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dissertation entitled

ROLE OF NADPH OXIDASE IN PERIPHERAL SYMPATHETIC AND SENSORY NEURONS IN HYPERTENSION

presented by

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has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Neuroscience

Major Professor's Signature

August 20, 2008

Date

ROLE OF NADPH OXIDASE IN PERIPHERAL SYMPATHETIC AND SENSORY NEURONS IN HYPERTENSION

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Neuroscience

2008

ABSTRACT

ROLE OF NADPH OXIDASE IN PERIPHERAL SYMPATHETIC AND SENSORY NEURONS IN HYPERTENSION

By

Xian Cao

The peripheral sympathetic division of the autonomic nervous system and the primary sensory nervous system modulate the splanchnic circulation. Abnormalities of these neurons, such as neurotransmitter mishandling, may contribute to neurogenic hypertension. NADPH oxidase is a superoxide anion ($O_2^{-\bullet}$)-producing enzyme that contributes to elevated vascular reactive oxygen species (ROS) production in hypertension. Its enzymatic activity has also been detected in prevertebral sympathetic ganglia. However, the role of NADPH oxidase in peripheral neurons in hypertension is not clear. This thesis is aimed to 1) localize NADPH oxidase in peripheral sympathetic and sensory ganglion neurons; 2) investigate the regulation of NADPH oxidase in sympathetic and sensory neurons in deoxycorticosterone acetate (DOCA)-salt hypertension; 3) examine the functional effects of ROS in sympathetic neurons by measuring norepinephrine transporter (NET) expression in response to NADPH oxidase-derived $O_2^{-\bullet}$ induced by endothelin-1 (ET-1).

Localization of NADPH oxidase. NADPH oxidase subunits are present in prevertebral sympathetic ganglia and sensory dorsal root ganglia (DRG). The NADPH oxidase protein expression was localized to not only the neuronal cell

bodies in the ganglia but also the perivascular nerve fibers originating from these neurons. The localization of NADPH oxidase to the two distinct functional compartments of peripheral neurons indicates that ROS may modulate neuronal properties via multiple discrete mechanims in the same neurons.

Regulation of NADPH oxidase. In DOCA-salt hypertension, the NADPH oxidase in sympathetic ganglia was found to be regulated differently compared to sensory ganglia. NADPH oxidase activity and expression were increased in sympathetic ganglia in hypertension but decreased in hypertensive DRG. The opposing regulation of NADPH oxidase in sympathetic and sensory neurons may have an impact on their innervation of the vasculature in hypertension.

Function of NADPH oxidase in sympathetic neurons. ET-1 increased $O_2^{-\cdot}$ production in PC12 cells, while also inducing a transient decrease in NET mRNA expression. Neither an induction of $O_2^{-\cdot}$ generation nor a downregulation of NET by ET-1 was observed in $p22^{phox}$ knockdown PC12 cells. These results indicate that NADPH oxidase derived ROS production in sympathetic neurons may modulate gene expression of the proteins that are critically involved in catecholamine handling, and thus contribute to hypertension.

In conclusion, NADPH oxidase is present in peripheral sympathetic and sensory ganglion neurons and nerve fibers. The enzyme is differently regulated in these two ganglia in DOCA-salt hypertension. NADPH oxidase derived-ROS decrease NET mRNA expression, which may contribute to perturbed sympathetic vascular innervation in hypertension. These findings may shed light on novel roles of NADPH oxidase and ROS in the peripheral neurons relating to the pathogenesis of hypertension.

DEDICATION

To my parents Lijiang Cao, Chunmei Yang and my husband Xuerui Yang

ACKNOWLEDGEMENTS

The year of 2003 turns out to be the most important year in my career life. After taking an overnight greyhound trip, with the passion of becoming a research scientist, I arrived at Michigan State University on a snowy day and knocked on Dr. David L. Kreulen's office door. It was then in August of 2003, I came back to Michigan State University as an incoming graduate student in Neuroscience Program and kicked off my science journey in Dr. Kreulen's lab. I have always been grateful to myself for what I chose to do in 2003. I feel more grateful to Dr. Kreulen, who became my research mentor and accompanied me with all the patience and kindness in this wonderful and painful world. Five years later now, I am sitting here finishing my doctoral disstertation. Sometimes I still cannot believe that I am truly becoming a PhD scientist. I cannot help thinking back of that girl in the greyhound bus. Thanks to Dr. Kreulen, the dream does come true. Thank you for being such a great advisor not only in science but also in life! Thanks for feeling my growing pain when I was frustrated! Thanks for sharing my happiness when I succeeded! I may not be your greatest student, but you will always be my greatest advisor!

In addition to Dr. Kreulen, I would also like to thank Dr. James Galligan, Dr. Greg Fink, Dr. Hongbing Wang and Dr. Lorraine Sordillo who served on my dissertation committee. Thank you for your valuable time and effort in helping with my dissertation project! Special thanks to Dr. Sordillo who kindly taught me the technique of RNA interference and patiently guided me throughout the project optimization and troubleshooting.

Many thanks to Neuroscience program director Dr. Cheryl Sisk. Thanks for including me in the program and being so supportive for my graduate study in the last five years. This is such a wonderful family. I am very proud of being a Neuroscience Alumni.

Friendship has always been one most important part in my life. I feel extremely lucky to be able to work with the crew of Dr. Kreulen's lab. Not only we work together, but also we make the best friends with each other. Erica Wehrwein, who recently became Dr. Wehrwein, was my deskmate in the lab for five years. Thanks for being such a great friend! I will always remember the days we spent together in the lab, in the dancing class, in the football game, in the coffee shop and in the Thai restaurant. I would also like to thank Xiaohong Wang, who came to the lab at the same time with me. It has truly been a great pleasure to work with you. There is much camaraderie among other lab members as well: Lindsay Parker, Anna Wright, Mohammad Esfahanian, Carmen Affonso, Ian Behr, Arun Nagaraju, Josh Mastenbrook, and Amy Albin.

Michigan State University and the last five years mean a lot to me not only because this is the place I get my PhD, this is also the place that I met my husband Xuerui Yang. You are the greatest thing that happened to me. You made my life colorful ever since we met. Thank you for being there for me!

Last but not the least, I would like to thank my parents Lijiang Cao and Chunmei Yang. Thanks for giving me endless unconditional love throughout the years! Countless times when I was crying over the phone, you told me to be strong; times when I was cheering for my success, you told me to be humble; times when I felt lonely and hopeless, you assured me that I am never alone in

the world because you are always there with me. You didnot teach me science, but you taught me the meaning and the beauty of life! Thank you!

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KEY TO SYMBOLS AND ABBREVIATIONS

Ang II	angiotensin II
ATCC	american type culture collection
BP	blood pressure
BLAST	basic local alignment and search tool
CBB	Coomassie brilliant blue
cDNA	complementary DNA
CG	celiac ganglia
CGRP	calcitonin gene related peptide
Cl	chloride
Ct	cycle threshold
Ctl	control
DEPC	diethylpyrocarbonate
DHE	dihydroethidium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DOCA	deoxycorticosterone acetate
DRG	dorsal root ganglia
EDTA	ethylenediaminetetraacetic acid
ET-1	endothelin-1
GAPDH	glyceraldehyde phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl) piperazine-1 ethanesulfonic acid
HRP	horseradish peroxidase

HT	hypertensive
IgG	immunoglobin G
IMG	inferior mesenteric ganglion
IP	immunoprecipitation
IR	immunoreactivity
Kd	kilo Dalton
KO	knockout mouse
KRH	Krebs-Ringer-Hepes
MA	mesenteric artery
mRNA	messenger ribonucleic acid
MA	Mesenteric artery
MV	mesenteric vein
Na	sodium
NADPH	reduced nicotinamide-adenine dinucleotide phosphate
NaCl	sodium chloride
NCBI	national center for biotechnology information
NE	norepinephrine
NET	norepinephrine transporter
NGF	nerve growth factor
no RT	no reverse transcriptase control
NOX	non-phagocytic oxidase
NPY	neuropeptide Y
NT	normotensive

NTC	no template control
$O_2^{-\bullet}$	superoxide anion
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC12	rat pheochromocytoma cell line
PCR	polymerase chain reaction
PKC	protein kinase C
PMSF	phenol methane sulfanyl fluoride
PVDF	polyvinylidene Fluoride
qPCR	quantitative real time polymerase chain reaction
Rac	small ras related G protein
REST	relative expression software tool
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
S6c	sarafotoxin 6c
SDS	sodium dodecyl sulfate
SHR	spontaneously hypertensive rat
TAE buffer	tris-glacial acetic acid-EDTA buffer
TEMED	tetramethylethylenediamine
TH	tyrosine hydroxylase
VMAT2	vesiscular monoamine transporter 2
WT	wild type

CHAPTER 1: INTRODUCTION

Approximately 65 million American adults are classified as hypertensive. Hypertension is known to be a risk factor for a variety of cardiovascular diseases, including atherosclerosis, heart failure and stroke, and accounts for 6% of adult deaths worldwide.

The neuronal regulation of splanchnic circulation is crucial in determining systemic blood pressure and therefore is important in hypertension development. The splanchnic circulation is composed of gastric, small intestinal, colonic, pancreatic, hepatic and splenic circulation (11). It stores 38% of total blood, of which up to 64% can be mobilized by the direct stimulation of sympathetic nerves (7). The splanchnic circulation is innervated by both the sympathetic division of autonomic nervous system (prevertebral sympathetic ganglion neurons, including celiac ganglia (CG), superior and inferior mesenteric ganglia (IMG)) and by the spinal sensory nerves (dorsal root ganglia neurons (DRG)).

Elevated sympathetic nervous system activation is one of the key pathophysiological changes observed in hypertension (5). It is characterized by diminished norepinephrine transporter (NET) reuptake in sympathetic neurons and elevation of plasma norepinephrine (NE) (6; 13). On the other hand,

alterations in the neuronal properties of peripheral sensory neurons that innervate the splanchnic circulation were also found to be significant in some types of hypertension (8; 16; 17). Although the exact mechanisms of how these changes in sympathetic or sensory neurons contribute to hypertension are not clear, a possible role of reactive oxygen species (ROS) was suggested in both scenarios (2) (15).

Increased generation of ROS is associate with many forms of hypertension (14), including deoxycorticosterone acetate (DOCA)-salt hypertension (1), in which endothelin-1 (ET-1) contributes to the pathogenesis of hypertension secondary to a low-renin state (10; 12). Among several enzyme systems that catalyze ROS production, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is believed to be the predominant source of superoxide ($O_2^{-\bullet}$), the precursor of all other ROS, in hypertension (9). ROS play essential roles in central nervous system -mediated regulation of cardiovascular function (18). In the peripheral nervous system, $O_2^{-\bullet}$ levels are elevated in CG and IMG of DOCA-salt hypertensive rats compared with normotensive rats and this $O_2^{-\bullet}$ is produced by NADPH oxidase (3) (4). However, the mechanisms underlying the elevation of NADPH oxidase-derived $O_2^{-\bullet}$ and the physiological consequences of increased ROS in peripheral neurons are not fully understood.

The goal of this study is to 1) examine the expression and the regulation of NADPH oxidase in peripheral sympathetic and sensory neurons in DOCA-salt

hypertension; 2) evaluate the functional effects of increased ROS in sympathetic neurons by measuring NET regulation induced by ROS.

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CHAPTER 2: LITERATURE REVIEW AND RATIONALE

Essential Hypertension

Hypertension, or high blood pressure (BP), generally means systolic BP consistently over 140 mmHg and/or diastolic BP over 90 mmHg. Essential, primary, or idiopathic hypertension is defined as high BP in which secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes of secondary hypertension or mendelian forms (monogenic) are not present. Essential hypertension accounts for 95% of all cases of hypertension (6). It is a common and powerful independent predisposing factor for development of coronary heart disease, stroke, peripheral arterial disease, and heart failure. Essential hypertension is heterogeneous and likely has many related contributing factors including, but not limited to, elevated sympathetic nervous system activity (49); altered properties of sensory innervation (20; 21); increased reactive oxygen species (ROS) production (31); and enhanced circulating endothelin-1 (ET-1) levels (48).

Elevated Sympathetic Nervous System Activation in Hypertension

Sympathetic nervous system activation has been implicated in both human hypertensive patients and hypertension animal models (40). The important role of peripheral sympathetic nervous system in hypertension is indicated by the fact that the arterial BP in DOCA-salt hypertensive animals can be decreased by ganglionic blockade with hexamethonium to a greater level than that in normotensive animals (18). Generally used as a measurement of sympathetic nerve activity, plasma norepinephrine (NE) levels were shown to be elevated in hypertensive individuals (17; 22). This indicates that altered NE handling in the peripheral sympathetic neurons may be attributed to faulty BP regulation. Mechanisms for the increased spillover of NE include sympathetic hyperinnervation, epinephrine cotransmission, increased nerve firing rates, and dysfunction of NE reuptake via neuronal NE transporter (NET) (16).

NET is a plasma membrane protein that belongs to the large gene family of Na+/Cl- dependent neurotransmitter transporters. Transporters in this family are responsible for clearance of NE, dopamine, and serotonin from the neuroeffector junction (57). NET is present in prevertebral sympathetic ganglion neurons and nerve terminals (35). NET is also localized to sensory neurons in dorsal root ganglia (DRG) (30) although its function in sensory neurons is unclear.

NET-mediated reuptake of NE increases when elevated nerve firing releases

more NE into the synapse (15). However, if NET function does not proportionally increase with NE release, excess NE would accumulate in the neuroeffector junction, sustain the constrictive stimulation on the blood vessels and spillover into the circulation (13). NET deficient mice showed a significant higher BP than control animals (28), which further indicates the role of NET in hypertension. However, the molecular mechanisms underlying the regulation of NET in hypertension are poorly understood. It is expected that alterations in the function of NET could be a result of one or several changes, including changes in the amount of NET inserted into the plasma membrane, changes in the transport affinity or capacity, and alterations in NET gene/protein expression and regulation. Interestingly, ROS displayed inhibitory effects on both NET function and expression *in vitro* (41). The purposed of this thesis is to examine whether NADPH oxidase-derived ROS modulates NET expression and function in peripheral neurons.

Sensory Neurons in Hypertension --- More than Just Baroreflex

The role of sensory neurons in BP regulation is best recognized in the baroreflex response. The baroreceptor reflex is a key regulator of BP; baroreceptors in the carotid sinuses and aortic arch detect changes in BP and trigger reflex circulatory adjustments that buffer or oppose the change in pressure.

The central initiation of this reflex begins with baroreceptive afferent release of glutamate to activate second-order neurons in the nucleus tractus solitarii. The reflex circus then modulates the sympathetic outflow and thus regulates BP. It is suggested that essential hypertension is accompanied by modification of the arterial baroreceptor reflex, that is, a resetting of the range of action of the reflex toward higher blood pressure value (23).

In fact, the abdominal circulation is sensitive to baroreceptor activation (24). However, not all sensory mediated reflexes on blood volume regulation are through baroreceptors. For example, intestinal distention can evoke an inhibitory junction potential (IJP) in the mesenteric blood vessels and this IJP mediates vasodilatation (42; 43). This peripheral reflex is mediated solely outside of the CNS and involves only pathways through peripheral sensory and sympathetic ganglia. In addition to its afferent properties, sensory neurons can synthesize and release vasoactive neuropeptide directly on the blood vessels and thus serve as an efferent mechanism. The vasodilatory neural transmitters released from the sensory fibers include calcitonin gene-related peptide (51), substance P (19), neurokinin A (14) and nitric oxide (58). Changes in expression or dynamics of these sensory neuropeptide have been associated with some types of hypertension animal models (27; 54; 56).

The involvement of peripheral sensory neurons in BP regulation was best documented in salt-sensitive hypertension. Neonatal degeneration of

capsaicin-sensitive sensory nerves in rats leads to a significant increase in BP when high salt diet was given (54), suggesting that sensory innervation plays significant roles in antagonizing the development of salt-induced hypertension. The interaction between sensory nerves and the renin-angiotensin system or the sympathetic nerve activation may be involved in mediating this effect but detailed mechanisms are not fully understood (53).

Oxidative stress can cause neuropathies in animal models of diabetes (52). During aging, the higher incidence of neuropathic pain in the elderly is suggestive of an association between progressive degeneration of primary sensory neurons and ROS generation (39). On the other hand, NADPH oxidase-derived ROS seem to have a beneficial effect on the maintenance of DRG neuron integrity and pain perception under normal physiological conditions (46). It is therefore reasonable to predict that DRG neuronal properties are under tight control of cellular redox homeostasis. However, the role of ROS in sensory cardiovascular regulation is very little studied.

Role of NADPH Oxidase and Endothelin-1 in Hypertension

ROS include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$) (12). Increased levels of ROS are correlated with numerous cardiovascular diseases including hypertension (5).

Among several enzyme systems that catalyze ROS production, NADPH oxidase is a predominant source of $O_2^{\bullet-}$, the precursor of all other ROS, in hypertension.

NADPH oxidase was first identified in phagocytes (neutrophils) (2), in which it plays a vital role in nonspecific host defense against pathogens by generating mill molar quantities of $O_2^{\bullet-}$ during the respiratory burst (50). The NADPH oxidase enzyme system consists of two integral membrane proteins $p22^{phox}$ and a NOX catalytic subunit (NOX1, $gp91^{phox}$, NOX3, NOX4, or NOX5), and several cytoplasmic regulatory elements that include $p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and a G protein (*Rac-1*). Activation of NADPH oxidase involves the translocation of regulatory elements from the cytoplasm to combine with catalytic subunits in the membrane (8) (Figure 2.1). In addition to phagocytes, NADPH oxidase is present in endothelial cells in blood vessels (3), vascular smooth muscle cells (34), kidney cortex (7) and nervous system (9; 29). Unlike those in neutrophils, the NADPH oxidase in these tissue make $O_2^{\bullet-}$ in small amounts for purposes of signaling under physiological conditions (25). However, excessive $O_2^{\bullet-}$ production will lead to a variety of intracellular signaling events that ultimately cause cell dysfunction (47).

Endothelin-1 (ET-1) is a potent 21-amino acid vasoconstrictor peptide produced by endothelium and to a lesser extent, the nervous system (36). ET-1 expression in vasculature is increased in salt-dependent hypertensive animal models as well as in human hypertensive individuals (48). Although most studies

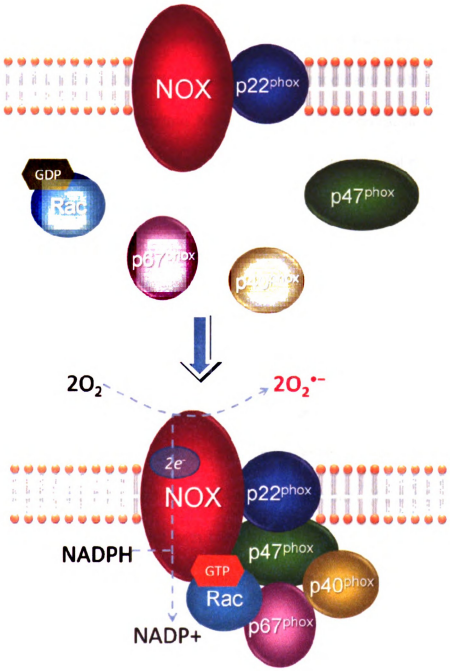

***Figure 2.1. Schematic diagram of NADPH oxidase structure.** At rest, NADPH oxidase contains two membrane-bound subunits NOX and p22phox and several cytosolic subunits p47phox, p40phox, p67phox and Rac-GDP. Upon activation, cytosolic subunits are recruited to the membrane and bind with the two membrane subuints. The assembly of the oxidase complex on the membrane initiates the production of $O2^{\cdot-}$ from O2 utilizing cytosolic NADPH as electron donors.

*Images in this dissertation are presented in color.

to date have been focused on vascular ET-1, several lines of evidence suggest that ETs may function as neurotransmitters or neuromodulators within the nervous system (11). ET-1 can potentiate NE-induced cardiac contractile response by either facilitating NE release from the sympathetic nerve terminal or impairing NE re-uptake by the neuronal NET (1). ET also modulates NE release in the posterior hypothalamus, a sympathoexcitatory region in the central nervous system that is involved in BP regulation (11).

ET-1 may be exerting its effects on the vasculature and nervous system by increasing the production of $O_2^{\bullet-}$, thereby altering the cellular redox environment and cell function. In the vasculature, ET-1 increases $O_2^{\bullet-}$ production via the activation of NADPH oxidase (37; 38; 55). The vascular NADPH oxidase activity and expression are increased in DOCA-salt hypertension (4), a salt-dependent hypertensive model associated with elevated ET-1 level (32; 33). In peripheral nervous system, $O_2^{\bullet-}$ levels are increased in inferior mesenteric ganglia (IMG) of DOCA-salt hypertensive rats compared to normotensive rats (10). In cultured celiac ganglia (CG) neurons treated with ET-1, $O_2^{\bullet-}$ production is significantly increased and this increase is blocked by NADPH oxidase inhibitor Apocynin (9), but not other oxidase inhibitors. Furthermore, NADPH oxidase enzymatic activity and ET_B receptor expression are both increased in CG from DOCA-salt hypertension compared to controls, indicating correlated upregulation of NADPH oxidase and ET-1 signaling in prevertebral sympathetic ganglion neurons (9; 10).

On the other hand, both ET-1 and its receptors are localized to DRG sensory neurons (44; 45). However, less is known about its role in BP regulation.

Summary

Hypertension is a multi-factorial disease and is an independent risk factor for various other cardiovascular disorders. Scientists have been working hard for decades trying to unravel the mysteries behind its mechanisms. However, although extensive progresses have been made so far, the etiology of hypertension is still unknown. Most studies showed pathophysiological features found in either animal models or human hypertensive individuals that are "correlated" with the onset of hypertension, with few of them clarifying whether those are actually "causative factors" or merely pathological changes secondary to increased BP. A typical example is the role of free radicals in hypertension. Elevated ROS levels have been reported from various types of hypertension yet anti-oxidant supplement showed little effect in lowering the risk for hypertension (26). More studies are clearly needed to determine the exact roles of ROS molecules in BP regulation.

In this thesis, experiments are designed to characterize the ROS-generating enzyme NADPH oxidase in peripheral sympathetic and sensory nervous system and its regulation in DOCA-salt hypertension, with the hope to provide possible

mechanisms related to elevated sympathetic activation and altered sensory properties in salt-sensitive hypertension. The functional effects of ROS in sympathetic neurons are studied in more detail under the hypothesis that ROS can affect catecholamine handling in sympathetic ganglion neurons via its modulation on NE reuptake by NET.

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CHAPTER 3: LOCALIZATION OF NADPH OXIDASE IN SYMPATHETIC AND SENSORY GANGLION NEURONS AND PERIVASCULAR NERVES

Abstract

Superoxide anion ($O_2^{-\bullet}$) production was previously reported to be increased in celiac ganglia in deoxycorticosterone acetate (DOCA)-salt hypertension, possibly via the activation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase. This suggested a role for neuronal NADPH oxidase in autonomic neurovascular control, but the expression and localization of NADPH oxidase in the peripheral neurons was not clear. The purpose of this study was to examine the mRNA expression and subcellular localization of NADPH oxidase in sympathetic and sensory ganglion neurons. Using reverse transcription-polymerase chain reaction, we determined that the mRNA of NADPH oxidase subunits NOX1, NOX2, NOX4, $p22^{phox}$ and $p47^{phox}$ were present in celiac gangia. The same subunits were also present in dorsal root ganglia with the exception that NOX4 levels were much lower. Immunohistochemical staining was performed to localize NADPH oxidase protein in sympathetic and sensory neurons by examining one of the membrane-bound subunit, $p22^{phox}$, and one of the cytosolic subunit, $p47^{phox}$. We found that in rat celiac ganglia and inferior mesenteric ganglia, there was intense immunostaining of $p22^{phox}$ associated with

ganglionic neuron somata and intercellular nerve fibers with no staining in satellite cells. $P22^{phox}$ also was localized to a subpopulation of dorsal root ganglia neurons that contain calcitonin gene related peptide. In mesenteric arteries, $p47^{phox}$ and $p22^{phox}$ were colocalized with neuropeptide Y or calcitonin gene related peptide in perivascular nerve terminals. A similar pattern of nerve terminal staining of $p47^{phox}$ and $p22^{phox}$ also was found in cultured celiac ganglia neurons and one-week nerve growth factor -differentiated PC12 cells. These data demonstrate a previously uncharacterized localization of NADPH oxidase in prevertebral sympathetic ganglia and sensory ganglia. The presence of a $O_2^{-\bullet}$ – generating enzyme in the close vicinity of the sites for neurotransmitter handling in these neurons may suggest novel reactive oxygen species-mediated mechanisms in peripheral sympathetic and sensory neurovascular control.

Introduction

Reactive oxygen species (ROS), such as superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) are signaling molecules which play important roles regulating cardiovascular function (14; 15). While first discovered in phagocytes, reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase is now thought to be a significant source of ROS in many cell types including smooth muscle cells (13; 41; 48), endothelial cells (38), fibroblasts (22) and neurons in the brain (11).

NADPH oxidase is a multi-subunit enzyme consisting of several cytosolic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and *Rac*) and a membrane-associated cytochrome *b558*, which is composed of $p22^{phox}$ and one NOX catalytic subunit. In phagocytes, enzyme activation requires the translocation of cytosolic subunits to the membrane where they associate with cytochrome *b558* and facilitate the transfer of electrons from NADPH via FAD and two heme moieties to molecular oxygen, resulting in $O_2^{-\bullet}$ formation. In non-phagocytic cells, intracellular pre-assembled NADPH oxidase may exist to facilitate $O_2^{-\bullet}$ production in the cytoplasm (28; 55).

Increased ROS production and NADPH oxidase activity are associated with cardiovascular dysfunction in hypertension (1), diabetes (17) and senescence (4). Studies of ROS in hypertension have focused primarily on vascular and endothelial ROS signaling (15). However, accumulating evidence indicates that peripheral neural components, including both sympathetic and sensory nerves, which innervate the splanchnic circulation, play a key role in regulating blood pressure, and that abnormalities in these neurons contribute to increased salt sensitivity and the development of hypertension (23; 33; 35; 49; 52). Enhanced NADPH oxidase activity in peripheral sympathetic neurons is associated with the onset of cardiovascular disorders (6; 32). This suggests a role of peripheral neuronal NADPH oxidase in the regulation of blood pressure.

Because of a short half life (1×10^{-6} sec), the direct actions of $O_2^{-\bullet}$ in the cell are confined to a limited region near the subcellular site of its production. In neurons, the major functional compartments–the cell body, dendrites, axons, and terminals– are separated by considerable distances; therefore it is important to evaluate the localization of NADPH oxidase in these compartments in order to fully understand the physiological consequences of $O_2^{-\bullet}$ production. In particular, transmission at the neuro-vascular junctions modulates vascular tone, the $O_2^{-\bullet}$ produced by NADPH oxidase in the cell body would not be expected to diffuse to the terminals; if NADPH oxidase were to influence neurotransmitter dynamics it would have to produce $O_2^{-\bullet}$ locally. Therefore, a systemic evaluation of the localization of NADPH oxidase in the peripheral sympathetic and sensory neurons is needed to address this issue.

A series of experiments were designed to localize NADPH oxidase subunits, $p22^{phox}$ and $p47^{phox}$, to prevertebral sympathetic ganglia and sensory ganglia as well as perivascular nerve fibers and endings to assess their anatomical location. Our results showed that NADPH oxidase subunits were present in both the cell bodies and the nerve fibers of these neurons. The presence of NADPH oxidase subunits in the neurons innervating the splanchnic circulation may have important implications in the role of NADPH oxidase in blood pressure regulation and hypertension.

Methods

Tissue Harvest and Cell Culture

All cell culture reagents are GIBCO® brand (Invitrogen, Carlsbad, CA) unless otherwise noted.

Primary sympathetic ganglion neuron culture

Celiac ganglia (CG) from postnatal 3 to 5-day-old Sprague-Dawley (SD) rats were harvested and enzymatically dissociated (2.5mg/ml collagenase 10 minutes at 37°C followed by 2.5mg/ml trypsin 45min at 37°C). Freshly dissociated neurons were plated as a monolayer on cover glass in culture dishes double coated with 100μg/ml poly-D-lysine (Sigma-Aldrich, St. Louis, MO) and collagen. Cells were maintained in N2 medium (49% DMEM, 49% F-12 nutrient mixture, 0.5mg/ml bovine serum albumin, 2mM L-glutamine, 1% N2 supplement, 100ng/ml nerve growth factor 2.5 (Millipore, Billerica, MA), 0.7% B-27) supplemented with 1% fetal bovine serum at 37°C in a 5% CO_2 humidified incubator. 0.24μg/ml 1β-arabinofuranosylcytosine (Ara-C) (Calbiochem, San Diego, CA) was added to N2 medium from the second day of culture to eliminate non-neuronal cell growth. Neurons were kept in culture for 7 days before immunostaining experiment to ensure full neurite outgrowth.

PC-12 Cell Culture

PC-12 cells are derived from a rat catecholamine-secreting chromaffin tumor. They can differentiate into cells with a sympathetic neuronal phenotype after one week of NGF treatment (12). PC-12 cells were obtained from American Type Culture Collection, and maintained at 37°C in a 5% CO_2 humidified incubator in RPMI 1640 medium supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, 100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml Fungizone. To differentiate PC12 cells, 50ng/ml NGF 2.5S (Millipore) was added to the medium for 7 days.

RNA Isolation

RNA was extracted from cultures of CG neurons, cultures of PC-12 cells, rat dorsal root ganglia (DRG) (spinal level T13 - L2), rat aorta, and rat cerebral cortex by using the standard TRIzol procedure (Invitrogen). The concentration/purity/integrity of RNA was ascertained spectrophotometrically (A_{260}/A_{280}). To eliminate residual genomic DNA in the preparation, total RNA samples were treated with 10 U/μl RNase-free DNase I (Roche, Nutley, NJ) for 30 min at 37°C, and DNase I was inactivated by heating for 10 min at 75°C.

Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

cDNA was synthesized from DNase-treated RNA using Superscript II mix (Invitrogen). The cDNA synthesized from 2μg total RNA was used in subsequent

PCR. All primers were derived from the *Rattus Norvegicus* gene (National Center for Biotechnology Information GenBank). Primer sequences are shown in Table 3.1. PCR products were electrophoresed on a 2.0% agarose gel for 60 minutes at 9V/cm gel. Bands corresponding to PCR amplicons were stained by ethidium bromide and visualized by UV light.

