats. At this point, DOCA-salt or sham rats were sacrificed with a lethal dose of pentobarbital intra peritoneal injection (100 mg/kg). The whole mesentery transferred into cold Krebs solution (mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 $NaHPO_4$, and 11, glucose. A section of tertiary mesenteric arteries (145-250 μm outside diameter) and veins (190-390 μm outside diameter) was dissected out and transferred to a recording chamber. The tissue was pinned flat with 50 μm-diameter stainless steel pins. Adipose and connective tissue was gently removed under dissecting microscope. The tissue was opened as a small window to allow stimulating and recording electrodes to be positioned on the vessel. The recording chamber was then mounted on the stage of an inverted microscope and tissue was superfused continuously (2.5 ml/min) with warmed (36°C), oxygenated (95% O_2, 5% CO_2) Krebs solution. Tissues were allowed to equilibrate for 30 minutes before beginning experiments. During this time the blood vessels relaxed to stable resting diameter. Video images were obtained using a black-and-white video camera (Hitachi model KP-111; Yokohama, Japan) connected to the microscope and fed to a Picolo frame grabber board (Euresys Inc., Itasca, IL) mounted

in a personal computer. Video images were analyzed using Diamtrak® edge tracking software (Adelaide, Australia). Diameter changes of 1 μm can be resolved.

4.3.4 Amperometric detection of norepinephrine (NE) release. A bipolar stimulating electrode made from silver wire was inserted into a 2-barrel glass capillary filled with Krebs solution. The electrode was mounted on a holder and positioned on the vessel surface using a micromanipulator. Sympathetic nerves were stimulated using trains of electrical stimulation (10 Hz, 0.5 ms pulse duration 6 s train duration, 60-80V). Released NE was detected as an oxidation current using amperometry with carbon fiber microelectrodes. Construction and preparation of carbon fiber microelectrodes was described previously (Park *et al.*, 2007). The microelectrode was positioned horizontally and pressed gently against the blood vessel surface so that NE flux from nearby release sites could be detected. The carbon fiber microelectrode was placed at a distance of 200μm from the stimulating electrode to minimize the stimulus artifact during the current recording. A commercial "no leak" Ag-AgCl reference electrode (3M KCl, model EE009, Cypress System Inc., USA) and a platinum wire counter electrode were also mounted in the chamber to complete the electrochemical cell. Continuous amperometric measurements were made with an Omni 90 analog potentiostat (Cypress Systems Inc.), a Minidigi analog-to-digital converter and a computer running Axoscope 9.0 (Axon Instruments, Union City, CA, USA). Data were obtained at a 100 Hz sampling rate. An applied potential of 0.6 volts was used to detect NE currents because this is the selective oxidation potential for NE at mass-transfer limited rate (Park *et al.*, 2007).

Currents were low pass filtered with at a time constant of 200 ms. Data were stored on the computer hard drive for further analysis.

4.3.5 Drug Application. Stock solutions of drugs were diluted to working concentrations with Krebs solution on the day of experiment. The vessels were initially performed 3 consecutive trains without drug to verify viability and response stability. Drugs were applied using a 3-way stopcock system and it took 3 minutes for drugs to reach the chamber. To obtain concentration response curves, agonists were applied 15 minutes before nerve stimulation with 45-minute wash between each concentration. Antagonists were applied for 30 minutes before agonist application.

4.3.6 Immunohistochemistry of mesenteric blood vessels. The whole mesentery was removed and transferred to a clear silicone bottom plate containing ice-cold phosphate buffered saline (PBS, 0.1 M). The tissues were pinned flat and cleaned of excess fat and connective tissue. Blood was flushed from the lumen by cold 0.9% saline perfusion with 27G needle into the superior mesenteric artery and vein. Tissues were fixed in ice-cold Zamboni fixative for 2 h and were washed 3x with PBS. Then, the tissues were incubated in 4% goat+sheep serum in PBS-0.1%Tritron-X at room temperature for 1 h. The vessels were cut into 1-cm length and incubated with primary antibodies against A_{2A}Rs (rabbit polyclonal, 1:100 dilution in PBS, Sigma) and tyrosine hydroxylase (TH) (mouse monoclonal anti-TH antibody, 1:100 in PBS, Millipore) overnight at 4°C. To control for non-specific binding, control vessels were incubated without primary antibodies. After overnight incubation, unbound primary antibody was

rinsed 3x with PBS and the blood vessels were incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (1:50) and Cy^3-conjugated goat anti-rabbit IgG (1:400, Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. Then, unbound secondary antibodies were rinsed 3x with PBS and 1x with deionized water to reduce PBS-produced autofluorescence. Specimens were mounted on microscope slides with anti-fade media (Prolong-Gold, Invitrogen) and viewed under a confocal microscopy (Ziess Pascal model).

4.3.7 Drugs. All drugs except cocaine and guanethidine were obtained from Sigma Aldrich, USA. Cocaine was acquired from Mallinckrodt, Missouri, USA. Guanethidine (CIBA Pharmaceutical, New Jersey) was a generous gift from Dr. Susan Barman. Forskolin, CGS21680 and SCH58261 were made as stock solutions in DMSO and kept at 0°C. The highest concentration of DMSO present in working solutions (0.001% vol/vol) did not affect vascular or sympathetic nerve function. Cocaine, guanethidine, and H-89 were dissolved in deionized water as concentrated stock solutions and kept at 0°C. All drug stocks were freshly diluted with Krebs buffer on the day of experiment.

4.3.8 Statistical Analysis. All data are reported as mean ± S.E., n values indicate the number of rats. Differences between groups were assessed using Student's t-test. Differences in agonist concentration response curves were assessed first by a 2 way ANOVA and Bonferoni's test for multiple comparisions (GraphPad Prism 5.0, LaJolla, CA). Agonist IC_{50} (half maximal inhibitory drug concentration) and maximum effect values were determined from individual concentration response curves using a non-

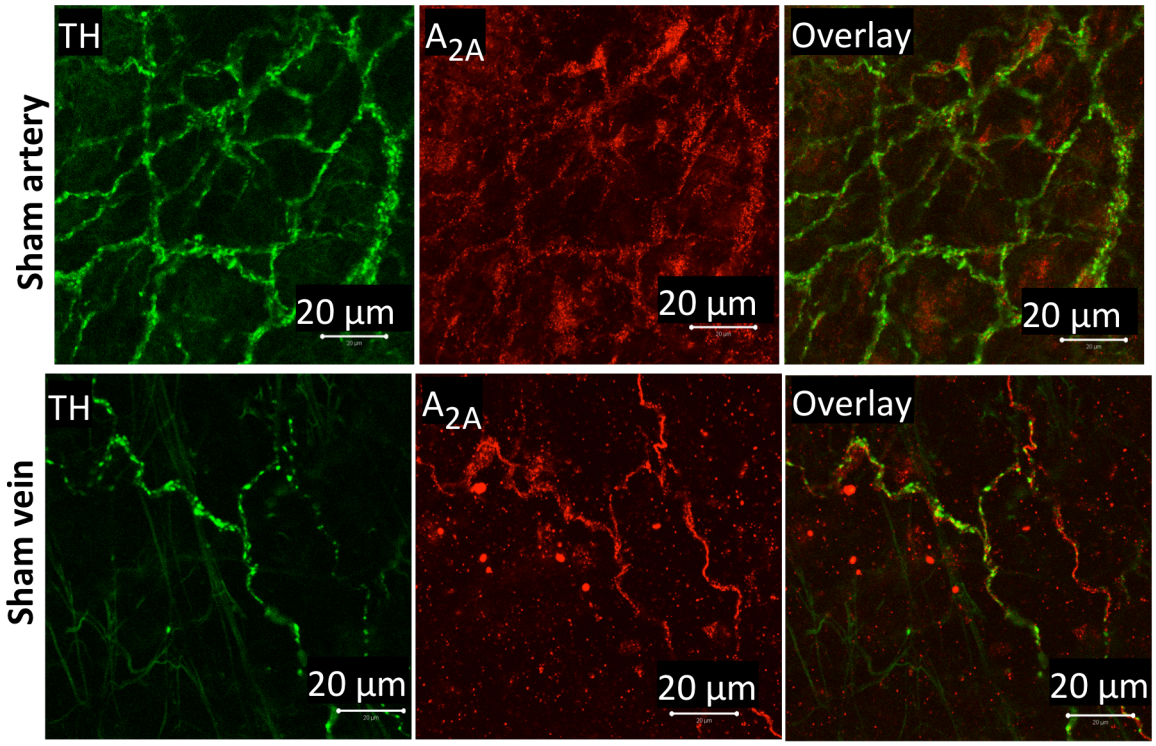
linear fitting routine and a logistic equation (Origin 8.0, Northampton, MA). IC_{50} and maximum effect values where then compared using Student's t-test. IC_{50} values are expressed as the negative log of the drug concentration that causes 50% of the maximum inhibitory response (pIC_{50}). $P<0.05$ was considered statistically significant. Kinetic of NE clearance is represented by *β*-ratio, which is calculated by area under the curve (AUC) of the pre-peak current (pre-I_{max}) divided by AUC of post-I_{max} (Fig. 33A). The lower *β*-ratio indicates the faster NE clearance.

