Superoxide anion is elevated in sympathetic neurons in DOCA-salt hypertension via activation of NADPH oxidase

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Dai, Xiaoling, Xian Cao, and David L. Kreulen. Superoxide anion is elevated in sympathetic neurons in DOCA-salt hypertension via activation of NADPH oxidase. Am J Physiol Heart Circ Physiol 290: H1019–H1026, 2006. First published October 7, 2005; doi:10.1152/ajpheart.00052.2005.—Superoxide anion (O$_2^-$) production is elevated in sympathetic ganglion neurons and in the vasculature of hypertensive animals; however, it is not known what enzymatic pathway(s) are responsible for O$_2^-$ production. To determine the pathway(s) of O$_2^-$ production in sympathetic neurons, we examined the presence of mRNA of NADPH oxidase subunits in sympathetic ganglionic neurons and differentiated PC-12 cells. The mRNAs for NADPH oxidase subunits p47$^{phox}$, p22$^{phox}$, gp91$^{phox}$, and NOX1 were present in sympathetic neurons and PC-12 cells, whereas the NOX4 homologue was present in sympathetic neurons but not PC-12 cells. Freshly dissociated celiac ganglion neurons from normal and DOCA-salt hypertensive rats were examined to determine the pathway(s) of O$_2^-$ production in sympathetic neurons, and in the vasculature of hypertensive animals, we examined the presence of mRNA of NADPH oxidase subunits in sympathetic ganglionic neurons and differentiated PC-12 cells. The mRNAs for NADPH oxidase subunits p47$^{phox}$, p22$^{phox}$, gp91$^{phox}$, and NOX1 were present in sympathetic neurons and PC-12 cells, whereas the NOX4 homologue was present in sympathetic neurons but not PC-12 cells. Freshly dissociated celiac ganglion neurons from normal rats and PC-12 cells produced O$_2^-$ when treated with the PKC activator PMA; O$_2^-$ production increased by 317% and 254%, respectively. The PMA-evoked increases were reduced by pretreatment with the NADPH oxidase inhibitor apocynin. These findings indicate that NADPH oxidase is the primary source of O$_2^-$ in sympathetic ganglion neurons. When celiac ganglia from hypertensive rats were incubated with apocynin, O$_2^-$ levels were reduced to the same levels as normotensive animals, indicating that NADPH oxidase activity accounted for the elevated O$_2^-$ levels in hypertensive animals. To test this latter finding, we compared NADPH oxidase activity in extracts of prevertebral sympathetic ganglia of DOCA-salt hypertensive rats and sham-operated rats. NADPH oxidase activities were 49.9% and 78.6% higher in sympathetic ganglia of DOCA rats compared with normotensive controls when using β-NADH and β-NADPH as substrates, respectively. Thus elevated O$_2^-$ levels in hypertension may be a result of the increased activity of NADPH oxidase in postganglionic sympathetic neurons.

Sympathetic ganglia; phorbol ester; reduced nicotinamide adenine dinucleotide phosphate oxidase; hypertension; deoxytocorticoesterone acetate

VASCULAR ENDOTHELIAL CELLS, smooth muscle cells, and fibroblasts generate reactive oxygen species (ROS), such as O$_2^-$ and H$_2$O$_2$. ROS play critical roles in both normal and pathophysiological states of vascular cells, including the modulation of redox-sensitive signaling pathways and gene expression, or in the pathophysiology of hypertension and atherosclerosis (16, 22, 26, 36). Several models of hypertension are associated with an increase in vascular O$_2^-$ (41, 42), and this increased O$_2^-$ may quench the endogenous vasodilator nitric oxide (NO) to cause a loss of NO bioactivity in the vessel wall and impair the endothelium-dependent vasorelaxation, resulting in hypertension (26). ROS-mediated endothelial dysfunction in ANG II-infused rats (24) and in DOCA-salt hypertensive rats (42) can be reversed by antioxidant enzyme, endothelial NO synthase (34), or superoxide dismutase (26). ROS can also induce the expression of cardiovascular-related genes, such as those for adhesion molecules and vasoactive substances. For example, the cytokine interleukin 1 activates VCAM-1 gene expression through a mechanism that is repressed ~90% by the antioxidants pyrroline dithiocarbamate and N-acetyl-l-cysteine (30).

