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Localization of NADPH Oxidase in Sympathetic and Sensory Ganglion Neurons and Perivascular Nerve Fibers

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Abstract

Superoxide anion ($O_2^{-\bullet}$) production was previously reported to be increased in celiac ganglia (CG) during DOCA-salt hypertension, possibly via activation of the reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase. This suggested a role for neuronal NADPH oxidase in autonomic neurovascular control. However, the expression and localization of NADPH oxidase in the peripheral neurons is not fully known. The purpose of this study was to examine the subcellular localization of NADPH oxidase in sympathetic and sensory ganglion neurons and perivascular nerve fibers. In rat CG, $p22^{phox}$ and neuropeptide Y (NPY) were colocalized in all neurons. $P22^{phox}$ was also localized to dorsal root ganglia (DRG) neurons that contain calcitonin gene related peptide (CGRP). In mesenteric arteries, $p22^{phox}$ and $p47^{phox}$ were colocalized with NPY or CGRP in perivascular nerve terminals. A similar pattern of nerve terminal staining of $p22^{phox}$ and $p47^{phox}$ was also found in cultured CG neurons and nerve growth factor (NGF)-differentiated PC12 cells. These data demonstrate a previously uncharacterized localization of NADPH oxidase in perivascular nerve fibers. The presence of a $O_2^{-\bullet}$ – generating enzyme in close vicinity to the sites of neurotransmitter handling in the nerve fibers suggests the possibility of novel redox-mediated mechanisms in peripheral 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 in regulating cardiovascular function (Griendling et al., 2000a; Griendling et al., 2000b). While first discovered in neutrophils, NADPH oxidase is now thought to be a significant source of ROS in many cell types, including smooth muscle cells (Griendling et al., 1994; Patterson et al., 1999; Ushio-Fukai et al., 1996), endothelial cells (Gorlach et al., 2000) and fibroblasts (Jones et al., 1994). NADPH oxidase has also been localized to the nervous system. For example, we previously showed that NADPH

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oxidase subunits were present in rat sympathetic and sensory ganglia (Cao et al., 2007; Dai et al., 2006).

Increased ROS production and NADPH oxidase activity are associated with cardiovascular dysfunction in hypertension (Beswick et al., 2001), diabetes (Gunes et al., 2005) and senescence (Chakravarti and Chakravarti, 2007). Studies of ROS in hypertension have focused primarily on vascular and endothelial ROS signaling (Griendling et al., 2000b). However, accumulating evidence indicates that peripheral neural components play a key role in regulating blood pressure. For example, mesenteric circulation is innervated by postganglionic sympathetic nerves and spinal sensory nerves. It can also be mobilized by peripheral reflex pathways that involve non-spinal peripheral sensory neurons (King and Szurszewski, 1989; Meehan and Kreulen, 1992). Abnormalities in the peripheral sympathetic and sensory neurons contribute to increased salt sensitivity and the development of hypertension (King et al., 2007; Mangiarua and Lee, 1990; Mathias, 1991; Wang and Li, 1999; Whitelaw and Smithwick, 1958). In particular, enhanced NADPH oxidase activity in peripheral sympathetic neurons is associated with the onset of cardiovascular disorders (Cao et al., 2007; Dai et al., 2004; Ma et al., 2006). 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 in the cell body would not be expected to diffuse to the terminals; if $O_2^{-\bullet}$ were to influence neurotransmitter dynamics it would have to be produced locally. However, it is not known if NADPH oxidase is localized to perivascular nerve fibers.

To address this issue, a series of immunohistochemical experiments were designed to localize NADPH oxidase subunits, $p22^{phox}$ and $p47^{phox}$, to the neuronal cell bodies in celiac ganglia (CG) and dorsal root ganglia (DRG) as well as perivascular nerve fibers on the mesenteric arteries. We found that NADPH oxidase localized to sympathetic and sensory neurons as well as periarterial nerve fibers and endings. The presence of NADPH oxidase subunits in fibers innervating the mesenteric circulation is novel and may have important implications in the role of NADPH oxidase in blood pressure regulation and hypertension.

Methods

Animals

Unless noted, all experiments were done using Sprague-Dawley rats from Charles River Laboratories (Portage, MI). Upon arrival at the animal care facility, animals were maintained according to standards approved by the Institutional Animal Care and Use Committee at Michigan State University. All experimental procedures were carried out in accordance with the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society.