4.4 Results

We first present the result of experiments that show expression of $A_{2A}R$ on sympathetic nerves supplying mesenteric arteries and veins from sham rats. The second section of the results describes $A_{2A}R$ function in enhancing NE clearance of sham rats. The third section we report the results of experiments that examine the disruption of $A_{2A}R$ in DOCA-salt hypertension. Finally, we present results of studies that involve downstream mechanism of $A_{2A}R$ activation and illustrates impaired Gs-stimulated adenylase cyclase (AC) step of DOCA-salt hypertension causing failure to enhance NE clearance during $A_{2A}R$ activation.

4.4.1 A_{2A}Rs are localized to sympathetic nerves supplying mesenteric arteries and veins. Tyrosine hydroxylase (TH), the rate-limiting enzyme in for the NE biosynthetic pathway, and A_{2A}Rs antibodies were used to identify perivascular sympathetic nerves and A_{2A}Rs. A_{2A}Rs were co-localized with TH in peri-arterial and peri-venous nerves (Fig. 39).

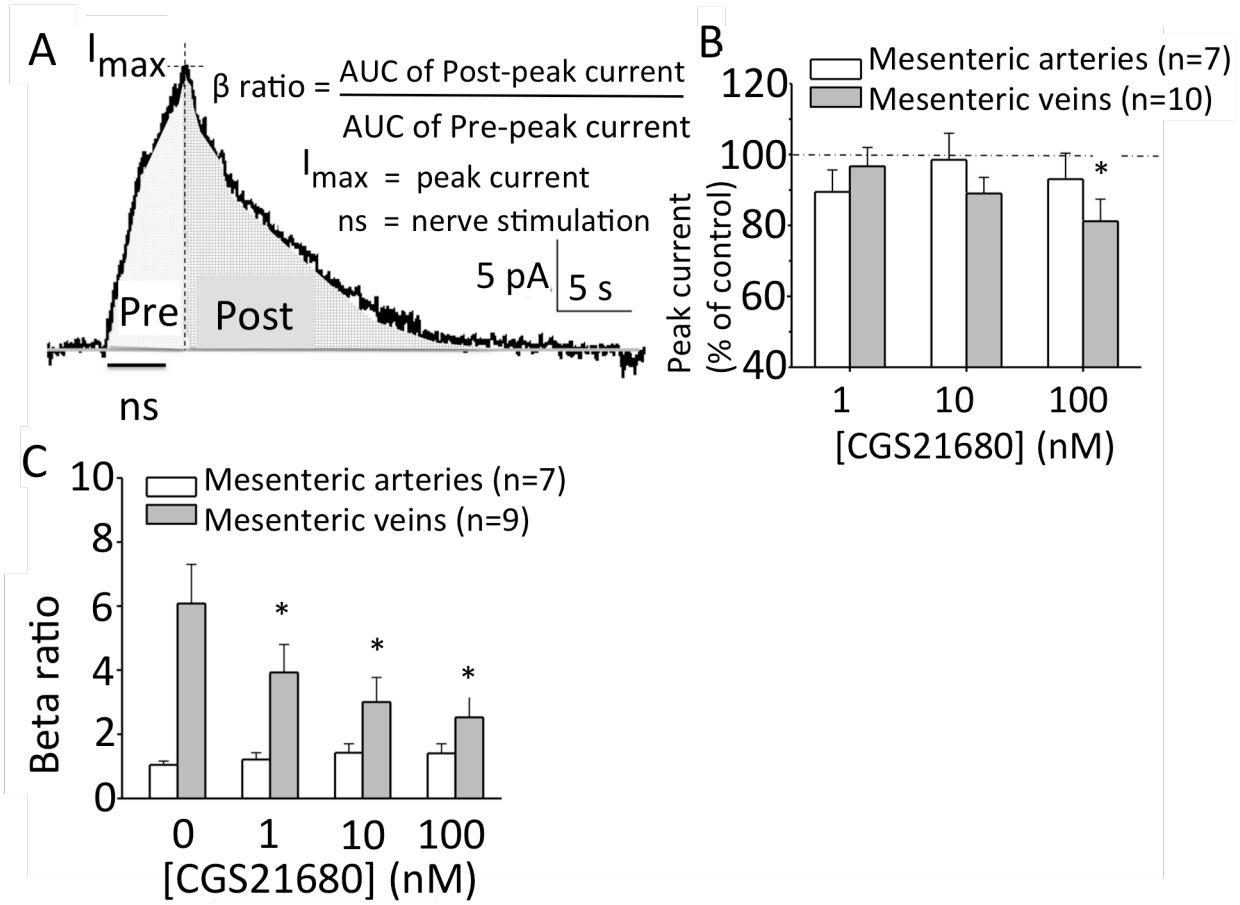
Figure 39. Localization of A_{2A} adenosine receptors (A_{2A}Rs) on sympathetic nerves supplying mesenteric artery and vein of sham rats. Mouse monoclonal TH (1:100) and rabbit polyclonal A_{2A}R (1:100) antibodies were used to identify sympathetic nerves and A_{2A}R, respectively. Images of whole mount preparations were acquired using confocal microscopy. TH is a protein marker for sympathetic nerves. Arteries (top panel) and veins (bottom panel) of sham rats contain TH (green) and A_{2A}R s (red). Merged images showed co-localization of TH and A_{2A}R (yellow). Scale bar=20µm. TH=tyrosine hydroxylase.



4.4.2 CGS21680 (selective $A_{2A}R$ agonist) enhanced NE clearance in mesenteric veins, but not arteries of normotensive rats. This experiment was designed to determine how $A_{2A}R$ activation modulated kinetics of NE release. The basal amplitude of NE oxidation currents in sham mesenteric veins was slightly higher than in sham arteries (15.6±2.2pA, n=10 *vs* 12.46±1.58pA, n=8, p>0.05). CGS21680 (1- 100nM) did not change peak oxidation currents in arteries and reduced this current in veins only at the highest concentration (Fig. 40B). Basal current half-life of mesenteric veins was longer than in arteries (16.9±7.4 s, n=10 *vs* 2.8±0.4 s, P < 0.05). The *β*-ratio is a measure of NE clearance rate. The lower *β*-ratio describes the faster NE reuptake. Basal *β*-ratios in mesenteric veins were significantly higher than in arteries (6.1 ± 1.2, n=9 *vs* 1.0 ± 0.1, n=7, P < 0.05) (Fig. 40C). CGS21680 caused a concentration dependent (1 – 100 nM) reduction in the *β*–ratio in veins, but not arteries (Fig. 40C).

Figure 40. CGS21680 enhanced NE clearance in mesenteric vein, but not artery of normotensive rats. Selective agonist CGS21680 was used to activate $A_{2A}R$. Concentration-response curves of CGS21680 (1-100nM) were performed and kinetics of NE oxidation currents was analyzed. A, each oxidation current was calculated for peak amplitude (I_{max}) and *β* ratio. *β* represents how fast NE was removed. The lower *β* ratio indicates the faster NE clearance. Basal NE clearance in perivenous nerves was dramatically slow compared to periarterial nerves since *β*-ratio in the absence of agonist was much greater in mesenteric veins (C). CGS21680 (1-10nM) did not alter peak current, but high concentration of CGS21680 slightly decreased peak current due to non-specific affinity toward A_1 adenosine receptors (B). CGS21680 also caused a reduction of *β* ratio in concentration-dependent manner (C). High concentration These CGS-mediated effects were specific to mesenteric vein, but not artery. Data are mean±S.E., n=number of rats, Data were performed repeated one-way ANOVA with Bonferroni multiple comparison post test. * indicates different from control ($p<0.05$).

