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Changes in the Subcellular Distribution of NADPH Oxidase Subunit p47^{phox} in Dendrites of Rat Dorsomedial Nucleus Tractus Solitarius Neurons in Response to Chronic Administration of Hypertensive Agents

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Abstract

NADPH oxidase-generated superoxide can modulate crucial intracellular signaling cascades in neurons of the nucleus tractus solitarius (NTS), a brain region that plays an important role in cardiovascular processes. Modulation of NTS signaling by superoxide may be linked to the subcellular location of the mobile NADPH oxidase p47^{phox} subunit, which is known to be present in dendrites of NTS neurons. It is not known, however, if hypertension can produce changes in the trafficking of p47^{phox} in defined NTS subregions, particularly the preferentially barosensitive dorsomedial NTS (dmNTS), or preferentially gastrointestinal medial NTS (mNTS). We used immunogold electron microscopy to determine if p47^{phox} localization was differentially affected in dendritic profiles of neurons from these NTS subregions of the rat in response to distinct models of hypertension, namely chronic seven-day subcutaneous administration of angiotensin II (AngII), or phenylephrine. In small (<1 μ m) dendritic processes, both AngII and phenylephrine produced a decrease in intracellular p47^{phox} labeling selectively in dmNTS neurons. In intermediate-size (1–2 μ m) dendritic profiles in the dmNTS region only, there was an increase in p47^{phox} labeling in response to each hypertensive agent, although these changes occurred in different subcellular compartments. There was an increase in non-vesicular labeling in response to AngII, but an increase in surface labeling with phenylephrine. Moreover, each of the changes in p47^{phox} targeting mentioned above occurred in dendritic profiles with, or without immunoperoxidase labeling for the AngII AT-1A receptor subtype (AT-1A). These results indicate that chronic administration of agents that induce hypertension can also produce changes in the subcellular localization in p47^{phox} in dmNTS neurons. Thus, systemic hypertension may produce alterations in the trafficking of proteins associated with superoxide production in central autonomic neurons, thus revealing a potentially important neurogenic component of free radical production and systemic blood pressure elevation.

Keywords

Angiotensin II; blood pressure; NADPH oxidase; phenylephrine; reactive oxygen species; superoxide

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Introduction

The nucleus of the solitary tract (NTS) is a critical central coordinator of cardiorespiratory processes that are known to modulate systemic arterial pressure (2), cerebral blood flow (1), respiration (18), and energy balance (15). The NTS receives the first central synapse of glutamatergic mechano- and chemoreceptor (2), as well as afferents from the caudal-most circumventricular organ the area postrema (45). The NTS also has extensive reciprocal connections with other areas of the brainstem-forebrain autonomic system, including the ventrolateral medulla (20), hypothalamus (20), and amygdala (52).

Within the NTS, autonomic processes are modulated by diverse signaling systems, including small amino acid transmitters, as well as catecholamine and peptide neuromodulators (25). One of the more potent modulators of cardiovascular processes mediated by the NTS is Angiotensin II (AngII), a peptide that acts locally via the angiotensin AT-1A receptor to inhibit the baroreflex (12). These actions may be mediated by production of reactive oxygen species (ROS), molecules that are emerging as an important molecular substrate of central autonomic regulation, in addition to their better known role in mediating hypertension-induced cellular plasticity in the systemic vasculature (7,10,22,29,53).

Superoxide generated by NADPH oxidase may be involved in the detrimental effects of neurogenic hypertension (14). NADPH oxidase was first described in phagocytes (24), but is also expressed in a wide range of cell-types (36,41,44), including NTS neurons (48). This enzyme consists of two membrane-bound subunits, gp91^{phox} and p22^{phox}, several mobile cytoplasmic subunits including, p40^{phox}, and p67^{phox}, and the essential p47^{phox}, as well as the small GTPase RAC (24,38). Superoxide production by NADPH oxidase is the result of a cascade of cellular events including increased intracellular calcium, such as that induced by AngII-induced stimulation of the AT-1A receptor, leading to phosphorylation and membrane translocation of p47^{phox} to the gp91^{phox} subunit (6,40).

The ability of superoxide to modulate intracellular signaling pathways is strongly related to its subcellular location, the latter of which is cell phenotype-dependent (43,51). In the dorsomedial NTS (dmNTS), an area that receives a high density of baroreceptors (9), the activation of AT-1A receptors produces a superoxide-dependent potentiation of calcium currents (47,48). In dmNTS, NADPH oxidase subunits are also present in membranous organelles in astroglia and dendritic processes of neurons (16). It is not known, however, if hypertension is associated with changes in the subcellular location of p47^{phox} in dendrites of dmNTS neurons. Dendrites of dmNTS neurons receive asymmetric excitatory-type synapses, indicative of glutamatergic neural transmission (32), from vagal visceral barosensitive afferents originating in the nodose ganglion (2). While glutamatergic visceral afferents target somata and proximal dendrites in the dmNTS, they occur preferentially at distal dendrites in this region (37,46). However, it is also not known if p47^{phox} trafficking in response to hypertension differs in distal or intermediate portions of dendrites.