Drug Treatment and Surgeries

Capsaisin-treatment (cap-tx)—Briefly, on days 1 and 2 of life, neonatal Wistar rats received capsaicin (50 mg/kg) subcutaneously, as described previously (Wang et al., 1998). Control rats were treated with equal volumes of vehicle solution (5% ethanol, 5% Tween 80

in saline). All treatments were performed with rats under ether anesthesia (Wang and Li, 1999).

Celiac ganglionectomy (CGx)—CGx was performed by locating the celiac plexus in between the aorta, celiac artery, and cranial mesenteric artery; dissecting it free from surrounding tissue; and removing it. Any additional nerves along these vessels in the area of the CG were also dissected free and transected (King et al., 2007). Treatments were performed in anesthetized adult Sprague-Dawley rats.

Antibodies

All antibodies used in this study are listed in Table 1.

Cell Culture

Unless noted, all cell culture reagents were GIBCO® brand (Invitrogen, Carlsbad, CA).

Primary ganglion neuron culture—CG and DRG from neonatal rats were harvested and enzymatically dissociated (2.5mg/ml collagenase for 10 minutes at 37°C followed by 2.5mg/ml trypsin for 45min at 37°C). Freshly dissociated neurons were plated as a monolayer on cover glass in culture dishes double-coated with poly-D-lysine (Sigma, 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 (NGF) 2.5S (Millipore, Billerica, MA), 0.7% B-27) supplemented with 1% fetal bovine serum at 37°C in a 5% CO_2 humidified incubator. From the second day of culture, 0.24μg/ml Ara-C (Calbiochem, San Diego, CA) was added to the N2 medium to eliminate non-neuronal cell growth. Neurons were kept in culture for 7 days before immunostaining to ensure full neurite outgrowth.

PC12 Cell Culture—PC12 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 (Greene et al., 1998). PC 12 cells were obtained from American Type Culture Collection (ATCC) and maintained at 37°C in a 5% CO2 humidified incubator in RPMI 1640 medium supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, 100U/ml penicillin, 100ug/ml streptomycin and 0.25ug/ml Fungizone. To differentiate PC12 cells, 50ng/ml NGF 2.5S was added to the medium for 7 days.

Immunocytochemistry of cultured ganglion neurons and PC12 cells

Cultured 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 X-100 in DPBS) for 30min. Cells were then incubated in DPBS with blocking solution (5% goat serum, 3% bovine serum albumin) for 1 hour at room temperature, followed by primary antibody incubation for overnight at 4°C. The next day, samples were washed in DPBS three times and then incubated with corresponding secondary antibodies 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) or Fluoview (Olympus, Center Valley, PA).

Immunostaining of Periarterial Nerve Fibers

Eight week old 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 diluted in Triton X-100 (1.0 %) for 1 hour. Tissues were then incubated with primary antibodies for 2 hours at 37°C. Next, tissues were washed 3 times in 0.1M PBS buffer and then incubated with secondary antibodies for 1 hour in a dark, humidified chamber at room temperature. Vessels were then washed 3 times with 0.1 M PBS at 5-minute intervals, and coverslips were mounted 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

P22^{phox} is localized in neuropeptide Y (NPY)-containing CG neurons and calcitonin gene related peptide (CGRP)-containing DRG neurons

In guinea pig prevertebral sympathetic ganglia, approximately 20% of all neurons contain IR to NPY and have been speculated to be vasoconstrictor neurons (McLachlan and Llewellyn-Smith, 1986). On the other hand, 18.9% of neurons in the IMG that innervate the inferior mesenteric artery are NPY-positive (Browning et al., 1999). In the rat CG, we found that all neuron cell bodies examined were immunopositive for NPY and the NADPH oxidase subunit p22^{phox} (Fig. 1A). The presence of both proteins was not limited to a subpopulation of neurons in rat CG. CGRP is a vasodilatory neuropeptide that is released from the sensory nerve fibers (Uddman et al., 1986). The synthesis of the peptide occurs in neuronal cell bodies of the DRG. CGRP immunostaining in rat DRG was primarily found in small-diameter neurons, while the distribution of p22^{phox} immunoreactivity was more universal throughout the ganglia (Fig. 1B). Staining for both CGRP and p22^{phox} was found in some cells, indicating colocalization of these two proteins in the same sensory neurons.