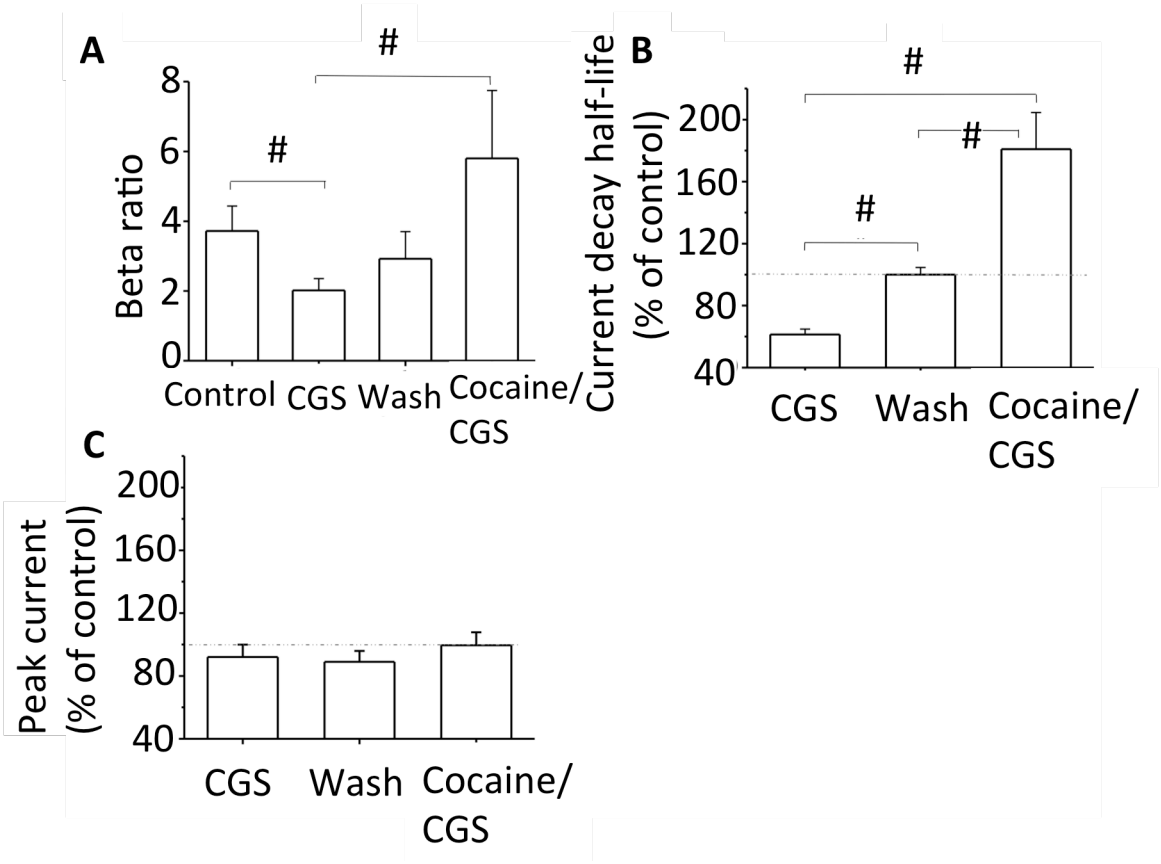
Figure 40. (cont'd)



4.4.3 Cocaine blocked CGS-induced effects.

The data above suggest that activation of $A_{2A}R$ increased NE clearance in veins. In the next experiment a NET blocker, cocaine, was used to determine if NE transport was involved in $A_{2A}R$-enhanced NE clearance. CGS21680 (10 nM) itself caused a reduction in *β*-ratio (Fig. 41A), and current half-life (Fig. 41B) without affecting the peak amplitude of NE oxidation current (Fig. 41C). The CGS-induced effects were prevented by cocaine (10 μM)(Fig. 41A-B).

Figure 41. NE transporters was involved in $A_{2A}R$-enhanced NE clearance in sham mesenteric veins. NE transporters (NETs) were blocked by cocaine to examine if $A_{2A}R$ enhanced NE clearance via NET. Series of paired experiments were performed. CGS21680 (10nM) enhanced NE clearance as it decreased β ratio (A) and current half-life (B). After 45 minute-wash, cocaine was incubated for 30 minutes before the agonist. Cocaine fully prevented CGS21680-induced effects (A-B). Neither cocaine nor CGS21680 affect peak amplitude of NE oxidation current(C). Data are mean±S.E., n=10. Data were analyzed by repeated one-way ANOVA with Bonferroni multiple comparison post test. # indicates different between groups (P<0.05).

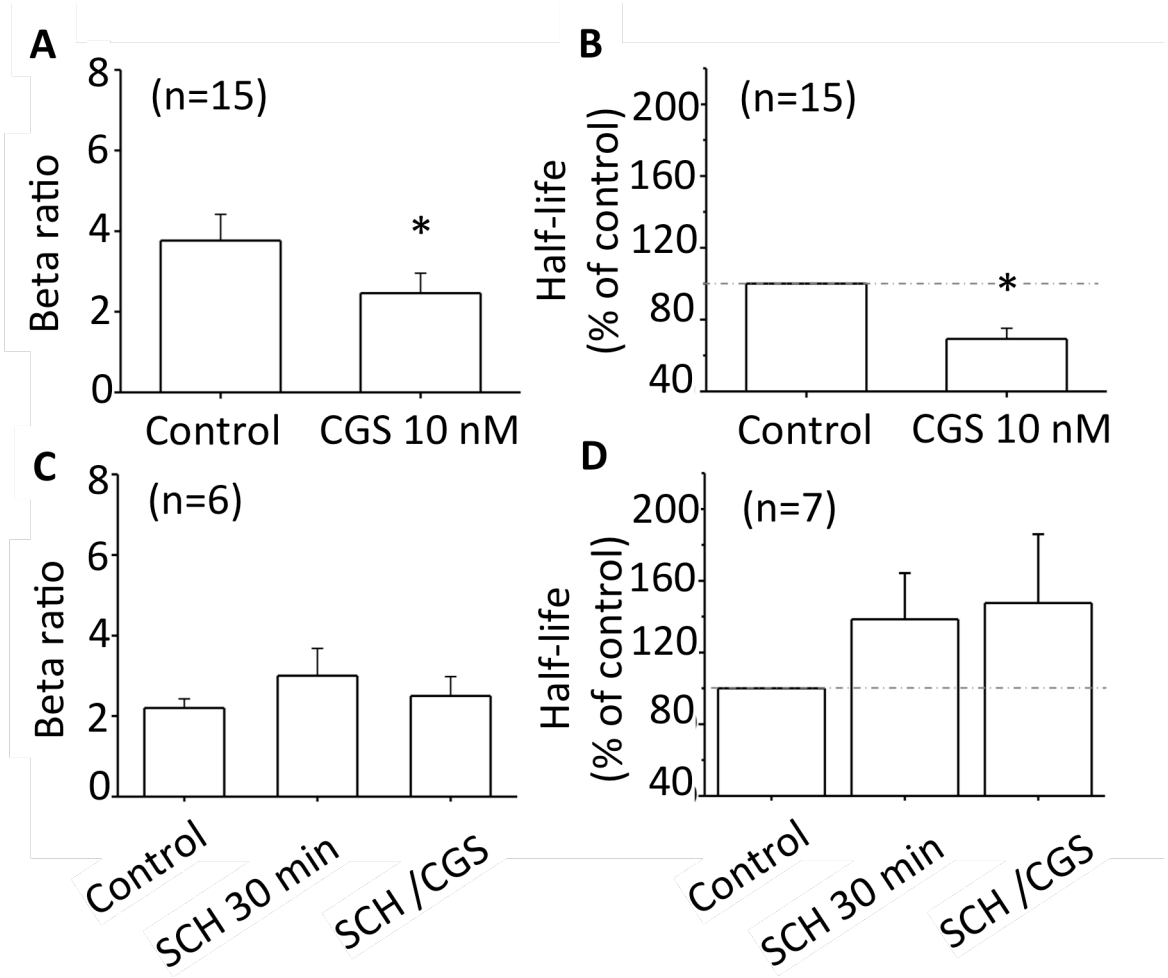


4.4.4 SCH58261 (selective $A_{2A}R$ antagonist) antagonized CGS-induced effects.

SCH58261 was used to examine if enhancement of NE clearance was mediated by $A_{2A}R$ activation. CGS21680 enhanced NE clearance (Fig. 42A,B) and this effect was blocked by SCH58261 (Fig. 42C,D).

