Reactive Oxygen Species in the Neuropathogenesis of Hypertension

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Current Hypertension Reports 2006, **8:**232–241
Current Science Inc. ISSN 1522-6417

New evidence that has emerged during the past several years clearly demonstrates that reactive oxygen species (ROS) in the brain play a crucial role in blood pressure regulation by serving as signaling molecules within neurons of cardiovascular control regions. In the forebrain, midbrain, and hindbrain, a key role for oxidant stress in the pathogenesis of angiotensin II–dependent and various other models of neurogenic hypertension has also been uncovered. As in the peripheral vasculature, NAD(P)H oxidase appears to be a major enzymatic source of brain ROS, and various homologues of the catalytic subunit of this enzyme appear to be differentially localized to cardiovascular-regulating nuclei in the brain. Recent studies have begun to elucidate the downstream effects of ROS in neurons, and it is now clear that ROS may interact with a number of well-described intracellular signaling pathways involved in neuronal activation. These exciting new discoveries have furthered our understanding of the pathogenesis of neurogenic hypertension and may ultimately lead to the development of new treatments. In this review, we discuss recent evidence in support of a role for brain ROS in the pathogenesis of hypertension and summarize current studies aimed at uncovering the complex mechanisms by which brain ROS regulate blood pressure in both health and cardiovascular disease.

Introduction

Cardiovascular-regulating nuclei in the brainstem and hypothalamus play a pivotal role in the enduring management of blood pressure and volume homeostasis by modulating sympathetic tone, the perception of thirst, and the release of hormones into circulation [1]. These nuclei sense fluctuations in the status of the cardiovascular system, in part through neural inputs received from circumventricular organs (CVOs), which are specialized brain regions strategically lacking a blood brain barrier (BBB) to enable the detection of systemic circulating effector hormones and blood osmolality [2]. Additional inputs are received from the peripheral nervous system, most importantly from baroreceptors, cardiopulmonary receptors, and chemoreceptors [3]. These central neural networks have emerged as primary culprits in various cardiovascular diseases [3]. For example, most individuals with hypertension exhibit increased sympathetic drive [4]. Excessive sympathetic nervous system activity is also implicated in metabolic disorders, such as diabetes and obesity [5], which carry increased risk for cardiovascular disease. Additionally, unbridled neurohumoral excitation is a hallmark of advanced heart failure [6]. Although much research has been focused on how dysregulation of cardiovascular-regulating nuclei may sustain hypertension and other cardiovascular diseases, recent advances in the tools of molecular genetics, coupled with improvements in physiologic analyses, have allowed a closer examination of the signaling pathways involved.

Since the discovery more than a decade ago that angiotensin II (Ang II) initiates the production of reactive oxygen species (ROS) through an NAD(P)H oxidase in vascular smooth muscle cells (VSMCs) [7••], much research has been devoted to understanding the roles of ROS in various tissue types in the pathogenesis of cardiovascular diseases. ROS are a diverse family of molecules that result from the reduction of molecular oxygen, and classically include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide, the hydroxyl radical, hypochlorous acid, and peroxynitrite. Although ROS arise as by-products of cellular metabolism, recent evidence suggests that most cell types are capable of the enzymatic production of ROS for use as signaling molecules [8]. During the past few years, work in our laboratory and others has shown that excessive production of ROS in the brain plays a crucial role in the pathogenesis of Ang II–dependent hypertension. Furthermore, excessive brain ROS production has been found in various other animal models of hypertension [8]. A clear understanding of the sources and downstream effects of ROS in cardiovascular-regulating nuclei in the central nervous system (CNS) will further our knowledge of the pathogenesis of hypertension and may eventually lead to the development of novel treatments

for this disease. In this review, we summarize the most recent evidence in support of a role for ROS in the pathogenesis of hypertension and discuss possible molecular mechanisms by which increased brain ROS may initiate a chronic rise in blood pressure.