P47^{phox} and p22^{phox} are present in the neurites of PC12 cells and cultured CG and DRG neurons

In order to examine the presence of NADPH oxidase in neuronal compartments outside of the cell bodies, we first immunostained 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. It was colocalized with secretogranin II (SGII), a darge dense core vesicle marker protein (Fischer-Colbrie et al., 1995), on the cell membrane and the neurites (Fig. 2A). In dissociated CG and DRG neurons, p47^{phox} was colocalized with SGII in both cell bodies and neurites (Fig. 2B and 2C). p22^{phox} was also found to be colocalized with SGII in CG and DRG cultured cells (data not shown). These findings suggest that in addition to cell bodies, NADPH oxidase is present in nerve fibers in cultured ganglion neurons.

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

In order to determine if NADPH oxidase subunits colocalize to sympathetic nerve fibers and endings in tissue, tertiary branches of mesenteric arteries were fixed and labeled with NPY and either anti-p22^{phox} (Fig. 3A) or anti-p47^{phox} (Fig. 3B). P22^{phox} or p47^{phox} and NPY were found in the same nerve fiber bundles. The overlay images show that p22^{phox} or p47^{phox} and NPY co-localize to some, but not all periarterial nerve fibers, indicating that NADPH oxidase is also localized to non-sympathetic nerve fibers on the blood vessels. Anti- tyrosine hydroxylase (TH) showed similar results to NPY-stained fibers (data not shown).

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. Fig. 4 shows that p22^{phox} or p47^{phox} was colocalized with CGRP in the same nerve fibers. The immunostaining for NADPH oxidase was not limited to CGRP positive fibers, indicating non-sensory source of NADPH oxidase on the nerve fibers. This is consistent with the findings of sympathetic localization of NADPH oxidase as was shown above. These results indicate that NADPH oxidase subunits are present in sensory periarterial nerve fibers.

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

Because sensory and sympathetic nerve fibers run close to each other, it is difficult to localize the precise labeling patterns of proteins to one or the other. Therefore, we chemically removed the sensory nerve fibers by treating animals with capsaicin to confirm the presence of NADPH oxidase in non-sensory fibers. Small mesenteric arteries removed from cap-tx animals were labeled with anti-p47^{phox} and anti-CGRP (Fig. 5). The absence of anti-CGRP labeling in cap-tx rats indicated that treatment was effective in depleting sensory nerve fibers. However, significant p47^{phox} immunostaining remained. The higher density of perivascular p47^{phox} staining in cap-tx rats may be due to an increase in p47^{phox}-containing sympathetic nerve sprouting, because long-term sensory denervation augments sympathetic neurotransmission (Ralevic et al., 1995). These results suggest that p47^{phox} exists at least partly in non-sensory peri-arterial nerve fibers.

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

Most sympathetic peri-arterial nerve fibers supplying mesenteric arteries originate in the CG, and spinal sensory nerves pass through the CG on their way to the DRG. Therefore, CGx was performed in adult rats to investigate possible presence of NADPH oxidase in perivascular nerve fibers that are neither sympathetic nor spinal sensory source. After CGx, TH staining on the mesenteric arteries was completely abolished (Fig. 6), so was CGRP staining (data not shown). These results indicate that the perivascular sympathetic and spinal sensory nerve fibers were successfully depleted by CGs. However, minor p47^{phox} immunoreactivity was still detected in some superficially-located nerve fibers. These remaining p47^{phox}-positive perivascular nerves may be either peripheral sensory fibers that were not abolished during the surgery or intestinofugal fibers, which originate in the myenteric plexus and terminate in prevertebral ganglia (Luckensmeyer and Keast, 1995).

Discussion

The primary finding of this study is that NADPH oxidase subunits are present in perivascular nerve fibers on mesenteric arteries. The localization of the same NADPH oxidase subunits in the neuronal cell bodies in CG and DRG indicates the sympathetic and sensory sources of these fibers. The presence of a local $O_2^{-\bullet}$-producing enzyme at the neurovascular junction may indicate roles of $O_2^{-\bullet}$ in the modulation of neurotransmission at the nerve terminals.

NADPH oxidase is a multi-subunit enzyme complex; the proteins p22^{phox} and p47^{phox} are key catalytic and regulatory components, respectively and have been used in this study to localize NADPH oxidase. Enzymatic activity requires the assembly of all or some the subunits centered around a NOX protein for which various isoforms have been identified, including NOX1, NOX2 and NOX4 (Lambeth et al., 2000). All of the isoforms of NOX produce ROS when expressed with p22^{phox} but only NOX2 requires p47^{phox} (Sumimoto et al., 2005). The localization of p47^{phox} in perivascular nerve fibers suggests that NOX2 is one of the isoforms present in these fibers. Although mRNAs for all of the NOX isoforms including NOX2 have

been identified in sympathetic ganglia and in DRG, the NOX isoform composition of perivascular nerve fibers is not known.