Immunogold electron microscopic cytochemistry has the spatial resolution necessary to detect protein immunolabeling at distinct subcellular localizations, and is also compatible with dual immunoperoxidase labeling for related antigens (8,27). We used immunogold electron microscopy to characterize the subcellular distributions of the p47^{phox} subunit in different size dendritic processes in response to chronic subcutaneous infusion of AngII. Peripheral administration of AngII increases systemic systolic blood pressure (34) and also activates central cardiovascular circuits, including the NTS (4,26,34,35). We compared the effects of AngII on p47^{phox} trafficking with phenylephrine. The latter agent is a peripherally acting α_1 -adrenergic receptor agonist that elevates systemic blood pressure (31), and can affect

activity of central cardiovascular pathways involving the NTS (50). When administered over a seven day period, AngII produces an elevation of systemic arterial pressure that is modest, slow, and not sufficient to produce cerebral, cardiac, or renal complications (49). The quantitative subcellular analysis of p47^{phox} immunolabeling in response to AngII and phenylephrine was carried out in two relatively distinct functional NTS subregions, the dmNTS, an area preferentially involved in barosensory function (9), and the mNTS, a region receiving prominent inputs from gastrointestinal afferents (11,30). In order to determine if these autonomic sensitive neurons were capable of responding to AngII, immunoperoxidase staining of the AngII AT-1A receptor subtype (AT-1A) was combined with immunogold p47^{phox} labeling in each NTS subregion.

Materials and Methods

Subjects, drug administration, and blood pressure measurements

Experimental protocols involving animals and their care were approved by the Institutional Animal Care and Use Committee at Weill Medical College of Cornell University and Rockefeller University conformed to NIH guidelines. Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 180–300 g were individually housed and maintained on a reverse 12-hr light/dark cycle (lights out 0700 hr). All rats had unlimited access to water and rat chow in their home cages. Rats were continuously infused with AngII (1.44 mg/kg/day; n=4), phenylephrine (30 mg/kg/day; n=4), or 0.9% saline (n=4) through subcutaneously implanted osmotic minipumps. To minimize stress and accustom rats to the procedure, systolic arterial pressure was measured daily for 7 days by means of tail-cuff plethysmography prior to the insertion of minipumps. For surgical implantation of minipumps, rats were anesthetized with isoflurane (induction 5%; maintenance 1.5% – 2%) and placed on a thermostatically controlled heating pad to maintain body temperature at 37 °C. After reaching a surgical level of anesthesia (unresponsiveness to tail pinch and lack of palpebral reflex) the scapulae area was shaved with a clipper and cleaned with Nolvasan solution. A small incision was made in the skin between the scapulae, and a small pocket formed by spreading apart the subcutaneous tissues. A previously filled osmotic minipump (Model # 2002; Alza Corporation, Palo Alto., CA) was placed in the pocket. Discomfort was minimized by infiltration of the surgical site with local anesthetics (0.25% bupivacaine) prior to incision. Animals were monitored closely for signs of pain and discomfort, and their arterial pressure was monitored daily with a non-invasive tail cuff sphygmomanometer. During daily sessions 10 blood pressure measurements were recorded and averaged. This value was the systolic pressure for that day. Although tail-cuff plethysmography underestimates absolute blood pressure values (19), this does not compromise our experiments since relative comparisons are made within groups of animals. After 7 days blood pressure was measured, and rats were then sacrificed in preparation for immunocytochemistry. All efforts were made to minimize the number of animals used and their suffering.

Tissue preparation

Rats were anesthetized with sodium pentobarbital (150 mg/kg, i.p.), and their brains were fixed by aortic arch perfusion sequentially with: (a) 15 ml of normal saline (0.9%) containing 1000 units/ml of heparin, (b) 50 ml of 3.75% acrolein in 2% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), and (c) 200 ml of 2% paraformaldehyde in PB, all delivered at a flow rate of 100 ml/minute. The brains were removed and post-fixed for 30 minutes in 2% paraformaldehyde in PB. Coronal sections 40 µm thick were cut with a vibrating microtome from brainstem at the level of the area postrema according to the atlas of Paxinos and Watson (1986). Tissue sections were next treated with 1.0% sodium borohydride in PB and then washed in PB. Sections then were immersed in a cryoprotectant solution (25% sucrose and 2.5% glycerol in 0.05 M PB) for 15 minutes. To enhance tissue permeability, sections were then

freeze-thawed in liquid freon and liquid nitrogen. Sections were next rinsed in 0.1 M Tris-buffered saline (TS, pH 7.6) and then incubated for 30 minutes in 0.1% bovine serum albumin (BSA) to minimize nonspecific labeling.

Antisera

To immunolabel NADPH oxidase subunits, a polyclonal goat anti-p47^{phox} antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) was used. This antiserum shows recognition of its epitope by Western Blotting, immunoprecipitation, and immunocytochemistry (Manufacturer's data). Preadsorption of each antisera with its respective blocking peptide prevented detection of immunolabeling (48). To label AT-1A receptors, we used an affinity purified rabbit anti-AT-1A receptor antiserum against the carboxy terminal peptide (amino acids 341–355), which was provided by Dr. Robert Speth (17,21). In rodents, AT-1A and AT-1B receptor subtypes are encoded by different genes, but have considerable amino acid homology. The antiserum employed, however, has been previously established to be specific for the AT-1A receptor subtype. This has been shown by exclusive immunolabeling with our AT-1A receptor antiserum in Chinese hamster ovary cells differentially transfected with either AT-1A, AT-1B, or AT-2 receptor (21). In addition, immunolabeling of this antisera is abolished when preadsorbed with AT-1A peptide (Huang et al. 2003).