Immunohistochemical Staining of Rat Inferior Mesenteric Ganglia and Cultured Celiac Ganglion Neurons and PC12 Cells

All antibodies used in these experiments are listed in Table 3.2. Postnatal 7 to 10-day-old SD rats were euthanized with sodium pentobarbital (50mg/kg). The inferior mesenteric ganglia (IMG) were surgically removed and maintained in Hank's Balanced Salt Solution (Invitrogen). The IMG were cleaned of surrounding connective tissue and blood vessels and the isolated ganglia were placed in fixative (4% paraformaldehyde, 0.1% Triton X-100 in Dulbecco's phosphate buffered saline (DPBS)) for 30min at room temperature. Cultured cells were cleaned from culture medium by three washes in DPBS and then placed into fixative for 30min. Samples (ganglia or cultured cells) were then incubated in DPBS with blocking solution (5% goat serum, 3% BSA) for 1 hour at room temperature, followed by primary antibody incubation for overnight at 4°C. The next day, samples were washed in DPBS for three times and then incubated with corresponding secondary antibodies in a dark chamber at room temperature for 1

Table 3.1 Primer sequences for NADPH oxidase subunits NOX, NOX2, NOX4, $p47^{phox}$ and $p22^{phox}$ and β-actin.

Gene	Sequence	Amplicon Length (bp)	NCBI accession Number
NOX1	For:5' TGAACAACAGCACTCACCAATGCC 3' Rev:5' AGTTGTTGAACCAGGCAAAGGCAC 3'	245	AF152963
NOX2	For:5' GTGGAGTGGTGTGTGAATGC 3' Rev:5' TCCACGTACAATTCGCTCAG 3'	324	AF298656
NOX4	For:5' ACCAGATGTTGGGCCTAGGATTGT 3' Rev:5' AGTTCACTGAGAAGTTCAGGGCGT 3'	261	AY027537
p47phox	For:5' GGCCAAAGATGGCAAGAATA 3' Rev:5' TGTCAAGGGGCTCCAAATAG 3'	221	AF260779
p22phox	For:5' TTGTTGCAGGAGTGCTCATC 3' Rev:5' TAGGCTCAATGGGAGTCCAC 3'	282	U18729
β-actin	For:5' GGCTACAGCTTCACCACCAC 3' Rev:5' TACTCCTGCTTGCTGATCCAC 3'	500	V01217

Table 3.2 Antibodies for immunohistochemical staining.

Primary antibodies

Antigen	Host species	Dilution	Source
p47phox (R360)	Rabbit	1:300	Dr. Mark T. Quinn (Montana State University)
p47phox	Rabbit	1:150	Santa Cruz Biotech., Inc., Santa Cruz, CA
p22phox^(H44.1)	Mouse	1:1000	Dr. Mark T. Quinn
p22phox (R5554)	Rabbit	1:300	Dr. Mark T. Quinn
NeuN^	Mouse	1:500	Millipore, Billerica, MA,
SGII^	Mouse	1:1000	Abcam Inc., Cambridge, MA
TH^	Mouse	1:150	Calbiochem, La Jolla, CA
NPY	Goat	1:300	Santa Cruz Biotech., Inc.,
CGRP	Sheep	1:1000	Abcam Inc., Cambridge, MA

SGII = secretogranin II; TH = tyrosine hydroxylase; NPY = Neuropeptide Y; CGRP = calcitonin gene-related peptide; ^=monoclonal antibodies

Secondary antibodies

Target species	Host species	Conjugated to:	Dilution
Mouse	Donkey	FITC	1:40
Rabbit	Donkey	Cy3	1:200
Sheep	Donkey	Cy3	1:200
Goat	Donkey	FITC	1:200
Mouse	Donkey	Cy3	1:200
Rabbit	Goat	Alexa 488	1:500
Mouse	Rabbit	Cy3	1:500

All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, except for goat anti-rabbit Alexa 488 was purchased from Invitrogen, Inc., Carlsbad, CA

hour, followed by three washes in DPBS. Samples were mounted onto glass slides using Prolong Gold anti-fade reagent (Invitrogen) for confocal laser scanning using Pascal (Zeiss, Thornwood, NY) or Fluoview (Olympus, Center Valley, PA).

Immunohistochemistry for Rat Celiac Ganglia and Dorsal Root Ganglia

Ganglia were dissected from adult SD rats and fixed in 10% formalin for 2 hours then transferred to 70% ethanol for storage ranging from several hours to overnight. Tissue was processed using a vacuum infiltration tissue processor (Thermo Electron Excelsior) with decreasing concentrations of ethanol followed by xylene. Tissues were embedded in paraffin, sectioned on a rotary microtome into 5 μm sections, and mounted on to glass slides (Corning Glass). Heat induced epitope retrieval (HIER) was used. Samples were blocked for endogenous elements with hydrogen peroxide/methanol for 30 minutes then rinsed. Due to the use of HIER, an additional blocking step of avidin and biotin was used with a 15-minute incubation. Normal goat serum or donkey serum (1:28, Vector Laboratories, Burlingame, CA) was used as a protein block followed by incubation primary antibodies for 60-minutes. Incubation of biotinylated goat-anti-rabbit secondary antibody (1:200, Vector Laboratories) for 30 minutes was followed by a 15 minute incubation with Nova Red chromagen (Vector Laboratories). Slides were counterstained with Lerner 2 hematoxylin then dehydrated. Images were

collected using standard bright field microscopy (Olympus BX60 with SPOT Insight Digital Camera, Olympus America Inc. Center Valley, PA). A "no primary control" was run in parallel without addition of primary antibody to assess antibody specificity. For fluorescent staining, slides were incubated with primary antibodies followed by incubation in fluorophore-conjugated secondary antibodies. Images were collected using Fluoview confocal microscope (Olympus).

Immunostaining of Periarterial Nerve Fibers

8 week old SD rats were euthanized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The mesentery was surgically removed and maintained in 0.1M phosphate-buffered saline (PBS). Mesenteric arteries were cleaned of adipose and connective tissue and cleared of blood via an intravascular PBS bolus. Tertiary branches were excised and isolated tissues were placed in Zamboni fixative (2% [vol/vol] formaldehyde and 0.2% [vol/vol] picric acid in 0.1M phosphate buffered saline, PBS) overnight (4 °C). The next day, the tissues were washed 3x with 0.1M PBS and then incubated in PBS with blocking serum (donkey) diluted in Triton X-100 (1.0 %) for 1 hour. Tissues were then co-incubated for 2 hours at 37 °C in diluted primary antibodies (in Triton-PBS). Next, tissues were washed 3x in 0.1M PBS buffer and then incubated for 1 hour in a dark, humidified chamber at room temperature in corresponding secondary antibodies. Vessels were then washed 3x with 0.1 M

PBS at 5-minute intervals and coverslipped with Prolong Gold anti-fade reagent for fluorescence confocal microscopy. Tissues were examined using a Leica TSL laser confocal microscope (Leica Microsystems Inc., Bannockburn, IL).

Results

Expression of NADPH Oxidase mRNA in CG, DRG and Differentiated PC-12 cells

PCR amplicons of NADPH oxidase catalytic core subunits NOX1 and NOX2 were detected in aorta, brain, dissociated CG neurons, DRG and differentiated PC-12 cells (Figure. 3.1 A, B and C) at the expected sizes of 245 and 324 bp, respectively. By contrast, NOX4 was present in aorta and CG neurons but not in brain or PC-12 cells (Figure. 3.1 A and B). NOX4 was present in rat DRG at very low level (Figure. 3.1C). Thus, NOX4 was the only homologue not found in all 4 cell types examined. PCR amplicons for NADPH oxidase regulatory subunits $p47^{phox}$ and $p22^{phox}$ were present in both rat CG and DRG (Figure 3.1 B and C). The sequenced PCR amplicons were aligned in GenBank. Greater than 99% of sequenced amplicons of $p47^{phox}$, $p22^{phox}$, NOX2, NOX1, and NOX4 in CG neurons and PC-12 cells matched published sequences.

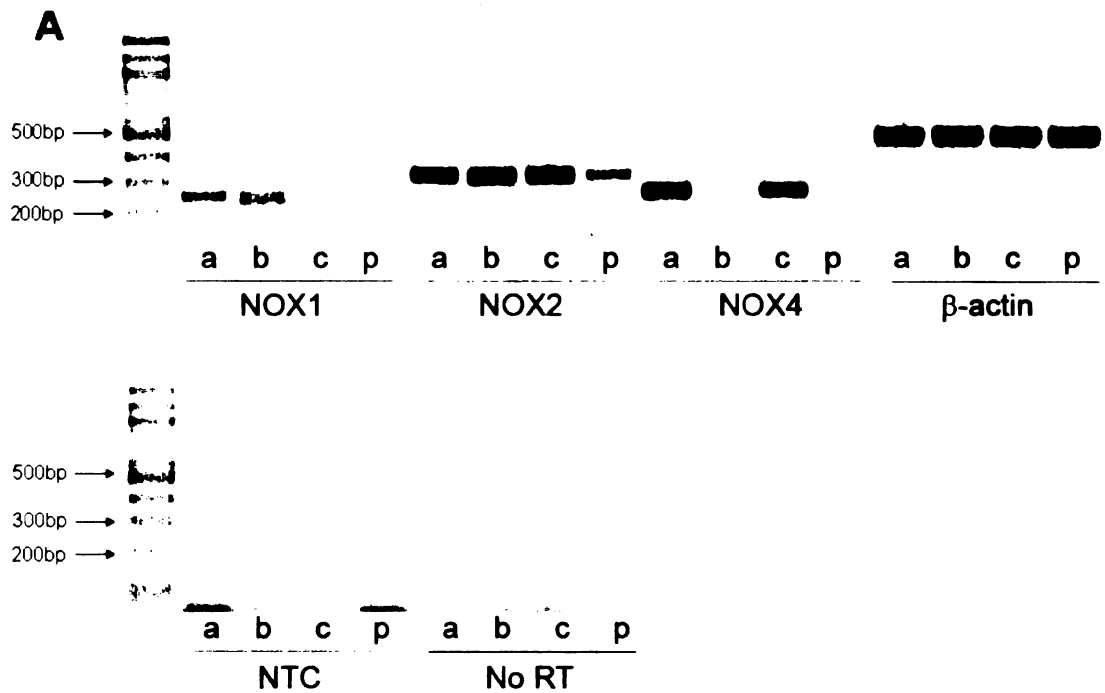
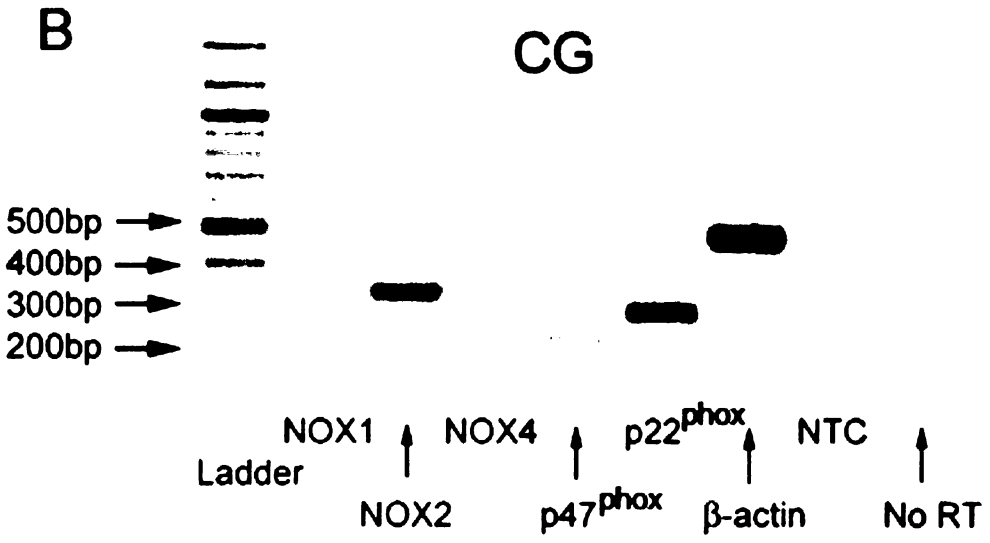
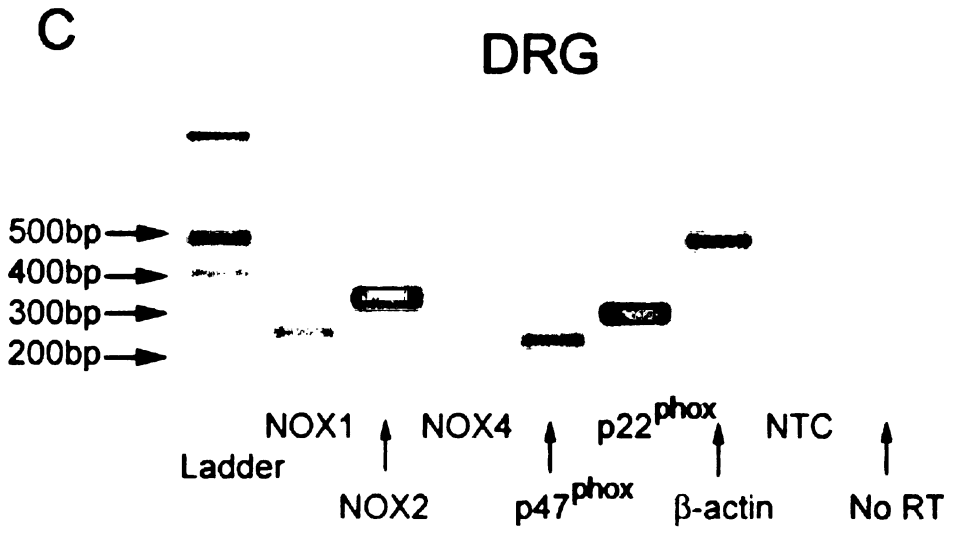


Figure 3.1. NADPH oxidase subunits in celiac ganglia (CG), dorsal root ganglia (DRG) and PC12 cells. PCR amplicons for catalytic core subunits NOX1, NOX2 were present on ethidium bromide-stained agarose gels from aorta (a), brain (b), CG (c) and PC12 cells (p) (A); NOX4 was found only in aorta and CG, not in brain or PC-12 cells (A). NOX4 was also found in DRG but at a very low level (C); NADPH oxidase regulatory subunits p47phox and p22phox were found in both CG (B) and DRG (C). β-actin was used as a loading control in all experiments. No-cDNA template control (NTC) and omission of the RT step (No RT) were both performed as a negative control.

Figure 3.1 continued





NADPH Oxidase Subunit p22^{phox} is Present in Neuronal Somata in Rat IMG.

In whole mount rat IMG, p22^{phox} immunoreactivity was found in ganglion neurons using two different antibodies targeting p22^{phox}. First, monoclonal mouse

anti-$p22^{phox}$ antibody (H44.1, see Table 3.2) staining showed that $p22^{phox}$ was localized to neuronal cell bodies in IMG with little staining in interneuronal structures (Figure 3.2). Second, in double labeling staining, $p22^{phox}$ (R5552, see Table 3.2) immunoreactivity was localized in Neuronal Nuclei (NeuN)-positive ganglion neurons (Figure 3.3). Although in some species, NeuN does not stain sympathetic neurons (53); in rat IMG NeuN immureactivity was localized primarily in the nuclei of neurons with lighter staining in the cytoplasm. There was no NeuN immunoreactivity in non-neuronal cells. The use of NeuN as a sympathetic neuronal marker was further verified in cultured CG neurons (Figure 3.4) The staining pattern of NeuN in both IMG and CG are identical to those found in other types of neurons (54). The colocalization of $p22^{phox}$ with NeuN indicates the presence of NADPH oxidase in sympathetic ganglion neuronal cell bodies in rat IMG.

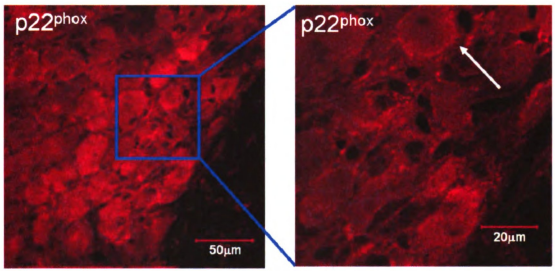


Figure 3.2. Immunolocalization of p22phox in rat inferior mesenteric ganglion (IMG). IMG was dissected from 3-week old male Sprague-Dawley rats and fixed immediately. The ganglion was incubated with mouse monoclonal antibody against p22phox (H44.1) followed by Cy3 tagged secondary antibodies. P22phox was localized in the cytosol and plasma membrane of ganglion neurons (arrow). Images were taken under confocal laser scanning microscopy.

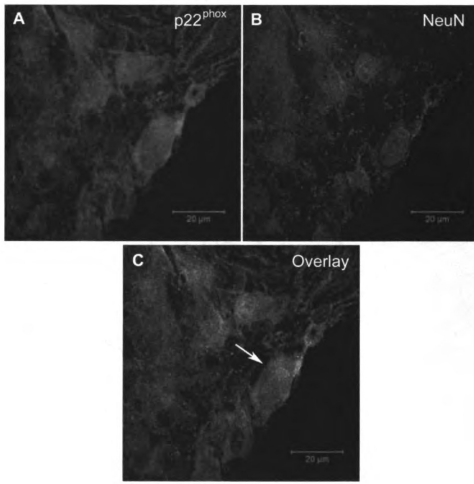


Figure 3.3. Colocalization of p22phox and NeuN in rat inferior mesentetic ganglia (IMG). IMG was removed from 10 day- old Sprague-Dawley rats and put in fixative immediately. The ganglion was incubated with (A)polyclonal rabbit anti-p22phox (R5554) and (B)monoclonal mouse anti-NeuN followed by secondary antibodies. (C)P22phox and NeuN immunoreactivity were found in the same neuron (arrow). Images were taken under confocal laser scanning microscopy. Scale bar is 20μm.

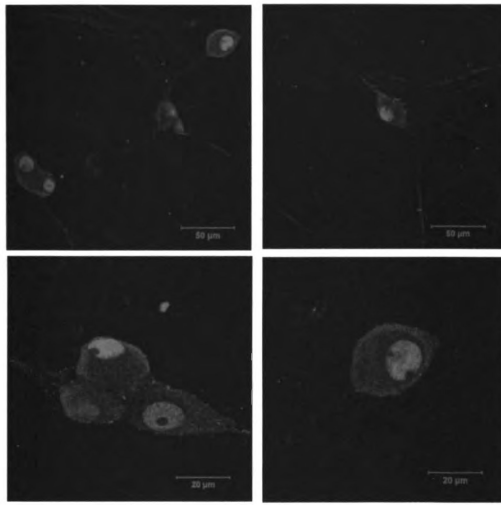


Figure 3.4 Immunostaining of NeuN in Cultured Celiac Ganglia (CG) Neurons. Freshly dissociated CG neurons were dissected from 3 to 5-day old Sprague-Dawley rats and were kept in culture medium for 7 days before immunocytochemical staining. Cells were incubated with mouse monoclonal anti-NeuN at a 1:500 dilution overnight at 4°C followed by incubation with Cy3 conjugated goat anti-mouse antibody. NeuN staining was found presumably in the nuclei of the neurons with less staining in the cytoplasm. Scale bar is 50µm in the upper pannels and 20µm in the lower pannels.

NADPH Oxidase Subunits $p22^{phox}$ and $p47^{phox}$ are Present in Rat CG Neurons.

Fixed CG tissue was cut into 5μm sections for immunohistochemisty. As shown in Figure 3.5 A, immunoreactivity for $p47^{phox}$ was found in all neurons in CG. The staining was presumably in neuronal cell bodies with little staining in non-neuronal structures. Similar staining pattern was also observed with $p22^{phox}$ antibody (data not shown). Previously, in guinea pig prevertebral sympathetic ganglia, approximately 20% of all neurons contain immunoreactivity to neuropeptide Y (NPY) and have been speculated to be vasoconstrictor neurons (36). On the other hand, 18.9% of neurons in the IMG that innervate the inferior mesenteric artery are NPY-positive (2). In order to identify whether NADPH oxidase is present in vasomotor neurons in CG, double-labeling of $p22^{phox}$ and NPY was performed. In the rat CG, all neuron cell bodies examined were immunopositive for NPY and $p22^{phox}$ (Figure 3.5 B). Thus, the presence of both proteins was not limited to a subpopulation of neurons in rat CG.

$P22^{phox}$ is localized in calcitonin gene-related peptide (CGRP) -positive neurons in DRG.

CGRP is a vasodilatory neuropeptide that is released from the sensory nerve fibers (47). The synthesis of the peptide occurs in neuronal cell bodies of the DRG. The intensity of CGRP immunostaining in rat DRG varied depending on the

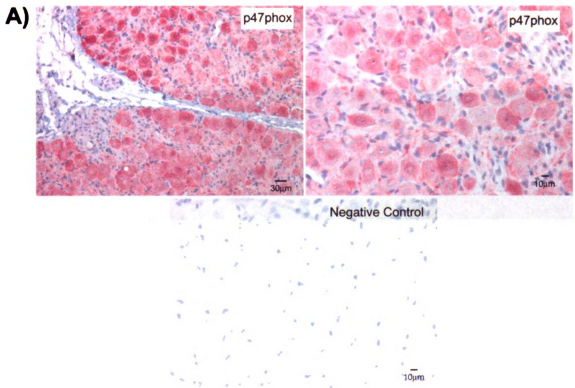
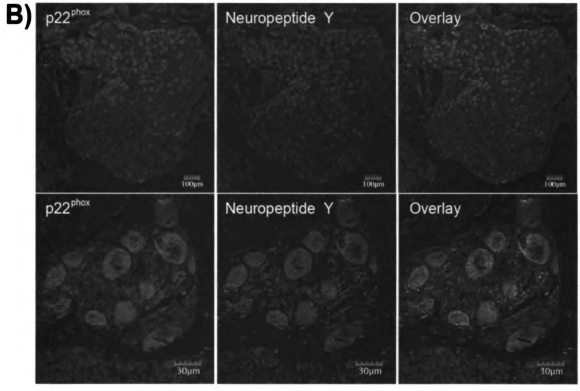


Figure 3.5. Immunolocalization of p47phox and p22phox in celiac ganglia (CG) neurons. CG were removed from adult SD rats and were fixed, embedded in paraffin and sectioned at 5μm for immunostaining. A) Polyclonal rabbit anti-p47phox (Santa Cruz Biotech) was used with NOVA RED chormagen such that p47phox immunoreactivity is shown in red. Images were captured using standard brightfield microscopy. P47phox immunoreactivity was found in nearly all neural cell bodies in CG although the intensity of staining varies across the tissue. This may suggest variable levels of NADPH oxidase expression among neurons in the same ganglia. Scale bar is 30μm in the upper left pannel and 10μm in the rest; B) CG sections were incubated with polyclonal rabbit anti-p22phox (R5554) and polyclonal goat anti-NPY followed by incubation with fluorophore-conjugated sencondary antibodies. Fluorescent images were taken using confocal laser scanning microscopy. Virtually all the neurons that contain NPY immunoreactivity showed positive staining for p22phox as well, indicating the colocalization of these two proteins in the same neurons in rat CG. Scale bar is 100μm in the upper pannel and 30μm in the lower pannel.

Figure 3.5 continued



size of the neurons (Figure 3.6), in which neurons in smaller sizes showed higher immunoreactivity to CGRP (18). The distribution of $p22^{phox}$ in DRG was identical to CGRP. Staining for $p22^{phox}$ was also found to be more concentrated in the cytoplasm of the neurons with smaller sizes, while staining was lighter and more diffuse in neurons with larger cell bodies. In addition to neurons, satellite cells that surround the neurons also showed immunoreactivity to both CGRP and $p22^{phox}$. These results indicate that the NADPH oxidase subunit $p22^{phox}$ and CGRP are colocalized in rat DRG neuronal cell bodies and some non-neuronal cells.

$P47^{phox}$ and $p22^{phox}$ are present in the neurites of nerve growth factor (NGF)-differentiated PC12 cells and cultured CG Neurons.

In order to examine the presence of NADPH oxidase in neuronal compartments outside of the cell bodies, I first did immunostaining of $p47^{phox}$ in NGF-differentiated PC12 cells. $P47^{phox}$ was present in PC-12 cell bodies, as well as the neurites that extended from the somata and was colocalized with secretogranin II (SGII), a large dense core vesicle marker protein (9), on the cell membrane and the neurites (Figure 3.7 A-C). In dissociated CG neurons cultured 7 days, $p47^{phox}$ and $p22^{phox}$ were colocalized with SGII in both cell bodies and neurites (Figure 3.7 D-F and H-J). We also used NPY and tyrosine hydroxylase (TH) to label the nerve fibers in CG cultures. All NPY immunoreactive fibers were

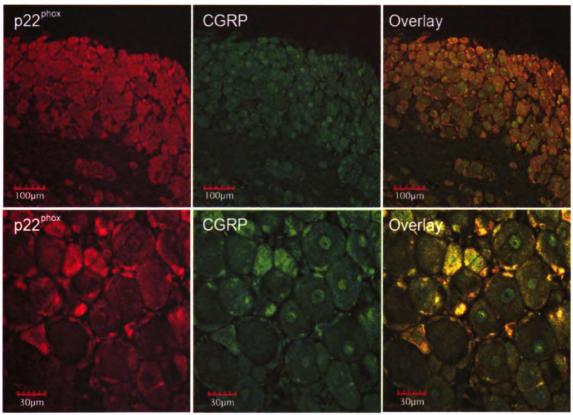


Figure 3.6. Immunolocalization of p22phox in dorsal root ganglia (DRG). DRG were removed from adult SD rats and were fixed, embedded in paraffin and sectioned at 5μm for immunostaining. DRG sections were incubated with polyclonal rabbit anti-p22phox (R5554) and polyclonal sheep anti-CGRP followed by incubation with fluorophore-conjugated sencondary antibodies. Fluorescent images were taken using confocal laser scanning microscopy. Immunoreactivities for p22phox and CGRP are localized in the same DRG neurons, both of which are more intense in the neurons with smaller sizes. In addition to neurons, satellite cells surrounding the neurons are also positive for p22phox and CGRP. Scale bar is 100μm in the upper pannel, 30μm in the lower pannel.

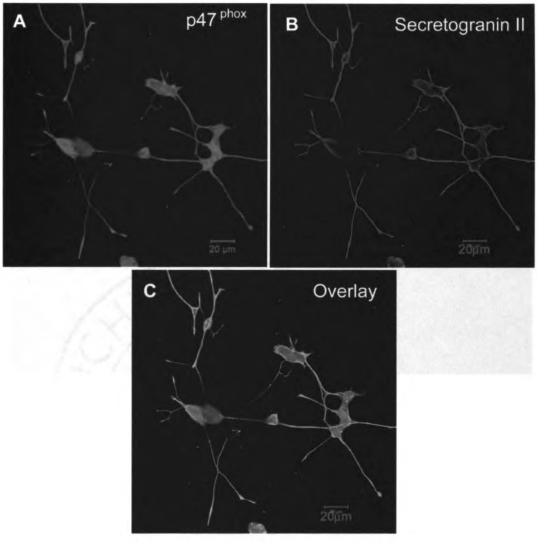


Figure 3.7.Immunoreactivity of p47phox and p22phox in NGF-differentiated PC12 cells and cultured celiac ganglia (CG) neurons. For immunostaining assay, PC12 cells were treated with NGF for 7 days to achieve neurite outgrowth. CG was removed from neonatal SD rats and ganglion neurons were freshly dissociated from the ganglia and were kept in culture medium for 7 days. Cells were incubated with polyclonal rabbit anti-p47phox (R360) or anti-p22phox (R5554) and monoclonal anti-secretogranin II, a marker for large dense core vesicles in sympathetic neuronal cell body and nerve endings. P47phox was co-labeled with secretogranin II in both cell bodies and neurites in PC12 cells (A-C) (Scale bar=20µm). P47phox and p22phox were co-localized with secretogranin II in CG neurons (D-F and H-J) (Scale bar=30µm).

Figure 3.7 continued

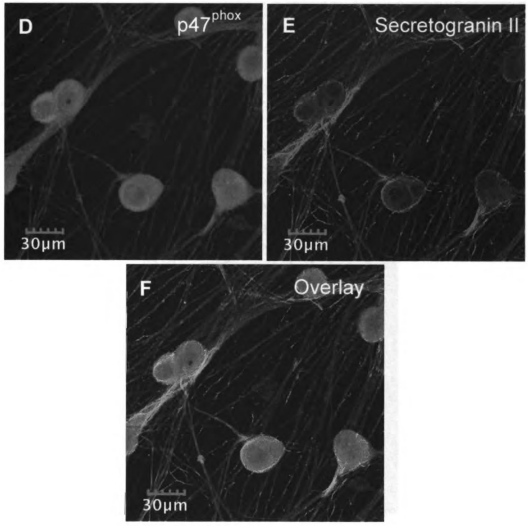
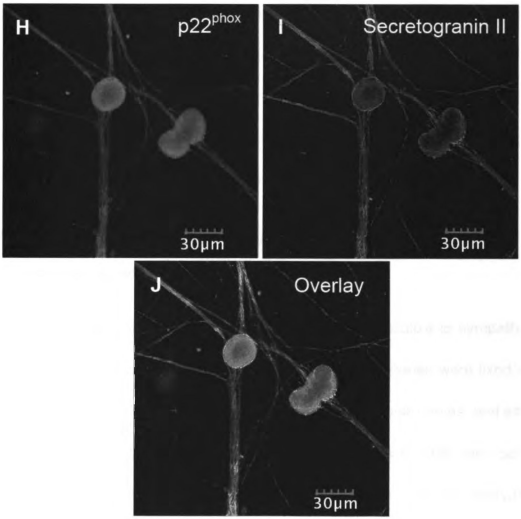


Figure 3.7 continued



positive for $p22^{phox}$ or $p47^{phox}$ (Figure 3.8), while TH immunoreactivity was found in some NADPH oxidase positive fibers but not others (Figure 3.9). The absence of TH in some of the nerve fibers from a sympathetic ganglion neuronal culture is unexpected. It is possible that some ganglia neurons developed non-adrenergic phenotype. For example, cholinergic property can be differentiated in sympathetic ganglia neurons under certain conditions (8). Further studies are needed to identify the TH-negative fibers in the culture. Nevertheless, these results suggest that in addition to cell bodies, NADPH oxidase is also present in nerve fibers.

NADPH oxidase subunits, $p47^{phox}$ and $p22^{phox}$ colocalize to NPY immunoreactive periarterial nerve fibers.