Recently, we showed that prevertebral sympathetic ganglion neurons generate O$_2^-$ and that O$_2^-$ production is elevated in hypertension (9). NADPH oxidase was first found and cloned in phagocytes (3). It is composed of a membrane-bound cytochrome b558 subunit consisting of a p22$^{phox}$-gp91$^{phox}$ heterodimer and several cytosolic subunits (p47$^{phox}$, p40$^{phox}$, p67$^{phox}$, and Rac-1). In phagocytes, this enzyme is normally silent, but on stimulation, cytosolic subunits are phosphorylated and translocate to the membrane and associate with cytochrome b558, resulting in the rapid activation of oxidase. NADPH oxidase in nonphagocytic cells such as endothelial cells and vascular smooth muscle cells exhibits significant differences from the phagocytic enzyme. In particular, there are a number of homologues of gp91$^{phox}$, termed “NOXs” (NADPH oxidase) (21), and it has been suggested that the substitution of gp91$^{phox}$ (also known as NOX2) by NOX1 or NOX4 may account for different behaviors of nonphagocytic enzymes (1, 22). NADPH oxidase is present in mouse superior cervical ganglion neurons where it has been shown to mediate apoptosis associated with NGF deprivation (45).

In hypertension, there are functional changes in the sympathetic nervous system, including increased sympathetic nerve activity and enhanced release of the vasoconstrictive neurotransmitters norepinephrine and ATP from nerve terminals. One of the factors might be ROS. ROS not only contribute to the oxidative damage and cell death in the nervous system (37) but also serve as signaling molecules to activate kinase pathways or mediate the effects of neuroactive substances (35). H$_2$O$_2$ can activate the PKC pathway (11), which is capable of facilitating neurotransmitter release from nerve terminals (29).

In addition, by causing a reduction in ganglionic NO levels, O$_2^-$ could reduce or eliminate the effects of NO, and this would result in altered excitability of sympathetic neurons (7). The ANG II/ROS signaling system in the central nervous system mediates the action of ANG II to increase blood pressure (50). In the peripheral nervous system, administration of H$_2$O$_2$ in the vicinity of sympathetic preganglionic neurons projecting to the adrenal gland results in the activation of...
sympathetic preganglionic neurons innervating the adrenal gland (27).

The source of the $O_2^-$ in prevertebral sympathetic neurons is not known. We thus examined freshly dissociated (<1 day) sympathetic neurons and differentiated PC-12 cells with sympathetic neuronal phenotype for the presence of NADPH oxidase in sympathetic neurons and differentiated PC-12 cells with sympathetic neuronal phenotype for the presence of NADPH oxidase by PKC activator PMA. Because of the increased $O_2^-$ production in sympathetic ganglia of DOCA-salt hypertensive rats (9), we further examined the effects of a NOX inhibitor on the increased $O_2^-$ production and the NOX activity in sympathetic ganglia of DOCA-salt hypertensive rats.

MATERIALS AND METHODS

Animals. DOCA-salt hypertensive rats were prepared as described previously (28). All animal procedures were followed in accordance with the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society and were reviewed and approved by the Animal Use and Care Committee of Michigan State University. Under pentobarbital sodium anesthesia (50 mg/kg i.p.; Sigma, St. Louis, MO), male Sprague-Dawley rats (175–200 g, Charles River, Portage, MI) were uninephrectomized and a DOCA (200 mg/kg; Sigma) pellet was implanted subcutaneously on the day of the surgery. Postoperatively, the rats were given a solution of 1% NaCl-0.2% KCl in the drinking water. Sham rats were uninephrectomized, received no DOCA, and drank normal tap water. Rats were housed in temperature- and humidity-controlled rooms with a 12:12-h light-dark cycle. Pellet rat chow and water were given ad libitum. Four weeks after surgery, the arterial blood pressure was measured with the tail cuff method. The rats with a mean arterial pressure of >150 mmHg were considered hypertensive. The mean arterial pressure for the DOCA-salt rats and sham rats were 204.5 ± 8.1 and 121.8 ± 2.4 mmHg, respectively ($n = 14$ in each group).