Immunocytochemical procedures

For single immunogold labeling of NADPH oxidase subunits, brainstem sections were incubated for 48 hours p47^{phox} antiserum (1:50). Sections were rinsed in 0.01 M PBS (pH 7.4), and blocked for 10 minutes in 0.5% BSA and 0.1% gelatin in PBS to reduce non-specific binding of gold particles. Sections then were incubated for 2 hours in donkey anti-goat IgG conjugated with 1 nm gold particles (1:50, AuroProbeOne, Amersham, Arlington Heights, IL). The sections were rinsed in PBS and incubated for 10 minutes in 2% glutaraldehyde. The bound gold particles were enlarged by a 6 minute silver intensification using an IntenSE-M kit (Amersham, Arlington Heights, IL). Sections were incubated in anti-goat IgG conjugated to biotin, rinsed in TS, and then incubated for 30 minutes in avidin-biotin-peroxidase complex (1:100, Vectastain Elite Kit, Vector Laboratories) in TBS. The bound peroxidase was visualized by reaction for 5–6 minutes in 0.2% solution of 3, 3'-diaminobenzidine and 0.003% hydrogen peroxide in TS.

For dual detection of immunogold labeling of the NADPH oxidase subunit and immunoperoxidase detection of AT-1A, brainstem sections were incubated for 48 hours in a solution containing a mixture of p47^{phox} antisera (1:50) and AT-1A (1:400) antibody. After incubation, sections were rinsed in TS and prepared first for peroxidase identification of AT-1A. Sections were incubated in anti-rabbit IgG conjugated to biotin, rinsed in TS, and then incubated for 30 minutes in avidin-biotin-peroxidase complex (1:100, Vectastain Elite Kit, Vector Laboratories) in TBS. The bound peroxidase was visualized by reaction for 5–6 minutes in 0.2% solution of 3, 3'-diaminobenzidine and 0.003% hydrogen peroxide in TS. Sections were then processed for immunogold labeling. Sections were rinsed in 0.01 M PBS (pH 7.4), and blocked for 10 minutes in 0.5% BSA and 0.1% gelatin in PBS to reduce non-specific binding of gold particles. Sections then were incubated for 2 hours in donkey anti-goat IgG conjugated with 1 nm gold particles (1:50, AuroProbeOne, Amersham, Arlington Heights, IL). The sections were rinsed in PBS and incubated for 10 minutes in 2% glutaraldehyde. The bound gold particles were enlarged by a 6 minute silver intensification using an IntenSE-M kit (Amersham, Arlington Heights, IL). There is the possibility that ABC/DAB reaction product will be intensified by silver enhancement. In our dual labeling experiments, the primary antisera labeled with a gold secondary was omitted as a control for this possibility (8). It should also be pointed out that peroxidase reaction product is typically not particulate, but somewhat

diffuse in nature, and thus with careful visual inspection not easily confused with silver-enhanced immunogold.

For light microscopy, sections were mounted on glass slides, dehydrated, and covered in resin beneath a glass coverslip. For electron microscopy, sections were postfixed in 2% osmium tetroxide in PB for one hour, and dehydrated in a series of alcohols, through propylene oxide, and embedded in EM BED 812 (EMS, Fort Washington, PA) between 2 sheets of Aclar plastic. Ultrathin sections from the surface of the tissue were cut with a diamond knife using an ultramicrotome (Ultratome, NOVA, LKB, Bromma, Sweden). These sections were collected on grids, and then counterstained with Reynold's lead citrate and uranyl acetate.

Ultrastructural analysis

From each of four animals per experimental group, vibratome sections of brainstem containing the dmNTS or mNTS at the level of the area postrema were thin sectioned and mounted on grids. One thin section from each vibratome section at the tissue-surface interface was selected for analysis to avoid differences in labeling due to differing penetrance of reagents. From this section five grid squares ($3025 \mu\text{m}^2/\text{square}$) were analyzed in a blind manner. The grid squares were visually scanned and any field containing at least one p47 labeled dendritic profile, with or without peroxidase reaction for AT-1A, was digitally captured and stored. These images were then analyzed to determine: (1) the number of labeled dendritic profiles, and (2) the number of gold-silver particles present in the cytoplasm, or in contact with the plasma membrane. Gold particles in contact with any portion of the surface membrane were considered as plasmalemmal. Morphological parameters, including surface area (perimeter) and cross-sectional area were measured using MCID software. The classification of dendrites was based upon descriptions by Peters and co-workers (32). Data were analyzed by one-way factorial ANOVA, and differences in means were analyzed by Fisher's LSD.