Figure 42. CGS21680 effects were antagonized by SCH58261. To confirm that A_{2A} activation enhances NE clearance via NET, a selective $A_{2A}R$ antagonist SCH58261 (400nM) was used to block CGS-mediated effect. A-B, control experiments illustrated that CGS21680 promoted NE clearance and CGS-induced effect was fully antagonized by a selective$A_{2A}R$ antagonist, SCH58261 (400nM, C, D). SCH58261 itself did not significantly affect basal NE clearance (C, D). Data are mean±S.E., n=6-15. Data were analyzed by ordinary one-way ANOVA with Bonferroni multiple comparison post test. * indicates different between groups ($P<0.05$).

Figure 42. (cont'd)

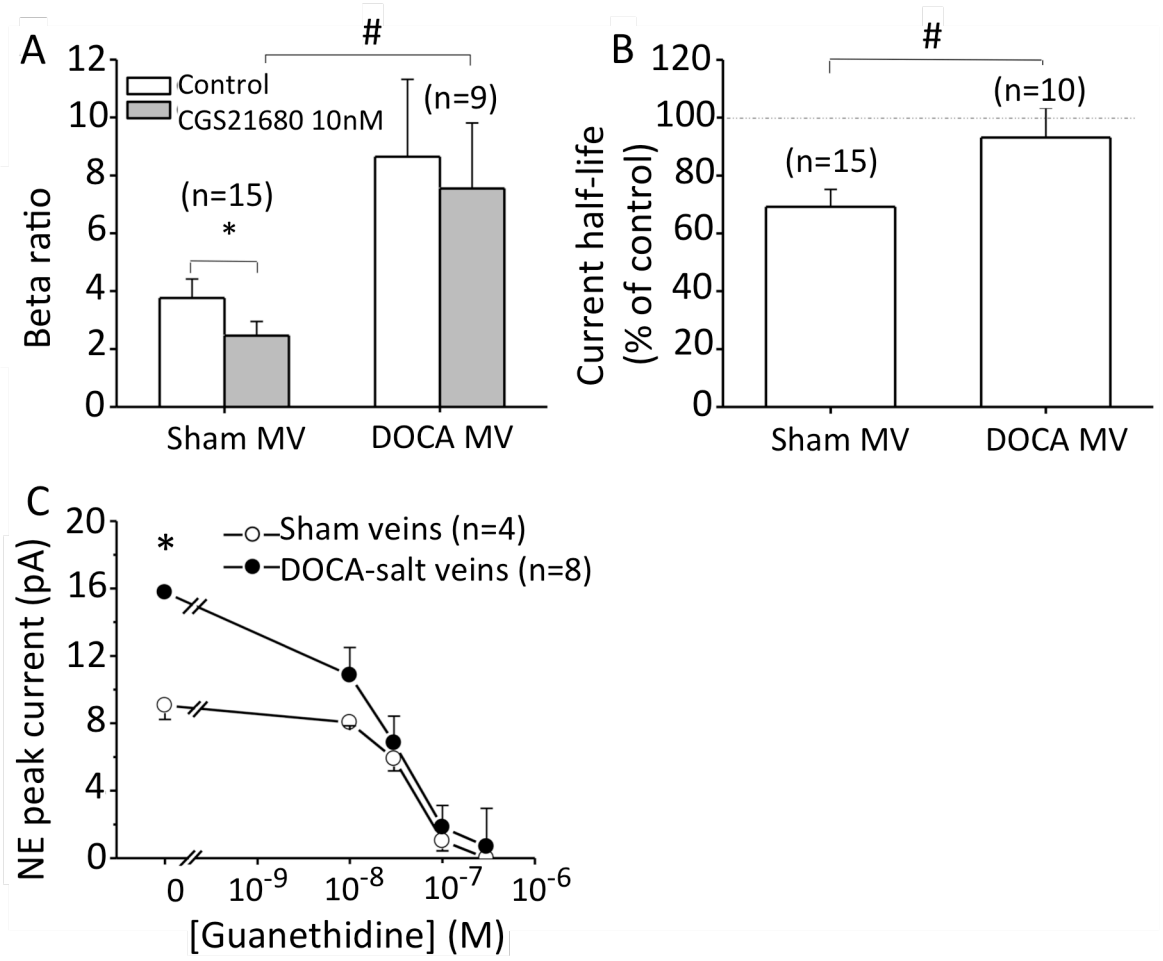


4.4.5 NE clearance is impaired in DOCA-salt hypertension

We next tested if $A_{2A}R$-induced enhancement of NE clearance is impaired in DOCA-salt hypertension. Only DOCA-salt veins were studied because CGS21680 did not alter NE clearance in arteries. The basal β-ratio and current half-life were higher in DOCA-salt compared to sham veins (Fig.43 A, B). DOCA-salt veins were less responsive to CGS21680 (10 nM) compared to sham veins (Fig 43A). CGS21680 decreased the β-ratio in DOCA-salt and sham veins by 13.06±4.9% and 49.05 ± 7.9%, ($P < 0.05$, Fig. 43A)) respectively. CGS21680 (10 nM) also shortened oxidation current half-life in DOCA-salt and sham veins by 10 ± 11%, and 40±7.2($P<0.05$), respectively (Fig. 43B).

The data above demonstrate that NE clearance in DOCA-salt veins is slower than in sham vein. The next experiment was performed to determine if impaired NET function contributes to inefficient NE clearance of DOCA-salt veins. Guanethidine, a substrate for NET was used to assess NET function. Guanethidine is taken up into sympathetic nerve terminals and then blocks NE release resulting in a reduction of NE oxidation current. Basal NE release in DOCA-salt veins was higher then in sham veins (9.1±2.1, n=4 vs 15.8±1.7pA, n=8, Fig. 43C). The concentration response curves for guanethidine-induced inhibition of NE oxidation currents were similar in sham and DOCA-salt veins (Fig. 43C). Maximal inhibition in sham and DOCA-salt veins were 100± 0.0%, n=4 and 95.4±3.3%, n=8, $P > 0.05$) respectively. The pIC_{50} values for guanethidine were similar in sham and DOCA-salt veins (7.4±0.0, n=4 and 7.7±0.2, n=8 Why is there a different “n” value here compared to the max inhibition n=4) respectively $P>0.05$).