In order to determine if NADPH oxidase subunits co-localize to sympathetic nerve fibers and endings, tertiary branches of mesenteric arteries were fixed and labeled with anti-NPY, as a marker for sympathetic perivascular nerves, and either anti-$p47^{phox}$ or anti-$p22^{phox}$. Upper rows in each panel are low power magnification images showing the meshwork pattern of nerve fibers innervating mesenteric arteries (40). Lower rows are high power magnifications, focusing on a single peri-vascular nerve bundle. $P47^{phox}$ and NPY were found in the same nerve fiber bundles (Figure 3.10A). Immunostaining for $p22^{phox}$ was also found in some of the same nerve fibers as NPY, but not all of them. The merged images show that $p22^{phox}$ and NPY co-localize to some, but not all periarterial nerve fibers.

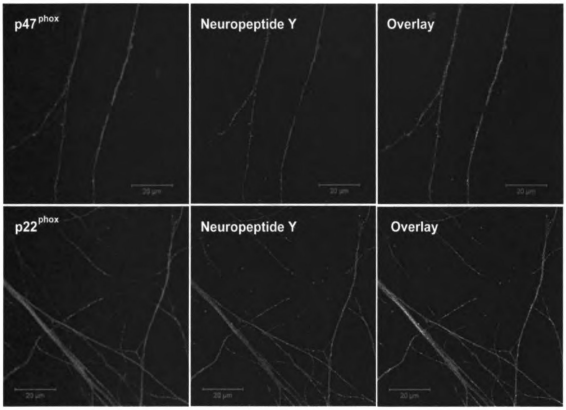


Figure 3.8. p47phox and p22phox colocalize with neuropeptide Y in nerve fibers in cultured celiac ganglia (CG) neurons. CG were removed from neonatal SD rats and neurons were dissociated and kept in culture medium for 7 days before immunostaining. After fixation, cells were incubated with polyclonal rabbit anti-p47phox (R360) or anti-p22phox (R5552) and polyclonal goat anti-NPY. Most nerve fibers in CG culture showed immunoreactivity for both p47phox or p22phox and NPY. Scale bar = 20μm.

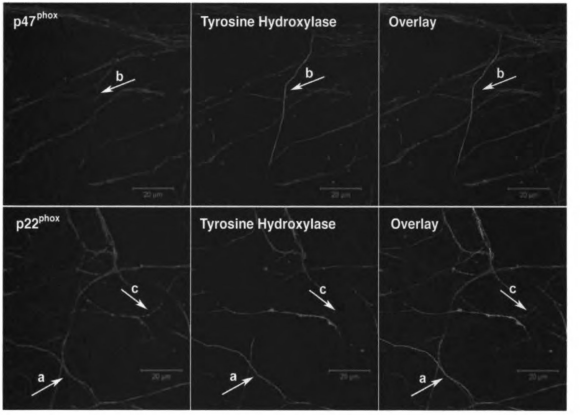


Figure 3.9. p47phox and p22phox colocalize with tyrosine hydroxylase (TH) in nerve fibers in cultured celiac ganglia (CG) neurons. CG were removed from neonatal SD rats and neurons were dissociated and kept in culture medium for 7 days before immunostaining. After fixation, cells were incubated with polyclonal rabbit anti-p47phox (R360) or anti-p22phox (R5552) and monoclonal anti-TH. In some nerve finbers, NADPH oxidase and TH were found to be colocalized in the same fiber (Arrow a). However, some fibers showed only staining for TH not NADPH oxidase (Arrow b) or NADPH oxidase not TH (Arrow c). Scale bar = 20μm.

A

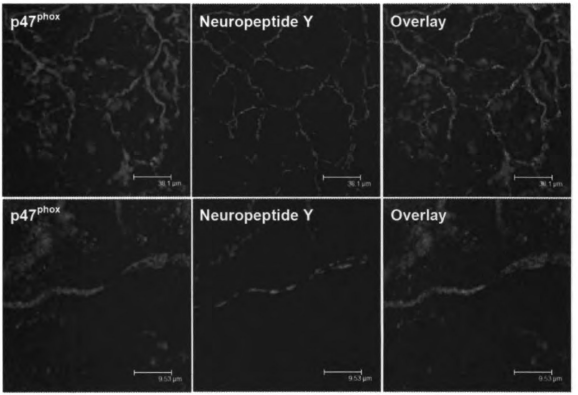
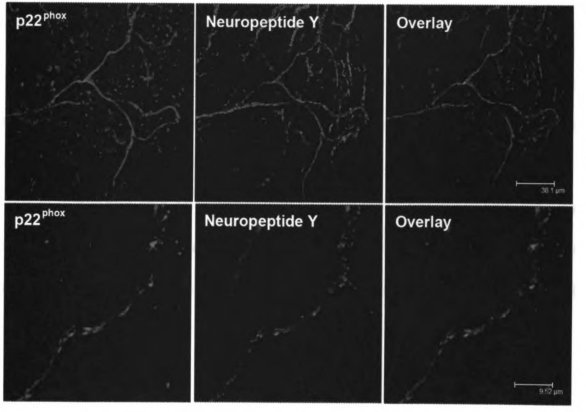


Figure 3.10. p47phox and p22phox colocalize with neuropeptide Y (NPY) in periarterial nerve fibers in mesenteric arteries. Tertiary branches of mesenteric arteries from adult SD rats were fixed and labeled with anti-NPY, as a marker for sympathetic perivascular nerves, and either anti-p47phox (R360) or anti-p22phox (R5554). Upper rows in each panel are low power magnification images showing the meshwork pattern of nerve fibers innervating mesenteric arteries. Lower rows are high power magnifications, focusing on a single peri-vascular nerve bundle. A) P47phox and NPY were found in the same nerve fiber bundles although the localization of the staining within the nerve fiber was variable. NPY staining appears to be more vesicular, while p47phox staining is diffuse throughout the nerve fibers. B) Immunostaining for p22phox was found in some of the same nerve fibers as NPY, but not all of them. The merged images show that p22phox and NPY co-localize to some, but not all periarterial nerve fibers. Scale bar is 38.1μm in upper panels, 9.53μm in lower panels.

Figure 3.10 continued

B



These results indicate that NADPH oxidase subunits are localized to sympathetic periarterial nerve fibers.

NAPDH oxidase subunits $p47^{phox}$ and $p22^{phox}$ colocalize to CGRP immunoreactive periarterial nerve fibers.

CGRP was used as a marker for sensory nerves on tertiary mesenteric arteries. Figure 3.11 A shows that $p47^{phox}$ was colocalized with CGRP in the same nerve fibers. Images in Figure 3.11 B shows the localization of $p22^{phox}$ and CGRP. $P22^{phox}$ immunostaining co-localized with CGRP positive fibers (yellow), but was also found in non CGRP containing fibers. These fibers may be non-sensory source such as sympathetic nerve fibers. These results indicate that NADPH oxidase subunits are present in sensory periarterial nerve fibers.

Discussion

In this study, I have shown that NADPH oxidase was expressed in sympathetic and sensory ganglion neuronal somata and perivascular nerve fibers originated from these neurons. 1) NADPH oxidase subunits NOX1, NOX2, $p22^{phox}$ and $p47^{phox}$ mRNA were expressed in NGF-differentiated PC12 cells and CG and DRG neuron, with the exception that detectable level of NOX4 mRNA was only found in CG but not in PC12 cells or DRG; 2) $p22^{phox}$ and $p47^{phox}$ expression were

A

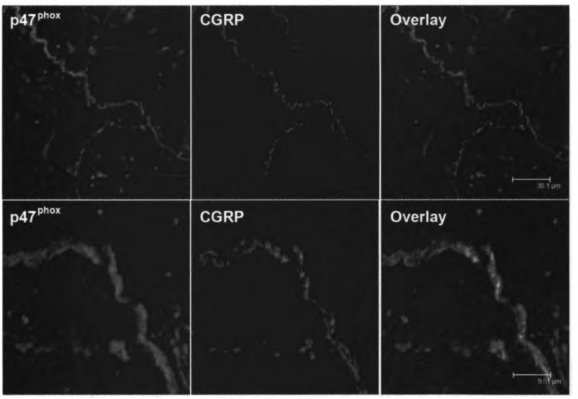
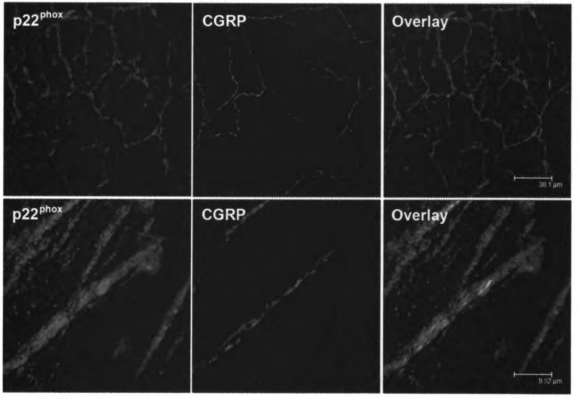


Figure 3.11. p47phox and p22phox colocalize with CGRP in periarterial nerve fibers in mesenteric arteries. Tertiary branches of mesenteric arteries from adult SD rats were fixed and labeled with anti-CGRP, as a marker for sensory perivascular nerves, and either anti-p47phox (R360) or anti-p22phox (R5554). Upper rows in each panel are low power magnification images showing the meshwork pattern of nerve fibers innervating mesenteric arteries. Lower rows are high power magnifications, focusing on a single peri-vascular nerve bundle. A) P47phox and CGRP were found in the same nerve fiber bundles. B) P22phox was found in CGRP immunoreactive nerve fibers. The p22phox positive fibers that lack immunoreactivity for CGRP may be sympathetic nerves. Scale bar is 38.1μm in upper panels, 9.53μm in lower panels.

Figure 3.11 continued

B



associated with neuronal cell bodies in rat IMG, CG and DRG; 3) the protein expression of $p22^{phox}$ and $p47^{phox}$ were present in NGF-differentiated PC12 cells as well as primary CG and DRG cultured neurons across the cell bodies and the neurites; 4) $p22^{phox}$ and $p47^{phox}$ were present in perivascular sympathetic and sensory nerve fibers on mesenteric arteries. This is the first study to systemically evaluate the NADPH oxidase expression in peripheral sympathetic and sensory nervous system. In addition to the verification of the presence of NADPH oxidase in prevertebral sympathetic ganglion neurons and primary sensory neurons, the localization of NADPH oxidase subunits to perivascular nerve fibers innervating the splanchnic circulation is novel, and this may have important implications in blood pressure regulation and hypertension.

The innervation of the mesenteric circulation consists of sympathetic neurons in celiac and mesenteric ganglia and sensory DRG neurons, respectively. The axons of the neurons travel to the mesenteric arteries and veins in the paravascular nerves, which divide in the adventitia of the blood vessels to form the perivascular nerve plexus(26; 27; 37). NADPH oxidase was previously shown to be localized to rat superior cervical ganglion (SCG) neurons and was suggested to be involved in regulating neuronal apoptosis (19; 46). However, since there is considerable heterogeneity in the morphological, neurochemical, and electrical properties of neurons from different sympathetic ganglia (21), the findings in SCG may not fully represent the case in other types of ganglia like. Therefore, a

complete understanding of the localization of NADPH oxidase in prevertebral sympathetic ganglia requires studies on other ganglia including CG and IMG. This study showed for the first time that $p22^{phox}$ and $p47^{phox}$ were present in rat CG and IMG neurons. The presence of NADPH oxidase in the neurons may provide novel mechanisms underlying the regulation of their activities. Evidence suggests that the neuronal properties, including firing rates and ion channel function, is indeed regulated by $O_2^{-\bullet}$ in the brain cardiovascular center (45; 51). There is also evidence showing that ROS modulate cellular gene expression (14), this also can be applicable to neurons. For example, we found that NADPH oxidase co-localizes with NPY in nearly all neurons in rat CG. Transcription factors such as activator protein 1 (AP-1) can be induced by ROS (24). Meanwhile, the modulation of NPY gene expression involves the activation of AP-1 signaling (20). Therefore, the coexistence of NADPH oxidase and NPY in the same neurons indicates a possible interaction between these two proteins. Given that NADPH oxidase can be activated by environmental stimulants like angiotensin II (Ang II) (13) or endothelin-1 (ET-1) (29), it is reasonable to hypothesize a possible mechanism by which NPY can be regulated by AngII or ET-1 via NADPH oxidase. On the other hand, sensory neurons located in the DRG revealed positive $p22^{phox}$ staining in the same neurons that were also positive for CGRP, a vasoactive neural peptide found in vasomotor sensory neurons. The colocalization of these two proteins may also indicate their potential interaction in the sensory neurons.

Noticeably, neurons have specific features that distinguish them from cells in other tissues, in which the chief functional compartments –the cell body, dendrites, axons, and terminals– are separated by considerable distance. Chemical/peptide transmission occurred at the neuro-vascular junctions directly determines the vascular tone. It is regulated at two levels: the electrical signals sent from the cell bodies and the local handling of neurotransmitters. In addition to its possible effects on neuronal firing as mentioned above, we would also like to know whether $O_2^{-\bullet}$ can more directly regulate the neurotransmission at the nerve terminal. However, $O_2^{-\bullet}$ is known to have an extremely short half-life and therefore, the direct actions of $O_2^{-\bullet}$ in the cell is greatly confined within a certain region that is determined by the subcellular site of its production. It is very unlikely that the $O_2^{-\bullet}$ produced up in the cell bodies can diffuse to the nerve terminals on the blood vessels. Most studies on the neuronal localization of NADPH oxidase to date have been limited to the cell bodies, with the exception that recent work from Picker's group showed a dendrite-associated NADPH oxidase staining in rat medial nucleus tractus solitarius (10; 11). We are by far the first group to investigate the localization of neuronal NADPH oxidase outside neural somata in the peripheral. Our novel findings of NADPH oxidase in perivascular sympathetic and sensory nerves suggest a possible role of NADPH oxidase-derived $O_2^{-\bullet}$ in the regulation of local neurotransmission.

The prototypical model of NADPH oxidase found in phagosome suggests that the catalytic core of the enzyme is a membrane-bound structure composed of a NOX subunit and $p22^{phox}$. Upon activation, it binds to NADPH and other cytosolic subunits at one side of the membrane while catalyzes the reduction of O_2 to $O_2^{-\bullet}$ to the other side (5). In addition to plasma membrane, several recent studies also suggested the presence of an intracellular vesicular membrane associated NADPH oxidase (30; 39). Based on these findings, we propose two possible locations of NADPH oxidase in the nerve fibers: the plasma membrane and the endosome/synaptic vesicle. The presence of these local NADPH oxidases could potentially have the following physiological significance. Take sympathetic nerve for example (Figure 3.12), it is suggested that nitric oxide (NO) can modulate sympathetic neurotransmission at the junction by reacting with norepinephrine (NE) resulting in its deactivation, thereby act as a protective effect against extra NE release from the sympathetic nerves (25). However, if there is a local $O_2^{-\bullet}$-producing site at the nerve terminal plasma membrane, like NADPH oxidase, the bioavailability of NO is then largely determined by the amount of $O_2^{-\bullet}$ on site because $O_2^{-\bullet}$ deactivates NO (16; 43). Therefore, the activity of NADPH oxidase at the sympathetic nerve terminal can potentially have an impact on the amount of NE being released to the neurovascular junction and the downstream vascular tone. In addition to release, sympathetic nerve varicosities are also the sites for the synthesis, storage and reuptake of NE. Once synthesized in the axonal

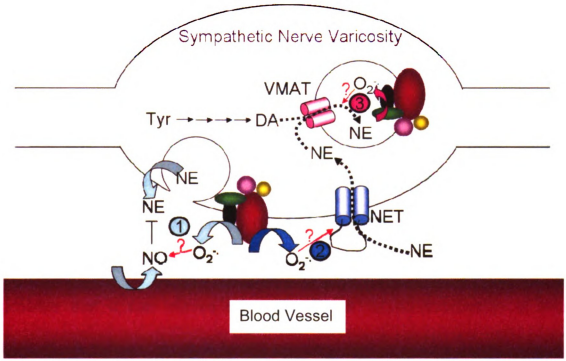


Figure 3.12. Schematic diagram of proposed mechanisms for the function of NADPH oxidase at the perivascular nerve terminal. The cartoon shows neurovascular junction forms by a single sympathetic varicosity and a blood vessel. The presence of NADPH oxidase at the nerve terminal may be located at the plasma membrane or the membrane of the synaptic vesicles. NADPH oxidsae may have an impact on noerpinephrine handling via three proposed mechanisms: 1) decrease bioavailability of nitric oxide (NO), and thereby limit the deactivation of NE by NO resulting in higher junctional NE level; 2) interfere with the reuptake of NE back to the presynaptic terminal via the modulation of norepinephrine transporter (NET) expression or function; 3) generate O2-• into the synaptic vesicle so as to affect the loading of NE into the vesicle via vesicular monoamine transporter (VMAT). Tyr=tyrosine; DA=dopamine.

cytoplasm, dopamine is loaded into synaptic vesicles by vesicular monoamine transporter 2 (VMAT2) and be further catalyzed into NE. After being released, NE is quickly cleared from the neurovascular junction mainly via uptake 1 through norepinephrine transporter (NET) on the plasma membrane and be further reloaded into synaptic vesicles via VMAT2. The function of VMAT2 and NET therefore plays indispensible roles in NE handling in the sympathetic varicosities. Interestingly, there is evidence showing that both VMAT2 and NET may be affected by ROS although the mechanisms underlying these effects have not been elucidated (7; 34). If NADPH oxidase is indeed present at the plasma membrane or synaptic vesicular membrane level at the nerve terminal, it is possible that it can involve in regulating the function/expression of these transporters thereby modulate the NE handling.

There are also non-sympathetic sources of NADPH oxidase in the nerve fibers surrounding the mesenteric arteries. The co-immunostaining with CGRP indicated its presence in sensory fibers, which is consistent with its staining in DRG neurons. Although the role of sensory nerves in blood pressure regulation is less defined as it is in sympathetic nerves, accumulating evidence has shown that sensory nerves play a counter-balancing role in preventing increases in blood pressure particularly in salt-induced hypertension (50), and there may be a ROS-associated mechanism as suggested by some recent studies including ours (3; 44). The presence of NADPH oxidase in the perivascular sensory fibers now

further provides insight into the role of locally produced $O_2^{-\bullet}$. NO also was shown to modulate sensory neurotransmission at the neurovascular junction (42). Therefore, the mechanism we proposed above for the role of $O_2^{-\bullet}$ in sympathetic neurotransmission may also apply to sensory nerves. Noticeably, the staining of NADPH oxidase was not yet limited to sympathetic and sensory nerve fibers. After both of them were depleted from the rats by celiac ganglionectomy (CGx), there was still substantial amount of remaining NADPH oxidase present in some nerve fibers (data not shown). These could either be some CGRP negative sensory fibers innervating the blood vessel without passing through the prevertebral ganglia which therefore cannot be abolished by CGx, or intestinofugal fibers which originate in the myenteric plexus, and terminate in prevertebral ganglia (31). More studies are needed to further identify the sources of these nerve fibers.

In summary, we have demonstrated that NADPH oxidase is expressed in sympathetic and sensory neurons as well as their perivascular nerve fibers. The findings of localized $O_2^{-\bullet}$ production at both the neuronal cell bodies and the prejunctional nerve terminals on the blood vessels are of great importance. These indicate that the regulation of NADPH oxidase by neurohormonal factors like Ang II or ET-1 in hypertension may have an impact on the activities of peripheral vasoactive neurons at two levels, which are by regulating gene expression in the cell bodies or modulating neurotransmission at the nerve terminals.

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CHAPTER FOUR

Xian Cao; Xiaoling Dai; Lindsay M. Parker; David L. Kreulen. (2007). Differential Regulation of NADPH Oxidase in Sympathetic and Sensory Ganglia in Deoxycorticosterone Acetate – Salt Hypertension. Hypertension. 2007 Oct; 50(4):663-71

CHAPTER 4: DIFFERENTIAL REGULATION OF NADPH OXIDASE IN SYMPATHETIC AND SENSORY GANGLIA IN DOCA-SALT HYPERTENSION

Abstract

We demonstrated recently that superoxide anion levels are elevated in prevertebral sympathetic ganglia of deoxycorticosterone acetate–salt hypertensive rats and that this superoxide anion is generated by reduced nicotinamideadenine dinucleotide phosphate oxidase. In this study we compared the reduced nicotinamide-adenine dinucleotide phosphate oxidase enzyme system of dorsal root ganglion (DRG) and sympathetic celiac ganglion (CG) and its regulation in hypertension. The reduced nicotinamide-adenine dinucleotide phosphate oxidase activity of ganglion extracts was measured using fluorescence spectrometry of dihydroethidine; the activity in hypertensive dorsal root ganglion was 34% lower than in normotensive DRG. In contrast, activity was 79% higher in hypertensive CG than normotensive CG. mRNA for the oxidase subunits NOX1, NOX2, NOX4, $p47^{phox}$, and $p22^{phox}$ were present in both CG and DRG; mRNA for NOX4 was significantly higher in CG than in DRG. The levels of mRNA and protein expression of the membrane-bound catalytic subunit $p22^{phox}$ and of the regulatory subunits $p47^{phox}$ and *Rac-1* were measured in CG and DRG in normotensive and hypertensive rats. $P22^{phox}$ mRNA and protein expression was

greater in CG of hypertensive rats but not in DRG. Compared with normotensive controls, $p47^{phox}$ mRNA and protein, as well as *Rac-1* protein, were significantly decreased in hypertensive DRG but not in CG. Immunohistochemical staining of $p47^{phox}$ showed translocation from cytoplasm to membrane in hypertensive CG but not in hypertensive DRG. This suggests that reduced nicotinamide-adenine dinucleotide phosphate oxidase activation in sympathetic neurons and sensory neurons is regulated in opposite directions in hypertension. This differential regulation may contribute to unbalanced vasomotor control and enhanced vasoconstriction in the splanchnic circulation.

Introduction

The splanchnic circulation is of great importance in regulating systemic blood pressure. It receives approximately 60% of the cardiac output and contains about one third of the total blood volume (26). The splanchnic circulation is innervated by both the sympathetic division of the autonomic nervous system (prevertebral sympathetic ganglion neurons, including celiac ganglia [CGs], superior and inferior mesenteric ganglia) and by spinal sensory nerves (dorsal root ganglia neurons [DRGs]). Elevated sympathetic nervous system activation has been shown in various types of hypertension (20; 36). In particular, sympathetic ganglionic blockade can reduce the arterial blood pressure increase in

deoxycorticosterone acetate (DOCA) -salt hypertension (16), indicating an important role of sympathetic ganglia in the development and maintenance of salt-induced hypertension. On the other hand, sensory nerves play a counter-regulatory role in preventing increases in blood pressure through either afferent baroreceptor-mediated mechanisms (8) or efferent release of vasodilatory neuropeptides, such as calcitonin gene–related peptide (CGRP) and substance P (SP) (15) (18). Altered synthesis or release of these vasodilator neuropeptides occurs in genetic and experimental hypertensive animal models (24; 40; 44).

Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase is an enzyme that catalyzes the production of superoxide anion ($O_2^{\bullet -}$) from oxygen and NADPH, and is considered the predominant source of $O_2^{\bullet -}$ in hypertension (45). It is a complex enzyme consisting of 2 membrane-bound components ($p22^{phox}$ and NOX) and 3 components in the cytosol ($p47^{phox}$ or NOXA1, $p67^{phox}$ or NOXO1 and $p40^{phox}$), plus a GTPase (*Rac-1* or *Rac-2*)(2). Activation of NADPH oxidase involves the translocation of regulatory elements from the cytoplasm to combine with catalytic subunits in the membrane (10). NADPH oxidase was first identified in phagocytes (4). It plays a vital role in nonspecific host defense against pathogens by generating large (millimolar) quantities of $O_2^{\bullet -}$ during the respiratory burst (41). More recently, the presence of NADPH oxidase in non-phagocyte cell types has been well identified. This is especially true in cardiovascular system

related tissues such as vascular endothelium (23), vascular smooth muscle (27), kidney cortex (7), and nervous system (25). Unlike in neutrophils, the NADPH oxidase in these tissues makes $O_2^{\bullet-}$ in small amounts for purposes of signaling under physiological conditions (22). However, excessive amounts of $O_2^{\bullet-}$ production leads to a variety of intracellular signaling events that ultimately may cause dysfunction of the system (35).This brings more attention to the pathophysiological role of this enzyme system in the regulation of cardiovascular diseases, such as hypertension.

Elevated NADPH oxidase-derived $O_2^{\bullet-}$ production in the vasculature (6) and the sympathetic neurons (13), accompanied by enhanced endothelin-1 (ET-1) signaling (28) and increased sympathetic system activity (16) are characteristic of DOCA-salt hypertension. Studies using this hypertensive animal model have shown that elevated arterial ET-1 levels lead to enhanced vascular $O_2^{\bullet-}$ production via the endothelin$_A$ (ET_A) receptor/NADPH oxidase pathway (29), while in prevertebral sympathetic ganglia, $O_2^{\bullet-}$ levels are increased due to enhanced activation of the ET_B/NADPH oxidase pathway (13). Although sensory neurons are known to participate in innervating the vasculature, the regulation of NADPH oxidase activity in sensory neurons has not been investigated in DOCA-salt hypertension. Possible differential regulation of $O_2^{\bullet-}$ in sympathetic and sensory ganglion neurons in cardiovascular diseases has been shown in apolipoprotein E

deficient mice, in which the levels of $O_2^{\bullet-}$ is increased in sympathetic ganglia neurons but not in nodose sensory neurons (31).

In this study, we measured the $O_2^{\bullet-}$ levels and the expression of NADPH oxidase subunits in sympathetic ganglia (CG) and sensory ganglia (DRG) and compared the expression levels in both normotensive and hypertensive conditions. We tested the hypothesis that NADPH oxidase is regulated differentially in sympathetic and sensory ganglia in DOCA-salt hypertension, in which the enzyme system is upregulated in CG but not in DRG.

Methods

Animals

All animal experiments were performed in accordance with the "Guide for the Care and Usage of Laboratory Animals" (National Research Council) and were approved by the Animal Use and Care Committee of Michigan State University. Adult male Sprague Dawley rats (250-300 g; Charles River Laboratories, Inc., Portage, MI) underwent uninephrectomy and subcutaneous implantation of DOCA (200 mg kg^{-1}) under isoflurane anesthesia. Post-operatively, the rats were given drinking water containing 1% NaCl and 0.2%KCl (herein, the DOCA-salt treated group is referred to as hypertensive (HT)). Normotensive controls (NT) to the hypertensive rats were uninephrectomized but were not given DOCA implantation

or salt drink. Four weeks after surgery, the arterial blood pressure was measured using the tail cuff method. Rats with a mean systolic arterial pressure of > 150 mmHg were considered hypertensive (30). The mean systolic arterial pressure for the HT rats and NT rats were 206.3±5.06 mmHg and 119.7±3.5 mmHg, respectively ($p<0.05$; n=20 in each group).

Tissue Harvest

Rats were sacrificed with a lethal dose of sodium pentobarbital (65 mg/kg *ip*); and the CG and DRG (spinal levels T13-L2) from HT and NT rats were removed and cleaned for further processing.

Measurement of NADPH Oxidase Activity

Activity of NADPH oxidase was measured using fluorescence spectrometry of dihydroethidine (DHE) in tissue homogenates of DRG and CG from NT rats and HT rats. DHE is oxidized to fluorescent ethidium by $O_2^{\bullet-}$. Ethidium will intercalate with DNA to further amplify the fluorescent signal and The intensity of the fluorescent signal is proportional to $O_2^{\bullet-}$ levels (5; 51).In a microtiter plate, freshly prepared DRG homogenates were incubated with DHE (10μmol/L), salmon testes DNA (0.5mg/mL, Stratagene, La Jolla, CA) and the substrate for NADPH oxidase, β-NADPH (0.1mmol/L, Sigma, St. Louis, MO), for 30 minutes at 37°C in a dark chamber. Salmon testes DNA was added to bind to ethidium and consequently

stabilize ethidium fluorescence, thereby increasing the sensitivity of $O_2^{\cdot-}$ measurement >40-fold (51). A parallel control group was analyzed in each run with no substrate added into the reaction. Ethidium-DNA fluorescence was measured at an excitation of 485±40nm and an emission of 590±35nm using a Biotek FL600 fluorescence plate reader (Bio-Tek Instruments, Inc., Winooski, VT). The enzyme activity was measured as total fluorescence units per minute per milligram tissue homogenate. Before statistical analysis, to eliminate the background fluorescence the no-substrate control readings were subtracted from the fluorescence readings of the wells with substrate. . NT rat ganglia were normalized to 100% in both CG and DRG independently. Experimental results are presented as the percent changes of fluorescence from NT to HT.

Reverse Transcription - Polymerase Chain Reaction (RT-PCR) and Quantitative Real-time RT-PCR (qPCR)

Fresh CG and DRG harvested from NT and HT rats were immediately placed in RNA*later* RNA stabilization Reagent (Qiagen, Valencia, CA). Total RNA was isolated from the ganglia using RNeasy Mini kit (Qiagen). cDNA was synthesized using Superscript II mix (Invitrogen, Carlsbad, CA). The cDNA synthesized from 2ug or 50ng total RNA was used in subsequent PCR or qPCR, respectively. All primers were derived from the *Rattus Norvegicus* gene (National Center for Biotechnology Information GenBank). Primer sequences are shown in Table 4.1.