Light Microscopy

For light microscopy, the mounted sections were viewed with a Nikon Microphot-FX microscope (Nikon, Garden City, NY) equipped with a digital CoolSNAP camera (Photometrics, Huntington Beach, CA). The light microscopic images were acquired through an interface between the camera and a Macintosh computer. These images were adjusted for contrast and brightness using Photoshop 6.0 software, and imported into Powerpoint 2001, to add lettering and prepare the composite light microscopic figure.

Electron Microscopy

Electron microscopic images were obtained using a digital camera (Advanced Microscopy Techniques) interfaced with a transmission electron microscope (Technai 12 BioTwin, FEI, Hillsboro, OR). For preparation of figures, images were adjusted for contrast and brightness using Photoshop 6.0 software, and imported into Microsoft PowerPoint to add lettering. For quantification of cross-sectional area, surface area, as well as minor and major axis length digital images were analyzed with Microcomputer Imaging Device software (MCID, Imaging Research Inc., Ontario, Canada).

Results

Systolic blood pressure in rats from the saline, AngII, and phenylephrine groups did not differ prior to treatment (Figure 1). However, continuous subcutaneous infusion of AngII or phenylephrine over a seven-day period produced a similar degree of hypertension relative to saline infused rats (Figure 1).

Electron microscopic imaging of p47^{phox} immunogold labeled dendritic profiles in the dmNTS of rats chronically administered saline, AngII, or phenylephrine

A combined total of 72,600 μm^2 of tissue was sampled from the dmNTS and mNTS at the level of the area postrema (Figure 2) for electron microscopic analysis of p47^{phox} and AT-1A receptor labeling in saline, AngII, and phenylephrine administered rats. Within the dmNTS of control and hypertensive animals, immunogold labeling of p47^{phox} was prominently present in dendritic processes, with or without AT-1A immunoperoxidase labeling. Within small ($<1 \mu\text{m}$) dendritic profiles, immunogold-silver deposits for p47^{phox} were associated with the surface membrane (Figure 3A) as well as intracellular sites including vesicular organelles characteristic of smooth endoplasmic reticula (sER), as well as the cytoplasm without apparent vesicular contact (Figures 3B–C). Immunolabeled dendrites were present in the neuropil populated by glial and neural profiles, including myelinated axons (Figures 3A and 3C), and were also found near blood vessels and surrounding endothelial cells (Figure 3B). Many p47^{phox} labeled dendritic profiles received one or more synapse from unlabeled axon terminals forming asymmetric excitatory-type synapses (Figures 3A–B). Immunogold labeling of p47^{phox} was frequently present in intermediate-size (1–2 μm) dendritic profiles. In these medium-size dendritic profiles, immunogold labeling was found in association with intracellular membranous organelles (Figure 4A), and the cytosol (Figure 4B), as well as the plasma membrane (Figure 4C). Similar to small profiles, intermediate-size dendritic processes were present near the neurovasculature (not shown) in addition to the neuropil populated by myelinated axons

Quantification of morphology and p47^{phox} immunogold labeling in dmNTS dendritic profiles from rats chronically administered saline, AngII, or phenylephrine

There were no significant differences in cross-sectional area (Figure 5A; $p=.39$), surface perimeter (Figure 5B; $p=.36$), minor axis length (Figure 5C; $p=.12$), or the ratio of major to minor axes (Figure 5D; $p=.53$) in dendritic profiles from rats administered saline, AngII, or phenylephrine. In addition, there were no differences in the total number of gold particles ($p=.24$) in dendritic profiles in animals from each of the treatment groups (Figure 6A). There were also no significant differences ($p=.9$) in the number of intracellular gold particles among saline, AngII, and phenylephrine administered rats (Figure 6B). However, there was a significant increase in the number of surface gold particles per unit surface perimeter in rats administered phenylephrine compared to saline ($p=.03$) and AngII ($p=.02$) treated animals (Figure 6C).

Subcellular p47^{phox} immunogold labeling was segregated into dendritic profiles with or without immunoperoxidase labeling for AT-1A (Table 1A). Although there were significant differences in total ($p<.0001$) and intracellular ($p<.0001$) immunogold p47^{phox} labeling between single p47^{phox} and dual p47^{phox} and AT-1A labeled dendritic profiles, there were no interactions between treatment and labeling type (i.e. single versus dual labeling) with respect to the total number of gold particles ($p=.76$) as well as intracellular ($p=.44$) or surface labeling ($p=.38$). Thus, while the density of total and intracellular labeling was higher in p47^{phox} single labeled, as compared to dual labeled dendrites, there were no differences in the subcellular distribution of this protein in either type of profile in saline, AngII, or phenylephrine groups.