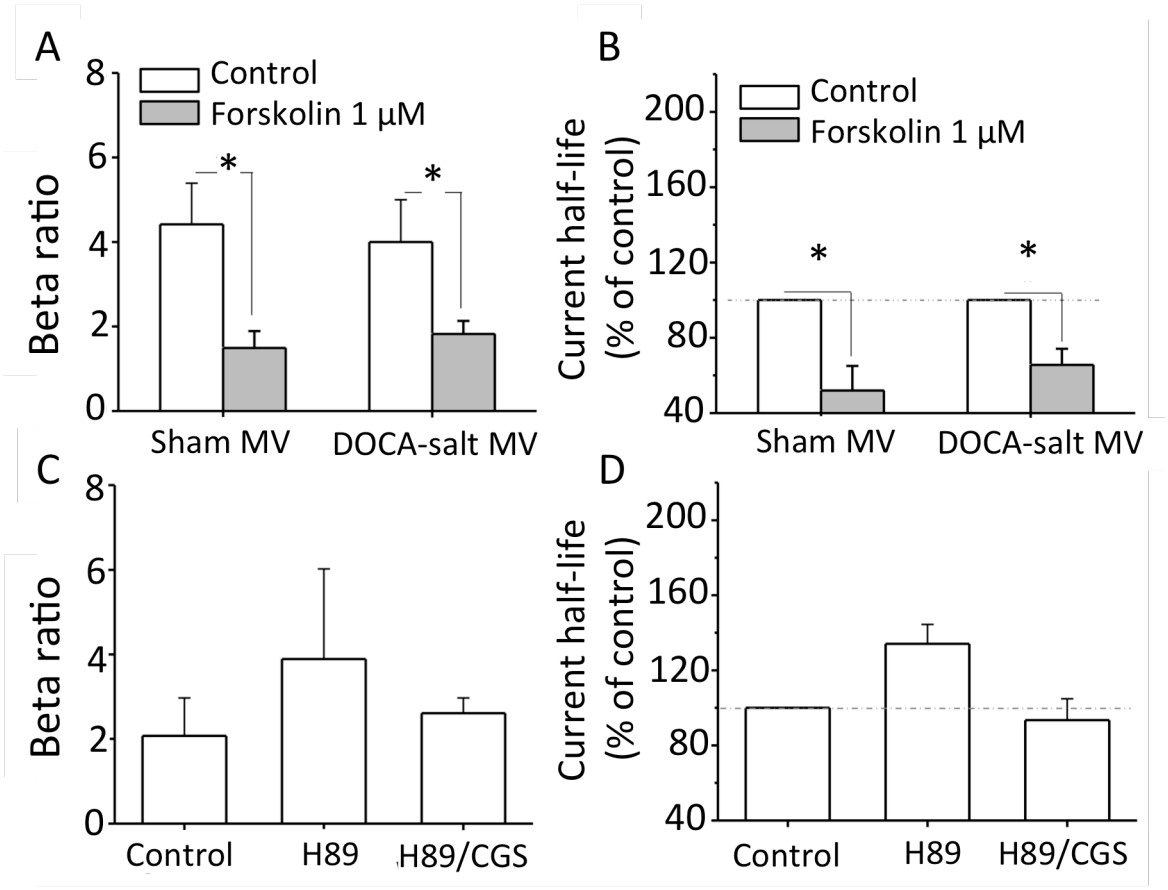
Figure 43. CGS21680 revealed impaired prejunctional $A_{2A}R$ function of DOCA-salts mesenteric veins. A, Basal β ratio of DOCA-salt mesenteric vein is higher than in control veins and DOCA-salt vein is relatively resisted to CGS21680-induced effect compared to control. B, CGS21680 shortened half-life of NE oxidation current in control vein, but not DOCA-salt vein. C, Guanethidine was used to determine NE transporter function. Guanethidine suppressed NE release in control and DOCA-salt veins with similar potency and efficacy, indicating that NET function is preserved in DOCA-salt vein. * indicates different between treatments of control rat (P<0.05). # indicates different between DOCA-salt and control rats(P<0.05).



4.4.6 Forskolin restored NE clearance of DOCA-salt hypertension

Since $A_{2A}R$ is a Gs-coupled receptor and the next experiment was designed to determine if the $A_{2A}R$-enhanced NE clearance used the adenylate cyclase/PKA/cAMP pathway. Forskolin (adenylase cyclase activator) decreased the *β*-ratio and NE oxidation current half-life in control veins. Forskolin also restored clearance in DOCA-salt veins (Fig. 44A,B). CGS21680 induced increases in NE clearance were blocked by H89, a PKA inhibitor (Fig. 44C,D).

Figure 44. Forskolin restored NE clearance mechanism of DOCA-salt mesenteric veins. PKA activator (forskolin) and inhib itor (H89) were used to reveal downstream mechanism of $A_{2A}R$ activation. Forskolin mimicked CGS-induced effects in control veins in lowering β-ratio and NE current half-life (A, B) and CGS-induced effects were prevented by H89 (C, D). Forskolin restored NE clearance mechanism in DOCA-salt veins suggesting that A2AR disruption occurs at G_s-adenylase cyclase coupling step. * indicates different between treatments (P<0.05).



4.5 Discussion

4.5.1 $A_{2A}R$ expression on sympathetic nerves supplying mesenteric arteries and veins.

Our results show that sympathetic nerves more densely supply mesenteric arteries compared to veins as shown previously (Birch *et al.*, 2008; Park *et al.*, 2007). We also show that $A_{2A}R$ immunoreactivity co-localization with TH a marker for sympathetic nerves in arteries and veins. We also detected some non-neuronal $A_{2A}R$ immunoreactivity. This is not surprising as $A_{2A}R$ couple to vasodilation in mesenteric artery (Hiley *et al.*, 1995) and coronary artery (Belardinelli *et al.*, 1998; Glover *et al.*, 1996), pial artery(Shin *et al.*, 2000) and renal artery (Hansen *et al.*, 2003; Rump *et al.*, 1987). Hence, the non-neuronal $A_{2A}R$ immunoreactivity is likely to be arterial smooth muscle cells. Conversely, there was far less non-neuronal $A_{2A}R$ immunoreactivity in mesenteric veins. We have shown previously that adenosine (Sangsiri *et al.*, 2012) constricts mesenteric veins, but not arteries suggesting that A_1R is more prominent in controlling venomotor tone directly.

4.5.2 Analysis of NE oxidation current kinetics.

The rise and fall of NE oxidation currents reflect NE release and clearance respectively. During the rising phase, a short train of focal stimulation causes NE release, which accumulates near the electrode producing the peak current (I_{max}). The rising phase consists of 2 components. Phase1 is rapid and is due to NE release from

multiple varicosities. Phase2 is slower and is influenced by exocytosis and the activation of α_2-adrenergeic and A_1-adenosine autoreceptors as NE accumulates in the junction causing a decline in the release rate (Park *et al.*, 2010). The decay phase is controlled largely by neuronal reuptake via NET, and diffusion away from release sites and the microelectrode. Diffusion is constant across different experimental conditions due to the constant solution flow rate and fixed microelectrode position. Thus, the current decay under different conditions reflects changes in NE clearance. Changes in the *β*-ratio could be due to changes in the rise and/or decay of the NE oxidation. However, none of the drugs used in this study altered the rising phase of NE current in mesenteric veins. Thus, changes in the *β*-ratio represent changes in NE clearance by NET.

4.5.3 Activation of A_{2A} receptors enhances NE clearance in perivenous, but not periarterial sympathetic plexus.

Recent studies have reported a novel mechanism of $A_{2A}R$ in modulating GABA and glutamate uptake via and action on GABA and glutamate transporters, respectively (Cristóvão-Ferreira *et al.*, 2011; 2009; Gonzalez *et al.*, 2006), (Matos *et al.*, 2012). In the present study, we have revealed a novelty of $A_{2A}Rs$ modulation of NE transporters in peripheral nervous system.

Activation of the prejunctional $A_{2A}R$ using CGS2168 enhances NE clearance. This effect was blocked by cocaine and the $A_{2A}R$ antagonist, SCH58261. This suggests that enhancement of NE transport is mediated by $A_{2A}R$ activation and that NET is

required. This mechanism may considered as a cross regulation of release of clearance by co-released neurotransmitters (NE and ATP) via their respective autoreceptors.