Tissue harvest and cell culture. Rats were euthanized with a lethal dose of pentobarbital sodium (65 mg/kg). Celiac ganglia (CG) or the inferior mesenteric ganglia (IMG) were removed from the animals and placed in PBS. CG neurons from normal rats were harvested, enzymatically dissociated (20 U/mL papain, 1 mg/mL collagenase, and 4 mg/mL dispase; Worthington Biochemical, Lakewood, NJ), and plated on culture dishes. Cells were maintained in feeding medium (minimal essential medium; Invitrogen, Carlsbad, CA) supplemented with 10% rat serum (Charles River), 1,000 U/mL penicillin-streptomycin (Invitrogen), 2 mM l-glutamine (Invitrogen), 0.3% glucose (Invitrogen), 10 mg/mL ascorbic acid (Sigma), 0.25 mg/mL glutathione (Invitrogen), 0.05 mg/mL 6,7-dimethyl-5,6,7,8-tetrahydropyridine (Sigma), 10 μM cytosine arabinoside (Sigma), 10 μM fluorodeoxyuridine (Sigma), 10 μM uridine (Sigma), and 50 ng/mL NGF (Chemicon International, Temecula, CA) at 37°C in a 5% CO$_2$ atmosphere agarose gel. The positive bands with predicted size were excised from the gel, extracted with using Qiagen DNA purification kit (Qiagen), and were developed with the use of Primer3 software (Massachusetts Institute of Technology) to generate several possible primer pairs. A NCBI Basic Local Alignment Search Tool (BLAST) search ensured the specificity of primer sequences for rats, and the primers were synthesized at the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University. Predicted sequences of PCR amplification products were aligned with other rat sequences in GenBank to examine the stringency. Primer sequences are shown in Table 1.

Sequencing. PCR amplicons were run on the low melting temperature agarose gel. The positive bands with predicted size were extracted from the gel by using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and further purified free of primers, nucleotides, enzymes, salts, agarose, ethidium bromide, and other impurities from DNA samples by using QIAquick PCR Purification Kit (Qiagen). The concentrations of purified DNA amplicons were determined spectrophotometrically. The identities of amplicons were confirmed by sequencing the mixture of 20-ng DNA amplicon and 30 pmol forward and reverse primers using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Genomic Technology Support Facility at Michigan State University.

Measurement of $O_2^-$ generation. The production of $O_2^-$ was measured in four different preparations: dissociated CG neurons, differentiated PC-12 cells, intact IMG, and homogenized CG. Relative levels of superoxide anion were determined by measuring the intensity of fluorescence emission of the oxidant-sensitive fluorescent probe dihydroethidine (DHE). DHE is oxidized to fluorescent ethidium by $O_2^-$ and will intercalate with DNA to further amplify the fluorescent signal. The intensity of the fluorescent signal is proportional to $O_2^-$ levels and can be used as a semiquantitative comparison of $O_2^-$ levels (4–6). To assure the reliability of the comparisons, we performed all studies where tissues were compared in parallel under the same conditions and at the same time.

Dissociated CG neurons and differentiated PC-12 cells were divided into three groups: 1) control (no treatment); 2) treated with the PKC activator PMA (1 μg/mL, Sigma) as the agonist for 30 min; and 3) treated with PMA plus pretreatment with NADPH oxidase inhibitor apocynin (10–4 mol/L, Sigma). All groups were prepared in parallel. Cells were incubated with DHE (2 × 10–6 mol/L; Molecular Probes, Eugene, OR) at 37°C for 30 min, washed three times with PBS buffer, mounted on slides, observed on a Zeiss LSM confocal microscope, excited with a 488-nm argon laser, and observed through a long-pass emission filter passing wavelengths above 560 nm. Parameters (laser
intensity and photomultiplier gain) for the confocal microscope were set up first in the PMA-treated group, and the same parameters were applied to other groups. Phase contrast images were also taken to visualize and localize cells. Confocal images consisting of a 0.36 μm optical slice through the approximate center of the cells were captured at a resolution of 1,024 × 1,024 pixels as 8-bit grayscale images with a brightness from 0 (black) to 255 (white). The fluorescent intensity of cells was analyzed by using the image analysis software ImagePro Plus (Media Cybernetics), and the total fluorescent intensity of each whole cell was measured by using a best fit circular region of interest. Each group was repeated four times; thus 42–119 of CG neurons or 89–187 of PC-12 cells were counted.

IMGs were gently dissected in oxygenated Krebs solution and divided into three groups: sham rats, DOCA-salt hypertensive rats, and PC-12 cells. The average fluorescent intensity of a circular region of interest encompassing the neurons and PC-12 cells matched published sequences.