When dendritic profiles were segregated by size, based upon minor axis length, into small ($<1 \mu\text{m}$), intermediate (1–2 μm), and large ($>2 \mu\text{m}$) categories, there were significant differences in the subcellular distribution of p47^{phox} gold labeling across treatment groups. In small dendritic profiles, there was a lower number of gold particles in intracellular sites in both AngII ($p=.006$) and phenylephrine ($p=.02$) treated rats when compared to saline administered animals (Figure 7A). The latter finding suggests that hypertension, independent of the causative agent, produces a reduction of intracellular p47^{phox} targeting in small dendritic profiles. In intermediate size dendritic profiles, however, there was an increase in p47^{phox} immunogold

labeling in rats chronically administered AngII or phenylephrine relative to those receiving saline, although these changes occurred in different subcellular locations. There was an increase in surface immunogold labeling in phenylephrine-treated rats as compared to animals given saline ($p=.01$) or AngII ($p=.016$) [Figure 7B]. However, there was an increase in p47^{phox} immunogold non-vesicular labeling in intermediate-size dendritic profiles from AngII administered rats compared to saline ($p=.0002$) and phenylephrine ($p=.0002$) treated animals (Figure 8A). Finally, there were no differences in total ($p=.76$), intracellular ($p=.53$), or surface ($p=.69$) p47^{phox} immunogold labeling in large dendritic profiles from the dmNTS of saline or hypertensive animals.

Electron microscopic imaging of p47^{phox} immunogold labeled profiles in the mNTS of rats chronically administered saline, AngII, or phenylephrine

Within the mNTS of control and hypertensive animals, immunogold labeling of p47^{phox} was present in numerous somata and dendritic profiles with or without AT-1A immunoperoxidase reaction product. Within dendritic profiles, immunogold-silver deposits for p47^{phox} were found in intracellular sites including small round vesicular organelles (Figure 9A). Immunogold particles for p47^{phox} were also associated with the surface membrane (Figure 9B) as well as, as well as the cytoplasm without apparent vesicular contact (not shown). Dendritic profiles showing surface and intracellular p47^{phox} labeling were present in the neuropil populated by unlabeled neuronal and astroglial profiles, but rarely myelinated axons. Dendritic profiles labeled for p47^{phox} were apposed by one or more axon terminal, but only infrequently received more than one synaptic contact (Figures 9A–C). Instead, p47^{phox} labeled dendritic profiles were more frequently apposed by small astrocytic processes (Figures 9A–B). In addition, to their presence in the neuropil, labeled dendritic profiles were also present near blood vessels (Figure 9C).

Quantification of p47^{phox} immunogold labeled dendritic profiles in the mNTS of rats chronically administered saline, AngII, or phenylephrine

Collapsed across all labeled dendritic profiles, there were no differences in cross-sectional area (Figure 5A; $p=.44$), surface area (Figure 5B; $p=.43$), minor axis length (Figure 5C; $p=.24$), or the ratio of major to minor axes (Figure 5D; $p=.76$) in mNTS neurons from saline, AngII, or phenylephrine treated rats. In addition, there were also no differences in total p47^{phox} gold particles per unit cross-sectional area (Figure 6A; $p=.11$), intracellular p47^{phox} particles per unit cross-sectional area (Figure 6A; $p=.2$), or surface p47^{phox} labeling per unit surface perimeter (Figure 6C; $p=.84$) in mNTS dendritic profiles.

Dendritic profiles in the mNTS were separated into single p47^{phox} labeled or dual labeled categories. There were no significant differences in intracellular p47^{phox} labeling per unit cross-sectional area ($p=.06$), or surface labeling per unit surface perimeter ($p=.06$) between single and dual labeled mNTS dendritic profiles (Table 1B). There also were no treatment effects on intracellular ($p=.9$) or surface ($p=.1$) labeling in p47^{phox} single or dual labeled dendritic profiles (Table 1B).

Dendritic profiles in the mNTS were divided into small, medium, and large sizes. There were no differences in p47^{phox} intracellular gold particles per unit cross-sectional area in small ($p=.17$), intermediate ($p=.2$), or large ($p=.2$) dendritic processes across the treatment groups (Figure 7C). In addition there were no differences in plasma membrane p47^{phox} gold labeling per unit surface perimeter in small ($p=.4$), intermediate ($p=.8$), or large ($p=.59$) dendritic profiles (Figure 7D). When p47^{phox} intracellular immunogold labeling was segregated into subcellular components, there were no differences in gold labeling in nonvesicular (Figure 8B), vesicular (not shown), or mitochondrial (not shown) sites.

Discussion

The present study provides the first immunocytochemical evidence that an equivalent level of systemic hypertension produced by two distinct pharmacological agents, AngII or phenylephrine, is associated with changes in the subcellular localization of p47^{phox} in the rat dmNTS, a brain region that preferentially receives cardiovascular afferents. These changes in p47^{phox} localization are not ubiquitous, since neither AngII nor phenylephrine had any effect on p47^{phox} localization in the mNTS, a region that is prominently innervated by alimentary/gastrointestinal afferents. Interestingly, the alteration in p47^{phox} localization may not be solely dependent on AngII signaling, since there is no difference in the targeting of this protein in dendritic profiles that only show p47^{phox} labeling and those that also express AT-1A receptor labeling. In addition, the changes in p47^{phox} subcellular localization induced by AngII or phenylephrine were not uniform, but instead were a function of each agent and the size of the dendritic profiles measured in our samples. Thus, changes in the subcellular trafficking of p47^{phox} in dendrites of dmNTS neurons are dependent on the particular hypertension-inducing agent, as well as the specific dendritic compartments expressing this protein.