Table 4.1 Primer sequences for NADPH oxidase subunits NOX1,NOX2, NOX4, p47phox and p22phox, β-actin and GAPDH

Gene	Sequence	Amplicon Length (bp)	NCBI accession Number
NOX1	For:5' TGAACAACAGCACTCACCAATGCC 3'	245	AF152963
	Rev:5' AGTTGTTGAACCAGGCAAAGGCAC 3'		
NOX2	For:5' GTGGAGTGGTGTGTGAATGC 3'	324	AF298656
	Rev:5' TCCACGTACAATTCGCTCAG 3'		
NOX4	For:5' ACCAGATGTTGGGCCTAGGATTGT 3'	261	AY027537
	Rev:5' AGTTCACTGAGAAGTTCAGGGCGT 3'		
NOX4	For:5' TCATGGATCTTTGCCTGGAGGGTT 3'	110	
(qPCR)	Rev:5' AGGTCTGTGGGAAATGAGCTTGGA 3'		
p47phox	For:5' GGCCAAAGATGGCAAGAATA 3'	221	AF260779
	Rev:5' TGTCAAGGGGCTCCAAATAG 3'		
p47phox	For: 5' AGGTTGGGTCCCTGCATCCTATTT 3'	95	
(qPCR)	Rev: 5' TGGTTACATACGGTTCACCTGCGT 3'		
p22phox	For:5' TTGTTGCAGGAGTGCTCATC 3'	282	U18729
	Rev:5' TAGGCTCAATGGGAGTCCAC 3'		
p22phox	For: 5' TGTTGCAGGAGTGCTCATCTGTCT 3'	150	
(qPCR)	Rev: 5' AGGACAGCCCGGACGTAGTAATTT 3'		
β-actin	For:5' GGCTACAGCTTCACCACCAC 3'	500	V01217
	Rev:5' TACTCCTGCTTGCTGATCCAC 3'		
GAPDH	For:5' ATCACTGCCACTCAGAAG 3'	317	NM017008
(qPCR)	Rev:5' AAGTCACAGGAGACAACC 3'		

PCR products were electrophoresed on a 2.0% agarose gel for 60 minutes at 9V/cm gel. Bands corresponding to PCR amplicons were stained by ethidium bromide and visualized by UV light. qPCR was performed using Mx3000P QPCR system (Stratagene). SYBR green was used as the fluorescence detector in the qPCR. Serial dilution was performed for each set of qPCR primers to determine its qPCR amplification efficiency (*E*) before the experimental run. A dissociation protocol (60-95 °C melt) was done at each end of the experiment to verify that only one amplicon was formed during the process of amplification. End point, used in qPCR quantification and Ct value, is defined as the PCR cycle number that crosses an arbitrarily placed signal threshold. The relative expression ratio of the target gene was calculated, based on its *E* and Ct difference (Δ) of sample versus control ($\Delta Ct_{control - sample}$) (Equation 1). Statistical analysis was performed by Pair Wise Fixed Reallocation Randomization Test© (http://www.gene-quantification.info) using Relative Expression Software Tool (REST) (34).

$$\text{Expression Ratio} = (E_{target})^{\Delta Ct}{}_{target(control - sample)} / (E_{GAP})^{\Delta Ct}{}_{GAP\ (control - sample)} \quad \text{--- 1}$$

Protein Isolation and Subcellular Fractionation

CG and DRG homogenates were extracted on ice with lysis buffer (10mM HEPES, 150mM NaCl, 1mM EDTA, 0.5% Triton X-100, protease inhibitor cocktail (1:100, Sigma, St. Louis, MO)). After homogenization the tissue lysates were

quickly centrifuged at 700×*g* for 5 min 4°C to pellet nucleic protein and any insoluble debris. The supernatant was saved for measurement of total protein. For subcellular fraction of CG protein, tissue was first harvested and homogenized in ice cold lysis buffer without Triton X-100. Tissue homogenates were then first centrifuged as described above, after which the supernatant were furthered centrifuged at 100,000 ×*g* for 60 min at 4°C. The resulting supernatant was saved as cytosolic protein. The pellet was resuspended in lysis buffer and saved as plasma membrane rich protein. Protein quantification was performed using Bradford protein assay (Bio Rad Laboratories, Hercules, CA).

Western Blotting

Equal amounts of protein were separated by 7.5%-15% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. After 2hrs of blocking in Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, membranes were incubated overnight at 4°C with specific primary antibodies: $p22^{phox}$(1:500, Santa Cruz Biotechnology, Santa Cruz, CA), $p47^{phox}$ (1:1000, Santa Cruz) and *Rac-1* (1:1000, Upstate, Charlottesville, VA). Following incubation in primary antibody, blots were washed and exposed to the HRP-linked secondary antibody for 1 hour at 4°C with rocking (anti-rabbit IgG, 1:2000; anti-mouse IgG, 1:5000; Santa Cruz). Finally, blots were washed and chemiluminescent detection of bands was performed (Pierce, Rockford, IL). To control for variation in protein

loading, membranes were stripped and reprobed with anti-α-actin (1:1000; Sigma) or with anti-Pan-Cadherin (1:1000, Zymed Laboratories Inc, South San Francisco, CA), a plasma membrane protein marker. CG subcellular fraction western blot was performed on one membrane with protein from 5 ganglia. Scanned films were analyzed for band density and area using NIH Image software.

Immunohistochemistry

Ganglia were dissected and fixed in 10% formalin for 2 hours then transferred to 70% ethanol for storage ranging from several hours to overnight. Tissue was processed using a vacuum infiltration tissue processor (Thermo Electron Excelsior) with decreasing concentrations of ethanol followed by xylene. Tissues were embedded in paraffin, sectioned on a rotary microtome into 5 μm sections, and mounted on to glass slides (Corning Glass). Heat induced epitope retrieval (HIER) was used. Samples were blocked for endogenous elements with hydrogen peroxide/methanol for 30 minutes then rinsed. Due to the use of HIER, an additional blocking step of avidin and biotin was used with a 15 minute incubation. Normal goat serum (1:28, Vector Laboratories, Burlingame, CA) was used as a protein block followed by incubation in $p47^{phox}$ primary antibody (1:150, Santa Cruz) for 60-minutes. Incubation of biotinylated goat-anti-rabbit secondary antibody (1:200, Vector Laboratories) for 30 minutes was followed by a 15 minute incubation with Nova Red chromagen (Vector Laboratories). Slides were

counterstained with Lerner 2 hematoxylin then dehydrated. Images were collected using standard brightfield microscopy (Olympus BX60 with SPOT Insight Digital Camera, Olympus America Inc. Center Valley, PA). A "no primary control" was run in parallel without addition of primary antibody to assess antibody specificity. Images were analyzed for staining density and area using NIH Image software.

Data Analysis

Data are presented as mean±SE of the mean. Statistical significance of NADPH oxidase activity, western blotting, immunohistochemistry data were assessed by Student *t* test using Prism 4.0 software (GraphPad Software, San Diego, CA). qPCR data statistical significance were assessed by Pair Wise Fixed Reallocation Randomization Test© using REST software. In both cases, $p<0.05$ indicates statistical significance.

Results

Expression of NADPH oxidase mRNA in normal rat DRG and CG

PCR amplicons of NADPH oxidase subunits $p47^{phox}$, $p22^{phox}$, NOX1, NOX2 and NOX4 were detected in RNA extracts of DRG and CG from normal rats that did not receive DOCA-salt treatments (Figure 4.1A). These amplicons were at the

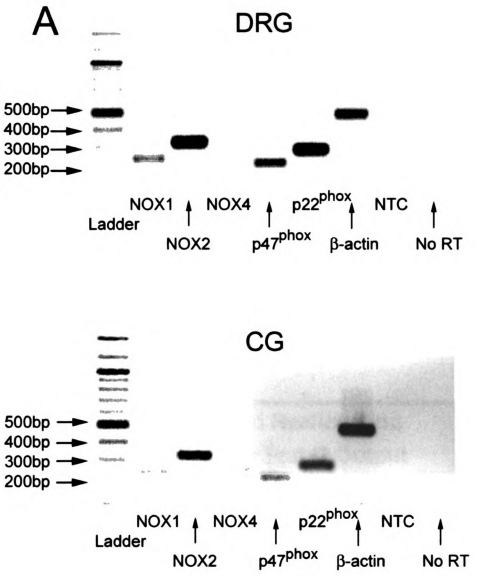
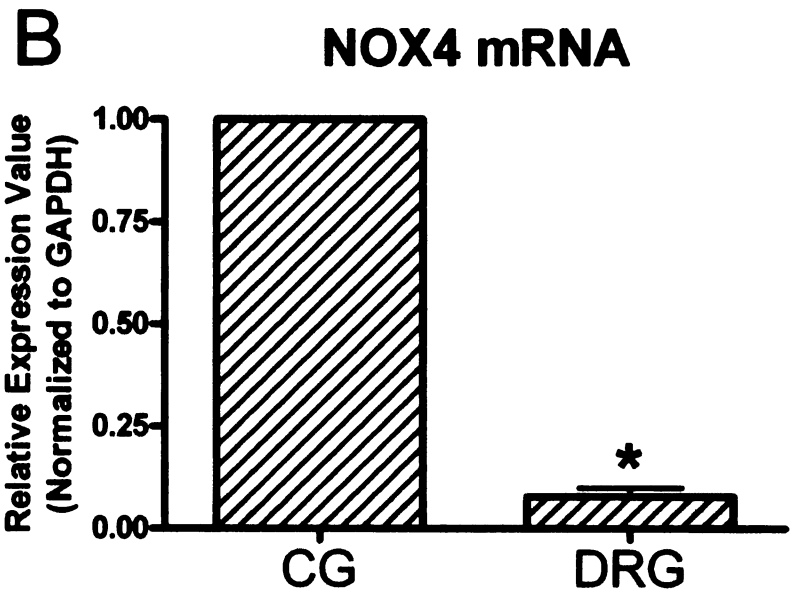


Figure 4.1. NADPH oxidase subunits are expressed in dorsal root ganglia (DRG) and celiac ganglia (CG). A) PCR amplicons for NOX1, NOX2, p47phox, p22phox and β-actin were present on ethidium bromide-stained agarose gels from DRG (top) and CG (bottom). PCR step with no cDNA template added (NTC) and reverse transcription step without adding the transcriptase enzyme (No RT) were performed as negative controls. B) qPCR results show NOX4 mRNA level is significantly lower in DRG then in CG in normal rats. The expression ratio of NOX4 in DRG vs. CG is 0.077 (n=7 normal rats). Relative expression value calculation and statistical analysis were performed by REST software. The randomisation test output from REST is listed in table format (bottom) attached to the bar graph. The significance ($P<0.05$) is indicated by * vs. CG.

Figure 4.1 continued



Pair Wise Fixed Reallocation Randomisation Test © Output		
Genes	GAPDH	NOX4
PCR Efficiencies	1.983	2.043
Control (CG) Ct Means	18.926	30.239
Sample (DRG) Ct Means	17.830	32.786
Expression Ratio		0.077
p-Value		0.001
Randomisations	2000 of 2000 done	

expected sizes of 221bp, 282bp, 324bp, 245bp and 261bp, respectively. PCR products from CG ganglia were consistent with our previous findings in dissociated CG neurons (12). However, DRG NOX4 mRNA was barely detectable on the regular PCR gel compared to CG. qPCR was then performed to determine the relative expression levels of NOX4 in normal DRG and CG. Results showed that the expression ratio of NOX4 in DRG and CG was 0.077 (Figure 4.1B) ($p<0.05$ vs CG; n=7).

NADPH oxidase activity in DRGs and CGs in NT and HT Rats

Tissue homogenates of DRGs and CGs from NT and HT animals were incubated with the NADPH oxidase substrate β-NADPH, and the formation of $O_2^{-\bullet}$ was detected in the reaction mixture. The NADPH oxidase activity of DRG homogenates from HT rats was 34% lower than the activity of homogenates from NT animals (Figure 4.2; $P<0.05$ versus NT; n=3). This result demonstrates that the NADPH oxidase enzymatic activity in tissue homogenates of HT DRGs is less than this activity in NT DRGs. Meanwhile, the NADPH oxidase activity in HT CGs is 78.6% higher than NT CGs ($P<0.05$ versus NT; n=6) (12).

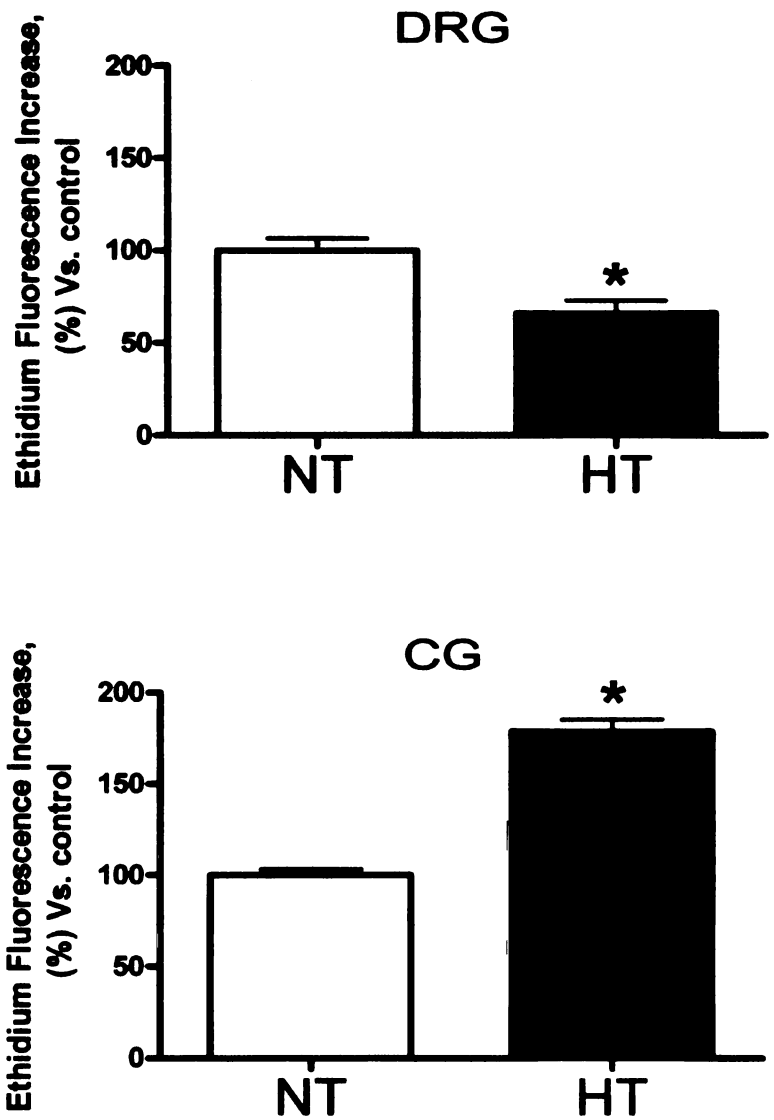


Figure 4.2: NADPH oxidase activity in DRG and CG. NADPH oxidase activity is lower in DRG from DOCA-salt hypertensive (HT) rats than from normotensive (NT) control rats, but is higher in HT CG than NT CG. β-NADPH was used as NADPH oxidase substrate. Results represent the percent changes of dihydroethidine (DHE) fluorescence intensity in the ganglia homogenate from no-substrate controls to substrate-treated groups in both HT and NT rats. The NADPH oxidase activity of DRG homogenates from HT rats was 34% lower than from NT animals (n=3) (top); meanwhile, the NADPH oxidase in HT CG is 78.6% higher than NT CG (n=6) (bottom). The significance ($P<0.05$) is indicated by * vs. NT.

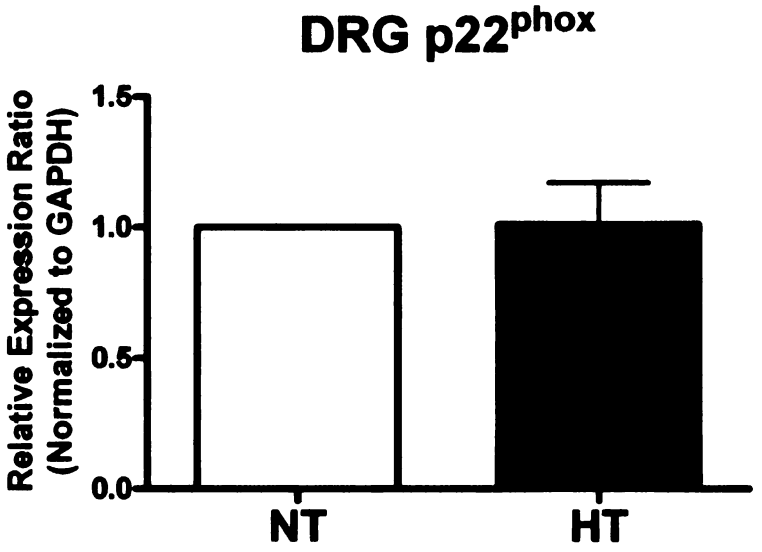
NADPH Oxidase Subunit mRNA Levels in DRGs and CGs in NT and HT Rats

$P22^{phox}$ and $p47^{phox}$ mRNA were both present in DRGs and CGs as shown above, and these subunits are critical in mediating the NADPH oxidase enzyme activity (1; 2) . We, therefore, compared the levels of $p22^{phox}$ and $p47^{phox}$ mRNA in RNA extracts of DRGs and CGs from NT and HT rats using qPCR. The mRNA level of $p22^{phox}$ in CG was significantly greater in HT animals compared with NT by the factor 1.776 ($P<0.05$ versus NT rats; n=7 NT rats; n=6 HT rats), whereas its level was unchanged in DRG (Figure 4.3A and B). On the other hand, $p47^{phox}$ mRNA was significantly lower in HT DRGs compared with NT DRGs. The relative expression ratio of $p47^{phox}$ mRNA in HT DRGs to NT DRGs is 0.379 ($P<0.05$ versus NT rats; n=7 NT rats; n=5 HT rats), whereas there was no significant difference between $p47^{phox}$ mRNA in NT CGs and HT CGs (Figure 4.3C and D).

NADPH Oxidase Subunit Protein Expression Levels in CGs and DRGs in NT and HT Rats

In addition to $p22^{phox}$ and $p47^{phox}$, we also measured *Rac-1* protein expression levels in the ganglia in NT and HT rats, because the protein expression of this regulatory factor has been associated with NADPH oxidase activity in the nervous system (49). The protein expression of $p22^{phox}$, $p47^{phox}$, and *Rac-1* in CGs and DRGs was examined by Western blotting analysis. The

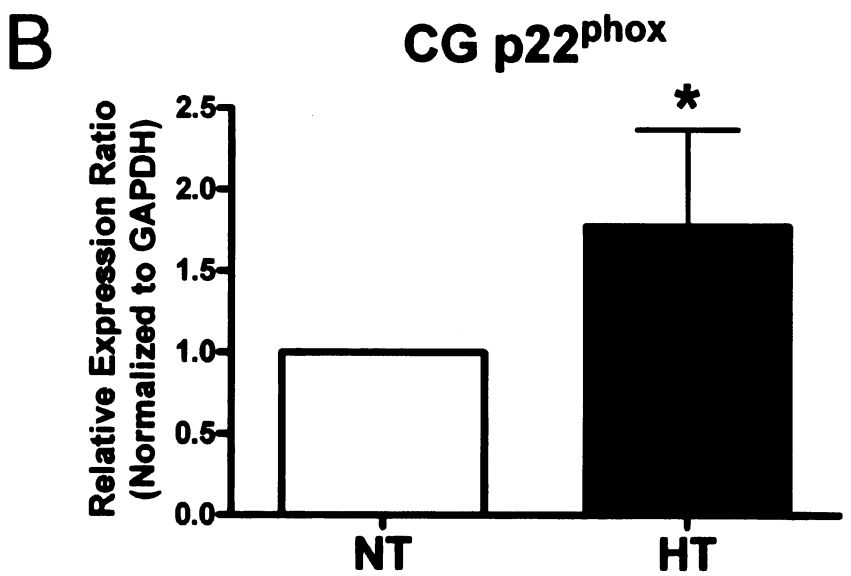
A



Pair Wise Fixed Reallocation Randomisation Test © Output		
Genes	GAPDH	$p22^{phox}$
PCR Efficiencies	1.983	2.107
Control (NT) Ct Means	17.711	22.014
Sample (HT) Ct Means	17.600	21.896
Expression Ratio		1.012
p-Value		0.955
Randomisations	2000 of 2000 done	

Figure 4.3: p22phox mRNA level is higher in CG in HT animals compare to NT controls, and p47phox mRNA is lower in HT DRG than in NT DRG. Panel A and B show the mRNA level of p22phox in CG was significantly greater in HT animals compared with NT by the factor 1.776 (n=7 NT rats, n=6 HT rats), while its level was unchanged in DRG. Panel C and D show p47phox mRNA was significantly lower in HT DRG compared to NT DRG. The relative expression ratio of p47phox mRNA in HT DRG to NT DRG is 0.379 (n=7 NT rats, n=5 HT rats), while there was no significant difference between p47phox mRNA in NT CG and HT CG. All qPCR data is normalized to GAPDH. Results are shown in table form from REST software analysis output (bottom), and in graphical form (top). The significance ($P<0.05$) is indicated by *vs. NT.

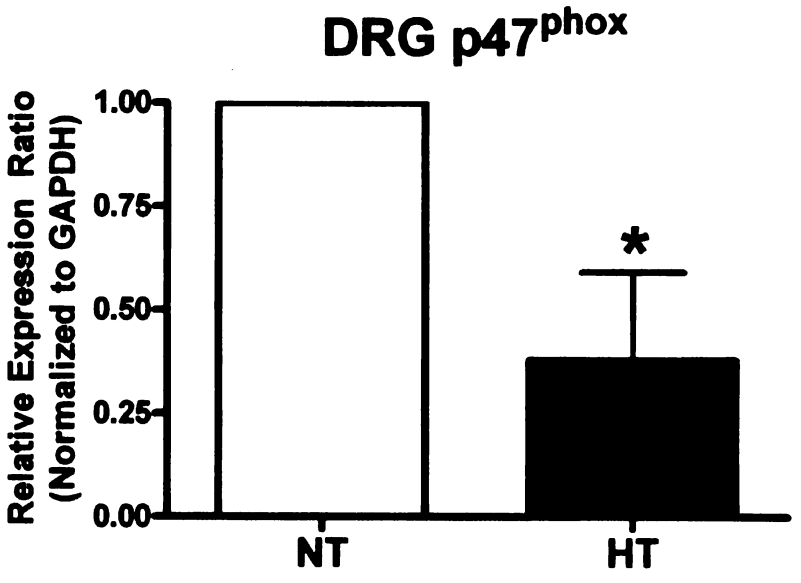
Figure 4.3 continued



Pair Wise Fixed Reallocation Randomisation Test © Output		
Genes	GAPDH	$p22^{phox}$
PCR Efficiencies	1.983	2.107
Control (NT) Ct Means	21.044	23.697
Sample (HT) Ct Means	22.200	23.988
Expression Ratio		1.776
p-Value		0.028
Randomisations	2000 of 2000 done	

Figure 4.3 continued

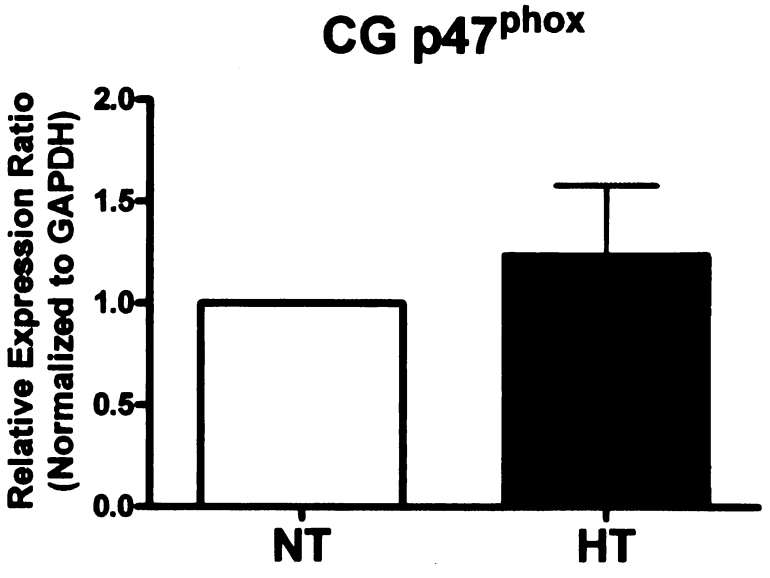
C



Pair Wise Fixed Reallocation Randomisation Test © Output		
Genes	GAPDH	$p47^{phox}$
PCR Efficiencies	1.983	2.038
Control (NT) Ct Means	18.559	27.264
Sample (HT) Ct Means	18.554	28.622
Expression Ratio		0.379
p-Value		0.048
Randomisations	2000 of 2000 done	

Figure 4.3 continued

D



Pair Wise Fixed Reallocation Randomisation Test © Output		
Genes	GAPDH	$p47^{phox}$
PCR Efficiencies	1.983	2.038
Control (NT) Ct Means	21.434	28.100
Sample (HT) Ct Means	22.765	29.083
Expression Ratio		1.235
p-Value		0.546
Randomisations	2000 of 2000 done	

expression of $p22^{phox}$ was greater in HT CGs than in NT CGs ($P<0.05$ versus NT rats; n=6), and this paralleled its greater mRNA levels shown above. Similar to its unchanged mRNA levels in DRGs, the $p22^{phox}$ protein expression was not significantly different between NT and HT rats (Figure 4.4A). In CGs, there was no difference between NT and HT rats in the amounts of $p47^{phox}$ and *Rac-1* in total protein fractions. Meanwhile, HT DRGs showed a different pattern in the expression of these 2 subunits. There was a profound downregulation of $p47^{phox}$ ($P<0.05$ versus NT rats; n=4; Figure 4.4B), as well as a significant decrease in *Rac-1* expression ($P<0.05$ versus NT rats; n=3; Figure 4.4C) in total protein preparation.

We also analyzed the $p47^{phox}$ expression in CGs and DRGs with immunohistochemistry. In both CGs and DRGs there was intense staining associated with the neuron cell bodies with little or no staining of intercellular elements. Compared with NT CGs, there was a significant redistribution of p47phox to the plasma membrane of neurons in the HT CGs ($P<0.05$ versus NT rats; n=7 neurons in NT CGs; n=14 neurons in HT CGs; Figure 4.5A and B). We observed a similar redistribution pattern in Western blotting of CG subcellular fractions; in HT CGs there was lower expression of $p47^{phox}$ in cytosolic fractions accompanied by greater $p47^{phox}$ expression in membrane fractions (Figure 4.5C). On the other hand, immunohistochemical staining of DRGs showed that the total

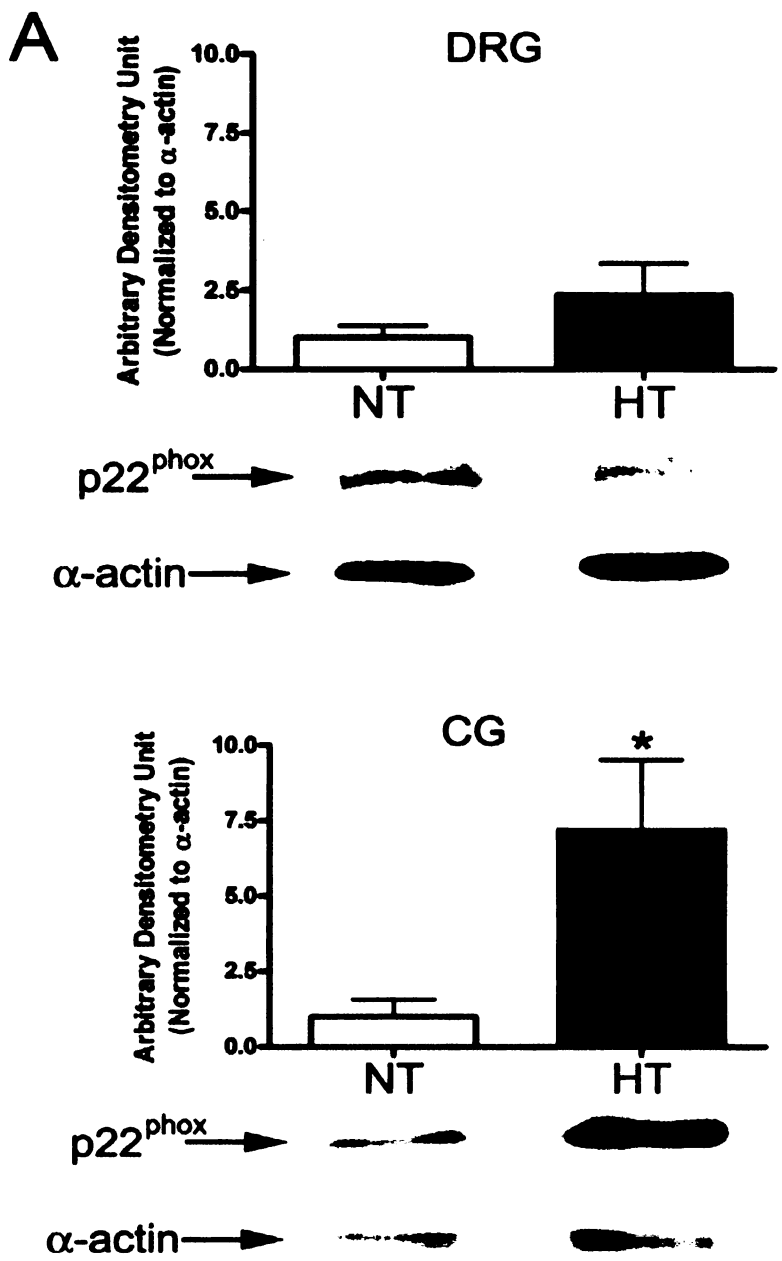


Figure 4.4: Western blot data from ganglia homogenate reveals that p22phox, p47phox and *Rac-1* are present in DRG and CG, and are differentially regulated in HT and NT animals. Panel A shows that there is no significant difference in the amount of p22phox protein expression in NT DRG versus HT DRG (top), while it is higher in HT CG than in NT CG (bottom) (n=6). Representative blots are shown below each figure. Panel B shows p47phox protein is significantly decreased in HT DRG compare to NT DRG (n=4) but is not different between NT and HT CG. Panel C shows the protein expression of *Rac-1* is lower in HT DRG than in NT DRG (n=3), and there is no significant difference between *Rac-1* protein levels in NT and HT CG. All data are normalized to β-actin before statistical analysis. The significance ($P<0.05$) is indicated by *vs. NT.