The involvement of NADPH oxidase in the pathogenesis of cardiovascular diseases has been characterized in the vasculature. NADPH oxidase-mediated superoxide anion ($O_2^{-\bullet}$) production is associated with hypertension or pro-hypertensive changes in different components of blood vessels. These include vascular smooth muscle cells (Griendling et al., 1994; Ushio-Fukai et al., 1996), endothelial cells (Gorlach et al., 2000), adventitial fibroblasts (Pagano et al., 1997) and perivascular adipose tissue (Gao et al., 2006). However, in spite of the presence of NADPH oxidase in sympathetic and sensory neurons that are known to innervate the vasculature (Cao et al., 2007), there has been no examination of its localization in perivascular nerves. We have previously shown that the activation and regulation of NADPH oxidase-derived $O_2^{-\bullet}$ in prevertebral sympathetic ganglia and primary sensory ganglia were associated with DOCA-salt hypertension (Cao et al., 2007; Dai et al., 2006). The two types of ganglia neurons both innervate the mesenteric vasculature. Axons of the neurons travel to the mesenteric arteries and veins in the paravascular nerves, which divide in the adventitia of blood vessels to form the perivascular nerve plexus (Kreulen, 2003; Meehan et al., 1991). In this study, we showed that the expression of NADPH oxidase in the peripheral sympathetic neurons and sensory neurons is not limited to the cell bodies in the ganglia, but is also found in the perivascular nerves, thus providing a possible additional source of $O_2^{-\bullet}$ on the blood vessels.

Changes in the activity of NADPH oxidase localized in the perivascular nerve terminals could influence neurovascular transmission. The prejunctional sympathetic nerve terminal is the site for synthesis, storage and release of the vasoconstrictor neurotransmitters norepinephrine (NE), NPY and ATP, while perivascular sensory nerves mediate vasodilatation by releasing CGRP, substance P and nitric oxide (NO) (Zheng et al., 1997). In DOCA-salt hypertension, NADPH oxidase activity and expression are upregulated in sympathetic ganglia and downregulated in spinal sensory ganglia (Cao et al., 2007). If the reciprocal changes of NADPH oxidase occur in the same manner in the terminals of these neurons, neurotransmission to the vasculature from sympathetic and sensory nerves may be differentially modulated by ROS in a manner that affects vascular tone in hypertension. For example, NO is a vasodilatory neurotransmitter released by perivascular sensory nerve fibers. In addition to being a direct vasodilatator, NO can also modulate neurotransmission at the sympathetic neurovascular junction by deactivating NE released from the sympathetic nerve terminals (Kolo et al., 2004). With the presence of NADPH oxidase at the nerve terminal, it can be predicted that the bioavailability of NO will be closely related to the activity of NADPH oxidase because $O_2^{-\bullet}$ rapidly inactivates NO (Gryglewski et al., 1986). In hypertension, increased sympathetic NADPH oxidase activity which would lead to higher $O_2^{-\bullet}$ production in the nerve terminals would diminish the effect from NO and thereby facilitate higher junctional NE levels and greater vasoconstriction. Sensory nerves, where NADPH oxidase activity is decreased, would play a compensatory role: the NO supply to the neurovascular junction would be elevated because of reduced NADPH oxidase activity.

Additional targets for nerve terminal $O_2^{-\bullet}$ are the neurotransmitter transporters norepinephrine transporter (NET) and vesicular monoamine transporter 2 (VMAT2), both of which are impaired by ROS (Elroy-Stein and Groner, 1988; Mao et al., 2004). When sympathetic NADPH oxidase activity is upregulated, as in hypertension, increased local $O_2^{-\bullet}$ at the nerve terminal may interfere with NE recycling and lead to increased junctional NE spillover and vasoconstriction. In DOCA-salt hypertensive rats, vesicular refilling of neurotransmitter ATP is impaired although it is not known if this is related to increased NADPH oxidase activity and elevated $O_2^{-\bullet}$ (Demel and Galligan, 2008). Furthermore, NET activity is present in perivascular sensory fibers (Zheng et al., 2000) and the activity of the transporter in these terminals may also be influenced by NADPH-generated $O_2^{-\bullet}$.