Recent Evidence for Increased Brain ROS in the Pathogenesis of Hypertension

Angiotensin II and brain ROS

Because the renin-angiotensin-aldosterone system is implicated in neurogenic mechanisms of hypertension, much research has been devoted to understanding the effects of both circulating and locally generated Ang II on brain cardiovascular nuclei. Because of its size, circulating Ang II is unable to cross the BBB and must utilize neurons of CVOs, which are rich in Ang II receptors, to influence the otherwise inaccessible cardiovascular regions inside the BBB [2]. Important early studies from Fink's group [9] demonstrated that lesioning the CVOs could attenuate the rise in blood pressure in certain experimental models of hypertension. More recently, Collister et al. [10] showed that lesioning the subfornical organ (SFO), a prominent forebrain CVO, prevented the hypertensive response to a chronic, peripheral Ang II infusion in rats. Recently, we have shown that the actions of peripheral Ang II on the SFO involve the production of $O_2^{\bullet-}$ [11••]. Using dihydroethidium staining to directly measure relative $O_2^{\bullet-}$ levels, we have detected increased production of $O_2^{\bullet-}$ in the SFO corresponding to the gradual rise in blood pressure following a peripheral, subpressor infusion of Ang II in mice. Moreover, scavenging of $O_2^{\bullet-}$ specifically in the SFO using adenoviral vectors to overexpress intracellular superoxide dismutase (Cu/ZnSOD) (an $O_2^{\bullet-}$ scavenging enzyme) abolished the rise in blood pressure [11••]. Interestingly, scavenging of extracellular $O_2^{\bullet-}$ in the SFO by overexpression of an extracellular-matrix targeted SOD (EcSOD) did not prevent this rise in blood pressure [11••], indicating the specificity of intracellular, and not extracellular, brain $O_2^{\bullet-}$ as a mediator of Ang II–induced hypertension.

In addition to responding to circulating Ang II, CVOs, as well as the majority of other cardiovascular-regulating nuclei that they influence, are also regulated by Ang II generated locally within the BBB through brain renin-angiotensin systems [12]. Neurons and glial cells in these regions are thought to secrete angiotensinogen and renin to produce Ang II in the interstitium, where its effects are limited locally by diffusion. Additionally, it is thought that certain neurons, especially those of the SFO, are also capable of synthesizing Ang II intracellularly in the somata, from where it is transported toward axon terminals and released as a neurotransmitter or neuromodulator [12]. In a variety of species, intracerebroventricular (ICV) Ang II induces a characteristic pressor, bradycardic, and dipsogenic response. Recent evidence has accumulated supporting a role for ROS in these actions of Ang II in the brain. Work from our lab has shown that overexpression of SOD targeted either to the cytoplasm (Cu/ZnSOD) or mitochondria (MnSOD) effectively scavenges Ang II–mediated increases in $O_2^{\bullet-}$ levels in the SFO, and prevents the physiologic responses to ICV Ang II in mice [13••]. The direct application of an acute dose of Ang II to the rostral ventrolateral medulla (RVLM), a major source of sympathetic outflow, elicits a pressor response concomitant with a local increase in $O_2^{\bullet-}$ production [14••]. Moreover, chronic infusion of a low dose of Ang II selectively to the RVLM, while failing to cause a significant increase in blood pressure, also increases $O_2^{\bullet-}$ production, increases renal sympathetic nerve activity, and impairs baroreflex sensitivity [15••].

Recent studies with tempol, a membrane-permeable SOD mimetic, also support a role for ROS in activation of the sympathetic nervous system by brain Ang II. In rats, ICV pretreatment with a large dose of tempol prevented central Ang II–induced increases in blood pressure, renal sympathetic nerve activity, and norepinephrine secretion from the posterior hypothalamus [16,17]. However, these studies with tempol are complicated by the fact that high, but not low doses of ICV tempol induce a marked hypotensive response. This suggests that ROS play a role in basal blood pressure regulation such that excessive $O_2^{\bullet-}$ scavenging results in a drop in blood pressure. Although it is possible that tempol lowers blood pressure partly through ROS-independent mechanisms, tempol has been shown to be an effective scavenger of ROS in the brain, and large doses of other SOD mimetics applied to the brain also result in a drop in baseline blood pressure [16,18]. Thus, it is likely that the hypotension induced by high doses of tempol is mediated at least in part through the scavenging of ROS. Indeed, given the evidence that ROS play an important role as signaling molecules in peripheral cells in the regulation of blood pressure [13••,19,20], it is not surprising that high doses of SOD or SOD mimetics can cause hypotension by interfering with normal signaling in the CNS. However, it should be noted that the role of ROS in the regulation of blood pressure in normotensive ranges is not fully understood. We have not seen a decrease in baseline blood pressure following brain gene transfer of Cu/ZnSOD, although our studies have targeted very specific brain regions [8,11••,13••]. Also, blocking the Ang II type 1 receptor in the RVLM with losartan improves baroreflex sensitivity and decreases renal sympathetic nerve activity without a decrease in $O_2^{\bullet-}$ production, suggesting, at most, a minimal role for Ang II–mediated ROS in modulating baseline cardiovascular function [15••]. Taken together, these studies suggest that the regulated production of ROS in the brain plays an important signaling role in the control of blood pressure in normotensive ranges, and, as such, a drop in pressure results only when ROS are scavenged at high levels in widespread regions of the brain. Furthermore, the excessive production of $O_2^{\bullet-}$ in