**RESULTS**

**Expression of NADPH oxidase mRNA in dissociated CG neurons and differentiated PC-12 cells.** PCR amplicons of NADPH oxidase subunits p47phox, p22phox, gp91phox and NOX1 were detected in both dissociated CG neurons and differentiated PC-12 cells (Fig. 1, A and B) at the expected sizes of 221, 282, 245, and 324 bp, respectively. By contrast, NOX4 was present in CG neurons but not in PC-12 cells. NOX4 was also present in the rat aorta but not in the brain (Fig. 2, A and B). The sequenced PCR amplicons were aligned in GenBank. Greater than 99% of sequenced amplicons of p47phox, p22phox, gp91phox, NOX1, and NOX4 in CG neurons and PC-12 cells matched published sequences.

**Fluorogenic detection of O2•− levels in dissociated CG neurons and PC-12 cells.** To determine whether the activation of PKC generates O2•− production in sympathetic neurons, and if so, whether this PKC-activated O2•− generation is mediated through NADPH oxidase, we examined the effects of PMA and PMA plus apocynin on O2•− production in sympathetic neurons and differentiated PC-12 cells. The enzyme activity was expressed as total fluorescent units per minute per milligram tissue homogenate of CG.

**Data analysis.** Data are presented as means ± SE for the number of animals or cells. Statistical significance was assessed by Student’s t-test or one-way ANOVA test with Dunnett’s multiple comparison post test with the use of Prism 3.0 (GraphPad Software, San Diego, CA) (P < 0.05 indicating statistical significance).

**Table 1. Primer sequences for NAD(P)H oxidase subunits gp91phox, Nox1, Nox4, p47phox and p22phox, and β-actin**

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<th>Amplicon Length, bp</th>
<th>NCBI Accession Number</th>
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<td>gp91phox (23)</td>
<td>AF298656</td>
</tr>
<tr>
<td>Forward: 5'-GAGGATGCTTCATGAGGC-3'</td>
<td></td>
</tr>
<tr>
<td>Reverse: 5'-TTGCAAGGGCTCCATAG-3'</td>
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<td>Nox1 (43)</td>
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<td>Nox4 (23)</td>
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</tr>
<tr>
<td>Reverse: 5'-TACTCTTGCTGCTGGATCCAC-3'</td>
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β-actin (12)

Forward: 5'-ACTTCCAGTGGATGGATGG-3'
Reverse: 5'-TCTGATGTTGGCACGGAAGCA-3'

500 V01217

**Measurement of NADPH oxidase activity.** Activity of NADPH was measured by using fluorescence spectrometry of DHE in tissue homogenates of CG from sham rats and DOCA rats (5, 51). In a microtiter plate, freshly prepared CG homogenates were incubated with DHE (10 μmol/l), salmon testes DNA (0.5 mg/ml, Stratagene, La Jolla, CA), and the substrates for NADPH oxidase β-NADH (0.1 mmol/l, Sigma) or β-NADPH (0.1 mmol/l, Sigma) for 30 min 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 O2•− measurement ≥40-fold (47, 51). Ethidium-DNA fluorescence was measured at an excitation of 485 ± 40 nm and an emission of 590 ± 35 nm with the use of a Bio-Tek FL600 fluorescence plate reader (Bio-Tek Instruments, Winooski, VT). The enzyme activity was expressed as total fluorescent units per minute per milligram tissue homogenate of CG.
same field as the confocal fluorescent images are shown adjacent to one another in Figs. 2 and 3.

Sympathetic ganglion cells incubated with PMA had a fluorescence intensity 317% greater than control untreated neurons [Fig. 2A; 46.6 ± 2.5 arbitrary fluorescence units (AFUs)], indicating elevated O$_2^·$ levels (Fig. 2B; 194.4 ± 2.7 AFUs). The response was limited to cells with typical neuronal morphology. Of 121 neurons counted in four sets of independent experiments, 119 (98.5%) were DHE positive when they were incubated with PMA, whereas no control neurons (45 neurons counted), which were not treated with PMA, exhibited fluorescence intensity above control levels. In cells pretreated with apocynin (Fig. 2C; 47.4 ± 3.0 AFUs), PMA treatment did not increase fluorescence intensity over control level. Similar results were obtained with PC-12 cells, which were incubated with PMA, whereas no control neurons (45 neurons counted), which were not treated with PMA, exhibited fluorescence intensity above control levels. In cells pretreated with apocynin (Fig. 2C; 47.4 ± 3.0 AFUs), PMA treatment did not increase fluorescence intensity over control level. Similar results were obtained with PC-12 cells, which were incubated with PMA, whereas no control neurons (45 neurons counted), which were not treated with PMA, exhibited fluorescence intensity above control levels.