The staining of NADPH oxidase was not limited to sympathetic and sensory nerve fibers. After both types of fibers were eliminated from the mesenteric circulation by CGx, there was still substantial amount of NADPH oxidase present in some nerve fibers. These could either be CGRP-negative sensory fibers that innervate the blood vessels 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 (Luckensmeyer and Keast, 1995). Further studies are necessary to 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 in their perivascular nerve fibers. The findings of NADPH oxidase in both neuronal cell bodies and prejunctional nerve terminals on blood vessels may indicate novel roles of locally-produced $O_2^{-\bullet}$ in the modulation of neurotransmission to blood vessels.

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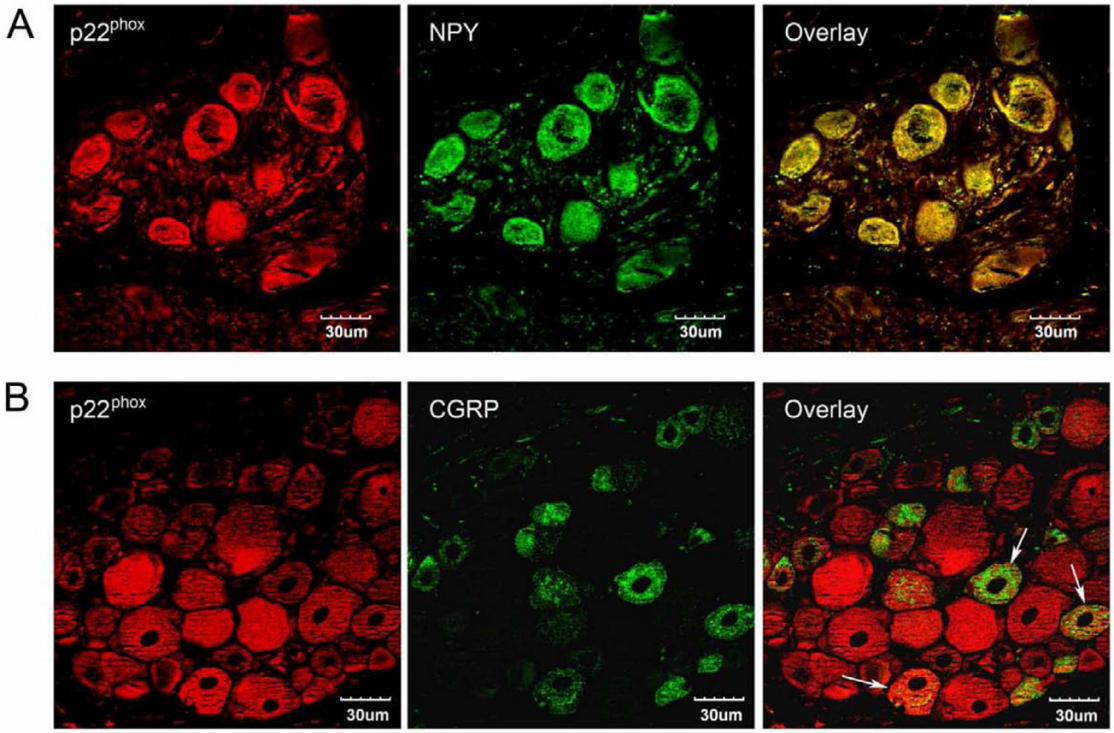

Fig. 1. Immunolocalization of p22^{phox} in CG and DRG neurons

CG and lumbar DRG from normal adult rats were cut to 5μm sections and immunostained with p22^{phox} and NPY or p22^{phox} and CGRP, respectively. A) All the neurons in CG that contain p22^{phox} (red) immunoreactivity showed positive staining for NPY (green); B) In DRG, p22^{phox} (red) and CGRP (green) colocalize to some neurons (arrows) but not all of them. Scale bar: 30μm. Single digital slice fluorescent images were taken under confocal microscopy.