the hypertensive state may be effectively scavenged with targeted lower doses of SOD to return ROS and blood pressure levels to baseline.

Role of brain NAD(P)H oxidase in hypertension

In defining a role for ROS in the neuropathogenesis of Ang II–dependent hypertension, it is important to consider the enzymatic source of ROS. Although various enzymatic and nonenzymatic sources of ROS exist in the cell [19,21], NAD(P)H oxidase is currently viewed as the most prominent enzymatic source of Ang II–derived ROS [19,22]. First described as a component of the respiratory burst in phagocytes, NAD(P)H oxidase is a multisubunit enzyme that catalyzes the reduction of molecular oxygen to $O_2^{\bullet-}$, using NAD(P)H as an electron donor [23••]. Enzyme complex assembly is initiated via a low-molecular weight G-protein (Rac1 or Rac2) and phosphorylation of the cytosolic $p47^{phox}$ subunit, recruiting the remaining cytoplasmic subunits (eg, $p67^{phox}$, $p40^{phox}$) to the plasma membrane, where interaction with the membrane-bound cytochrome b_{558} (comprising $p22^{phox}$ and $gp91^{phox}$) completes activation of the enzyme [22,23••]. Within the past few years, an entire family of homologues of the catalytic subunit $gp91^{phox}$ (recently renamed Nox2) have been identified and shown to have different functions in various cardiovascular tissues [22]. In peripheral cardiovascular cells, Ang II uses the enzyme containing Nox1, Nox2, and/or Nox4 as the catalytic subunit to drive the production of $O_2^{\bullet-}$ as a signaling molecule [22,23••]. Interestingly, we have recently found that, just like peripheral cells, the pattern of distribution and regulation of Nox homologues in brain cardiovascular nuclei is complex [24,25]. For example, we have found that Nox2 and Nox4 are the most abundantly expressed homologues in forebrain and hindbrain CVOs in the mouse brain [25], although their relative expression differs in specific nuclei in various models of cardiovascular disease, suggesting that these Nox homologues may play unique roles in the various cardiovascular sites in the CNS. Importantly, we have recently discovered a role for NAD(P)H oxidase–dependent $O_2^{\bullet-}$ production in the physiologic responses to brain Ang II by demonstrating that the $O_2^{\bullet-}$ production and the physiologic responses to ICV Ang II are prevented by inhibiting Rac1 activation in the brain with adenoviral-mediated overexpression of a dominant-negative mutant of this small GTPase [26••]. Recall that Rac1 is necessary for the assembly and activation of NAD(P)H oxidase [23••]. In addition to our studies localizing Nox homologues in brain regions, recent studies have localized other NAD(P)H oxidase subunits to numerous cardiovascular nuclei in the brain, and have demonstrated a functional significance for this enzyme in Ang II–mediated signaling. Colocalization of $gp91^{phox}$ and the Ang II type 1 (AT_1) receptor to neuronal processes in the nucleus tractus solitarius (NTS), an important mediator of sympathetic outflow, has recently been reported [27••]. In the rabbit RVLM, ICV Ang II upregulates the expression and protein levels of NAD(P)H oxidase subunits $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ and increases NAD(P)H-dependent $O_2^{\bullet-}$ production [15••]. Additionally, application of Ang II to the rat RVLM results in immediate $p47^{phox}$ phosphorylation [14••]. Pretreatment with the NAD(P)H oxidase inhibitors apocynin or diphenyleneiodonium (DPI), or with antisense oligonucleotides to $p22^{phox}$ or $p47^{phox}$, prevents Ang II–induced $O_2^{\bullet-}$ production in the rat RVLM [14••]. Furthermore, the pressor effect of Ang II microinjection in the RVLM is attenuated by co-administration of DPI [14••], and pretreatment with the specific NAD(P)H oxidase inhibitor gp91ds-tat [28] blunts the physiologic responses to ICV Ang II [29••]. Taken together, these studies confirm a potential role for NAD(P)H oxidase as a source of Ang II–mediated $O_2^{\bullet-}$ production and Ang II actions in the brain.