Figure 4.4 continued

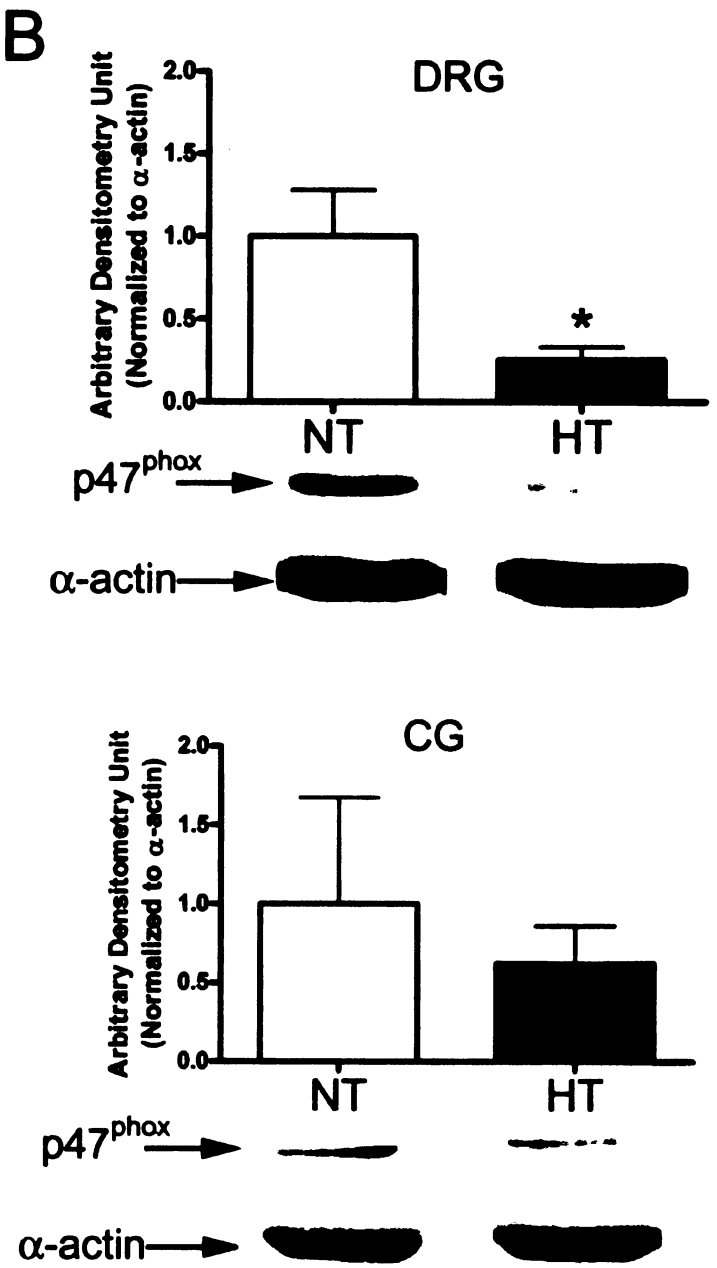


Figure 4.4 continued

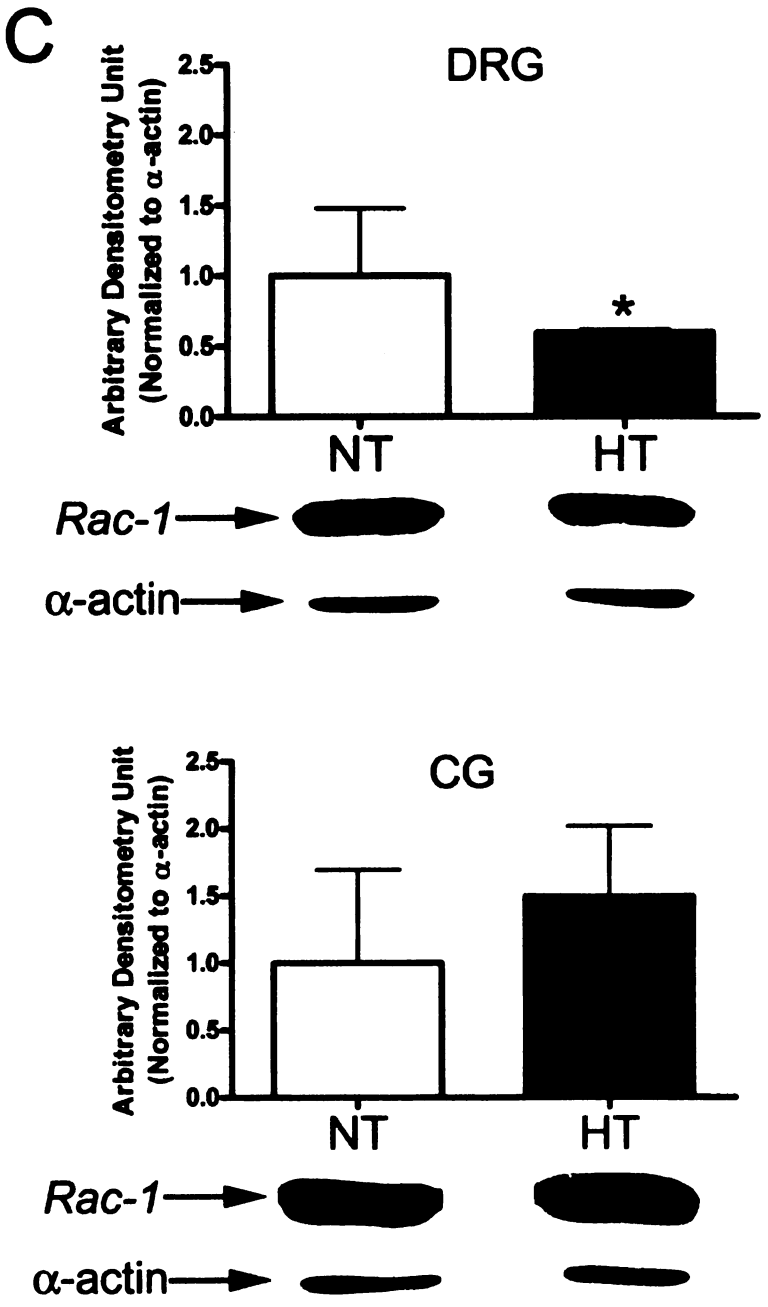


Figure 4.5 p47phox protein is redistributed in the CG neurons in HT animals compared to NT controls. A) Immunohistochemistry reveals p47phox protein localization in CG neurons (shown in red). Left three panels are CG from NT rats and right three panels are HT CG (top to bottom: 20x magnification, 100x oil objective, no primary antibody controls). Representative images show p47phox protein is present in most cells within the ganglia. The membrane localization of p47phox is significantly higher in HT CG than in NT CG (arrow), indicating a translocation of this protein from the cytoplasm to the plasma membrane. Scale bars are 50μm. Plots of the density measurements are shown in panel B): In HT CG there was a significant increase in plasma membrane density (*$p<0.05$ vs NT; n=7 neurons in NT CG, n=14 neurons in HT CG) , but no change in total (plasma membrane + cytoplasm) staining density; C) Western blot performed on CG subcellular fraction protein shows that in cytosol fraction, NT CG has higher p47phox protein expression than HT CG. However, in membrane-rich fraction, HT CG has more p47phox than NT CG. Membranes are stripped and re-probed with Pan-cadherin, a plasma membrane marker, indicating membrane-rich protein preparation (n=5, pooled ganglia).

A

CG

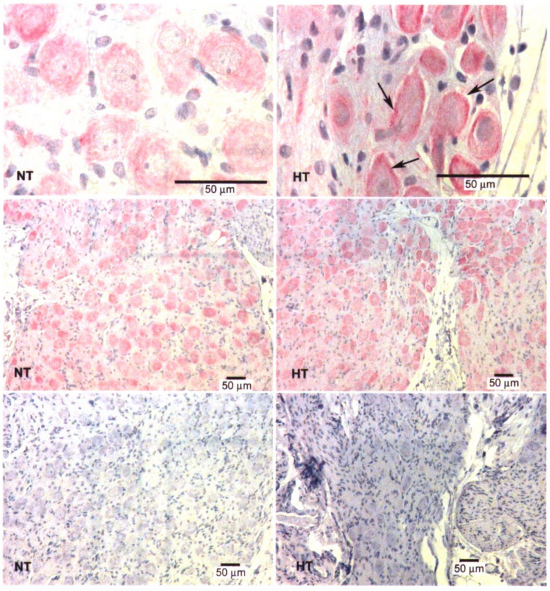
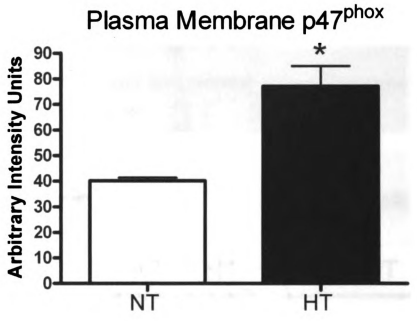


Figure 4.5 continued

B



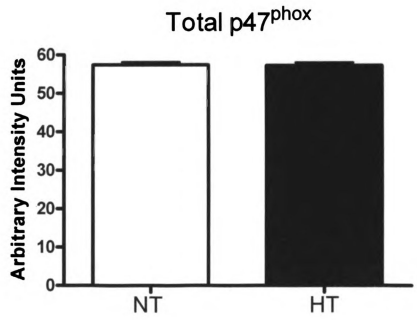
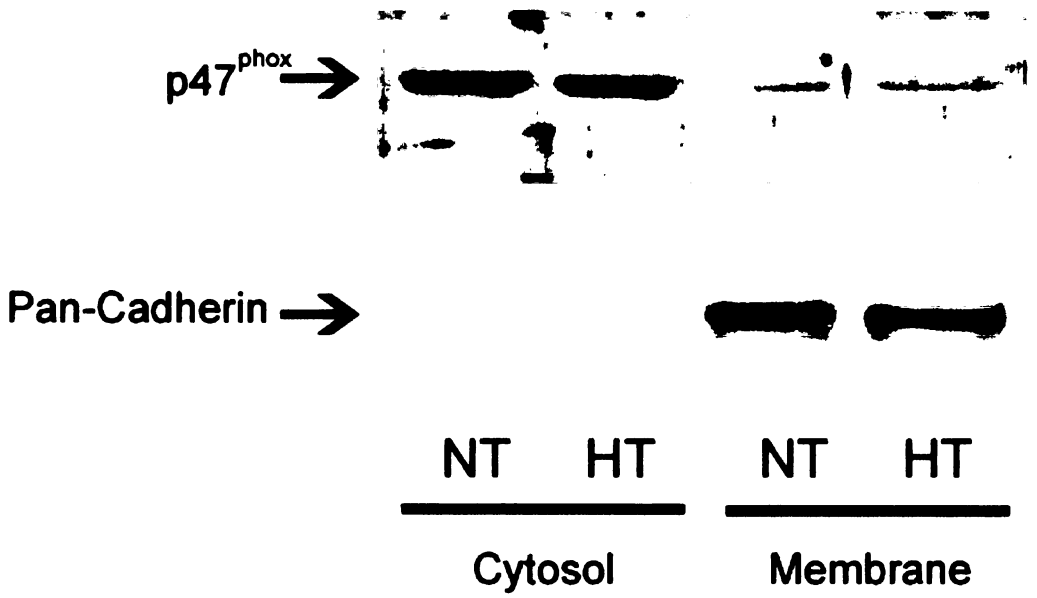


Figure 4.5 continued

C



$p47^{phox}$ staining was decreased in HT DRGs as compared with NT DRGs ($P<0.05$ versus NT rats; n=61 neurons in NT DRGs; n=91 neurons in HT DRGs; Figure 4.6). This is consistent with our Western blotting data in which there was decreased $p47^{phox}$ protein expression in HT DRGs (see Figure 4.4). However, there was no $p47^{phox}$ redistribution from cytosol to membrane in HT DRGs. This suggests that the translocation of $p47^{phox}$ from the cytoplasm to the plasma membrane may contribute to the elevated NADPH oxidase activity in HT CGs, whereas in HT DRGs the lack of this translocation, as well as the decreased expression of total $p47^{phox}$ protein, could contribute to the lower activity level of the enzyme.

Discussion

In this study, we have shown for the first time that, in DOCA-salt hypertension, NADPH oxidase– derived reactive oxygen species production is regulated in opposite directions in sympathetic ganglion neurons and in primary sensory neurons. Whereas $O_2^{-\bullet}$ production and NADPH oxidase activity are increased in sympathetic ganglia in HT rats (12; 13), they are decreased in DRGs. The expression of NOX4 is much higher in CGs than in DRGs. Furthermore, $p22^{phox}$ is increased in HT CGs, whereas $p47^{phox}$ and *Rac-1* are

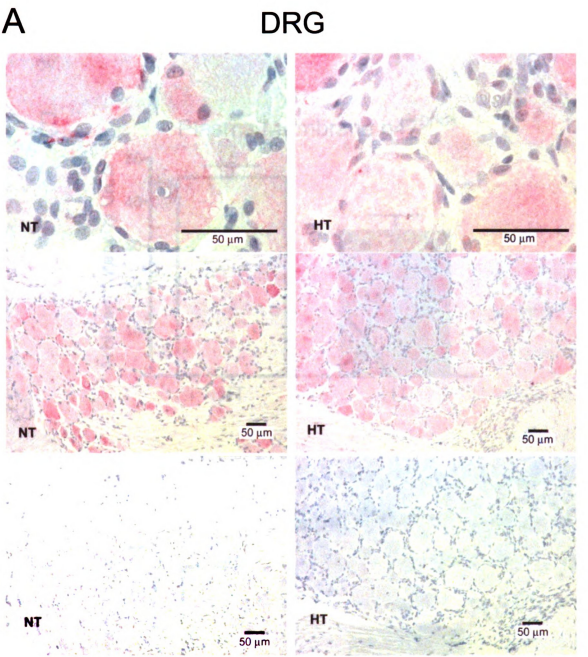
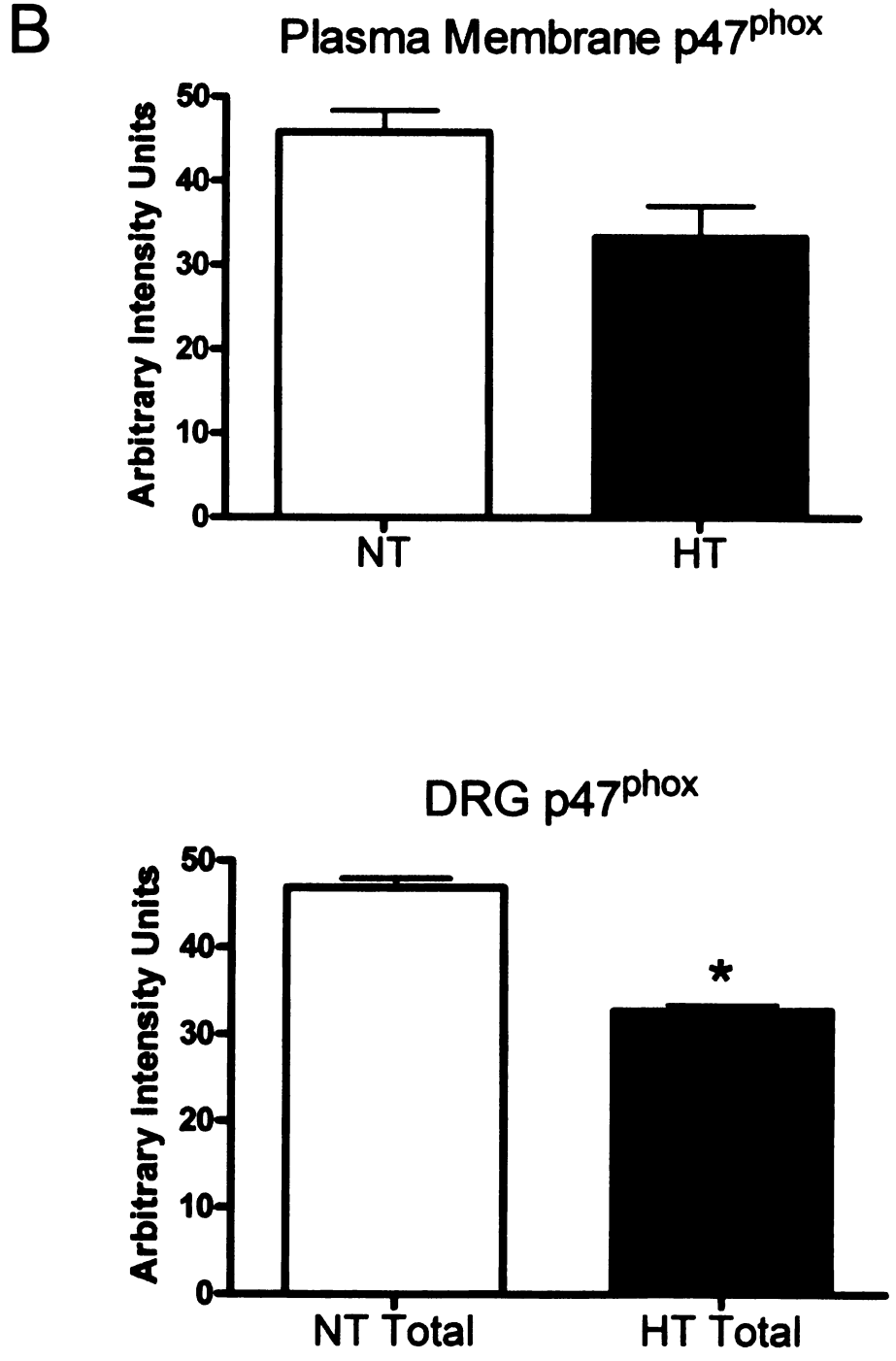


Figure 4.6: Immunohistochemistry reveals p47phox localization in DRG neurons. A)Left three panels are DRG sections from NT rats and right three panels are from HT rats (top to bottom: 20x magnification, 100x oil objective, no primary antibody controls). The micrographs show p47phox staining (red) throughout the cytoplasm of the ganglion cell bodies with limited membrane localization in both NT and HT DRG. Scale bars are 50μm. Plots of the density measurements are shown in panel B): The total amount of p47phox is significantly lower in HT DRG than in NT. (*$p<0.05$ vs NT; n=61 neurons in NT DRG, n=91 neurons in HT DRG).

Figure 4.6 continued



decreased in HT DRGs. Finally, $p47^{phox}$ is translocated from the cytoplasm to the plasma membrane in HT CGs but not in DRGs.

NADPH oxidase activity can be determined by 2 major factors: the capability of different NOX isoforms to catalyze electron transfer reactions and/or the availability of the cytosolic regulatory subunits. The expression pattern and level of the core protein, as well as the regulatory subunits, can affect the enzyme activity level. First, the differential regulation of NADPH oxidase activity in CGs and DRGs in hypertension may be because of their differences in the expression of NOX isoforms. The formation of the catalytic core of NADPH oxidase between either one of the NOX isoforms and $p22^{phox}$ is essential for the production of $O_2^{-\bullet}$ (39). However, whereas the activation of catalytic complexes made with NOX1/NOX2 and $p22^{phox}$ requires the addition of cytosolic regulatory subunits, such as $p47^{phox}$ or the GTPase *Rac* (3), NOX4-$p22^{phox}$ produces $O_2^{-\bullet}$ constitutively without combining with other subunits (32). Different from the other 2 isoforms, the expression of NOX4 is much higher in CGs than in DRGs. It is then conceivable that, because a large part of the oxidase in CGs where NOX4 expression is high contains only NOX4-$p22^{phox}$, the $p22^{phox}$ increase that we observed in HT CGs may be responsible for the elevated oxidase activity even if the expression levels of regulatory subunits $p47^{phox}$ and *Rac-1* were unchanged.

Second, differences in the availability of regulatory subunits can affect NADPH oxidase activity. For example, in NOX1- or NOX2-based NADPH

oxidase, $O_2^{-\bullet}$ generation is regulated by the concentration of $p47^{phox}$ and *Rac-1* (9; 11; 14), and inhibition of $p47^{phox}$ or *Rac-1* expression can result in a decrease in $O_2^{-\bullet}$ production (38; 49). Therefore, the decreased expression of $p47^{phox}$ and *Rac-1* in HT DRGs, where the NOX1 and NOX2 dominate, is likely to result in lower oxidase activity. Moreover, NADPH oxidase activation involves the translocation of regulatory subunits from the cytoplasm to combine with catalytic core in the membrane. The redistribution of regulatory subunits can be another indicator for oxidase activity level. There is increased membrane-bound $p47^{phox}$ in HT CG but not in HT DRG, indicating that the translocation of $p47^{phox}$ may contribute to enhanced oxidase activity in HT CG, whereas the lack of this translocation accompanied by decreased total $p47^{phox}$ expression may explain the attenuated oxidase activity in HT DRG.

In hypertension, enhanced NADPH oxidase activity and expression occur in various tissue types, including vasculature (29; 37), kidney (7), and the nervous system (12; 50). There is a positive correlation between reactive oxygen species levels in the nervous system and sympathetic neuronal activity in hypertension. For example, removal of extracellular $O_2^{-\bullet}$ or reactive nitrogen species within the rostral ventrolateral medulla by microinjection of superoxide dismutase reduces sympathetic nervous system activity in animals subjected to oxidative stress (46); also, intravenous administration of the superoxide dismutase mimetic Tempol lowers mean blood pressure and renal sympathetic nervous system activity in the

DOCA-salt hypertensive model (43). Activation of ET_B receptors increases $O_2^{-\bullet}$ production in prevertebral sympathetic ganglia both in vitro (13) and in vivo (17). In these ganglia, ET_B receptor expression and NADPH oxidase-derived $O_2^{-\bullet}$ generation are elevated in DOCA-salt hypertension (13). Because DOCA-salt hypertension is characterized by sympathetic hyperactivation, elevated $O_2^{-\bullet}$ levels in sympathetic ganglia may directly or indirectly contribute to the hypertension.

The relationship of changes in reactive oxygen species levels in sensory neurons to blood pressure regulation is not known but could be related to interactions between sensory neurons and sympathetic ganglionic neurons (26; 48) or of sensory nerves directly with the vasculature. In salt-sensitive hypertension, synthesis and release of vasoactive neuropeptides from sensory ganglia innervating the splanchnic circulation are increased (40; 42), and this may play a role in blood pressure regulation (33), but it is not known whether these are related to the observed decreases in the activity of NADPH oxidase.

One of the important findings of the present study is that NADPH oxidase activity is decreased in extracts of spinal sensory ganglia in hypertension; this is in contrast to sympathetic ganglia, where it is increased in hypertension. Both types of ganglia are made up of neurons and satellite cells, but in both types, the presence of the enzyme appears limited to the neurons. Dorsal root ganglia are a mixture of neurons with different functional and neurochemical properties, and only a subset of the neurons innervates the vasculature and release

neuropeptides. Sensory nerve fibers that innervate the systemic blood vessels contain the vasodilatory neuropeptides calcitonin gene-related peptide and substance P (19), and subsets of dorsal root ganglion neurons are labeled with calcitonin gene-related peptide (33%) substance P (23%) (21) or NO synthase (12%) (47). Thus, if decreased NADPH oxidase activity in dorsal root ganglia is associated with changes in activity of the peptide-containing vascular neurons, it is possible that these changes could contribute to hypertension.

The splanchnic vasculature is innervated by sympathetic nerves, which are vasoconstrictor, and by sensory nerves, which are vasodilator. The NADPH oxidase system that is responsible for generation of $O_2^{-\bullet}$ is regulated differently in these 2 types of nerves in DOCA-salt hypertension. We suggest that $O_2^{-\bullet}$ overproduction evoked by the increased NADPH oxidase in sympathetic ganglia may play a role in the increased neurogenic vasoconstriction. Decreased oxidase activity in sensory ganglia may also enhance blood vessel tone or it may be a response to increased blood pressure. Further studies are needed to unravel the mechanisms underlying the fine tuning of NADPH oxidase-derived reactive oxygen species levels and neuronal activities in these ganglia and how these contribute to the development and maintenance of DOCA-salt hypertension.

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CHAPTER 5: ENDOTHELIN-1 TRANSIENTLY DOWNREGULATES NOREPINEPHRINE TRANSPORTER VIA THE ACTIVATION OF $P22^{PHOX}$-CONTAINING NADPH OXIDASE IN PC12 CELLS

Abstract

Dysfunction of norepinephrine (NE) reuptake via norepinephrine transporter (NET) in the sympathetic nerve endings contributes to the elevated sympathetic drive to the vasculature in hypertension. In deoxycorticosterone acetate (DOCA)-salt hypertension, superoxide anion ($O_2^{-\bullet}$) levels are increased in sympathetic ganglion neurons; this increase is mediated in part by endothelin-1 (ET-1) which activates NADPH oxidase. The purpose of this study was to determine whether NET is regulated by NADPH oxidase-derived $O_2^{-\bullet}$ in response to ET-1 in sympathetic neuronal cell line PC12 cells. It was hypothesized that ET-1 can downregulate NET; and this effect can be diminished by knockdown of $p22^{phox}$, an indispensable component of NADPH oxidase. PC 12 cells were treated with ET-1 (100nM) from 30 minutes to 24 hours. Compared to no treatment control, intracellular $O_2^{-\bullet}$ levels measure by the intensity of dihydroethidium fluorescence were elevated by 360.7% after 1 hour of ET-1 incubation ($n=3$; $p<0.05$), while NET mRNA was decreased and reached its minimum at 2 hours (39.6% decrease, $n=8$; $p<0.01$). Apocynin (100μM) or actinomycin D (5μM) pretreatment abolished NET mRNA decrease in response to ET-1, indicating a role of NADPH oxidase in transcriptional downregulation of

NET. Two lines of short hairpin RNA (shRNA) stably transfected PC12 cells, shRNA-p22 #1 and shRNA-p22 #2, were generated by transfecting undifferentiated PC12 cells with two shRNA-constructs targeting distinct parts in the $p22^{phox}$ sequence. As compared to normal PC12 cells, $p22^{phox}$ mRNA levels were decreased in shRNA-p22 #1 and shRNA-p22 #2 by 45.9% and 79.1%, respectively. $P22^{phox}$ immunoreactivity was also significantly diminished in shRNA-p22 #1 (40.2% decrease) and shRNA-p22 #2 (46.3% decrease). In contrast to normal PC12 cells or scramble control, neither $O_2^{-\bullet}$ production nor NET mRNA expression was significantly changed by ET-1 in shRNA-p22 cells. The results indicate that 1) ET-1 down-regulates NET transcription in PC12 cells; 2) the effects of ET-1 on $O_2^{-\bullet}$ production and NET mRNA expression are $p22^{phox}$-dependent. These suggest that NADPH oxidase-derived reactive oxygen species as a result of enhanced ET-1 signaling in sympathetic neurons may contribute to NET dysfunction in hypertension.

Introduction

Endothelin-1 (ET-1), a 21-amino-acid peptide originally isolated from the supernatant of cultured endothelial cells from blood vessels, is the most extensively studied endothelin isoform in the cardiovascular system(45; 50). In addition to endothelial cells, ET-1 is also produced by other tissue including kidney and neurons (13; 23; 36). In the central nervous system, ET-1 is present in cerebral perivascular nerves and sympathetic and sensory ganglia neurons (39). The ET-1 mRNA and protein levels from these neurons were shown to be

higher in spontaneously hypertensive rats than in normal Wistar Kyoto rats(42), which suggests that neuronal ET-1 is important in hypertension. In the peripheral nervous system, our lab reported that endothelin B receptor (ET_B), one of the ET-1 binding receptors, is upregulated in prevertebral sympathetic ganglia from deoxycorticosterone acetate (DOCA)-salt hypertensive rats as compared to normotensive controls. This also indicates enhanced neuronal ET-1 signaling in hypertension (12). On the other hand, relatively little is known about the functional consequences of elevated ET-1 signaling in neurons. Cao et al is among the few studies who reported that ET-1 has a neuro-excitatory effect on vasomotor neurons in the rat brain (9). However, the mechanisms underlying this effect are not fully understood.

Since elevated catecholamine levels were reported in patients with primary hypertension more than thirty years ago (14), sympathetic neuronal norepinephrine (NE) handling in hypertension has been intensively studied. Higher neuronal firing activity (26) and impaired nerve terminal NE reuptake via norepinephrine transporter (NET) (19) were reported from hypertensive patients, both of which can contribute to increased junctional NE and NE spillover in hypertension. Interestingly, recent studies showed that ET-1 regulates NE release and reuptake via the ET receptors located on the cardiac sympathetic nerve terminals (4; 30). This suggests the potential effect of ET-1 on sympathetic neuronal activity through the regulation of NE handling.

Our lab has previously reported that ET-1 induced superoxide anion ($O_2^{-\cdot}$) production through the activation of ET_B/NADPH oxidase pathway in primary celiac ganglionic neurons and rat pheochromocytoma PC12 cells *in vitro*(12). Lau

et al used systemic administration of a selective ET_B agonist, sarafotoxin 6c (s6c), and observed a similar elevation of $O_2^{-\bullet}$ levels in sympathetic ganglion *in vivo*, although the source of $O_2^{-\bullet}$ was not specified in that study(37). More recently the same group reported that chronic activation of ET_B in rats causes sustained hypertension (21), which may be associated with the increased ganglionic $O_2^{-\bullet}$. Meanwhile, the involvement of $O_2^{-\bullet}$ in the regulation of NET was first suggested by Mao et al who showed that in PC12 cells NET activity and protein expression were inhibited when cellular oxidative stress level was increased(41). These studies together implicate a potential functional link among ET-1, $O_2^{-\bullet}$ and NET in sympathetic neurons.

NADPH oxidase is a multi-subunit enzyme system that contains two membrane-bound catalytic subunits, NOX and $p22^{phox}$, and several cytosolic regulatory subunits, namely $p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and *Rac* (3). We have previously shown that NADPH oxidase activity was increased in sympathetic ganglion in DOCA-salt hypertension (11) and this elevated enzyme activity was accompanied by an upregulation of $p22^{phox}$ expression (10). In this study, we sought to investigate the effect of ET-1 on neuronal NET. In addition to primary celiac ganglion neurons, we also used PC12 cells, which endogenously express ET_B and $p22^{phox}$-containing NADPH oxidase (11; 12) as well as the machinery for synthesis, release and reuptake of NE(15), as a model to further study the NADPH oxidase-mediated part of ET-1's effect on NET by genetically manipulating the expression of $p22^{phox}$ in PC12 cells using short interference RNA technique (22).We tested the hypothesis that in sympathetic neurons, ET-1

can downregulate NET; and this effect can be diminished by knockdown of NADPH oxidase subunit $p22^{phox}$.

Methods

Cell Culture

The rat pheochromocytoma PC12 cell line was obtained from American Type Culture Collection (ATCC), and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, 100U/ml penicillin, 100ug/ml streptomycin, and 0.25ug/ml Funizone in a humidified incubator at 37 °C and 5% CO_2 atmosphere. Medium was changed every other day and the cells were passaged every 7 days. All experiments were performed using cells under passage 15^{th} to avoid cell line drift. For drug treatment, cells were plated as a monolayer on multi-well cell culture plates coated with 100μg/ml poly-D-lysine (Sigma-Aldrich, St. Louis, MO) one day prior to the experiment. All drugs were prepared as stock solutions in DMSO or H_2O. Aliquots were stored at -80°C and protected from light before use: ET-1 (Bachem Americas, Inc., Torrance, CA), BQ788 (Sigma), BQ610 (Sigma), apocynin (Sigma), actinomycin D (Sigma).