Dendritic p47^{phox} labeling in dmNTS neurons of saline administered rats

Within the dmNTS of saline treated rats, immunogold labeling of p47^{phox} was present in somata, dendrites, and astroglia, a pattern that parallels immunolabeling for this and other NADPH oxidase subunits in normal rats (16). Within dendrites, the major site of NADPH oxidase subunit immunolabeling in dmNTS neurons, p47^{phox} immunoreactivity was associated with intracellular organelles that are involved in protein transport and calcium signaling, particularly ER-like tubulovesicular organelles, which were frequently associated with the outer mitochondrial membrane. Thus, p47^{phox} immunolabeling in dendritic smooth ER, which are sites for calcium storage and release, is consistent with physiological evidence that calcium currents in dmNTS neurons are modulated by superoxide produced by NADPH oxidase (23, 47,48). In addition to intracellular sites, p47^{phox} immunolabeling was also seen at synaptic and extra-synaptic areas of the plasmalemma, similar to other reports in the literature indicating that NADPH oxidase has a synaptic distribution in other brain regions (41).

Effect of chronic AngII on dendritic p47^{phox} immunogold labeling in dmNTS

In rats treated chronically with AngII, subcellular p47^{phox} immunogold labeling in the dmNTS was qualitatively similar to that seen in saline administered animals. Immunolabeling for p47^{phox} was present in association with a variety of intracellular organelles including ER and mitochondria, as well as the plasma membrane. However, there was a quantitative difference in the accumulation of dendritic p47^{phox} immunogold labeling in particular subcellular compartments, in a manner that depends upon profile size. Across all p47^{phox} labeled dendritic processes, there were no differences in total, intracellular, or plasma membrane immunogold labeling in response to AngII. When dendritic profiles were further compartmentalized by size, there were significant differences in p47^{phox} labeling in small and intermediate processes. In small, presumably distal dendritic profiles, there was a decrease in intracellular p47^{phox} labeling. However, in intermediate-size dendritic profiles, there was an increase in intracellular p47^{phox} immunogold labeling, and this change was solely due to an increase in nonvesicular immunogold particles. Thus, the administration of AngII over seven days, which produces elevated systemic blood pressure, is associated with a redistribution of p47^{phox} immunolabeling away from intracellular organelles in the distal dendritic compartment, and toward non-vesicular targets in less distal, intermediate areas of dmNTS neurons.

Effect of chronic phenylephrine on dendritic p47^{phox} immunogold labeling in dmNTS

In animals receiving chronic phenylephrine, immunolabeling of p47^{phox} in the dmNTS was qualitatively similar to that seen in saline- as well as AngII-treated rats. However, there were

prominent quantitative differences in the subcellular distribution of p47^{phox} immunogold labeling in dendritic profiles of dmNTS neurons in animals given phenylephrine as compared to saline. Whereas there were no differences in total or intracellular dendritic gold labeling, there was a significant increase in plasmalemmal p47^{phox} labeling in dmNTS dendritic profiles in rats treated with phenylephrine compared to those receiving saline. Segregating dendritic profiles into small, intermediate, and large categories revealed an important effect of size on the subcellular redistribution of p47^{phox} immunoreactivity. In small dendritic profiles, there was a significant decrease in intracellular p47^{phox} immunogold labeling relative to saline administered rats, an effect similar to that occurring with AngII. Unlike the case of AngII, however, there was an increase in plasma membrane gold labeling in intermediate-size dendritic profiles of phenylephrine-treated animals relative to saline- and AngII-treated rats. Thus, chronic administration of phenylephrine, which produces increases in systolic blood pressure, was associated with a repartitioning of p47^{phox} immunolabeling away from intracellular organelles in distal dendritic areas, and toward the plasma membrane of intermediate dendritic areas of dmNTS neurons.

Repartitioning of p47^{phox} in dendritic profiles of dmNTS neurons induced by chronic AngII or phenylephrine: Functional considerations

Production of superoxide within central autonomic circuitry is considered to be a potentially important substrate of neural cardiovascular regulation (53). While the precise mechanisms linking superoxide production, intracellular signaling, and modulation of neural cardiovascular regulatory circuitry are unclear, the subcellular localization of NADPH oxidase-mediated superoxide production may have a significant impact on intracellular signaling pathways (24, 51). Within the framework of the classic neutrophil model, NADPH oxidase requires stimulation for assembly and superoxide production at the plasma membrane, however the particular subcellular location of active NADPH oxidase depends upon cellular phenotype (24,28). In the NTS, previous ultrastructural evidence indicates that NADPH oxidase subunits p47^{phox}, gp91^{phox}, and p22^{phox} are present in association with endomembranes, as well as the plasmalemma in neurons (16). In addition, previous neurophysiological results also indicate that functional NADPH oxidase potentiates AT-1A mediated calcium currents in dissociated second-order neurons in the dmNTS (47,48). The present results demonstrate that the subcellular distribution of p47^{phox}, and presumably functional NADPH oxidase-mediated superoxide production, is not spatially static, but subject to repartitioning in dendrites of dmNTS neurons in response to regimens of AngII or phenylephrine that produce a similar degree of hypertension. Thus, the production of superoxide and its metabolites, such as hydrogen peroxide, are likely to be subject to subcellular plasticity in dendrites of dmNTS neurons in response to AngII or phenylephrine.