Evidence for brain ROS in other animal models of neurogenic hypertension

In addition to its role in Ang II–dependent hypertension, it has become clear that increased ROS production in the brain is also common to various other animal models of hypertension. Recent studies have demonstrated increased oxidative stress in the brain of the spontaneously hypertensive rat (SHR) and the stroke prone SHR (SHRSP) [18,30,31]. Using electron spin resonance, levels of oxidative stress in whole SHR brains were found to be higher than in controls, and even higher in SHRSP [30]. ROS levels are markedly elevated in the RVLM of SHR and SHRSP, and blood pressure is reduced in SHR by scavenging $O_2^{\bullet-}$ in the RVLM with MnSOD, or with moderate doses of the SOD mimetics tempol or Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) [18,31]. In addition, total SOD levels are decreased in the RVLM of the SHRSP [31], suggesting that increased brain ROS in SHRSP may arise from an imbalance of endogenous ROS-scavenging mechanisms. However, the possibility of dysregulation of an enzymatic source of brain ROS in these animals has not been extensively studied. Another intriguing possibility is that brain ROS in SHR are generated by increased sensitivity to Ang II, as these rats show increased neuronal activation to both central and peripheral Ang II infusions [32], as well as Ang II receptor upregulation in the NTS [33]. Additionally, treatment with losartan alters antioxidant enzyme activity in the brains of SHR but not control animals [34]. A recent study from Raizada's group [35] has also implicated a novel pathway for $O_2^{\bullet-}$ production in these hypertensive animals. Microarray profiling revealed enhanced brain expression of soluble epoxide hydrolase (sEH) in the SHR brain. In the periphery, sEH converts epoxyeicosatrienoic acids (EETs), powerful vasodilators derived from arachidonic acid, to the less potent dihydroxyeicosatrienoic acids (DHETs). ICV delivery of an inhibitor of sEH results in a dramatic rise in both

blood pressure and heart rate in SHR, and this effect was prevented by pretreatment with an inhibitor of NAD(P)H oxidase [35]. Thus, inhibiting sEH in the SHR brain might result in increased levels of EETs that are capable of initiating NAD(P)H oxidase-dependent ROS production, which in turn results in sympathoexcitation. The authors suggest that upregulation of sEH in SHR may be an attempt to decrease ROS levels by diminishing EETs as a source. However, because brain oxidative stress in these animals arises from multiple factors, sEH upregulation is not a complete compensatory mechanism [35].

Consistent with the idea that a lack of endogenous antioxidant enzymes is a predisposing factor in the pathogenesis of hypertension, a recent study has implicated a role for adrenomedullin in the regulation of central sympathetic outflow. Adrenomedullin is an endogenous antioxidant peptide whose production and receptors have been localized to various cardiovascular-regulating nuclei [36]. Mice deficient in adrenomedullin demonstrated an enhanced pressor response to ICV hypertonic saline, as well as an increase in $O_2^{\bullet-}$ levels in the paraventricular nucleus [37]. Although the mechanisms underlying ICV hypertonic saline-induced sympathoexcitation are not fully understood, this study demonstrates that the pressor effect of this treatment involves $O_2^{\bullet-}$ production. Furthermore, adrenomedullin may play a role in cardiovascular homeostasis by limiting ROS to basal levels.

Downstream Effectors of Brain ROS in the Pathogenesis of Hypertension

The physiologic responses to increased brain ROS likely arise ultimately from increases in neuronal firing within cardiovascular control regions. In neurons, the binding of Ang II to its receptors initiates a number of well-described intracellular signaling cascades, resulting in the modulation of ion channel conductance, changes in membrane depolarization, and neuronal activation [38]. Recent studies have placed ROS as central players at crucial points along these signaling pathways leading to neuronal activation. We briefly summarize recent work demonstrating how ROS are capable of interacting with these intracellular cascades in cardiovascular control regions to signal an increase in blood pressure (Fig. 1).