$A_{2A}R$ enhanced NE clearance is more prominent in perivenous compared to periarterial sympathetic nerves. Artery-vein differences in the arrangement of sympathetic nerves could partly explain this difference in clearance. The sympathetic nerve plexus is denser in mesenteric arteries compared to veins. Periarterial nerves could clear NE more efficiently than perivenous nerves because NET expressed by neighboring or varicosities near release sites could contribute to NE clearance. This is analogous to "lateral inhibition" mediated by α_2AR autoreceptors (Brock, 1995). Lateral inhibition does not occur in perivenous nerves since their varicosities are too far from one another to detect NE released from adjacent varicosities or nerve fibers. This would explain the slow basal NE clearance in mesenteric veins. Secondly, the periarterial plexus resides at the adventitial and media border while the perivenous plexus spreads deeper into muscle layer (Birch *et al.*, 2008). Thus, neurogenic venous contraction occurs more rapidly than arterial contraction because of the closer proximity of varicosities to the muscle cells based on nerve geography (Park *et al.*, 2007). Third, veins are more sensitive to NE-mediated contraction than arteries (Pérez-Rivera *et al.*, 2004). Therefore, small increases in NE availability would constrict veins and reduce capacitance. Finally, prolonged α_1-adrenergic receptor activation causes desensitization in mesenteric veins, but not arteries (Pérez-Rivera *et al.*, 2004; Xu *et al.*, 2007), indicating a need for regulation of NE availability at the neuroeffector junction. Thus, although veins do not make efficient use of the $A_{2A}R$ autoreceptor to regulate NE

availability they may use regulation of NE clearance to buffer junctional NE. This mechanism may permit veins to re

One may question about the source of adenosine that modulates basal perivenous sympathetic transmission. Is it from neuronal or non-neuronal sites? An α_1-adrenergic receptor blocker, prazosin, completely blocked neurogenic constriction in veins, but only partly reduces arterial contraction (Park *et al.*, 2007). In addition, purine receptor blockers do not affect neurogenic constrictions in veins while markedly inhibiting arterial constrictions (Park *et al.*, 2007). These results support the idea that NE is the major transmitter released from perivenous sympathetic nerves. It has been argued that although $P2X_1$ receptor agonist, α, *β*-methylene ATP failed to contract mesenteric vein, ATP and UTP, an agonist for $P2Y_2$/$P2Y_4$ receptors could induce venous contraction, and this effect were prevented by sumarin a P2Y2 receptor antagonist (Galligan *et al.*, 2001). Moreover, Bobalova et al detected electrically-evoked ATP release from canine and guinea-pig mesenteric veins. It should be noted that basal neurogenic ATP release was much lower than basal NE release, particularly in canine mesenteric veins (approximately 10 times lower than NE)(Bobalova *et al.*, 2001a; Bobalova *et al.*, 2001b). Thus, it is possible that ATP is co-released with NE in perivenous nerves and the small amount of ATP released may provide the adenosine needed to modulate adrenergic transmission. Postjunctional P2Y receptors may only be activated when high concentrations of ATP are achieved such as might occur during cell injury (Yin *et al.*, 2007).

Since activation of $A_{2A}R$ requires brief, but high (micromolar) concentrations of adenosine (Olah *et al.*, 1995), enhanced NE clearance by $A_{2A}R$ action may serve as a rescue mechanism to shut down sympathetic nerve activity to reduce oxygen consumption. Adenosine is known as a key regulator of vasomotor tone during hypoxia (Tabrizchi *et al.*, 2001). Unlike previous studies showing that $A_{2A}R$ facilitate NE release in the rat tail artery (Fresco *et al.*, 2004), we did not see facilitation of NE release through $A_{2A}R$ activation in mesenteric arteries or veins. It is possible facilitation of NE release could be masked by A_1R-coupled NE release inhibition since we did not preincubate the tissue with A_1R antagonist. One should keep in mind that although CGS21680 is considered as selective $A_{2A}R$ agonist, it is likely to distinguish $A_{2A}R$ well from $A_{2B}R$ but not from the other subtypes. CGS21680 is 170-fold selective for A_{2A} over A_1R in rat and its selectivity is far lower in humans (Klotz, 2000). A more selective $A_{2A}R$ agonist is needed.

4.5.4 Impaired $A_{2A}R$ function in DOCA-salt hypertension occurs at Gs-protein coupling step.

We studied $A_{2A}R$ function in DOCA-salt mesenteric veins because CGS21680 did not alter arterial NE clearance in sham rats. In DOCA-salt veins, CGS21680 failed to enhance NE clearance suggesting that the prejunctional $A_{2A}R$ function is impaired in

DOCA-salt hypertensive veins. $A_{2A}R$ dysfunction probably occurs at the G_s-stimulated adenylase cyclase step because forskolin could restore NE clearance in DOCA-salt mesenteric veins. Previous work showed impaired NET function in DOCA-salt arteries, but not veins (Dong, 2009), which is consistent with our data showing that NET function is not altered in DOCA-salt veins. Western blot analysis revealed overexpression of NET in mesenteric arteries, veins, and superior cervical and stellate sympathetic ganglia in DOCA-salt rats (Luo *et al.*, 2003). This suggests that NET overexpression in DOCA-salt arteries may occur to compensate for impaired NET function. Upregulation of NET in DOCA-salts veins may result from impaired prejunctional $A_{2A}R$ stimulation of NET activity. Overall, $A_{2A}R$ dysfunction accounts for impaired NE clearance DOCA-salt veins and increased NE availability to act at postjunctional receptors. The present study is the first to show disruption of prejunctional receptors on perivenous sympathetic nerves. Relatively high sensitivity to NE-induced constriction plus elevated NE level due to slow clearance mechanism in DOCA-salt mesenteric veins could contribute to increased venomotor tone and a decrease in vascular capacitance (Fink, 2009).

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CHAPTER 5

GENERAL DISCUSSION,

PERSPECTIVES AND FUTURE DIRECTIONS

5.1 Result Summary

Sympathetic nervous system hyperactivity is associated with salt-sensitive hypertension. However, the mechanism of sympathetic nerve overactivity is not fully understood. From the autoreceptor perspective, it is possible that local regulations of neurotransmitter release and clearance by prejunctional receptors at sympathetic nerve varicosities are compromised (deChamplain *et al.*, 1987; Dong, 2009). This leads to an increase in sympathetic transmitter release and gradual removal of transmitters from the neuroeffector junction causing sustained vascular contraction, thereby an increase in arterial peripheral resistance and a rise in blood pressure. These present studies focus on role of an ATP degradation product adenosine as a neuromodulator in sympathetic neurotransmission of mesenteric circulation. In these studies, DOCA-salt hypertensive rats were used, as this model is a low renin form of salt-sensitive hypertension with an increase in sympathetic nerve activity. Video camera and continuous amperometry with carbon fiber microelectrode were used to detect vasoconstriction and real-time NE release from sympathetic nerve varicosities in response to brief trains of electrical nerve stimulation. The overall finding of these present studies is that DOCA-salt hypertension is associated with disruption of prejunctional adenosine receptor function. Decreased activity of prejunctional adenosine A_1 receptors coupled to inhibition of NE release leads to increased NE release from sympathetic varicosities. Additionally, dysfunction of prejunctional adenosine A_{2A} receptors linked to increasing NE transporter activity results in increased NE availability at the neuroeffector junction to act on the postjunctional

receptors. These findings indicate a fine-tuning cross-regulation between purinergic and adrenergic neurotransmission and reveal a local dysregulation of sympathetic transmission at the neuroeffector junction, which occurs independently from the central nervous system. The key findings of these present studies include:

5.1.1. Prejunctional adenosine A_1 receptors (A_1Rs) inhibits NE release from perivascular sympathetic nerves and impaired A_1R function contributes to increased NE release in DOCA-salt hypertension. This finding is consistent with previous published studies reporting that A_1Rs couple to inhibition of NE release from sympathetic nerves supplying several vascular beds including rat tail arteries (Diniz *et al.*, 2004) , rat caudal arteries (Shinozuka *et al.*, 1988), and human forearm vessels (Rongen *et al.*, 1996). In the present study, it is found that activation of the prejunctional A_1Rs located on periarterial and perivenous sympathetic nerves inhibits NE release from sympathetic nerve varicosities supplying mesenteric arteries and veins. The function of the prejunctional A_1Rs is predominant in periarterial nerves under normal physiologic condition. Previous work has shown that the prejunctional α_2-adrenergic autoreceptor function is impaired in DOCA-salt hypertension (deChamplain *et al.*, 1987). My data show that this effect is not specific for the α_2-adrenergic autoreceptor as A_1 autoreceptor function is also impaired. Similar to the prejunctional α_2-adrenergic

receptors, A_1R function is impaired specifically in periarterial, but the receptor function is preserved in perivenous sympathetic nerves.