In small, presumably distal dendritic processes, where the density of p47^{phox} immunolabeling is highest under basal conditions (16), there was a significant decrease in immunogold particles in animals administered AngII or phenylephrine, relative to saline administered rats. This decrease occurs exclusively in the intracellular compartment. Small, distal dendrites receive a high degree of input from vagal-originating axon terminals that form asymmetric excitatory-type synapses (37,46), indicating that hypertension produces a select translocation of p47^{phox} in the dendritic compartment preferentially targeted by visceral afferents. Since similar changes in p47^{phox} immunolabeling occurred specifically in small dendritic profiles in response to both AngII and phenylephrine, the decrease in p47^{phox} immunolabeling would appear to be the result of the common hypertensive effects of each agent. Thus, it is possible that heightened baroreflex activation accompanying hypertension produces increased activity of glutamatergic cardiovascular afferents, which in turn contributes to a decreased accumulation of p47^{phox} in distal dmNTS dendrites.

Chronic infusion of AngII and phenylephrine were associated with an increase in immunogold particles in intermediate-size dendritic profiles in dmNTS neurons, but these changes occurred in different subcellular compartments. The mechanism mediating this effect is unclear, although it should be pointed out that AngII and phenylephrine, while inducing hypertension, can also produce differing effects on other physiological processes. Thus, these differences may help to account for the divergence of p47^{phox} cellular targeting in intermediate dendritic profiles. One area where AngII and phenylephrine produce contrasting effects is in the area of fluid balance. Angiotensin II is a potent dipsogenic agent (13), while centrally infused phenylephrine inhibits drinking induced by central administration of AngII (5). In addition, AngII and phenylephrine also produce differences in neural activation in brain areas involved in fluid homeostasis. For example, in rabbit, AngII induces immediate early gene activation in the subfornical organ (SFO), and organum vasculosum of the lamina terminalis (OVLT), whereas phenylephrine does not (3). The effects of AngII on drinking behavior are mediated by hypothalamic circuits (39) that have connections with the NTS (33). Interestingly, descending projections from the hypothalamus form symmetric inhibitory-type synapses (42), which would be expected to target more proximal regions of dendrites (32), an area where there were differing effects of AngII and phenylephrine on subcellular p47^{phox} localization. Whatever the underlying substrates for these diverse changes, they suggest that NADPH oxidase-induced superoxide production in dendrites of dmNTS neurons would be shifted intracellularly, in the case of AngII, and near the plasma membrane in the case of phenylephrine.

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Figure 1.

Blood pressure is elevated in response to chronic administration of AngII or phenylephrine. Rats infused with AngII (n=4) or phenylephrine (n=4) over a seven day period develop similarly elevated levels of blood pressure as compared to a saline (n=4) control group. *p<0.05

Figure 2. Schematic diagram of the caudal medulla. The trapezoids illustrate the relative size and location of the areas containing the dmNTS (right) and mNTS (left, inverted) in which ultrathin sections were obtained for electron microscopic analysis in separately processed brainstem sections. AP= area postrema, DMX= dorsal motor nucleus, ts= solitary tract.

Figure 3.

Visualization of the subcellular localization of p47^{phox} immunogold labeling in small (<1 μ m) dendritic profiles from the dmNTS of saline, AngII and phenylephrine administered rats. (A). A small single labeled dendritic profile (p47-d) from a saline treated rat contains immunogold particles (small arrows) and receives asymmetric synapses (large arrows) from unlabeled axon terminals (ut1–2). Immunogold-silver deposits are present on the surface membrane, including the extrasynaptic plasmalemma near an asymmetric synapse made by an unlabeled axon terminal (ut-2). An unlabeled myelinated axonal profile (m-ax) characteristic of the dmNTS neuropil is present nearby. (B). A single labeled dendritic profile (p47-d) from an AngII treated rat contains p47^{phox} immunogold particles (small arrows) that are associated with diverse

structures. In addition to labeling near the surface, an immunogold particle is found near a small vesicular organelle (vo). This dendritic profile is contacted by an unlabeled axon terminal (ut) forming an asymmetric synapse (large arrow). The dendrite and axon terminal are proximal to a blood vessel as indicated by the presence of an endothelial cell (edth) surrounding a lumen (lu). (C). A single labeled dendritic profile (p47-d) from a phenylephrine treated rat contains intracellular and surface p47^{phox} immunogold labeling (small arrows). A single gold particle is present on the plasma membrane apposed to an unlabeled axon terminal (ut) forming an apparent asymmetric type synapse (large arrow) and another is present intracellularly. A myelinated axon (m-ax) is present in the nearby neuropil. Scale Bars =0.5µm

Figure 4.