RNA Isolation and Reverse Transcription-Quantitative Polymorease Chain Reaction (RT-qPCR)

Total RNA was isolated from the cells using RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was synthesized using Superscript II mix (Invitrogen).

qPCR analysis was then performed using Mx3000P QPCR system (Stratagene, La Jolla, CA). SYBR green was used as the fluorescence detector in the qPCR reaction (Applied Biosystems, Foster City, CA). All primers were derived from the Rattus Norvegicus gene (National Center for Biotechnology Information GenBank). NET forward: 5'-GCC TGA TGG TCG TTA TCG TT-3'; reverse: 5'-CAT GAA CCA GGA GCA CAA AG-3'. GAPDH forward: 5'-GGA GTC TAC TGG CGT CTT CAC-3'; reverse: 5'-GGT TCA CAC CCA TCA CAA AC-3'.

Design of shRNA Insert

Target sites for rat $p22^{phox}$ were selected following the guidelines for effective shRNAs (18). Two complementary DNA oligonucleotides incorporating each chosen target site, a loop sequence, and the corresponding reverse complement of the target site were designed. Xho I and Xba I overhangs were added at the end of the two oligonucleotides, respectively. The transcriptional termination signal for 5 T's was added at the 3' end of the inverted repeat (Figure 5.1A). The pair of oligonucleotides was then annealed and ready to be ligated into the plasmid vector. A scramble sequence with no known targeting sites in the rat genome was selected as control. Target sequences for $p22^{phox}$ and scramble control sequence are listed in Table 1.

ShRNA Expressing Cassette Construction

Vector-based short hairpin RNA (shRNA) expressing cassette was constructed by cloning the $p22^{phox}$ shRNA insert into linearized pSuppressorNeo plasmid with neomycin/kanamycin selection cites and Sal I and Xba I cloning

A

shRNA-p22 #1

XhoI Overhang — Loop — Stop

5' TCGA GACGCTTCACGCAGTGGTA **GAGTACTG** TACCACTGCGTGAAGCGTC TTTTT 3'

3' CTGCGAAGTGCGTCACCAT **CTCATGAC** ATGGTGACGCACTTCGCAG AAAAA GATC 5'

XbaI Overhang

shRNA-p22 #2

XhoI Overhang — Loop — Stop

5' TCGA GCTTCACGCAGTGGTACTTTG **TTCAAGAGA** CAAAGTACCACTGCGTGAAGC TTTTT 3'

3' CGAAGTGCGTCACCATGAAAC **AAGTTCTCT** GTTTCATGGTGACGCACTTCG AAAAA GATC 5'

XbaI Overhang

Scramble Ctl

XhoI Overhang — Loop — Stop

5' TCGA TTTCAGCACGTATATGGTCGT **GAGTACTG** ACGACCATATACGTGCTGAAA TTTTT 3'

3' AAAGTCGTGCATATACCAGCA **CTCATGAC** TGCTGGTATATGCACGACTTT AAAAA GATC 5'

XbaI Overhang

B

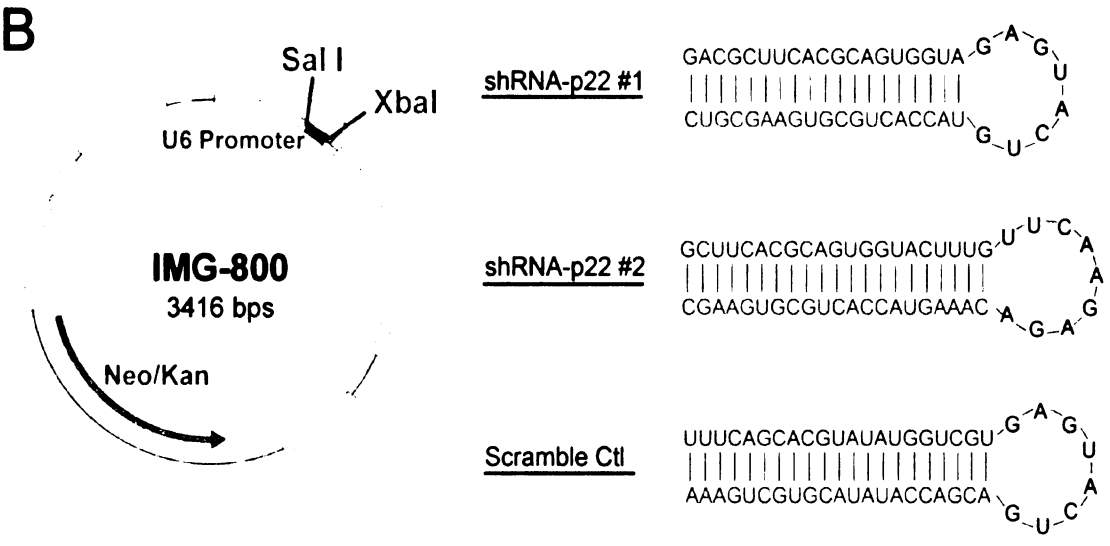


Figure 5.1. Plasma-based expression of shRNA-p22. A) Deoxyoligonucleotides used in the construction of shRNA-p22 #1, shRNA-p22 #2 and scramble control are shown as they would be paired after anneling. The color-coded labels above and below the oligonucleotides indicate the source or the function of the nucleotides in the indicated regions. B) Left: plasmid map of pSuppressorNeo plasmid with neomycin/kanamycin selection cites and Sal I and Xba I cloning sites from Imgenex Corp. shRNA constructs insertion site is marked in red. Right: the predicted stem-loop structure of shRNA-p22 and scramble ctl expressed from the plasmid.

Table 5.1 Target sequences for NADPH oxidase subunit p22phox for shRNA constructs.

	Sequence
shRNA-p22 #1	5'- GACGCTTCACGCAGTGGTA -3'
shRNA-p22 #2	5'- GCTTCACGCAGTGGTACTTTG -3'
Scramble Ctl	5'-TTTCAGCACGTATATGGTCGT -3'

sites (Imgenex, San Diego, CA) (Figure 5.1 B). Plasmids were transformed into One Shot® Chemically competent *E. coli* (Invitrogen). Cells were plated on LB plates containing 100μg/ml kanamycin (Sigma). Kanamycin resistant colonies were selected and amplified in LB medium and plasmids were isolated and purified using PureYield™ Plasmid Midiprep System (Promega, Madison, WI). The resulting plasmid was sequenced to ensure that the insert was present and correct. The predicted shRNA expressed by this construct is shown in Figure 5.1B.

Transfection and selection of stably transfected PC12 cells

An early passage of PC12 cells were plated as monolayer on 6-well cell culture plate pretreated with poly-D-lysine one day prior to the transfection. Cells (1×10^6) were transfected with 4 μg of plasmid constructs containing either $p22^{phox}$ shRNA sequences or scramble sequence as described above using Lipofectamine 2000 (Invitrogen). One day after transfection the cells were replated at a lower density and selected with 400 μg/ml G418 (Sigma) for 35 days until discrete colonies were formed. Individual colonies were isolated, grown up and maintained in RPMI medium containing 200μg/ml G418. Colonies with effective $p22^{phox}$ knockdown were defined as shRNA-p22 #1 or #2 and were used in later experiments.

Immunocytochemistry of $p22^{phox}$ in PC12 Cells

Normal and transfected PC12 cells were plated onto poly-D-lysine coated cover glasses and were maintained in culture medium for one day before

immunostaining. The cells were cleaned from culture medium by three washes in Dulbecco's phosphate buffered saline (DPBS) and then placed into fixative (4% paraformaldehyde, 0.1%triton-X100 in DPBS) for 30min at 4°C. Cells were then incubated in DPBS with blocking solution (5% goat serum, 3% bovine serum albumin (BSA)) for 1 hour at room temperature followed by incubation with mouse monoclonal $p22^{phox}$ antibody (H44.1, kindly provided by Dr. Mark Quinn) at a 1:500 dilution in DPBS with 1%BSA for overnight at 4°C. The next day, samples were washed in DPBS for three times and then incubated with Cy3 conjugated secondary antibody in a dark chamber at room temperature for 1 hour followed by three washes in DPBS. Samples were mounted onto glass slides using Prolong Gold anti-fade reagent (Invitrogen) for confocal microscopy using Pascal (Zeiss, Thornwood, NY). In order to compare immunostaining intensity across samples on different slides, samples in the same comparing group were prepared on the same day and the images were taken under the same microscopy parameters. Images were analyzed using Image J software (NIH).

Measurement of $O_2^{-\bullet}$ Level

Intracellular $O_2^{-\bullet}$ levels were examined by measuring fluorescence signal intensity resulting from intracellular probe oxidization. Normal or transfected PC12 cells were plated on poly-D-lysine coated cover glasses one day prior to the experiment. Cells were pretreated with ET-1 (100nM) for 1 hour, and then loaded with the dihydroethidine (DHE) (10μM) (Invitrogen) and incubated at 37°C for 45 minutes before measuring fluorescence (excitation: 514nm; emission: 560nm) with confocal microscopy (Zeiss). DHE was used as an intracellular $O_2^{-\bullet}$

probe. Upon its reaction with $O_2^{-\bullet}$, DHE is oxidized to fluorescent ethidium. Ethidium will then intercalate with DNA in the nucleus to further amplify the red fluorescent signal and the intensity of the fluorescent signal is proportional to $O_2^{-\bullet}$ levels (5; 53). Confocal images consisting of a 0.36μm optical slice through the approximate center of cells were captured and analyzed using Image J software (NIH).

Data Analysis

Data are presented as mean±SE of the mean. Statistical significance of NADPH oxidase activity, immunohistochemistry data were assessed by Student *t* test using Prism 4.0 software (GraphPad Software, San Diego, CA). qPCR data statistical significance were assessed by Pair Wise Fixed Reallocation Randomization Test© using REST software. In both cases, $p<0.05$ indicates statistical significance.

Results

ET-1 Induced Transient Decrease in Norepinephrine Transporter mRNA Levels in PC12 Cells

When 100nM ET-1 was applied to PC12 cells, there was a 39.6% decrease in NET mRNA expression at 2 hours. The relative expression ratio of NET mRNA at 2 hours to control is 0.6 ±0.1 ($n=8$; $p<0.01$). After the transient

decrease, the levels of NET mRNA recovered to normal levels at 12 and 24 hours of ET-1 incubation (Figure 5.2).

Apocynin Abolished NET mRNA decrease following ET-1 Treatment

To determine whether the transient decrease of NET mRNA in response to ET-1 was mediated through NADPH oxidase-derived $O_2^{-\bullet}$, apocynin (4-hydroxy-3methoxy-acetophenone) was used as an NADPH oxidase inhibitor (49). Cells were incubated with apocynin (100μM) for 1 hour before the addition of ET-1 (100nM). Pretreatment of PC12 cells with apocynin completely abolished the decrease of NET mRNA in 2-hour ET-1 treated groups (Figure 5.3) (n=6; p=0.36). This indicates that ET-1 may induce NET mRNA decrease via the activation of NADPH oxidase.

Actinomycin D prevented the decrease of NET mRNA in response to ET-1

NADPH oxidase derived $O_2^{-\bullet}$ was shown to reduce mRNA stability of certain proteins (52). Actinomycin D was used in this study to block transcription in PC12 cells in order to test whether the reduction of NET mRNA in response to ET-1 was due to a decrease in NET mRNA stability. Normal PC12 cells were pre-treated with 5 μM actinomycin D 1 hour prior to the addition of ET-1 (100nM). Total RNA was collected for RT-qPCR for NET. Figure 5.4 shows that no NET mRNA decrease was found in cells treated with actinomycin D and ET-1. These results indicated that ET-1 reduces NET mRNA expression via the modification of NET transcription rather than its mRNA stability.

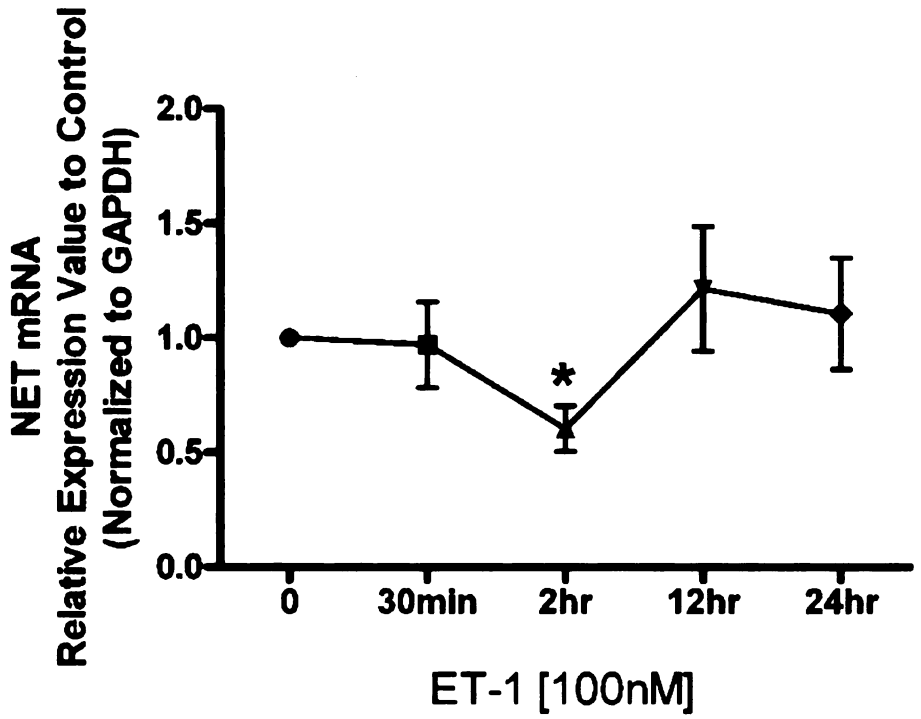


Figure 5.2. ET-1 induces a transient decrease of NET mRNA expression in PC12 cells. Undifferentiated PC12 cells were incubated with ET-1(100nM) for 30 minutes, 2 hours, 12 hours or 24 hours. Cells were collected at the end of each time point. Total RNA was isolated for the purpose of RT-qPCR for NET mRNA measurement. Relative expression value to calibration (control) was calculated in MxPro™ QPCR Software (Stratagene). All data points were normalized to their own GAPDH levels. The amplification efficiency of NET (99.1%) and GAPDH (95.0%) primers in PC12 cells were identified using serial dilutions. As compared to no treatment control, cells treated with ET-1 for 2 hours showed a significant decrease in NET mRNA levels. This decrease was recoverd to normal levels after 12 hours or 24 hours treatment. Data are expressed as the mean relative expression value ±SEM (n=8 cell preparations). * indicates significance by a paired Student's *t* test ($p<0.01$) at 2hr versus 0.

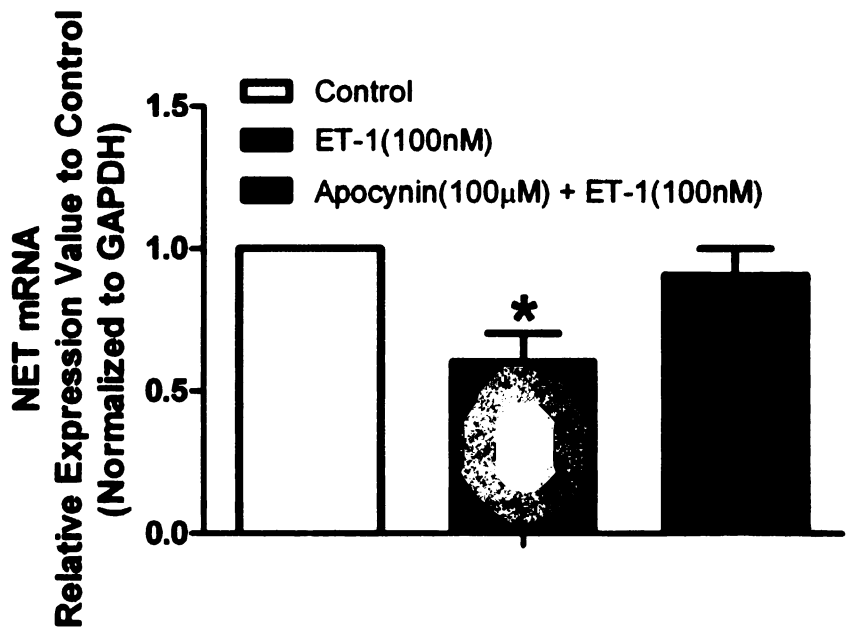


Figure 5.3. Apocynin abolishes the decrease of NET mRNA in response to ET-1 in PC12 cells. PC12 cells were either not treated or treated with ET-1 (100nM) for 2 hours. ET-1-treated cells were preincubated with apocynin (100μM) or vehicle 1 hour before the addition of ET-1. Total RNA was harvested from the cells for RT-qPCR analysis for NET mRNA. Relative expression ratio calculation and statistical analysis were performed by Pair Wise Fixed Reallocation Randomization Test© (http://www.gene-quantification.info) using Relative Expression Software Tool (REST) {Pfaffl, 2002 6906 /id}. All data points were normalized to their own GAPDH levels. As compared to control, cells treated with ET-1 showed a significant decrease in NET mRNA expression while this decrease was absent in cells pretreated apocynin. Data are expressed as the mean relative expression value ±SEM (n=6 cell preparations). * indicates significance $p<0.01$ at ET-1 versus Control.

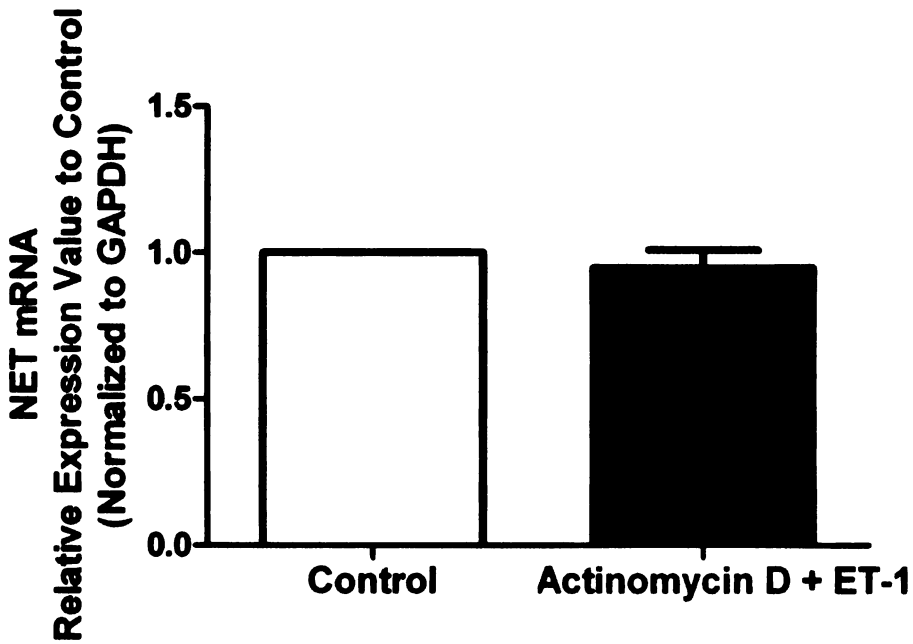


Figure 5.4. Actinomycin D abolished NET mRNA decrease in response to ET-1 in PC12 cells. Normal PC12 cells were pretreated with actinomycin D (5μM) for 1 hour followed by ET-1 (100nM) incubation for 2 hours. Total RNA was harvested for RT-qPCR analysis for NET mRNA. Relative expression ratio calculation and statistical analysis were performed by Pair Wise Fixed Reallocation Randomization Test© (http://www.gene-quantification.info) using REST software. All data points were normalized to their own GAPDH levels. No significant difference of NET mRNA expression was found in treated cells as compared to controls. Data are expressed as the mean relative expression value ±SEM (n=9 cell preparations).

$P22^{phox}$ knockdown in PC12 cells by RNA interference

PC12 cells transfected by plasmids containing shRNA sequences (Seq #1 or Seq #2, see Table 5.2) against $p22^{phox}$ were selected for the subsequent experiments. They were defined as shRNA-p22 #1 and shRNA-p22 #2, respectively. PC12 cells transfected by scramble sequence-containing plasmid were defined as shRNA-scramble. RT-qPCR results revealed that the levels of $p22^{phox}$ mRNA were significantly decreased in both shRNA-p22 #1 (45.9±6.3% knockdown) and shRNA-p22 #2 (79.1±10.0% knockdown) (Figure 5.5A). The $p22^{phox}$ mRNA level was not affected by scramble sequence transfection. Off-target effects of RNAi was evaluated by measuring the mRNA level of signal transducers and activator of transcription protein-1 (STAT-1) because there is evidence of sequence-independent off target effects via interferon response that signals through STAT (31). Figure 5.5B shows that STAT-1 mRNA expression was not significantly different between transfected cells and control normal PC12 cells. This indicates the signaling pathway through STAT-1 was not activated by shRNA transfection.

$P22^{phox}$ protein levels in shRNA-p22 cells were measured by immunocytochemistry. The staining of $p22^{phox}$ in normal PC12 cells was located in both the plasma membrane and the cytoplasm (Figure 5.6A). Using the same microscopy setting (gain and offset), shRNA-p22 cells revealed lower immunostaining intensity as compared to normal PC12 cells or scramble control cells (Figure 5.6). The decreases of mean fluorescent intensity in shRNA-p22 #1

A

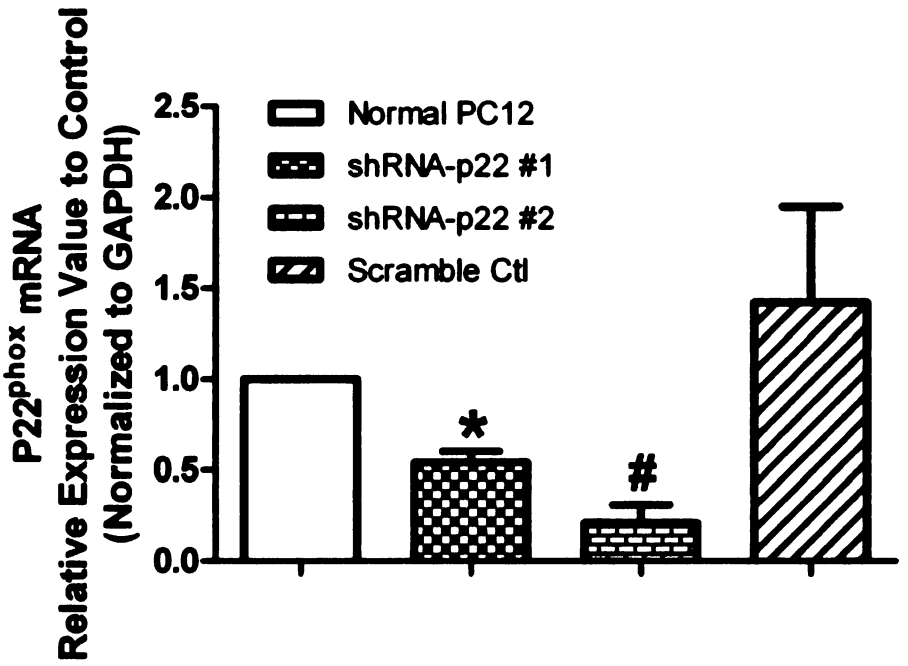
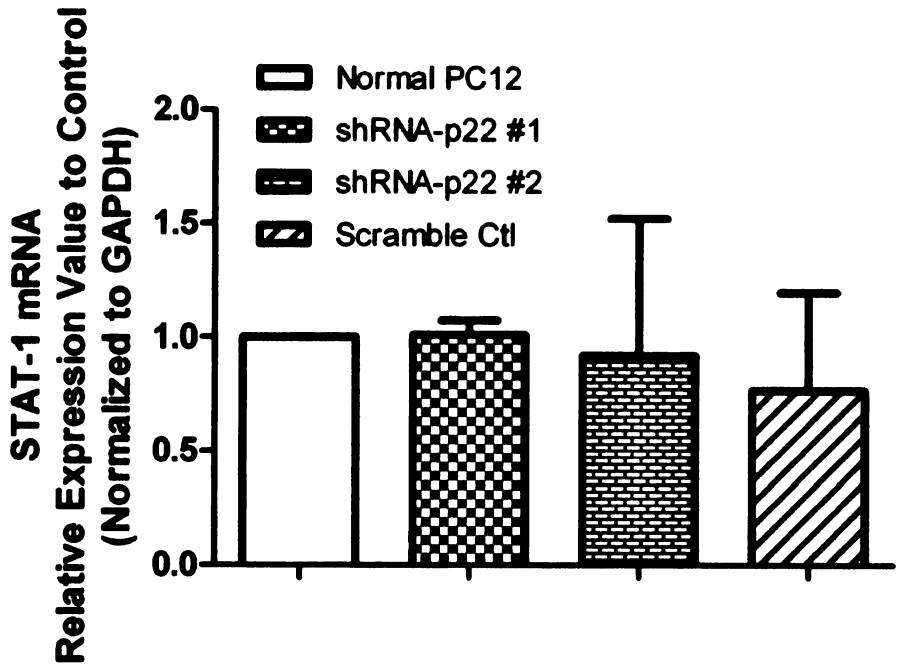


Figure 5.5. P22phox mRNA expression level is suppressed by RNA interference in PC12 cells. PC12 cells stably transfected by two shRNA sequences targeting p22phox were selected and defined as shRNA-p22 #1 and shRNA-p22 #2. Control cells were stably transfected by a scramble sequence with no known target in the rat genome and was defined as scramble ctl. Total RNA was harvested for RT-qPCR analysis for p22phox and STAT-1 mRNA. Relative expression ratio calculation and statistical analysis were performed by Pair Wise Fixed Reallocation Randomization Test© (http://www.gene-quantification.info) using REST software. All data points were normalized to their own GAPDH levels. A) As compared to normal PC12 cells, shRNA-p22 #1 and shRNA-p22 #2 both have significant decreased levels of p22phox expression. The knockdown level of p22phox mRNA were 45.9% and 79.1%, respectively. Meanwhile, scramble ctl cells have a similar level of p22phox expression with normal cells. B) As compared to normal PC12 cells, STAT-1 mRNA levels were not significantly different in shRNA-p22 #1, shRNA-p22 #2 and scramble control cells. Data are expressed as the mean relative expression value ±SEM (n=6 cell preparations). * indicates significance $p<0.01$ at shRNA-p22 #1 vs Normal; # indicates significance $p<0.01$ at shRNA-p22 #2 vs Normal.

Figure 5.5 continued

B



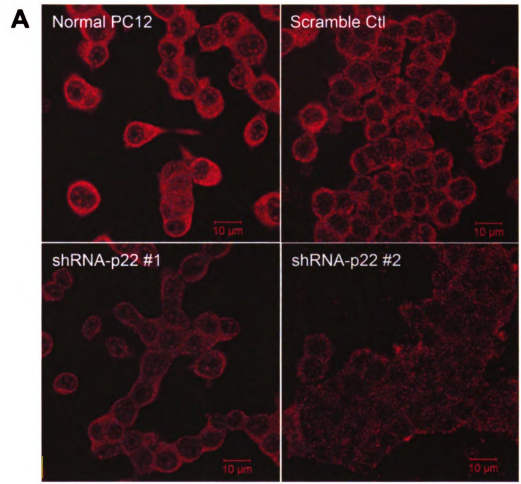
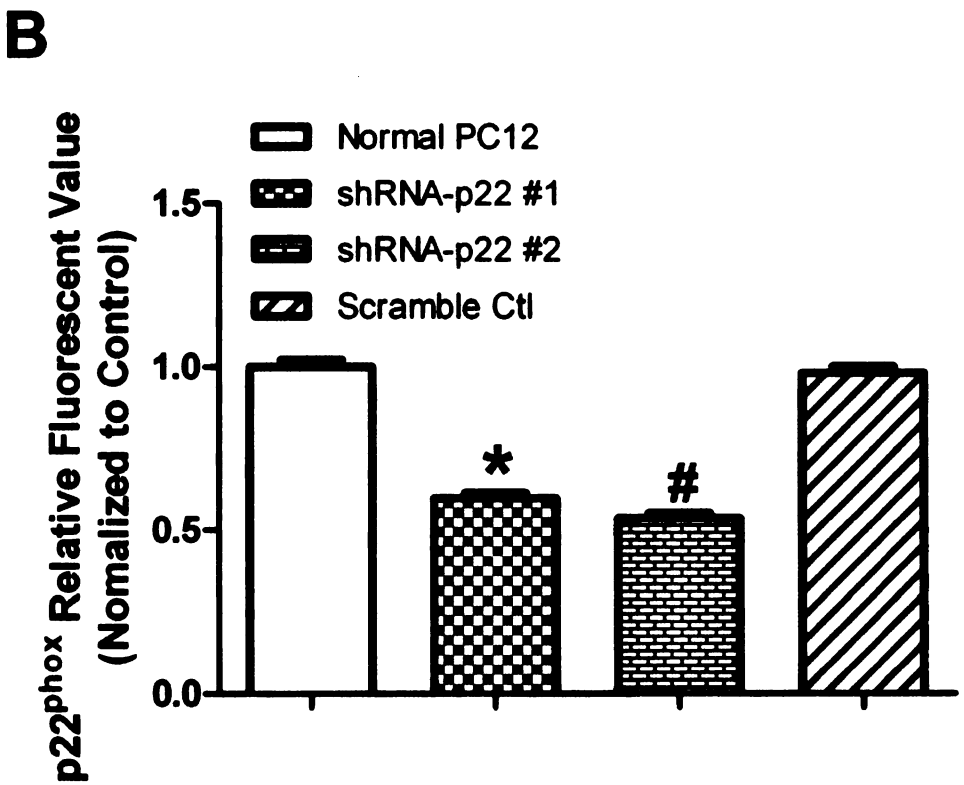


Figure 5.6. P22phox immunoreactivity in shRNA-p22 PC12 cells. Immunocytochemical staining of p22phox was performed in normal PC12 cells, shRNA-p22 #1 and #2, scramble control PC12 cells. A) Confocal images show the immunoreactivity of p22phox (red) in PC12 cells. The microscope was focused on the center of the monolayer of the cells. All images were taken on the same day with the same gain and offset settings on the scope. B) The fluorescent intensity of p22phox staining was analysized using Image J software. Relative fluorescent intensity to normal PC12 cells were calculated and plotted. As compared to normal PC12 cells, shRNA-p22 #1 and shRNA-p22 #2 showed 40.2% and 46.3% decrease in mean fluorescent intensity, respectively. No significant change was found in scramble control cells. Data are expressed as the mean relative fluorescent value ±SEM. Numbers of cells counted in each group are 44 (normal PC12), 102 (shRNA-p22 #1), 62 (shRNA-p22 #2) and 75 (scramble ctl). *indicates significance $p<0.01$ at shRNA-p22 #1 vs normal PC12; #indicates significance $p<0.01$ at shRNA-p22 #2 vs normal PC12.