5.1.2. Adenosine A_{2A} receptor ($A_{2A}R$) stimulation enhances sympathetic NE clearance and this response is impaired in DOCA-salt hypertension. Herein, the novel mechanism of $A_{2A}Rs$ in modulating NE uptake via NE transporter in peripheral nervous system is revealed. $A_{2A}Rs$ are part of a local mechanism to regulate NE clearance from the neurovascular junction. Unlike A_1R function, which is predominant in periarterial nerves, the $A_{2A}R$ activation enhances NE clearance occurring prominently in perivenous nerves. This mechanism provides such a precise regulation of NE availability to perivenous sympathetic neuroeffector junction, where low efficiency of NE clearance occurs. This mechanism also permits rapid removal of α_1-adrenergic receptor desensitization in response to NE providing efficient dynamics of venomotor tone to maintain normal blood pressure during postural hypotension. Prejunctional $A_{2A}R$ function is impaired in DOCA-salt hypertension and impairment probably occurs at the G_s-protein coupling step as forskolin (an adenylyl cyclase activator) restored NE clearance in DOCA-salt veins. This work is the first showing the disruption of prejunctional receptors on perivenous sympathetic nerves.

5.2 General Discussion

5.2.1 Why perivascular sympathetic nerves contain and release more than one transmitter?

Firstly, co-stored transmitters may be a simple way to balance ions within synaptic vesicles. The major transmitter NE with positively charged amine might be neutralized by negatively charged ATP and/or NPY molecules. Secondly, the pattern of sympathetic nerve firing can vary. In some circumstances, the physiological function of an organ might require short burst of high frequency that release certain transmitters, while during other periods it might require a slow tonic release of another transmitter. For instance, sympathetic nerves innervating the vas deferens contain NE, ATP, and NPY as cotransmitters. Under low frequency nerve stimulation, NE and ATP, but not NPY are released. NPY is likely to be released during intense stimulation (Kennedy *et al.*, 1997) to produce strong contraction and enhance action of NE (Ekblad *et al.*, 1984). Thirdly, release of multiple transmitters may provide synergism and flexibility of postjunctional responses. For example, NE and ATP are co-released by perivascular sympathetic nerves onto the postjunctional α_1 adrenergic and P2 receptors, respectively to synergistically stimulate contraction. In arteries, stimulation of the postjunctional P2X receptors by ATP elicits rapid arterial contraction following by sustained contraction mediated by the action of NE on the metabotropic α_1 adrenoceptors. Moreover, NE action on the α_1 adrenoceptors is likely to desensitize quickly in mesenteric veins. Conversely, adenosine, an ATP degradation product, provides sustained venous contraction

through activation of A_1 adenosine receptors (see chapter3). This indicates a rescue mechanism from receptor desensitization by using multiple transmitters to activate different effector pathways. Moreover, while neuronal ATP acts on P2 receptors, NE-induced activation of *α*1 adrenoceptors also causes the release of ATP from vascular smooth muscle cells to potentiate the contraction indicating flexibility to produce neurogenic contraction (Billaud *et al.*, 2011). Lastly, co-transmitters modulate release of one another to provide precise control of neurotransmission. For example, NE provides the autoinhibition to inhibit further NE release, NE also inhibits ATP release (Demel *et al.*, 2008a). Likewise, adenosine, an ATP metabolite, interacts with the prejunctional A_1 adenosine receptor to inhibit NE (Sangsiri *et al.*, 2010) and ATP release (Demel *et al.*, 2008a). Prejunctional adenosine receptors may serve as a spare mechanism to control major transmitter release under pathologic conditions (hypertension, for example). One should keep in mind that the co-transmitters released by perivascular sympathetic nerves are not fixed and could be altered by aging and pathologic diseases (Burnstock, 1990). Periarterial sympathetic nerves in DOCA-salt hypertension have decreased purinergic transmission due to ATP depletion (Demel *et al.*, 2008b). Conversely, adrenergic transmission is likely to be enhanced, as tyrosine hydroxylase is upregulated (Grobecker *et al.*, 1976) and increased plasma NE is detected (Bouvier *et al.*, 1986). Furthermore, NE and NPY bioavailability is increased, but purinergic transmission is decreased in

perivascular sympathetic nerves of spontaneously hypertensive rats (SHR) (Burnstock, 1990) .

5.2.2 Is G-protein coupling a key pathological target?

As my work suggests the impaired function of the presynaptic A_1R, and $A_{2A}R$, likely to occurs at the receptor-G-protein coupling step. This work supports the hypothesis that failure of the receptor-G-protein interaction may account for the impaired negative modulation of NE release provided by the prejunctional receptors on perivascular sympathetic nerves.

Autoinhibitory feedback is a general mechanism for control of neurotransmitter release throughout the nervous system. The basic mechanism of α_2-AR inhibition is based on reduced opening probability of Ca^{2+} channel by the inhibitory $\beta\gamma$ subunit of G_O-protein leading to decreased Ca^{2+} currents and inhibition of exocytosis. Lipscombe and co workers showed that NE-induced α_2-AR activation strongly inhibited the N-type, but not L-type Ca^{2+}channels in frog sympathetic ganglia (Lipscombe *et al.*, 1989). Likewise, Yawo and Chuhma showed that adenosine inhibited ω-conotoxin-sensitive Ca^{2+} channels (Yawo *et al.*, 1993) suggesting that the N-type Ca^{2+}channels are the target for adenosine receptor-mediated autoinhibition as ω-conotoxin is a selective inhibitor of the N-type Ca^{2+} channels. It should be noted that the concentration of ω-conotoxin in

their study was 10 μM which if far above the concentration that is selective for N-type channels (Yawo *et al.*, 1993). Demel showed that N-type Ca^{2+}channel function is preserved in DOCA-salt hypertensive rats (Demel, 2008). Thus, it is logical to imply that autoinhibitory feedback failure of the α_2-ARs and A_1Rs found in DOCA-salt arteries probably occurs somewhere upstream the activation of Ca^{2+}channels which could be either extracellularly at agonist-receptor binding step or intracellularly at the receptor-G protein interaction step. Fardoun and coworkers have reported that reactive oxygen species (ROS) interfere with the coupling of dopamine D_1 receptors and the target G-protein by causing an increase in the G protein receptor kinase (GRK) level in old Fischer 344 rats (Fardoun *et al.*, 2006). GPCRs are substrates for GRK phosphorylation, which promotes receptor downregulation via a *β*-arrestin-dependent pathway. It is possible that changes in GRK level or activity would alter the activity of the GPCRs and their coupling activity with G-proteins. Growing evidence suggests that increased GRK level is involved with impaired receptor-G protein coupling resulting in reduced adenylyl cyclase activation (Gros *et al.*, 1997). GRK2 is highly expressed in immune cells and its expression is affected by oxidative stress (Lombardi *et al.*, 2002). The role of GRK2 in phosphorylation of *β*-adrenergic receptors, which link to Gs/ adenylyl cyclase/ cAMP pathway under normal and hypertensive conditions has been studied widely. However, the study of the GRK mediated phosphorylation of the α_2-adrenergic, A_1, and A_{2A} adenosine receptors in hypertension has not been undertaken. Fortunately, the

process of A_1R desensitization has been studied intensively and it has been shown that GRK2 is also responsible for A_1R desensitization, where the GRK2 phosphorylation site is located in the third intracellular loop (Nie *et al.*, 1997) on serine and threonine residues (Jajoo *et al.*, 2010) and at the threonine residue on the C-terminus (Palmer *et al.*, 1996). $A_{2A}R$/Gs couple receptor desensitization is also mediated by GRK2 phosphorylation, and tumor necrosis factor (TNF)-*α* prevented $A_{2A}R$ desensitization and this TNF-*α* induced-effect occurs similarly in *β*-adrenergic receptors. TNF-*α* prevents receptor desensitization by blocking translocation of GRK2 to the phosphorylation site of the receptor (Khoa *et al.*, 2006). This phenomenon may account for the selective impairment of the function of prejunctional G_i-, but not G_s-coupled receptors in DOCA-salt arteries.

Interestingly, GRK4 is associated with salt-sensitive hypertension in humans and in animal models (Gros *et al.*, 1997; Gros *et al.*, 2000; Zeng *et al.*, 2008). Increased levels of GRK lead to hyperphosphorylation of the GPCR allowing *β*-arrestin binding, which in turn prevents the G-protein interaction with the receptor. Differential increases of GRK protein subtypes may account for differential disruption of receptor-G protein coupling as observed in arteries versus veins.