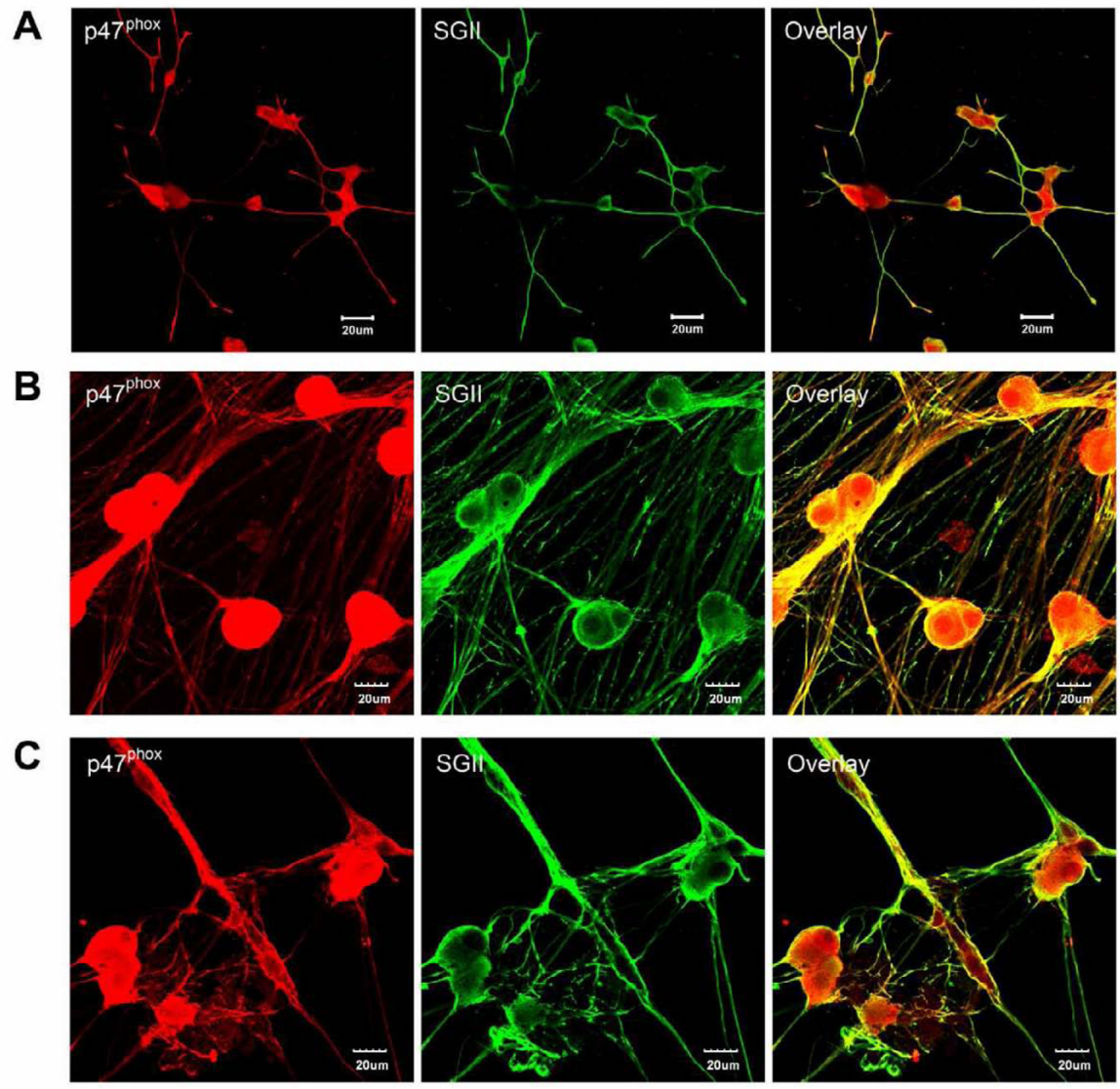

Fig. 2. Immunolocalization of p47^{phox} in PC12 cells and cultured CG and DRG neurons

NGF-differentiated PC12 and dissociated CG and DRG neurons were immunostained with antibodies against p47^{phox} and secretogranin II (SGII), a marker for large dense core vesicles in neuronal cell body and nerve endings. P47^{phox} was co-localized with SGII in the cell bodies and the neurites in PC12 cells (A), CG neurons (B) and DRG neurons (C). Scale bar: 20μm. Single digital slice fluorescent images were taken under confocal microscopy.