Figure 5.6 continued



and #2 were 40.2±1.7% and 46.3±1.6%, respectively. These results indicate the protein expression of $p22^{phox}$ was diminished in shRNA transfected cells.

$O_2^{-\bullet}$ production induced by ET-1 was attenuated in shRNA-p22 PC12 cells

ET-1 increases intracellular $O_2^{-\bullet}$ production by NADPH oxidase in PC12 cells via the activation of ET_B receptor (12). To test the function of NADPH oxidase in shRNA-p22 cells, we measured $O_2^{-\bullet}$ production in response to ET-1. In normal PC12 cells and scramble control cells, $O_2^{-\bullet}$ production was increased by 360.7±60.7% and 106.5±14.1% by ET-1, respectively (Figure 5.7). However, no significant difference in DHE fluorescence was found in shRNA-p22 #1 or shRNA-p22 #3 cells treated with or without ET-1. These results showed that $O_2^{-\bullet}$ production in response to ET-1 treatment was diminished in shRNA-p22 cells but not scramble control cells. This indicates that ET-1 induces $O_2^{-\bullet}$ production in PC12 cells via NADPH oxidase. The difference in baseline $O_2^{-\bullet}$ was unexpected. More studies are needed to evaluate the source of that $O_2^{-\bullet}$.

ET-1 showed no effect on NET mRNA expression in shRNA-p22 cells

ET-1 (100nM) was applied to normal PC12 cells, shRNA-p22 #1, #2 and scramble control cells for 2 hours. NET mRNA levels were significantly

Figure 5.7. ET-1-incduced O2–• production in PC12 cells is attenuated in shRNA-p22 cells.
A) Representative confocal fluorescent images of normal PC12 cells, shRNA-p22 #1 and #2 cells and scramble control cells incubated with DHE. Red fluorescence indicates the presence of O2–•. In each group, control cells without ET-1 treatment are listed in the left row. ET-1 treated cells are listed in the right row. B) and C) Relative DHE fluorescence is analyzed using Image J software. Results are expressed as mean ±SEM. (n=3 cell preparations for each group). The statistical significance ($p<0.05$) is indicated by *vs control.

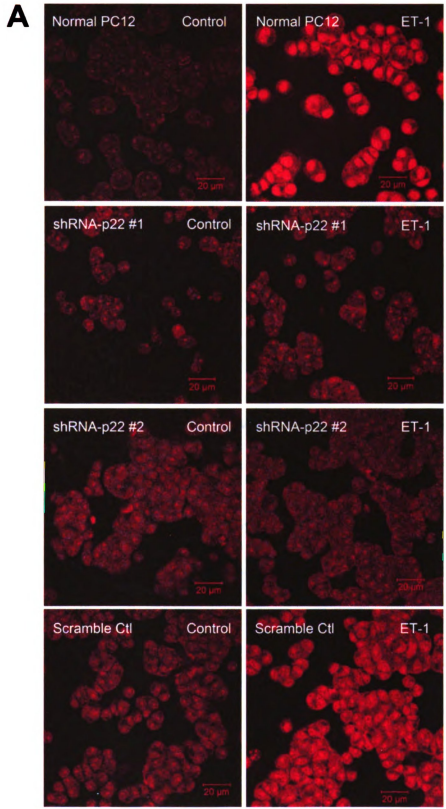

A
Normal PC12
Control
Normal PC12
ET-1
shRNA-p22 #1
Control
shRNA-p22 #1
ET-1
shRNA-p22 #2
Control
shRNA-p22 #2
ET-1
Scramble Ctl
Control
Scramble Ctl
ET-1
20 μm

Figure 5.7 continued

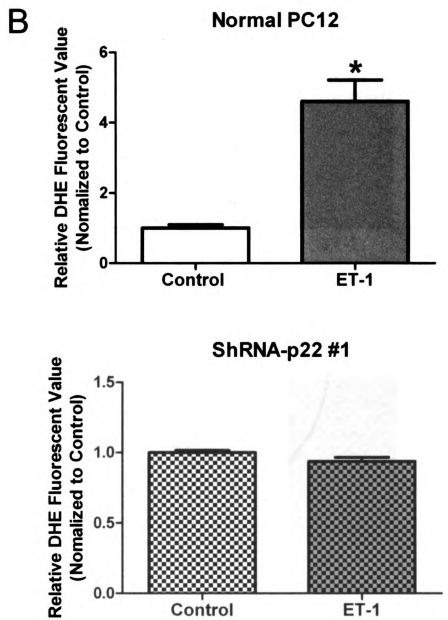
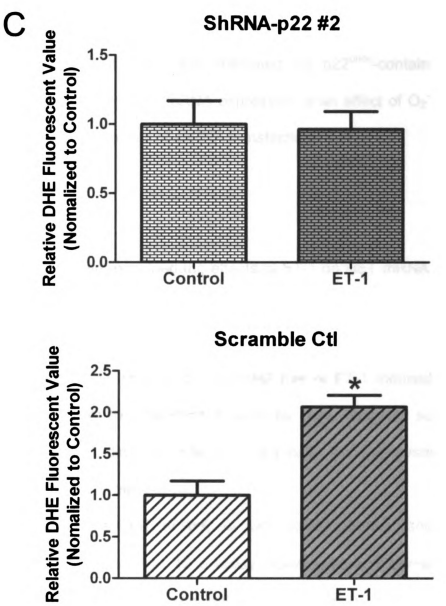


Figure 5.7 continued



decreased in normal PC12 as well as scramble control cells (Figure 5.8). No change in NET mRNA was found in shRNA-p22 #1 or #2 cells. These data, together with the apocynin results, further indicate that the effect of ET-1 on NET mRNA in PC12 cells was mediated via $p22^{phox}$-containing NADPH oxidase. The reduction in NET mRNA expression is an effect of $O_2^{-\bullet}$ production on a side effect of the process of shRNA transfection.

Discussion

In this study, we examined the effects of ET-1 on NET mRNA expression. We found that ET-1 induced a transient decrease in NET mRNA levels and this decrease was mediated through an NADPH oxidase-dependent pathway. The findings from this study implicated a potential role of ET-1 induced $O_2^{-\bullet}$ in the regulation of catecholamine handling in neuronal cells. This may suggest novel approach in understanding the role of sympathetic nervous system in blood pressure regulation in hypertension.

NET belongs to a family of sodium- and chloride-coupled transporters. It is a 617 amino acid protein with 12 α-helical transmembrane domains that are interrupted by alternating intra- and extracellular loops. Its major function is to presynaptically terminate NE signaling at the neuroeffector junction (2) and therefore serves as an indispensible determinant of sympathetic innervation. There is in fact evidence for an impaired NE clearance by NET in hypertensive patients (19), suggesting that altered NET function might contribute to the elevated sympathetic tone in hypertension. NET was previously shown to be

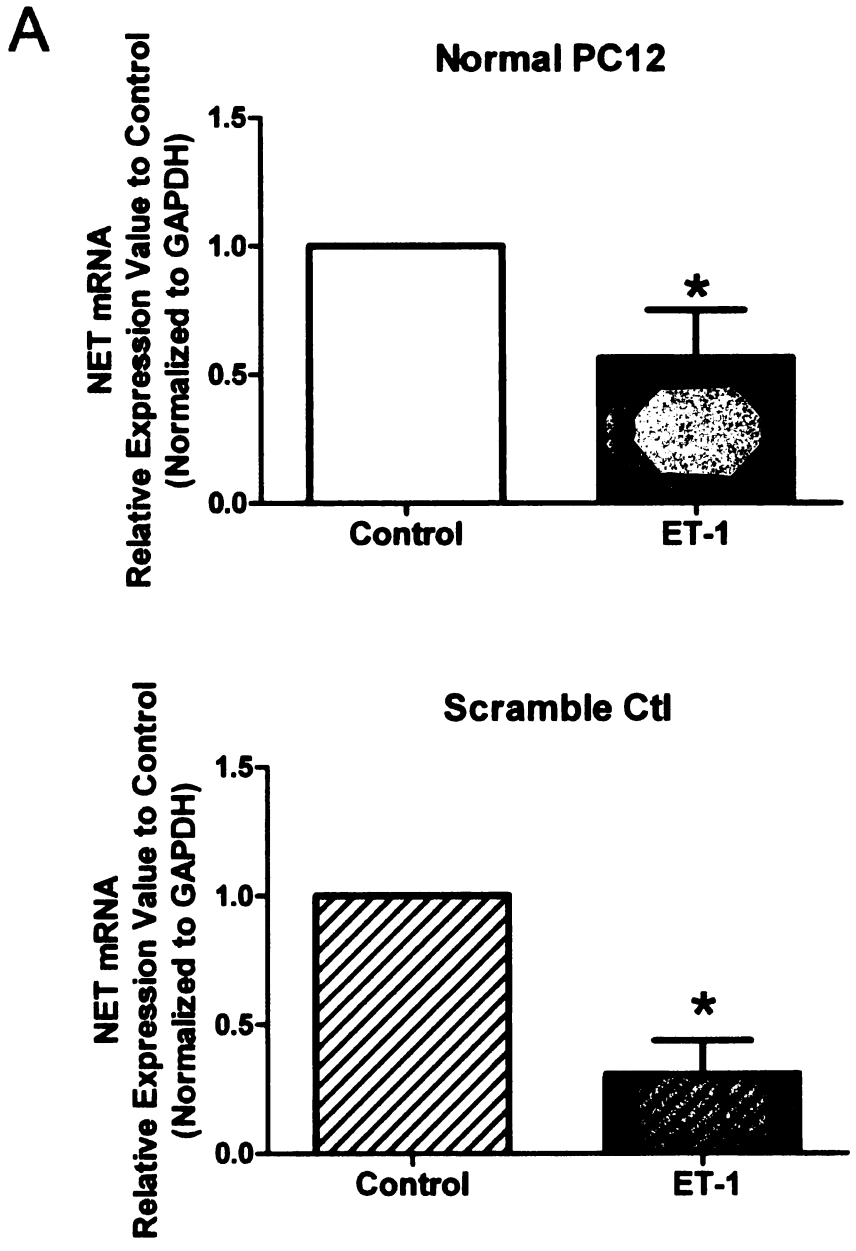
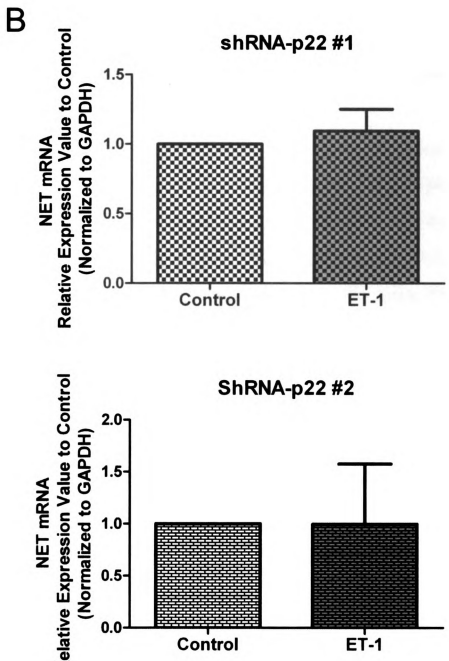


Figure 5.8. NET mRNA is reduced by ET-1 in normal PC12 cells but not shRNA-p22 cells. Normal PC12 cells, shRNA-p22 #1, #2 and scramble control cells were either treated with ET-1 (100nM) or vehicle for 2 hours. Total RNA was harvested for RT-qPCR analysis for NET mRNA. Relative expression ratio calculation and statistical analysis were performed by Pair Wise Fixed Reallocation Randomization Test© (http://www.gene-quantification.info) using REST software. All data points were normalized to their own GAPDH levels. ET-1 treatment elicited a significant reduction in NET mRNA expression in normal PC12 cells and scramble control cells (A). No change in NET mRNA in response to ET-1 was found in shRNA-p22 #1 and shRNA-p22 #2 cells (B). Data are expressed as the mean relative expression value ±SEM (n=6 cell preparations). * indicates significance $p<0.05$ vs control.

Figure 5.8 continued



acutely or chronically regulated by variant stimulants such as insulin (20), angiotensin II (40), nitric oxide (33), desipramine (47) and nerve growth factor (29). The modification of NET may involve subcellular redistribution of the transporter, acute alterations in its transport efficiency (51) or regulation on the transcriptional level of the NET gene (40).

ET receptors located on the adrenergic nerves have been suggested to modulate NE release and reuptake (48) (30) (4). However, the mechanisms are not fully understood. We have previously shown that ET_B activation in the prevertebral sympathetic ganglia resulted in an increased production of $O_2^{-\bullet}$ by NADPH oxidase in hypertension (11; 12). Meanwhile, a study using PC12 cells reported that NET function and expression were down-regulated by an oxidative stress -mediated mechanism (41). Therefore, we speculated that the increased $O_2^{-\bullet}$ in the sympathetic neurons we found might be involved in NE mishandling in hypertension.

PC12 is a clonal cell line derived from rat pheochromocytoma cells (25). These cells synthesize NE and have the capacity to take up NE by a Na^+-dependent transporter, NET, and thus are frequently used as model system for studying NE handling and NET regulation (8). The rat NET gene and its promoter have been successfully cloned in PC12 cells (8) (43). The presence of several transcription factor binding sites in the promoter region reveals the putative mechanisms of transcriptional regulation of the NET gene. In this study, we found a significant increase in intracellular $O_2^{-\bullet}$ production in PC12 cells treated with ET-1, and this was accompanied by a reduction in the NET mRNA expression. This decrease was not found in cells pretreated with actinomycin D, indicating the

effect of ET-1 on NET mRNA is a transcriptional modification. The suppression of NET transcription could possibly be achieved by the following mechanisms: 1) increased binding of inhibitory transcription factor to the NET promoter and thus suppresses NET expression. One of these factors could be Oct-2 which acts in neuronal cells as an inhibitor for the expression of specific genes (34), however Oct-2 has not been directly coupled with NET so far; 2) the binding of enhancer factors to certain NET promoter region is attenuated and therefore decrease the transcription of NET. For example, neuron specific enhancer *cis*-element was identified in the intron sequence upstream to the NET gene (35). It is suggested that by inhibiting the action of enhancing factor that bind to this region, NET transcription might be compromised (29); 3) DNA methylation-dependent silencing pathway could also potentially repress NET transcription because the NET promoter region is rich in CpG dinucleotides, which renders it susceptible to methylation-related gene silencing (16).

However, in the experiments performed by Mao et al. (41), NET mRNA levels were found to be unchanged in response to oxidative stress despite the decreased membrane protein expression or uptake function. The author concluded that the decrease of NET was a post-transcriptional modification. Noticeably, in their study the unchanged NET mRNA was found in a setting where the cells were incubated in an oxidative challenging medium for 24 hours. In our experiment, the significant decrease in NET mRNA level following 2 hours of ET-1 treatment was indeed recovered and stayed at normal level after 12 to 24 hours of incubation. Interestingly, they also reported a peak intracellular $O_2^{-\bullet}$ levels after 2 hours of incubation but the parallel NET mRNA levels were not

measured. It is therefore possible that an early NET mRNA modification was not caught in the study. The recovery of NET mRNA level may be due to 1) inducible upregulation of defense mechanisms against free radicals, such as superoxide dismutase or catalase (46), in response to prolonged oxidant challenge; 2) ROS-sensitive transcriptional factors enhance NET transcription to overcome the initial reduction in NET mRNA. For example, nuclear factor κB (NF-κB) and activator protein-1 (AP-1) can both be activated by ROS (38) and their binding regions are present in NET promoter (43).

Apocynin pretreatment abolished the NET mRNA decrease in response to ET-1. This was the first clue that the modification of NET mRNA is through an NADPH oxidase mediated pathway. However, there has been accumulative evidence questioning the specificity of the effects of apocynin. It is suggested that apocynin only serves for an NADPH oxidase inhibitor in the presence of myeloperoxidase (MPO) (28), which many non-phagocyte cells that contain NADPH oxidase do not express, for example vascular smooth muscle cells or endothelial cells. It is then heavily questionable if apocynin is a good candidate for NADPH oxidase inhibitor in those systems. In the nervous system, on the other hand, MPO is reported to be expressed in neuronal cell bodies in the brain (24) as well as in PC12 cells (27). It is thus still promising to use apocynin as an effective inhibitor to NADPH oxidase in these cells. Nevertheless, no direct evidence has been provided to confirm the involvement of MPO in apocynin's effects in neurons. We therefore used RNA interference (RNAi) as a more direct approach to investigate the role of NADPH oxidase in this pathway.

RNAi can be elicited transiently in mammalian cells by transfection with small interfering RNAs (siRNAs) (17). Long term RNAi can be achieved by transfecting the cells with plasmids expressing short hairpin RNAs (shRNA) followed by selecting transfected cells that have stably incorporated the shRNA plasmid into the genome (7; 44). The former method is more applicable to cells that have high transfection efficiency. Although it takes substantial investment in time to derive stably transfected cell lines, the latter shRNA method is clearly a better choice to elicit RNAi in hard-to-transfect cells, such as PC12 cells. The selection with drugs allows the survival and study only of transfected cells. These cells are homogeneous, and their knockdown phenotype can be maintained in culture for months. In this study, we established two stably transfected cell lines that have diminished $p22^{phox}$ levels in PC12 cells. The reasons we chose $p22^{phox}$ as the target are as following: 1) $p22^{phox}$ associates with NOX catalytic subunits (NOX1, NOX2, NOX3, NOX4, or NOX5) and is required for their functioning (1); 2) $p22^{phox}$provides a binding site for the regulatory subunit $p47^{phox}$ (32), and the binding between $p22^{phox}$ and $p47^{phox}$ is a key step for NADPH oxidase activation (3); 3) specific upregulation of $p22^{phox}$ expression has been found in both vascular tissue and sympathetic ganglia in ET-1 dominant hypertension (6) (10). Taken together, $p22^{phox}$ is an indispensible component of the NADPH oxidase enzyme system and therefore by knocking down $p22^{phox}$ one would predict an efficient silence in overall NADPH oxidase activity. The two knockdown cell lines were derived by transfecting PC12 cells with shRNA-expressing plasmids containing two distinct sequences against rat $p22^{phox}$ gene. We also developed a cell line that was transfected by the same plasmid containing a scramble

sequence to serve as a parallel control. Our results first showed that in the two shRNA cell lines, the $p22^{phox}$ mRNA and protein levels were significantly lower than normal PC12 cells or scramble control cells. Second, the $O_2^{-\cdot}$ production in response to ET-1 were decreased in knockdown cells. These together confirmed that ET-1 induced $O_2^{-\cdot}$ generation was through NADPH oxidase in PC12 cells and this effect was attenuated by $p22^{phox}$ knockdown. In consistence with the results from apocynin treatment, NET mRNA in shRNA-p22 cells did not decrease after ET-1 incubation, which further verified the involvement of NADPH oxidase in ET-1/NET signaling.

In summary, we have demonstrated in this study that ET-1 acutely downregulated NET transcription in PC12 cells. We used both pharmacological and molecular biological tools to verifiy that this effect was mediated through the activation of NADPH oxidase. These findings may indicate novel mechanisms for the regulation of catecholamine handling in sympathetic neurons. Future studies are needed to address the remaining questions from this work. For example, whether the transient downregulation of NET mRNA by ET-1 is reflected by any functional change in NET protein in sympathetic neurons, or whether the chronic regulation of NET in hypertension follows the same mechanisms.

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CHAPTER 6: CONCLUSION AND DISCUSSION

Hypertension

Despite advances in research in the past several decades, about 90 to 95 percent high blood pressure cases are still reported to have "no known causes" according to the latest description of hypertension from American Heart Association (www.americanheart.org, March 2008). Diuretics, β-adrenergic blockers (β-blockers), and angiotensin converting enzyme inhibitors (ACEI) are the typical agents that fall into the category of initial drug choices of the treatment of hypertension. However, most of them alone can only lower blood pressure (BP) in about 50% to 60% of hypertensive patients (73). The poor outcome of single agent treatment proves the heterogeneity of underlying mechanisms in essential hypertension. This makes hypertension an extreme challenging and interesting topic to study.

Volume retention and activation of the renin angiotensin system (RAS) are two well-characterized features found in many hypertensive patients. Extensive research has been focused on the kidney and cardiovascular tissue, which are the primary organs involved in regulating fluid balance and RAS. However, regulation of BP is a complex integrated response involving multi-organ interaction. The crosstalk among different organ systems should not be neglected.

In particular, most organs in the body including kidney and blood vessels are innervated by nerves, whose activities directly affect the performance of the target tissues. This gives the nervous system an indispensable role in regulating BP.

Neurogenic Hypertension: Role of the sympathetic nervous system

It is clear that in response to stressors, sympathetic activation can cause elevation in systemic BP characterized by a transient increase in plasma norepinephrine (NE) levels (27). However, although studies assessing plasma catecholamine, NE spillover, microneurography, and heart rate variability have indicated that the sympathetic activation is greater in hypertensive patients than controls (25) (69) (35), the exact role of sympathetic nervous system in sustained hypertension is not fully established. Especially the factors that stimulate sympathetic nerve activity in essential hypertension are poorly understood. Some of the current suggested causes of elevated sympathetic tone in hypertension include: 1) impaired baroreceptor reflex response (13); 2) increased RAS activity (89); 3) salt mishandling (96); 4) genetic factors (12); and 5) psychological factors or hyper-responsiveness to stress (12). Yet almost all of the above failed to serve as an independent causative factor of increased sympathetic tone in essential hypertension.

The neural control of the circulation operates via two efferent arms of the autonomic nervous system – the sympathetic and the parasympathetic nerves.

The sympathetic nervous system is intimately involved in BP regulation. Systemic BP is largely controlled by the background level of sympathetic tone present at rest. The network that sets this background is presumably located in the rostral ventrolateral medulla (RVLM), the hypothalamus and the nucleus of the solitary tract (NTS) in the central nervous system (CNS). This core sympathetic network is regulated by sensory afferents projected to the NTS (for example, baroreceptors) and by circulating hormones such as angiotensin II (AngII) (18) and endothelin-1 (ET-1) (17). All sympathetic preganglionic neurons, including the neurons that synapse on postganglionic neurons which directly innervate blood vessels, receive monosynaptic inputs from overlapping subsets of neurons located in the above regions. It has been shown that there is increased neuronal activity found in this core network in hypertension (87). In animal models of neurogenic hypertension, a large and rapid drop in BP was found after inhibiting hypothalamic or RVLM neurons (1; 86). These indicate that increased sympathetic outflow from these regions is very crucial in the hypertensive process in neurogenic hypertension (44).

However, less is known about the peripheral sympathetic nervous system and its role in hypertension. As mentioned above, sympathetic preganglionic neurons receive excitatory synaptic inputs from brain regions like RVLM. These cholinergic preganglionic neurons then send axons to innervate postganglionic neurons in the sympathetic ganglia (para- or pre-vertebral sympathetic ganglia), adrenal gland or other neural networks (enteric or cardiac ganglionic networks) located outside CNS. The sympathetic ganglionic neurons that control

cardiovascular targets are primarily noradrenergic. They innervate the target organs by releasing NE, ATP and/or other vasoactive neuropeptides such as neuropeptide Y (NPY). Thus, the neural pathway connecting the CNS sympathetic core and the target blood vessels involve multiple steps of neurotransmission: 1) CNS neurons to preganglionic neurons; 2) preganglionic neurons to ganglionic neurons; 3) ganglionic neurons to blood vessels. Pharmacological therapies of hypertension targeting the reduction of sympathetic activity include ganglionic blockers such as hexamethonium which blocks cholinergic (nicotinic) transmission in the ganglia; agents acting on sympathetic nerve terminals such as guanethidine which inhibits presynaptic Mg^{++}ATPase (74). These agents all showed potent effect on reducing BP, indicating that every step in the neural pathway from the CNS through the ganglia to the blood vessels is crucial in mediating the sympathetic control of BP.

It is generally assumed that increased sympathetic outflow from the CNS can result in increased vasoconstriction at the target organ. However, is it really that simple? Is the pathway described above simply a relay for all the neural activation sent from the CNS or is every step in the pathway actually a setting for re-integration of the signals? Magee *et al.* showed that there is an enhanced neurotransmission through sympathetic ganglia in spontaneously hypertensive rats as compared to controls (68). More recently, ganglionic long-term potentiation (gLTP) has been shown to be associated with the induction or the aggravation of hypertension (40). This further suggested that the sympathetic ganglia may serve more than just a simple "relay station" in conveying the central

sympathetic outflow.

Among all the sympathetic ganglia, prevertebral sympathetic ganglia such as celiac ganglia, superior and inferior sympathetic ganglia are in particular important in regulating systemic BP since they contain ganglionic neurons that innervate the splanchnic circulation. The splanchnic circulation is composed of gastric, small intestinal, colonic, pancreatic, hepatic and splenic circulation (83). It receives about 60% of the cardiac output and contains about one third of the total blood volume. The rises in splanchnic vascular resistance is associated with human essential hypertension (81). It has also been estimated that innervation to the non-hepatic splanchnic organs accounts for half of the total NE released in the entire body (4). Thus, the properties of those neurons in prevertebral sympathetic ganglia and their alterations in association with essential hypertension are important in understanding neurogenic hypertension.

Role of sensory neurons in the regulation of BP

One often easily neglected part in the research of neural control of splanchnic blood flow is the sensory innervation of the blood vessels. Cell bodies of primary sensory neurons are located in the spinal dorsal root ganglia (DRG). They send nerve fibers in not only the central direction, in which they serve as "afferent" neurons to transmit information to the CNS, but also the peripheral direction, in which they act as "effector" neurons by releasing peptide transmitters at the sensory nerve terminals (50). In contrary to sympathetic innervation which

causes vasoconstriction, local activation of sensory nerves evokes vasodilatation (76). This is mediated by vasodilatory neural transmitters released from the sensory fibers, which include calcitonin gene-related peptide (CGRP) (103), substance P (SP) (38), neurokinin A (NKA) (30) and nitric oxide (NO) (112). It has been shown that in several hypertension animal models alterations in certain neuropeptide-containing sensory neuron properties contribute significantly to the development and maintenance of elevated BP (57; 105; 109).

Primary sensory nerves and sympathetic nerves that innervate mesenteric circulation can cause antagonistic effects on blood vessel diameter. In addition to causing direct vasodilatation, sensory afferents from the mesenteric vessels can also depolarize neurons in prevertebral sympathetic ganglia and may evoke vasoconstriction responses through this pathway (61; 62; 85). The cross talk between sensory and sympathetic nerves may also occur at the level of perivascular fibers because both types of nerve fibers are physically in close association with one another and therefore may directly interact with each other (30). This crosstalk between sensory and sympathetic nerves may play important roles in regulating BP.

The research in this dissertation was designated to study the pathologic events that occur in peripheral sympathetic and sensory neurons in hypertension, which may help us better understand the role of these neurons in the development and maintenance of neurogenic hypertension.

NADPH Oxidase --- Where, How and What

Reactive Oxygen Species

Free radicals are defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (29). The unpaired electron gives a considerable degree of reactivity to the free radical. The primary free radical derived from oxygen is superoxide anion ($O_2^{-\bullet}$), the formation of which is by adding one electron to the dioxygen molecule. Other reactive oxygen species (ROS) are generated from $O_2^{-\bullet}$ either directly or through enzyme- or metal-catalyzed processes (29). These include hydrogen peroxide (H_2O_2), hydroxyl radical (·OH) and peroxyl radical (HOO·). In addition, $O_2^{-\bullet}$ can also react with nitric oxide (NO·) and form peroxynitrite anion ($ONOO^-$). The nitrogen-containing molecules are also very reactive due to their unpaired electrons or strong oxidizing capability (84). They are defined as reactive nitrogen species (RNS).

The steady state of redox homeostasis in biological cells and tissues is maintained by the balance between the rates of ROS production and the rates of their clearance by various antioxidant compounds and enzymes (29). Under certain conditions, however, ROS production is increased more strongly and persistently, and the antioxidative response may not be sufficient enough to reset the system to its original level of redox homeostasis. This chronic shift in the level

of homeostasis is considered "oxidative stress" and has been seen in pathological processes like aging (45) and hypertension (111).

The production of $O_2^{-\bullet}$ occurs mostly within the mitochondria of a cell (11). It is produced from both Complexes I and III of the electron transport chain during energy transduction. It is suggested that under physiological conditions, about 1-3% of all electrons in the transport chain "leak" to oxygen prematurely and generate $O_2^{-\bullet}$ in that manner. However, the anionic $O_2^{-\bullet}$ is too strongly charged to readily cross the mitochondrial membrane, almost no detectable levels of $O_2^{-\bullet}$ can escape from intact mitochondria (80). Mitochondrial dysfunction is involved in certain diseases such as degenerative disorders or aging (32; 75) that are shown to be associated with increased mitochondria-derived $O_2^{-\bullet}$ production.

Unlike the mitochondrial electron transfer chain, nicotine adenine dinucleotide phosphate (NADPH) oxidase produces $O_2^{-\bullet}$ more directly in response to environmental stimuli. The enzyme was first characterized in neutrophils, where its production of $O_2^{-\bullet}$ generates the "respiratory burst" necessary for bacterial destruction (8). The enzyme complex consists of two membrane-bound subunits, NOX2 and $p22^{phox}$, which comprise cytochrome b558, the catalytic core of the enzyme. After activation, cytosolic components, involving $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small G coupled protein Rac translocate to the membrane to form the active enzyme complex. More recently, the presence of NADPH oxidase in non-phagocyte cell types have been identified in various tissue types, especially in cardiovascular tissues like vascular endothelium (56), vascular smooth muscle (63), kidney cortex (21), and nervous system (59).

Unlike those in neutrophils where the activation of NADPH oxidase generates "respiratory burst" by producing massive amounts of antimicrobial and tumoricidal ROS, the NADPH oxidase in cardiovascular tissues makes $O_2^{-\bullet}$ in relative smaller amounts for purposes of signaling (46). Fourteen years ago, Griendling *et al* first reported that in the vascular smooth muscle cells, AngII stimulated intracellular $O_2^{-\bullet}$ production via the activation of NADPH oxidase (42). Soon after that, functional NADPH oxidase was discovered and studied in vascular endothelial cells (56), kidney cells (55) and cardiac myocytes (94). Accumulating evidence suggested that NADPH oxidase-derived ROS are critical intracellular signaling molecules that are involved in the process of cardiovascular diseases such as hypertension (65) and atherosclerosis (107).