**Fluorogenic detection of O$_2^·$ levels in IMG.** O$_2^·$ levels were evaluated in intact IMG (n = 4 rats in each group) incubated with DHE in vitro. O$_2^·$ levels in both neurons and satellite cells were greater in IMG from a DOCA-salt rat (Fig. 5B) than from a sham rat (Fig. 5A), and the fluorescence intensity was 267% higher in neurons and 186% higher in satellite cells (Fig. 5D). Neurons displaying elevated O$_2^·$ levels were distributed throughout the ganglia. DOCA IMG in vitro treated with apocynin showed no difference in O$_2^·$ fluorescence (Fig. 5C) compared with sham IMG (Fig. 5A). This indicates that there is higher O$_2^·$ production in neurons and glia in prevertebral sympathetic ganglia from DOCA than from sham rats and that the increased O$_2^·$ production in prevertebral sympathetic ganglia from DOCA rats can be blocked by treatment with the NADPH oxidase inhibitor apocynin.

**NADPH oxidase activity in CG from sham and DOCA-salt hypertensive rats.** Tissue homogenates of CG from sham rats and DOCA-salt hypertensive rats (n = 6 sham rats; n = 6 DOCA-salt hypertensive rats) were incubated with either of the NADPH oxidase substrates β-NADH or β-NADPH, and the formation of O$_2^·$ was detected in reaction mixtures. O$_2^·$ production was greater in homogenates from DOCA ganglia than from sham ganglia regardless of the substrate (Fig. 6). NADPH oxidase activities of sham and DOCA homogenates were 482.7 ± 42 and 723.3 ± 42 fluorescence intensity units (FIUs)/min·mg⁻¹·wet wt tissue⁻¹, respectively, when using β-NADH as the NADPH oxidase substrate, and 586.3 ± 19.0 and 1,047.3 ± 37.7 FIUs/min·mg⁻¹·wet wt tissue⁻¹, respectively, when using β-NADPH as the NADPH oxidase substrate (Fig. 6). NADPH oxidase activities are 49.9% and 78.6% higher in DOCA CG than in sham CG when using β-NADH and β-NADPH as the substrate, respectively. The no-substrate control group showed very low fluorescence in-
tensity, which was ~6.0% of the average of experimental groups and considered to be background. These results indicate that tissue homogenates of CG produce $\cdot{\mathrm{O}_2}$ by using either $\beta$-NADH or $\beta$-NADPH as NADPH oxidase substrates and that the NADPH oxidase enzymatic activity is greater in CG from DOCA rats than from sham rats.

**DISCUSSION**

These results provide the first evidence that mRNAs of NADPH oxidase subunits NOX1, p22$^{\text{phox}}$, and p47$^{\text{phox}}$ are present in rat sympathetic postganglionic neurons and differentiated PC-12 cells. Also, we have shown that NOX4 mRNA is present in CG neurons but not in differentiated PC-12 cells. The activation of NADPH oxidase by PMA administration in sympathetic postganglionic neurons and PC-12 cells increased $\cdot{\mathrm{O}_2}$ production, which was blocked by pretreatment with NADPH oxidase inhibitor apocynin. $\cdot{\mathrm{O}_2}$ production was higher in sympathetic ganglia from DOCA-salt rats than from sham rats, and this difference was eliminated by treatment with the NADPH oxidase inhibitor apocynin. The increased $\cdot{\mathrm{O}_2}$ production in sympathetic ganglia from DOCA-salt hypertensive rats may arise from an upregulated NADPH oxidase activity.

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**Fig. 2.** NADPH oxidase activation by PMA elevates $\cdot{\mathrm{O}_2}$ levels in dissociated CG neurons in vitro, and this increase is attenuated by pretreatment with NADPH oxidase inhibitor apocynin. Confocal fluorescent images (left) and phase-contrast microscopy images (right) of control group (A), PMA (B), and PMA plus apocynin (C). Calibration bar in C applies to all panels.

**Fig. 3.** NADPH oxidase activation by PMA elevates $\cdot{\mathrm{O}_2}$ levels in differentiated PC-12 cells in vitro, and this increase is attenuated by pretreatment with NADPH oxidase inhibitor apocynin. Confocal fluorescent images (left) and phase-contrast microscopy images (right) of control group (A), PMA (B), and PMA plus apocynin (C). Calibration bar in C applies to all panels.