Visualization of the subcellular distribution of $p47^{phox}$ labeling in intermediate-size (1–2 μm) dendritic profiles from the dmNTS of saline, AngII and phenylephrine treated rats. (A). A dendritic profile (p47-d) from a saline treated rat has gold particles for $p47^{phox}$ (small arrow) in diverse intracellular sites, including a vesicular organelle (vo). This profile is contacted by an unlabeled terminal (ut). An unlabeled myelinated axon (m-ax) is present in the adjacent neuropil. (B). A dendritic profile (p47-d) from an AngII-treated rat, contains exclusively intracellular $p47^{phox}$ gold particles (small arrows) in the cytosol. This profile is contacted by an unlabeled axon terminal (ut). (C). A single labeled dendritic profile (p47-d) from a phenylephrine infused animal contains gold-silver deposits for $p47^{phox}$ (small arrows) mainly

associated with the plasma membrane. A myelinated axonal profile (m-ax) is present in the adjacent neuropil. Scale Bars =0.5 μ m.

Figure 5. Quantitative morphology of p47^{phox} immunogold labeled dendritic profiles from the dmNTS and mNTS of rats chronically administered saline, AngII, or phenylephrine.

Figure 6.

Subcellular distribution of p47^{phox} immunogold particles in dendritic profiles of neurons in the dmNTS and mNTS of rats chronically infused with saline, AngII, or phenylephrine. (A–B). There are no differences in total or intracellular labeling in either NTS region across any of the groups. (C). There is an increase in surface labeling in dendritic profiles solely in dendritic profiles from the dmNTS of phenylephrine administered rats. * p < .05

Figure 7.

Contrasting changes in p47^{phox} labeling in small- and intermediate-size dendritic profiles as a function of hypertensive agent specifically in dendritic profiles of dmNTS neurons. (A). In the dmNTS, hypertension, independent of the stimulating agent, results in decreased intracellular p47^{phox} immunogold labeling in small (<1 μm), presumably distal dendritic profiles. (B). In the dmNTS, medium size dendritic profiles (1–2 μm) show an increase in surface p47^{phox} labeling in phenylephrine treated animals compared to saline and AngII treated rats. (C–D). There are no differences in intracellular or surface p47^{phox} immunogold labeling in mNTS dendritic profiles. * $p < .05$

Figure 8.

Differences in non-vesicular p47^{phox} labeling in intermediate-size dendritic profiles in dmNTS neurons. (A). There is a significant increase in labeling associated with non-vesicular sites in intermediate-size dendritic profiles of rats treated with AngII relative to saline and phenylephrine treated rats. (B). There are no significant differences in the degree of non-vesicular labeling in dendritic profiles from the mNTS in any of the three groups of animals.

Figure 9.

Visualization of the subcellular distribution of p47^{phox} labeling in small (<1 μ m) dendritic profiles from the mNTS of rats chronically administered saline, AngII, or phenylephrine. (A). A dendritic profile (p47-d) from a saline treated rat has gold particles for p47^{phox} (small arrows) near a vesicular organelle (vo) as well as the plasmalemma. This dendritic profile is contacted by astroglia (asterisks) as evidenced by the presence of glial fibers (f). Note the presence of multiple unlabeled glial (ug) and unmyelinated axonal (ua) profiles characteristic of the neuropil of the mNTS. (B). A dendritic profile (p47-d) from an AngII treated rat contains exclusively surface p47^{phox} gold particles (small arrows). This profile is contacted by a small unlabeled axon terminal (ut), but is mainly sheathed by astrocytic processes (asterisks). (C).

A dendritic profile (p47-d) contains gold-silver deposits for p47^{phox} (small arrows) associated with a vesicular organelle (vo) and the plasma membrane. This profile is contacted by unlabeled axon terminals (ut 1–2), one of which forms an apparent asymmetric synapse (large arrow). This complex is closely apposed to the neurovasculature, as indicated by the presence of an endothelial cell profile (edth) surrounding a vessel lumen (lu). Scale Bars =0.5 μ m.

Table 1

Table 1A. Subcellular distribution of p47 ^{phox} immunogold labeling in dendritic profiles showing single labeling for p47 ^{phox} or dual labeling for p47 ^{phox} and the AT-1A receptor in dmNTS neurons						
	p47 ^{phox}			p47 ^{phox} +AT1A-receptor		
	Intracellular/Area	Surface/Perimeter	Non-Ves./Area	Intracellular/Area	Surface/Perimeter	Non-Ves./Area
Saline	.8±.09	.04±.009	.54±.06	.5±.03	.045±.01	.32±.03
AngII	.76±.07	.05±.01	.56±.07	.59±.04	.04±.008	.47±.04
Phenylephrine	.7±.07	.08±.01	.47±.06	.57±.05	.05±.01	.4±.05

Table 1B. Subcellular distribution of p47 ^{phox} immunogold labeling in dendritic profiles showing single labeling for p47 ^{phox} or dual labeling for p47 ^{phox} and the AT-1A receptor in mNTS neurons						
	p47 ^{phox}			p47 ^{phox} +AT1A-receptor		
	Intracellular/Area	Surface/Perimeter	Non-Ves./Area	Intracellular/Area	Surface/Perimeter	Non-Ves./Area
Saline	.65±.05	.08±.008	.46±.03	.57±.06	.03±.009	.43±.05
AngII	.75±.05	.065±.009	.49±.04	.6±.04	.06±.015	.47±.04
Phenylephrine	.75±.07	.08±.01	.58±.06	.66±.06	.06±.015	.49±.06