In the brain, ROS are best known for their association with neurological disorders such as stroke, Alzheimer disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS) (reviewed in (16; 53; 60)) . More recently, the important effects of ROS in central and peripheral neural mechanisms that regulate cardiovascular function have started to be recognized, particularly those that involve AngII signaling. Zimmerman *et al* pioneered the research in unraveling the role of neuronal NADPH oxidase in cardiovascular regulation. They reported that AngII-stimulated pressor and bradycardic responses were abolished in mice whose circumventricular organs were transduced with Adenoviral vectors encoding a dominant-negative inhibitor (N17Rac1) form of Rac1, a pivotal molecule for activating NADPH oxidase (113). This strongly suggested that a Rac-1 dependent NADPH oxidase is activated by

AngII in the brain and is a key source of $O_2^{-\bullet}$ in the central oxidant signaling cascade that is crucial in mediating the systemic responses to AngII.

This thesis was focused on understanding the role of NADPH oxidase in the peripheral sympathetic and sensory nervous systems in hypertension by addressing three questions: **1) Where** is NADPH oxidase localized in the peripheral neurons? **2) How** is NADPH oxidase regulated in these neurons in hypertension? **3) What** does NADPH oxidase-derived $O_2^{-\bullet}$ do in the neurons?

The "Where": NADPH Oxidase and its Localization in the Peripheral Sympathetic and Sensory Neurons

The presence of NADPH oxidase in peripheral sympathetic neurons was first demonstrated by Tammarielle *et al* who showed that all of the subunits of NADPH oxidase are expressed in primary rat sympathetic ganglion neurons and the activity of NADPH oxidase was closely associated with programmed cell death (100). The same research group later verified the localization of NADPH oxidase proteins in cultured superior cervical ganglion neurons from neonatal rats (49). Around the same time, my thesis research lab (Dr. David Kreulen's laboratory) started to investigate ROS in peripheral sympathetic neurons that innervate the splanchnic circulation. The first study was published in 2004 where they showed that an increased level of $O_2^{-\bullet}$ production was found in inferior mesenteric ganglia from deoxycorticosterone acetate (DOCA)-salt hypertensive rats than from normotensive controls, and this increase might be related to an up-regulation of ET-1 signaling via ET_B receptor in the ganglion neurons (24). The

study sets the foundation of this thesis project, in which the source of $O_2^{-\bullet}$ production in response to ET-1 in the prevertebral ganglion neurons and the regulation of NADPH oxidase in hypertension ganglia were examined.

In this thesis, I examined the expression and regulation of NADPH oxidase in rat prevertebral sympathetic ganglia and sensory dorsal root ganglia both of which are involved in regulating mesenteric circulation and thus are important in determining systemic BP as reviewed earlier in this chapter. My findings in this specific project can be concluded as following: 1) NADPH oxidase subunits NOX1, NOX2, $p47^{phox}$, $p22^{phox}$ and GTPase *Rac-1* are expressed in celiac ganglia and lumbar level dorsal root ganglia (T13-L2). NOX4 is expressed at a much higher level in CG than in DRG; 2) NADPH oxidase are localized not only to neuronal cell bodies in prevertebral sympathetic ganglia (CG and IMG) and DRG but also to perivascular sympathetic and sensory nerve fibers.

There are two interesting points from this study. First, CG and DRG do not express the same subunits of NADPH oxidase in the neurons. While DRG have higher levels of NOX1, NOX2, $p47^{phox}$ and $p22^{phox}$ than CG, the NOX4 expression is significantly lower in DRG. In fact, NOX4 has some unique characteristics in the NOX protein family. For example, NOX4 is the only core protein that does not require the binding of regulatory subunits to be functional (72) and it can produce ROS without any stimuli (91). The presence of an adequate level of NOX4 in CG but not DRG may indicate different mechanisms of $O_2^{-\bullet}$ generation or regulation in the two types of peripheral neurons, although they both contain NADPH oxidase. Second, NADPH oxidase subunits are localized to perivascular

sympathetic and sensory nerve fibers in addition to the neuronal cell bodies. The perivascular nerves are the sites for the storage and release of neurotransmitters or neuropeptides. In sympathetic nerve terminals, the two major neurotransmitter molecules NE and ATP are synthesized at the nerve terminal as well. $O_2^{-\bullet}$ is known to have an extremely short half life and therefore, the direct actions of $O_2^{-\bullet}$ in the cell is greatly confined within a certain region that is determined by the subcellular site of its production. The localization of NADPH oxidase at the nerve fibers may suggest a role of local $O_2^{-\bullet}$ in neurotransmitter handling. Unlike the mitochondrial electron transport chain, the activation of NADPH oxidase can be directly linked to environmental stimuli. For example, the NADPH oxidase localized to the plasma membrane on the presynaptic nerve terminal can potentially be activated by AngII or ET-1 through the activation of their receptors located nearby (52; 115). The $O_2^{-\bullet}$ produced by NADPH oxidase can either directly react with pre- or post-synaptically localized molecules involved in NE handling such as adrenergic receptors or NET; or indirectly affect the intracellular signaling after diffusing back to the nerve fiber in the form of H_2O_2. These hypotheses were not tested in this thesis but will be of great interest in future research.

The "How": Differential regulation of NADPH oxidase in sympathetic ganglia and sensory ganglia in DOCA-salt hypertension

Oxidative stress resulted from elevated NADPH oxidase activity or expression has been proposed to be an important pathological factor contributing

to hypertension. Cumulative evidence has pointed out the association between an increased $O_2^{-\bullet}$ and the onset of hypertension in animal models. The areas of the studies range from vasculature (10), kidney (21), and more recently the nervous system (23; 114). In this thesis, I found that the NADPH oxidase was upregulated in prevertebral sympathetic ganglia in DOCA-salt hypertension. This upregulation of the enzyme includes two components: the increased expression of $p22^{phox}$ and the translocation of $p47^{phox}$ from the cytoplasm to the plasma membrane.

$p22^{phox}$ is the membrane-bound subunit of NADPH oxidase that is associated directly with the catalytic core NOX protein. It was first recognized in phagocytes as the 22-kDa α-subunit of cytochrome b_{558}. In the neutrophils, it physically binds to the β-subunit $gp91^{phox}$ (later re-defined as NOX2) and is required for the $O_2^{-\bullet}$-producing function of cytochrome b_{558} (7). $P22^{phox}$ was later proven to be the "stabilizer" subunit on the membrane with other isoforms of NOX core protein such as NOX1 and NOX4 (2) in non-phagocyte cells that also contain NADPH oxidase. The amount of $p22^{phox}$ expression has been associated with the amount of NOX in the same tissue (64) and NOX is the core protein that produces $O_2^{-\bullet}$, the level of $p22^{phox}$ therefore directly affects the tissue's capability of $O_2^{-\bullet}$ production. Another important role of $p22^{phox}$ in the enzyme complex is to provide an anchoring site for cytosolic factor $p47^{phox}$ at its C-terminal cytoplasmic tail. After $p47^{phox}$ is phosphorylated upon stimulation, its Src homology 3 domain binds to the proline-rich region of the $p22^{phox}$ tail and thereby initiates the formation of the active NADPH oxidase (97). Therefore, the upregulation of

$p22^{phox}$ can yield two results, both of which contribute to elevated $O_2^{-\bullet}$ production: 1. increased stabilization or expression of NOX core proteins; 2. more available binding sites for $p47^{phox}$.

The increased gene expression of $p22^{phox}$ was in particular associated with hypertension as compared to other subunits. It was first reported to be upregulated in aorta from an AngII-induced hypertension model (37). In other types of hypertension, for example low-renin DOCA-salt hypertension, $p22^{phox}$ was also shown to be increased in the vasculature (10). The upregulation of $p22^{phox}$ is not limited to the blood vessels. It was also observed in the kidney (20). Interestingly, *in vivo* short interference RNA (siRNA) targeting $p22^{phox}$ was shown to reduce the oxidative stress level and the AngII slow-pressor response in the AngII-infused animals (78). In addition, polymorphisms of $p22^{phox}$ gene or its promoter have been associated with human cardiovascular diseases (6; 39; 79; 88). In this thesis, the finding of the increased $p22^{phox}$ expression in the prevertebral ganglia in further supports the indispensable role of $p22^{phox}$ in hypertension.

The mechanisms of the up-regulation of $p22^{phox}$ are not fully understood. Some evidence suggested that $p22^{phox}$ itself can be regulated in a redox-sensitive pathway (28) such that the expression of $p22^{phox}$ can be augmented by an increased intracellular $O_2^{-\bullet}$ production from the initial activation of NADPH oxidase in the same cell. Although no clear mechanisms were provided in that paper explaining the pathways of this feed-forward upregulation, recent report on the AP-1-dependent transcriptional regulation of NADPH oxidase may indicate

the involvement of redox-sensitive transcriptional factors. AP-1 was shown to enhance $p22^{phox}$ promoter activity possibly via the direct binding of c-Jun/c-Fos to the AP-1 binding site on the promoter sequence of $p22^{phox}$ (70). Meanwhile, AP-1 can be activated by ROS and is one of the major molecules mediating redox-dependent transcriptional regulation (67). Therefore, it is reasonable to propose that upon stimulation, the initial increase in $O_2^{-\bullet}$ production is from the quick assembly of NADPH oxidase complex. This $O_2^{-\bullet}$ later activates AP-1, facilitates its binding to $p22^{phox}$ promoter and thereby increases the gene expression of $p22^{phox}$. Elevated $p22^{phox}$ expression further enhances the activity of NADPH oxidase by either docking more NOX core protein to the membrane or/and providing more anchoring sites for cytosolic factor $p47^{phox}$. This feed-forward mechanism may help sustain the high levels of ROS against antioxidants in the cell and eventually result in oxidative stress. To date, most studies were performed in the vascular cells. Whether the similar mechanisms exist in the neurons is not known. Future studies can be designed to explore this possibility.

So far, it seems like the regulation of NADPH oxidase is limited to a "unidirectional" upregulation towards more $O_2^{-\bullet}$ production everywhere in the body in hypertension. However, is it true? Is NADPH oxidase solely a bad player? This thesis challenges the view by showing that in rat DRG, the activity and expression of NADPH oxidase were actually decreased in DOCA-salt hypertension. This finding argues against the traditionally accepted idea of "universal oxidative stress" in hypertension. This is probably one of the reasons why anti-oxidant therapy has not yet shown exciting outcome in preventing or

treating hypertension in human despite the publication of hundreds of papers listing the association of $O_2^{-\bullet}$ and high BP in the past decade (51). The fact that not all tissues showed upregulation of NADPH oxidase at least requires a more sophisticated method in anti-oxidant therapy of hypertension. For example, the use of target-specific approach should be considered.

One important fundamental question that has not been well addressed in this field is "$O_2^{-\bullet}$ or hypertension, which comes first?" Is $O_2^{-\bullet}$ a causative factor of hypertension or is high BP leading to more $O_2^{-\bullet}$ production? Although generally more appreciated, no direct evidence has supported the former view. Therefore, the possibility that the regulation of $O_2^{-\bullet}$ production subjects to the onset of high BP still exists. In that case, NADPH oxidase in different parts of the body may respond differently to increased BP. For example, sensory nerves serve as compensatory mechanism to antagonize the increased vasoconstriction by elevated sympathetic innervation in hypertension. The decreased activity and expression of NADPH oxidase in DRG in DOCA-salt hypertension may be part of this mechanism: It has been shown that the synthesis and release of the vasodilatory peptide CGRP from the DRG neurons were increased in salt-sensitive hypertension (98; 108). On the other hand, CGRP displayed inhibitory effect on $O_2^{\bullet-}$ production in neutrophils (101). Although the pathway has not been tested in the sensory neurons, its presence is possible. Therefore, the $O_2^{-\bullet}$ decrease in DOCA-salt DRG may be a result from compensatory increase of CGRP and this will favor the view of "high BP regulates $O_2^{-\bullet}$ production".

This thesis provides the descriptive findings of differential regulation of NADPH oxidase in two different parts of the peripheral nervous system. It is the first evidence showing a decreased oxidase activity associated with hypertension. These findings provide a different angle for us to consider the role of ROS in hypertension.

The "What": Regulation of sympathetic neuronal NET by NADPH oxidase-derived $O_2^{-\bullet}$

It is well documented that increased ROS levels are associated with various pathological disorders including hypertension. However, little is known about the exact role of ROS in modifying neuronal function in hypertension. It is well characterized in the phagocytes that NADPH oxidase plays a crucial role in host defense by producing $O_2^{-\bullet}$ that are intended to kill invading microbes. In non-phagocytic cells, on the other hand, $O_2^{-\bullet}$ is produced by NADPH oxidase in a much smaller amount for the purpose of inter- or intracellular signaling. In vascular system, multiple functions have been reported for $O_2^{-\bullet}$ generated by NADPH oxidase, including directly scavenging nitric oxide (NO) (106), mitogenic signaling (22) and oxygen sensing (54). However, less is known about the function of $O_2^{-\bullet}$ in the nervous system that is involved in cardiovascular regulation.

Although not directly linked, the studies on ROS in neurodegenerative diseases may shed light on exploring ROS function in cardiovascular diseases. Oxidative stress has long been a discussion point for age-related neurodegenerative diseases including Alzheimer's disease, Parkinson's disease

and amyotrophic lateral sclerosis (ALS). Virtually all cellular biomacromolecules (lipids, sugars, proteins, and polynucleotides) in the neurons are potential targets for ROS damage. The so-called neurotoxicity results from multiple aspects of the functions of elevated ROS in the nervous system: mediating neuroinflammatory response, causing faulty protein modification (formation of unfolded or misfolded proteins), modulating gene expression via its effects on transcription factors, interfering with cellular signal transduction or disrupting membrane function via lipid peroxidation (affecting maintenance of membrane potential and synaptic signaling), etc (reviewed in (90)). "Whether oxidative stress is a consequence of degenerative processes initiated by other factors or it is an early event that contributes integrally to the etiology of the disease" has also been a central question in this area. There is already evidence for a primary contribution of oxidative stress to neuronal cell death. Take ALS for example, a good fraction of the disease is caused by mutations in the antioxidant enzyme cytosolic Cu,Zn-superoxide dismutase (SOD1) (26). Mouse models of ALS based on overexpressed mutant human SOD1 experience predictably staged, age-dependent motor neuron degeneration with profound cellular and biochemical damage to nerve fibers and spinal cord tissue (43). Redox perturbations resulted from SOD1 mutation causes irreversible oxidative protein modifications and leads to protein malfunction (3), and also propagate or modify signal transduction that facilitates the formation of neuroinflammation (48). All these evidence support a primary role of oxidative stress in the pathologic process. This approach may help us unravel the relationship between oxidative stress and cardiovascular

diseases. In fact, the association between polymorphism of NADPH oxidase $p22^{phox}$ and cardiovascular diseases in human already suggested a potential primary contribution from ROS in the disorders (6; 88). Future studies using genetic manipulation in animal models can further strengthen this view.

It is noteworthy though that the situation in hypertension is different from it is in neurodegenerative diseases. While neuronal cell death is the common pathological feature in neurodegeneration as a result of oxidative stress, neurons are not necessarily to be killed by ROS in hypertension. At least, there is no clear evidence so far showing significant neuronal death associated with any kind of cardiovascular diseases. In neurodegeneration, mitochondria-derived oxidative stress is considered a primary pathological event (66). As mentioned previously, the production of $O_2^{-\bullet}$ occurs mostly in the mitochondria of the cell as a byproduct of the electron transport chain. In other words, as long as oxidative phosphorylation is functioning in the mitochondria to power ATP synthesis for the cell, a certain amount of $O_2^{-\bullet}$ is produced at the same time. Mitochondrial dysfunction or perturbation of mitochondrial membrane permeability therefore leads to profound amount of ROS leakage into the cytoplasm from this source. On the other hand, elevated ROS in hypertension are thought to be mainly from enhanced NADPH oxidase activity or expression. Although activated NADPH oxidase in the phagocytes is able to produce great amount of $O_2^{-\bullet}$ at a time, NADPH oxidase in the non-phagocytic cells only produces $O_2^{-\bullet}$ in a very low amount and this process is highly controlled by regulatory factors. The high ROS levels in neurodegeneration are indeed causing "stress" to the cells and thus be

detrimental for cellular viability, but ROS produced by NADPH oxidase in cardiovascular diseases are probably more suited for involvement in signal transduction or redox signaling rather than causing oxidative stress. Therefore, when we seek to understand the role of ROS in hypertensive neurons, we should orient ourselves more towards the potential contributions of oxidative signaling to typical functional features found in the neurons in hypertension rather than a universal neuronal damage caused by oxidative stress.

One of the functional alterations found in the peripheral sympathetic neurons in hypertension is catecholamine mishandling. NE is the major catecholamine neurotransmitter that is released by the sympathetic nerve terminals to the blood vessels. The vasoconstrictor effect of NE is greatly limited by NE clearance via the prejunctional norepinephrine transporter (NET). The direct physiological consequence of NET malfunction is increased level of neurovascular junctional NE content and thus elevated vasoconstriction. Impaired nerve terminal NE reuptake via NET has been found in essential hypertension (34). Therefore, NET was selected as the molecule of interest in this thesis to help investigate the potential relationship between sympathetic neuronal function and ROS.

The lessons we learned from neurodegenerative diseases include 1) ROS can modulate protein structure/function, and 2) ROS modifies gene expression. To be followed is the discussion of the potentialities of NET being regulated by ROS from each of the above aspects.

NET protein

NET belongs to a family of sodium- and chloride-coupled transporters. The rat NET is a 617 amino acid protein with 12 α-helical transmembrane domains (TMD) that are interrupted by alternating intra- and extracellular loops (14). As illustrated in Figure 6.1, three sites on the NET amino acid sequence can potentially render NET to ROS regulation. They are cysteine residues at amino acid 28, 44, 176, 185 and 351; asparagine residues at amino acid 184 and 192; and serine residues at amino acid 259 and 579.

Cysteine is an important structural and functional component of many proteins because of the high reactivity of its thiol. Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between the thiol groups (-SH) and low molecular weight thiols, in particular GSH (*S*-glutathiolation) (95). The cysteine residues at amino acid 28 and 44 of rat NET are located intracellularly while 176 and 185 are located in the second extracellular loop. Therefore, the ROS produced at either side of the cell membrane can potentially regulate NET protein function by modulating the disulphide bonds formation from cysteine residues. Since the formation of disulphides is mostly reversible, its impact on NET regulation should be considered acute or transient. The acute modification of NET protein has been observed in various aspects including rapid subcellular redistribution or alterations in its transport efficiency (110). Although there is no direct evidence showing a functional relationship of these effects with disulfide formation on cysteine residues, the possibilities exist. In addition to

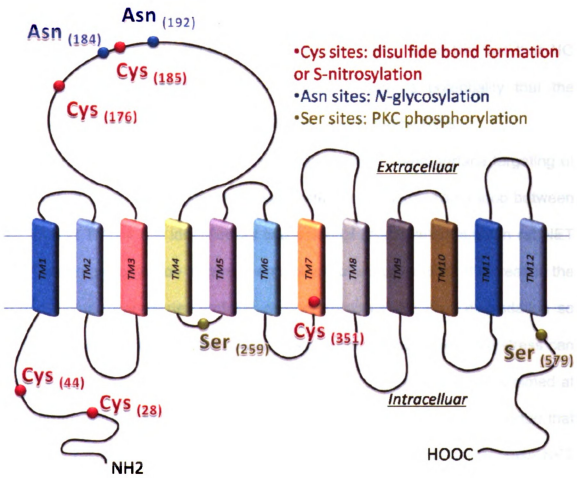

Figure 6.1. Topological model of rat NET protein. The cartoon illustrates the proposed structure of rat NET with 12 transmembrane (TM1-12) domains. N- and C- temini are located intracellularly and a large extracellular loop is positioned between TM3 and TM4. Red solid cirles represent cysteine (Cys) residues, which are potential sites for disulfide bond formation or *S*-nitrosylation. Blue solid circles represent asparagine (Asn) residues, which are potential *N*-glycosylation sites. Green solid circles represent serine (Ser) residues, which are potential phosphorylation sites for protein kinase C (PKC). Numbers in the parentheses indicate the amino acid number in the NET sequence.

cytosolic or extracellular loop sequences, the TMDs of NET also contain several cysteine residues. In particular, the TMD 7 has been suggested to play a key role in mediating catecholamine transport by catecholamine transporters (15). A key cysteine residue (amino acid 351) in the TMD 7 of NET was shown to be regulated by nitric oxide (NO) via *S*-nitrosylation (58). Given the fact that NO levels are closely related to ROS production, there is potentiality that the nitrosylation of Cys 351 can also be indirectly modulated by ROS.

N-glycosylation is important for physical maturation and surface targeting of NET protein (77). Asparagine residues located in the extracellular loop between TMD 3 and TMD 4 are identified as two potential glycosylation sites in rat NET (14). Protein glycosylation occurs in endoplasmic reticulum (ER). It increases the properly folded transporter proteins and/or protects them from degradation so that they can be efficiently trafficked to plasma membrane. Oxidative stress can cause ER stress, which leads to rapid reduction in protein biosynthesis aimed at lowering the load of ER's client proteins including NET. It has been reported that ER stress resulted from intracellular ROS challenge was able to decrease NET function and expression via the perturbation of its *N*-glycosylaton (71). Meanwhile, NADPH oxidase has been localized in ER compartment (2; 104). The presence of ROS-producing enzyme in the close vicinity of the sites where NET protein undergoes critical post-translational regulation indicates possible interaction between the two molecules.

The third group of amino acid residues in NET protein that is crucial to its regulation is the serine residues 259 and 579 located in the intracellular loop of

the protein. They are the two potential phosphorylation sites for protein kinase C (PKC). Multiple studies have demonstrated that PKC pathway is the most common intracellular mechanism through which monoamine transporters can be regulated. Phosphorylation of NET at these sites is followed by the internalization of the transporter into the cytoplasm from the plasma membrane (5). Thus, PKC regulates NET function by rapidly altering the equilibrium between the plasma membrane and the cytoplasmic fractions of transporters. PKCs contain unique structural features that are susceptible to oxidative modification. Its N-terminal domain contains zinc-binding, cysteine-rich motifs that are readily oxidized by peroxide while its C-terminal domain contains several reactive cysteines that are target for various antioxidants such as vitamin E analogues (41). The oxidization of the N-terminal domain of PKC by ROS stimulates PKC activity while the modification of the C-terminal cysteines by antioxidants decreases its activity. Therefore, PKC activity is tightly regulated by intracellular redox state. When increased NADPH oxidase activation occurs in the cell, the shift of the oxidant/antioxidant balance could potentially stimulate excess PKC activity and thereby decrease NET surface expression.

NET gene expression

The regulation of NET gene expression in response to ROS production is the topic of interest in this thesis (see Chapter 5). Using ET-1 as an agonist, enhanced intracellular ROS generation was observed in PC12 cells. This ROS production was through the activation of NADPH oxidase. NET mRNA levels were decreased in response to ET-1 after 2 hours of incubation, and this was

followed by a recovery after 12 to 24 hours. When NADPH oxidase key subunit $p22^{phox}$ expression was attenuated by RNA interference in PC12 cells, ET-1 no longer elicited any suppressive effect on NET mRNA expression. This indicated that NET transcription in PC12 cells can be transiently inhibited by NADPH oxidase derived ROS.

The rat NET gene and its promoter have been cloned in PC12 cells (14) (82). The presence of several transcription factor binding sites in the promoter region reveals the putative mechanisms of transcriptional regulation of the NET gene. Certain transcription factors can alter their activity, depending on cellular redox conditions. Redox-dependent change in transcriptional output facilitates the cell to respond appropriately to the perceived oxidative stress in various disease conditions. Activator protein -1 (AP-1) (Fos and Jun), nuclear factor κB (NF-κB) and cAMP-response element-binding protein (CREB) are the major redox-sensitive transcription factors found in mammalian cells (67). Their bindings sites are also identified in the promoter sequence of NET gene (82). Therefore, it is likely that NET transcription can be regulated in response to intracellular ROS production via the activation/deactivation of these transcription factors. However, the direct link between these factors and NET expression has not been clarified. It is thus not clear whether the NET mRNA decrease in response to ROS was mediated through these redox-sensitive transcriptional factors.

An important feature of NET promoter sequence is that it is rich in CpG dinucleotides, which renders it susceptible to methylation-related gene silencing (31). DNA methyltransferases that modify CpG sites by adding a methyl group at

position five of cytosine base residues, are responsible for the methylation of CpG islands (9). Once the CpG motif is altered in this manner, it is bound by methylcytosine specific proteins, which can interfere with the binding of transcription factors, beginning the process of epigenetic silencing (36). Hypermethylation of NET promoter was found in panic disorder patients and was suggested to mediate NET gene silencing (33). There is also a potential influence of ROS on DNA methylation pattern as reported in cancer research (19). It is therefore possible that ROS can have an impact on NET gene methylation to attenuate its expression. However, it is very questionable whether acute NET mRNA suppression can be caused by hypermethylation. Although there is no direct evidence indicating the actual time course for the effects of methylation on gene silencing to occur, with the fact that DNA methylation involves multiple-step mechanisms, it is unlikely to be an acute modification. Therefore, hypermethylation may not serve as an appreciable mechanism to explain the NET mRNA decrease followed by 2 hours of ET-1 treatment in this thesis. The potential chronic modulation of NET methylation pattern by ROS should still be considered in disease state like hypertension.

ROS has also been shown to attack RNA molecule directly. Nucleic acids can be damaged by hydroxyl radicals produced from $O_2^{-\bullet}$ and peroxide by the Fenton reaction (47). Various types of oxidatively altered bases have been detected in DNA or RNA, among which 8-hydroxydeoxyguanosine in DNA or 8-hydroxyguanosine in RNA appear to be the most deleterious ones (102). 8-hydroxyguanine can incorrectly pair with adenine or thymine at similar or higher

efficiency than with cytosine, resulting in nucleotide mis-incorporation during RNA synthesis (99). Oxidized or modified mRNAs are subsequently removed from the functional RNA pool by RNA surveillance mechanisms and therefore will not participate in active protein translation. It was reported that 8-hydroxyguanine is present in 30-70% of the mRNA found in the brains of Alzheimer's patients (93). The oxidation of mRNA may prime cell death by reducing the expression of critical proteins (92). It seems very appealing to think that the NET mRNA can be damaged by ET-1 induced $O_2^{-\cdot}$ and therefore causes a decrease in its mRNA levels. However, when actinomycin D, a transcription inhibitor, was applied to the cells prior to ET-1 addition, the decrease of NET mRNA was abolished. This suggests the downregulation of NET mRNA occurs through a transcriptional modification rather than direct damage on RNA molecules by ROS.

In this thesis, I showed evidence for an NADPH oxidase mediated pathway in the acute downregulation of NET gene expression. However, no further effort was made to explore the detailed mechanisms of the transcriptional regulation of NET under the influence of ROS production. Therefore, limited information can be provided at this point to explain the above descriptive results. More studies are clearly needed to fully understand the mechanisms.

Summary

This thesis has provided these novel findings: 1) NADPH oxidase is expressed in prevertebral sympathetic ganglia and sensory ganglia. NADPH

oxidase is also present in sympathetic and sensory perivascular nerves on mesenteric arteries. 2) NADPH oxidase is upregulated in sympathetic ganglia but downregulated in sensory ganglia in DOCA-salt hypertension. 3) NADPH oxidase mediates ET-1 downregulation of NET mRNA transcription, and this effect can be blocked by $p22^{phox}$ knockdown.

The presence of NADPH oxidase in both the neuronal cell bodies and the nerve fibers of sympathetic and sensory ganglia strongly indicate a close relationship between the NADPH oxidase derived ROS and the properties of the neurons (Figure 6.2). The conventional concept of oxidative stress as a universal character in the disease is being challenged because $O_2^{-\bullet}$ levels are not increased in all tissues in hypertension. Instead of exerting toxic damages to the cells, ROS may modulate cellular function via complex signal transduction in response to environmental stimuli. The functional consequences of ROS production in peripheral neurons in hypertension may include modification of gene expression for certain proteins that are critically involved in blood vessel innervation, such as NET. These findings may shed light on novel roles of neuronal redox signaling in hypertension.

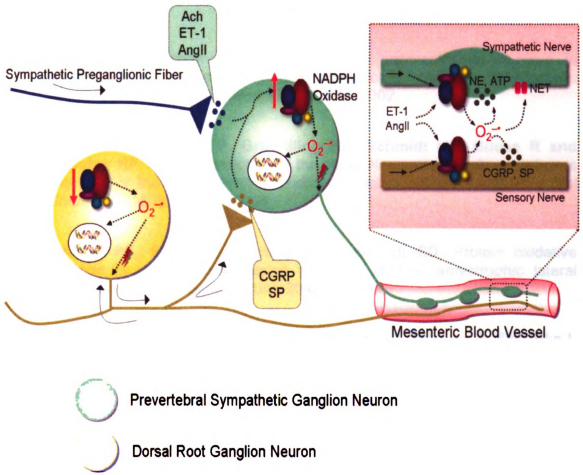

Figure 6.2. Diagram of NADPH oxidase in the sympathetic and sensory innervation of mesenteric blood vessels in hypertension. NADPH oxidase is localized to both prevertebral sympathetic ganglia and dorsal root sensory ganglia. The activity and expression of NADPH oxidase in the sympathetic ganglia is increased in hypertension. This could be due to an enhanced neurotrasmission from preganglionic nerve fibers or sensory fibers. The elevated level of $O_2^{\cdot-}$ in the ganglia could potentially affect the gene expression in the neuron or/and modulate neuronal firing properties. On the other hand, NADPH oxidase is downregulated in dorsal root ganglia in hypertension. The decreased level of $O_2^{\cdot-}$ may be a compensotary effect of elevatd blood vessel tone. $O_2^{\cdot-}$ in the sensory neurons could also potentially be involved in the regulation of gene expression and neuronal firing properties. NADPH oxidase is also localized to sympathetic and sensory perivascular nerve fibers. Their activities may be modulated by nerve firing signals sending from the neuronal cell bodies or hormonal factors released from the blood vessels. $O_2^{\cdot-}$ produced at the neurovascular junction may have an impact on neurotransmitter release from the nerve teminals or modulate neurotransmitter reuptake. Ach: acetylcholine; ET-1: endothelin-1; AngII: angiotensin II; CGRP: calcitonin gene related peptide; SP: substance P; NE: norepinephrine; NET: norepinephrine transporter.

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