**Fig. 4.** Effects of NADPH oxidase activation on relative $\cdot{\mathrm{O}_2}$ levels in CG neurons and PC-12 cells in vitro. Results are expressed as means ± SE in fluorescence intensity units. Control (CTRL) cells received no PMA treatment. #Significance ($P < 0.05$) vs. CTRL in CG cells and PC-12 cells ($n = 4$ dishes in each treatment group).
The subunits p22

NADPH oxidase, which was first described in phagocytes, consists of and NOX4 are present in freshly dissociated rat sympathetic
dermal neurons to 70 DOCA-salt neurons and 496 sham satellite cells to 543
neurons are still a mystery. Under physiological conditions, the
neurons is under
regulation of ROS in normal cells, including neurons, is under
tight homeostatic control and does not alter the redox state of
neurons, a profile that is distinct from both phagocytes and
vascular cells. Because of the absence of the NOX4 message in
differentiated PC-12 cells, this profile is also distinct from that
found in PC-12 cells. One important difference between the
cultures of these two preparations is the presence of satellite
cells in the CG cultures and their absence in the PC-12
cultures. O$_2^\cdot$ is generated in both neurons and satellite cells in
IMG (9); therefore, it is possible that mRNAs of NOX4 come from
ganglionic satellite cells but not neurons. Although
p67phox is present in astrocyte cultures (35) representative of
central nervous system glia, the presence of NADPH subunits
has not been examined in satellite cells, which are the glia of
peripheral ganglia.

NADPH oxidase in sympathetic neurons can be activated to
generate O$_2^\cdot$. One required step for NADPH oxidase activation is
the phosphorylation of p47phox by PKC, which permits
p47phox to interact with the cytoplasmic tail of membrane-bound
p22phox and initiate the formation of an active and
membrane-bound enzyme complex (17). PMA activates a PKC
pathway independent of receptor stimulation (38) and the
activated PKC phosphorylates p47phox to form a functional
NADPH oxidase (44). Apocynin, a selective NADPH oxidase
inhibitor, impedes the assembly of NADPH oxidase complex
(31), and its ability to block O$_2^\cdot$ production is diagnostic of the
presence of a functional NADPH oxidase. PMA-induced O$_2^\cdot$
production is concentration dependently inhibited by apocynin
in purified rat phagocytes via an action on NADPH oxidase
(40). This is similar to the present observation in sympathetic
neurons and PC-12 cells where PMA-induced O$_2^\cdot$ production
was blocked by apocynin treatment. These findings confirm the
presence of a functional NADPH oxidase in sympathetic
neurons, like the NADPH oxidase in phagocytes (3) and vascular
cells (25).

The rationale for increased O$_2^\cdot$ production in sympathetic
neurons is still a mystery. Under physiological conditions, the
generation of ROS in normal cells, including neurons, is under
tight homeostatic control and does not alter the redox state of

Fig. 5. O$_2^\cdot$ levels, indicated by dihydroethidine fluorescence intensity in
colfocal images of inferior mesenteric ganglia (IMG), are higher in IMG from
DOCA-salt rats than from sham rats, and this increase is attenuated by
pretreatment with NADPH oxidase inhibitor apocynin. Cells with a soma
diameter of 15–35 μm were identified as neurons, and cells with a diameter of
5–10 μm were identified as satellite cells. Arrows indicate examples of
neurons and arrowheads indicate examples of satellite cells. A: IMG from a
sham rat. B: IMG from a DOCA-salt rat. C: apocynin-pretreated IMG from a
DOCA-salt hypertensive rat. D: comparison of mean fluorescence intensity of
157 sham neurons to 70 DOCA-salt neurons and 496 sham satellite cells to 543
DOCA satellite cells (n = 4 rats in each group). Fluorescence of both types of
cells was significantly (#P < 0.05) greater in DOCA ganglia compared with
sham. Calibration bar in C applies also to A and B.