Moreover, it has been reported that ROS induced a rapid transient increase of intracellular Ca^{2+} ($[Ca^{2+}]_{in}$) in various cell types including smooth muscle cells. The rapid increased $[Ca^{2+}]_{in}$ is caused by ER store leakage as

ROS inhibit the ATP-dependent Ca^{2+} pump (SERCA) on the ER membrane (Thannickal *et al.*, 2000). Thus, the cells could not maintain a low concentration (10-100 nM) of free $[Ca^{2+}]_{in.}$ The rapid increase of $[Ca^{2+}]_{in}$ is followed by a slow increased $[Ca^{2+}]_{in}$ mostly derived from extracellular site (Thannickal *et al.*, 2000). This mechanism could partly account for a slightly depolarized resting membrane potential of DOCA-salt arterial smooth muscle cells compared to sham (Demel, 2008) .

5.3 Therapeutic significance by modulation of perivascular sympathetic transmission

These results suggest that sympathetic nerve overactivity in salt-sensitive hypertension is partly independent from the central control.

The physiological relevance of perivascular sympathetic transmission has been studied by several groups (Brock *et al.*, 1997; Bucher *et al.*, 1992; Donoso *et al.*, 1997; Ekblad *et al.*, 1984; Nilsson *et al.*, 1985; Ralevic, 1995; Shinozuka *et al.*, 1988; Stjarne, 1989; Westfall *et al.*, 1995). However, the pharmacological differences between arteries and veins, and the possible pre-and postjunctional interactions between drugs and the different neurotransmitter system at arterial *vs* venous nerve varicosities have not been widely studied. For example, one drug may exert pharmacological action differently by periarterial *versus* perivenous nerves. In this case, cocaine (NE transporter blocker) and CGS21680 ($A_{2A}R$ agonist) provide a good illustration. Cocaine

Alternately, how can a drug that prevents one co-transmitter's postjunctional effect also abolish the contraction effect mediated by the other co-transmitters? Specifically, why would the pharmacological action of prazosin significantly lower blood pressure *in vivo*, while PPAD ($P2X_1$ receptor antagonist) does not (Demel, 2008)? Prazosin strongly inhibits vascular contraction by two major mechanisms. First, prazosin blocks the α_1 adrenergic receptors (α_1AR) so that NE is no longer produce vascular contraction. Second, the postjunctional α_1AR blockade prevents a large amount of ATP release from vascular smooth

muscle cells through pannexin1 channels. Conversely, PPAD failed to lower blood pressure *in vivo* as PPAD only blocks the P2X receptors, but the cotransmitter NE still activates α_1AR and produce arterial contraction.

Moreover, how would a drug exert multiple effects on the same transmitter release system? Specifically, low concentrations of dipyridamole (adenosine transporter blocker) cause a decreased synaptic concentration of NE. Hence, the NE-dependent postjunctional effects would be decreased. Alternatively, high concentrations of dipyridamole produce an increased concentration of NE in the neurovascular junction, which would increase NE-dependent postjunctional effects including the non-neuronal release of ATP.

The evidence above indicates that differential nerve organization between arteries and veins, and the complex interaction between cotransmitters activating multiple pre- and postjunctional mechanisms certainly make pharmacological investigation more intricate. This complexity provides wider opportunities for more precise modification of the neurotransmission process, which could ultimately constitute a potential therapeutic advantage. As the alterations in the prejunctional adenosine receptor function in DOCA-salt hypertension occurs, drugs selective for presynaptic adenosine receptors may have some usefulness in the treatment of salt-sensitive hypertension. Currently, it is unclear whether the prejunctional adenosine receptor dysfunction is involved in human salt-sensitive hypertension

5.4 Future directions

5.4.1 Measurement of ATP release by excitatory junction potential (EJP) recording

As ATP is co-released with NE from perivascular sympathetic varicosities, it is important to further investigate how purinergic transmission is regulated. In arteries, ATP acts on the $P2X_1$ receptors on smooth muscle cells. The P2X1 receptors are ion channels permeable to cations. The influx of cations in response to $P2X_1$ stimulation causes membrane depolarization and vascular contraction. To record EJPs one applies submaximal electrical nerve stimulation to avoid contraction and loss of the microelectrode impalement during vascular contraction. EJP recordings have some limitations. The EJP is not a direct measurement of ATP release, but it measures membrane depolarization-induced by ATP action on the P2X ion channels. In addition, EJPs do not occur in mesenteric veins, as the majority of the postjunctional ATP receptors in the veins are the P2Y G-protein coupled receptors. Demel has revealed decreased ATP bioavailability and EJP rundown in DOCA-salt arteries. She also proposed that impaired purinergic transmission of DOCA-salt rats is not due to α_2-adrenergic and A_1-adenosine autoreceptors as exogenous NE, UK14304 (α_2 agonist), and CPA (A_1R agonist) produce EJP inhibition equally in both sham and DOCA-salt arteries (Demel *et al.*, 2008a). It is possible that the neuronal ATP release-induced EJP results might be masked by the non-neuronal ATP release. Kügelgen and Starke proposed that approximately one third of stimulation-evoke

ATP release is from sympathetic nerves, and the other two third of released ATP is from smooth muscle cells (guinea pig vas deferens), where ATP is released by neurogenic NE-stimulated α_1 adrenoceptors (Kügelgen *et al.*, 1991). As mentioned earlier, pannexin1 channels are crucial in facilitating intracellular ATP release in response to the postjunctional α_{1D} receptor activation (see chapter 1). NE is also co-released during the nerve stimulation and acts on α_{1D} receptors. Thus, ATP from neuronal and non-neuronal sources could produce the EJPs. Sympathetic NE: ATP release ratio is approximately 7:1 indicating that neuronal ATP release is quite small compared to NE (Lagercrantz, 1976). PPAD (P2X1 antagonist), dramatically inhibit neurogenic contraction in mesenteric artery indicating that ATP is a major vasoconstrictor in arteries. Thus, it is possible that ATP-induced contraction is mainly from non-neuronal site. The large amount of non-neuronal ATP may mask the neuronal ATP-mediated EJP and conceal the difference between DOCA-salt and sham responses to NE, and CPA in modulating purinergic neurotransmission. However, this could be argued that the time course of non-neuronal ATP release may be too slow to produce EJP and the direct effect of pannexin channel blockade on EJP amplitude has not been evaluated.

5.4.2 Measurement of endogenous adenosine

To provide insight into how adenosine modulates sympathetic neurotransmission, direct measurement of adenosine concentration is needed. Recently, amperometry with an enzymatic-based biosensor has been used to

measure real-time ATP release and its degradation product (adenosine) in several brain regions. Unfortunately, my attempt to measure adenosine concentration from sympathetic nerves supplying mesenteric arteries by using a commercial adenosine biosensor (Sarissa Biomedical™, Conventry, UK) failed. The failure to detect adenosine by using the commercial biosensors might related to the small amount of ATP release from the peripheral sympathetic nerves compared to the amount of ATP released in the CNS (Frenguelli *et al.*, 2003; Gourine *et al.*, 2008; Llaudet *et al.*, 2005). Thus, small amounts of the ATP degradation product, adenosine, from a mesenteric artery may be below the sensitivity threshold of the biosensor. It should be noted that the amplitude of adenosine concentration detected in the brain slices during hypoxia was high up to 30 μM (Frenguelli *et al.*, 2003). Further development of an adenosine biosensor with high sensitivity is needed. Cechova and Venton have introduced fast-scan cyclic voltametry with carbon fiber microelectrodes to detect endogenous adenosine and dopamine simultaneously from rat cuadate-putamen. As adenosine and dopamine have clearly different oxidation potentials (1.5 *vs* 0.6 Volts, respectively (Cechova *et al.*, 2010)), it permits one to detect two-transmitters released simultaneously (Cechova *et al.*, 2008). This study broadens the opportunities to detect adenosine or even simultaneous multi-detection of NE and ATP and/or adenosine by using amperometry or fast scans cyclic voltametry. Combination of amperometry with carbon fiber electrodes at the potential 1.5 Volts could permit adenosine to be detectable as an oxidation current. Additionally, fast scan cyclic voltametry with potential ranging for NE and ATP/or

adenosine oxidation would allow one to measure multiple transmitters simultaneously. However, my major concern would be about the small proportion of ATP/adenosine release compared to NE (see chapter1) might be below the detection threshold.

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