ROS-mediated regulation of ion channels and intracellular signaling cascades in neurons

Some of the most exciting work on the role of brain ROS in the pathogenesis of hypertension has come from recent studies demonstrating that ROS may directly influence ion channel opening and membrane ionic currents in neurons. Ang II is a known activator of neuronal Ca^{2+} currents (Fig. 1) [38]. We have recently demonstrated that an Ang II–mediated influx of extracellular Ca^{2+} in neurons depends on increased $O_2^{\bullet-}$ production by a Rac1-dependent NAD(P)H oxidase [39]. Wang et al. [27••] showed that Ang II–mediated upregulation of L-type Ca^{2+} currents in neurons isolated from the NTS is inhibited by scavenging ROS with MnTBAP or by inhibiting NAD(P)H oxidase with gp91ds-tat or apocynin, implicating a role for NAD(P)H-derived $O_2^{\bullet-}$ in the activation of Ca^{2+} channels in this cardiovascular nucleus. Although it is possible that Ang II–induced ROS may trigger the opening of Ca^{2+} channels indirectly through induction of signaling cascades, the time course of activation suggests that ROS may open Ca^{2+} channels directly. As discussed by Wang et al. [27••], ROS may potentially achieve this by altering the structure of membrane proteins, either directly by the oxidation of sulfhydryl groups, or indirectly by the peroxidation of membrane lipids.

In addition to Ca^{2+} channels, it has been well established that Ang II regulates neuronal activity by influencing potassium conductance. Work during the past decade by Raizada and Sumners' group [29••] has been instrumental in defining the molecular mechanisms involved. Briefly, Ang II binding to the G-protein–coupled AT_1 receptor activates phospholipase C (PLC), resulting in the production of diacylglycerol and inositol-1,4,5-phosphate (IP_3)-mediated release of Ca^{2+} from intracellular stores, with subsequent activation of protein kinase C (PKC) (Fig. 1). An increase in intracellular [Ca^{2+}] also results in the activation of calcium/calmodulin kinase II (CaMKII) which, along with PKC, inhibits a delayed rectifying potassium current (I_{Kv}), resulting in membrane depolarization [38]. A recent study from this group has demonstrated a role for $O_2^{\bullet-}$ in this pathway. Using inside-out patch clamping techniques, Sun et al. [29••] showed that increased $O_2^{\bullet-}$ production in neurons specifically and directly closes potassium channels to inhibit I_{Kv}. $O_2^{\bullet-}$ generated by either Ang II–mediated NAD(P)H-oxidase or by xanthine/xanthine-oxidase has the same effect on I_{Kv}, indicating that $O_2^{\bullet-}$ may influence membrane conductance regardless of its source [29••]. The authors speculate that Ang II binding to its receptor may activate NAD(P)H oxidase via phosphorylation by CaMKII and/or PKC, and that the resulting increase in $O_2^{\bullet-}$ production mediates neuronal firing by closure of potassium channels. Furthermore, recent evidence suggests that, in hippocampal neurons, $O_2^{\bullet-}$ may enhance CaMKII activity by directly inhibiting protein phosphatases involved in CaMKII inactivation [40], suggesting an additional mechanism by which ROS may mediate neuronal activation (Fig. 1).

Interestingly, Raizada and Sumners' [41,42] group has also recently reported a potential "off" switch for Ang II–mediated signaling that may involve ROS. Ang II binding to its receptor in neurons increases intracellular concentrations of macrophage migration inhibitory factor (MIF), which then inhibits further neuronal activity. MIF contains thiol-oxidoreductase activity, capable of scavenging ROS [41]. Overexpression of recombinant MIF prevents an increase in Ang II–induced $O_2^{\bullet-}$ production in cultured