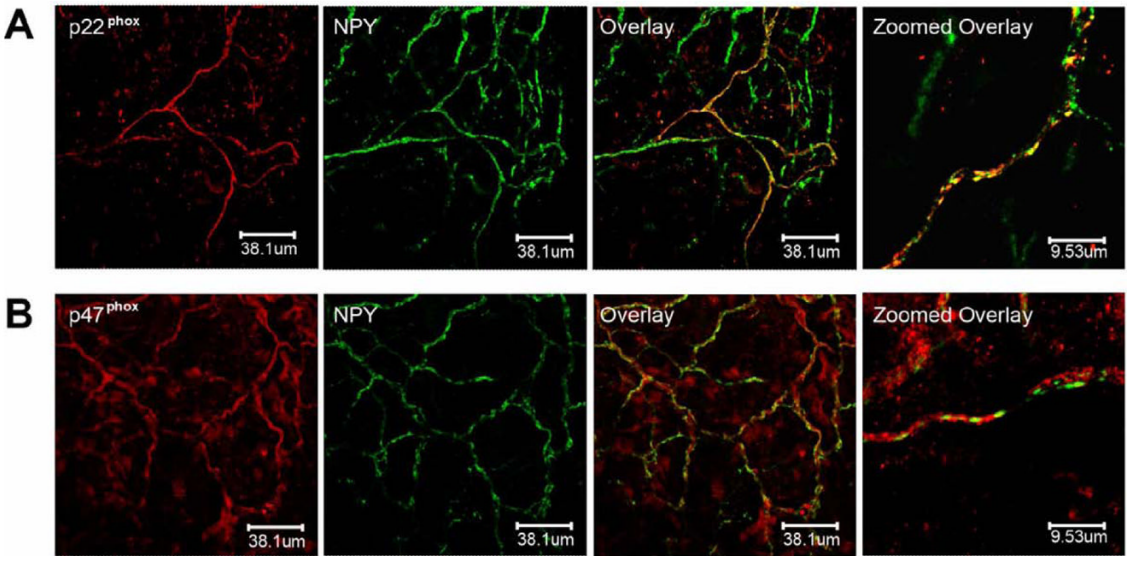

Fig. 3. p22^{phox} and p47^{phox} colocalize with NPY in periarterial nerve fibers in mesenteric arteries

Tertiary branches of mesenteric arteries from adult rats were fixed and labeled with anti-NPY, as a marker for sympathetic perivascular nerves, and either anti-p47^{phox} or anti-p22^{phox}. In each panel, three low power magnification images on the left show the meshwork pattern of nerve fibers innervating mesenteric arteries. The zoomed overlay images on the right are high power magnifications, showing colocalization in a single peri-vascular nerve bundle. A) Immunostaining for p22^{phox} was found in some of the same nerve fibers as NPY. The overlay images show that p22^{phox} and NPY co-localize to some, but not all periarterial nerve fibers. B) p47^{phox} and NPY were found in the same nerve fiber bundles although the localization of the staining within the nerve fiber was variable. NPY staining was discontinuous, while p47^{phox} staining distributed throughout the nerve fibers. Single digital slice fluorescent images were taken under confocal microscopy.

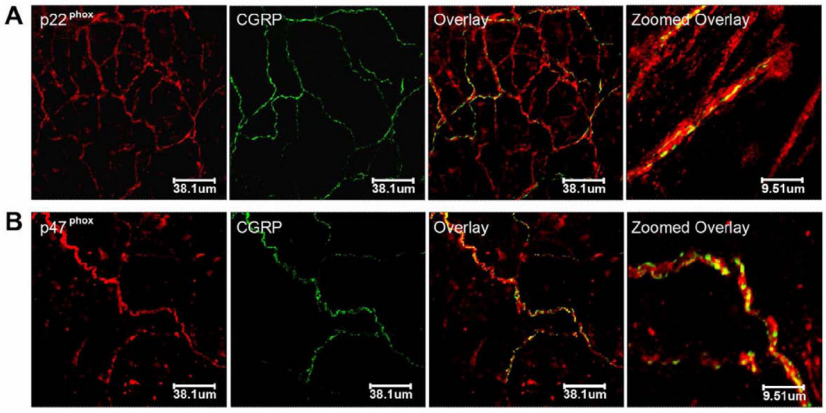

Fig. 4. p22^{phox} and p47^{phox} colocalize with CGRP in periarterial nerve fibers in mesenteric arteries

Tertiary branches of mesenteric arteries were fixed and labeled with anti-CGRP, as a marker for sensory perivascular nerves, and either anti-p22^{phox} or anti-p47^{phox}. In each panel, three low power magnification images on the left show the meshwork pattern of nerve fibers innervating mesenteric arteries. The zoomed overlay images on the right are high power magnifications, showing colocalization in a single peri-vascular nerve bundle. A) p22^{phox} was found in some of the CGRP immunoreactive nerve fibers; B) p47^{phox} and CGRP were found in the same nerve fiber bundles. Single digital slice fluorescent images were taken under confocal microscopy.