Fig. 6. NADPH oxidase activity is higher in CG from DOCA-salt hyperten-
sive rats than from sham rats. NADPH oxidase activities of CG from sham rats
and DOCA-salt hypertensive rats are 482.7 ± 42 and 723.3 ± 42 fluorescence
intensity units·min⁻¹·mg wet tissue⁻¹, respectively, when using β-NADH as
the NADPH oxidase substrate, and 586.3 ± 19.0 and 1,047.3 ± 37.7 fluores-
cence intensity units·min⁻¹·mg wet tissue⁻¹, respectively, when using
β-NADPH as the NADPH oxidase substrate. *Significance (P < 0.05) vs.
sham (n = 6 sham rats; n = 6 DOCA-salt hypertensive rats).
cells, which have large reserves of reducing agents, notably reduced glutathione, as well as biological antioxidant defense mechanisms, such as SOD, catalase, and peroxidases (13, 19, 22). This reducing intracellular environment allows ROS to function as second messengers (22). Vascular cells produce low amounts of ROS that stimulate transcription factors as well as signaling cascades via the activation of kinases and inhibition of tyrosine phosphatases. This is in contrast to the cytotoxic amounts of superoxide generated by phagocytes (11, 22). Similar to what is happening in vascular cells, ROS may also operate as second messengers in neurons to mediate the effects of neuroactive substances. In central nervous system neurons, the ROS signaling system mediates the action of ANG II to increase blood pressure (50). Furthermore, there is an interesting interactive and feed-forward relationship between PKC and ROS whereby PKC activation induces ROS production and ROS also can activate PKC pathways. Hydrogen peroxide activates PKC isozymes α, β, and γ (20), and the oxidative activation of PKC may enhance or facilitate the release of vasoconstrictor neurotransmitters, such as norepinephrine and ATP, from peripheral sympathetic nerves (11, 29). Particularly, with the consideration of a high sympathetic outflow present in hypertension (2), such as in DOCA-salt hypertensive rats (39), ROS-activated PKC may result in elevated neurotransmitter release from nerve terminals innervating blood vessels and, accordingly, increase blood pressure.

$O_2^-$ levels are higher in sympathetic ganglia from DOCA-salt hypertensive rats than from sham rats (9), and this increase may come from the increased NADPH oxidase activity. NADPH oxidase activity was higher in CG homogenates from DOCA-salt hypertensive rats. Several factors including nonhemodynamic factors, such as hormones or cytokines, may be responsible for enhanced NADPH oxidase activity in hypertension. For example, ANG II treatment increases $O_2^-$ production by increasing NADPH oxidase activity in cultured vascular smooth muscle cells (15). Endothelin-1 (ET-1) may also be important in the development and maintenance of DOCA-salt hypertension. For example, there is more endothelial ET-1 mRNA and higher basal release of endogenous endothelin in DOCA-salt hypertensive rats (32, 48), and chronic endothelin receptor blockade treatment decreases blood pressure to the normal range (10). ET-1 is a potential endogenous stimulating factor for $O_2^-$ production in sympathetic ganglia (9). In DOCA-salt hypertensive rats, there is increased expression of ETB receptors, which mediate ET-1 effects in this tissue (9). In carotid arteries, there is more ET-1 mRNA and peptide in endothelial cells in DOCA hypertension, and ET-1 increases vascular NADPH oxidase activity (26).

Alternatively, the increased NADPH oxidase activity may come from the upregulated expression of NADPH oxidase. In aorta of DOCA-salt hypertensive rats, p22phox mRNA is increased, accompanied with an increased NADPH oxidase activity (49). In vivo ANG II treatment (7 days) in rats upregulates the expression of NADPH oxidase subunits and significantly increases NADPH oxidase activity (33). The changes in NADPH oxidase mRNA and protein expression in sympathetic ganglia from DOCA-salt hypertensive rats have not been investigated.

This study demonstrates that mRNA for NADPH oxidase subunits p47phox, p22phox, gp91phox, and NOX1 is present in sympathetic neurons and differentiated PC-12 cells. Furthermore, PMA treatment results in increased $O_2^-$ levels in sympathetic postganglionic neurons and PC-12 cells, and this increase can be attenuated by pretreatment with the specific NADPH oxidase inhibitor apocynin. Finally, NADPH oxidase activity is upregulated in sympathetic ganglia from DOCA-salt hypertensive rats, resulting in elevated $O_2^-$ production. We propose that $O_2^-$ production evoked by the active oxidase may play roles in the increased sympathetic excitability and pathogenesis in DOCA-salt hypertension. We speculate that ROS in the sympathetic nervous system may be an important target for therapeutic treatment of hypertension.

**REFERENCES**

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H1026

SUPEROXIDE AND NADPH OXIDASE IN SYMPATHETIC GANGLIA