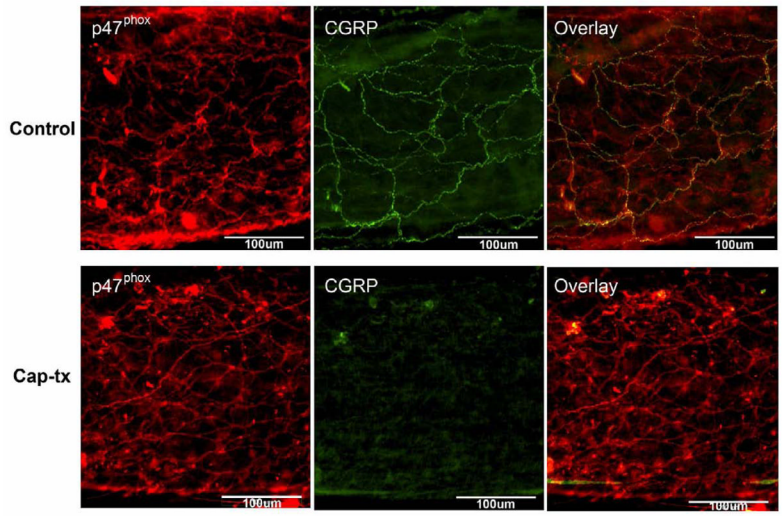

Fig. 5. Perivascular fiber p47^{phox} staining remains after capsaicin treatment (cap-tx)

Small mesenteric arteries removed from cap-tx animals were labeled with anti-p47^{phox} and anti-CGRP. Control animals were sacrificed and vessels were run at the same time as cap-tx animals. A) Vessels from control animals showed immunoreactivity for both p47^{phox} and CGRP on the perivascular nerve fibers. Colocalization of p47^{phox} and CGRP are present on some of the fibers but not all of them. B) The absence of anti-CGRP labeling in cap-tx rats indicates that treatment was effective in depleting sensory nerve fibers. However, p47^{phox} immunostaining remains. This suggests that p47^{phox} exists partly in non-sensory peri-arterial nerve fibers. Scale bar: 100μm. Images were taken using conventional fluorescence microscopy.

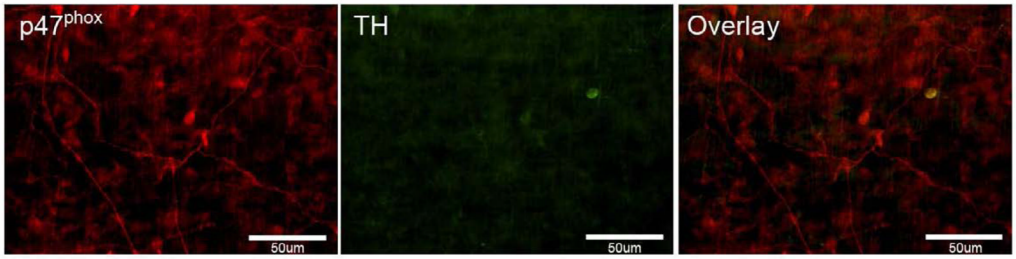

Fig. 6. Periarterial fiber p47^{phox} staining remains two weeks after celiac ganglionectomy (CGx)

After CGx, perivascular TH staining was completely abolished. This indicates that sympathetic nerve fibers were successfully depleted after CGx. However, few superficial p47^{phox}-positive fibers remain. Scale bar: 50μm. Images were taken using conventional fluorescence microscopy.

Table 1

Antibodies for Immunohistochemical Staining

Primary antibodies			
Antigen	**Host species**	**Dilution**	**Source**
$p47^{phox}$ (R360)	Rabbit	1:300	(De Leo et al., 1996)
$p22^{phox}$ (R5554)	Rabbit	1:300	(Jesaitis et al., 1990)
SGII^	Mouse	1:1000	Abcam Inc., Cambridge, MA
TH^	Mouse	1:150	Calbiochem, La Jolla, CA
NPY	Goat	1:300	Santa Cruz Biotech., Inc., Santa Cruz, CA
CGRP	Sheep	1:1000	Abcam Inc., Cambridge, MA
Secondary antibodies			
Target species	**Host species**	**Conjugated to:**	**Dilution**
Mouse	Donkey	FITC	1:40
Rabbit	Donkey	Cy3	1:200
Goat	Donkey	FITC	1:200
Mouse	Donkey	Cy3	1:200
Rabbit	Goat	Alexa 488	1:500
Sheep	Donkey	Rhodamine Red	1:500
Rabbit	Donkey	FITC	1:500

SGII = secretogranin II; TH = tyrosine hydroxylase; NPY = Neuropeptide Y;

CGRP = calcitonin gene-related peptide; ^=monoclonal antibodies

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