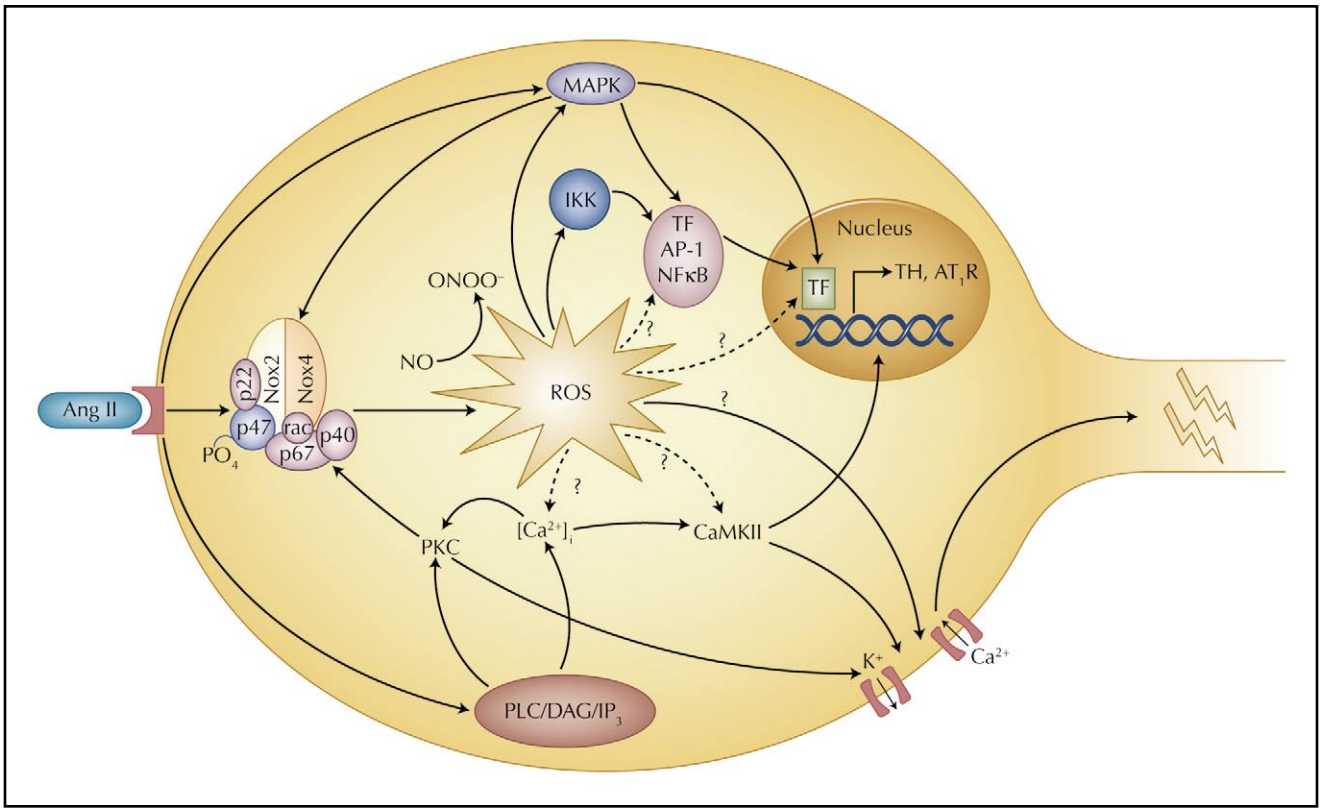

Figure 1. Diagram summarizing the roles of ROS in angiotensin II–dependent signaling pathways in neurons of cardiovascular-regulating nuclei. Ang II binding to its receptors activates a number of intracellular signaling pathways in neurons that are mediated by ROS or result in increased ROS production through activation of NAD(P)H oxidase. Crosstalk between these various pathways involves the utilization of ROS as signaling intermediates. Ang II directly activates a Rac1-dependent NAD(P)H oxidase to initiate the production of ROS [26••]. Ang II also stimulates the MAPK and PLC/IP_3/PKC pathways [14••,38], both of which may induce NAD(P)H oxidase to increase ROS production [14••,23••]. Additionally, activation of the MAPK pathway by Ang II is ROS dependent [14••]. PKC, as well as CaMKII, mediate the immediate actions of Ang II on neuronal firing by regulating membrane ion currents [38]. It is also possible that ROS may directly modify K^+ and/or Ca^{2+} channels [27••,29••]. Ang II–dependent changes in the neuron over the long-term depend on transcription factor activation by MAP kinases, the IKK complex, and CaMKII, all of which are sensitive to ROS [14••,38,40,57]. ROS may also mediate neuronal signaling through interactions with nitric oxide, although the precise mechanisms are not fully understood [64]. The central role of ROS in these multiple signaling pathways demonstrates the importance of ROS in the regulation of blood pressure in both normotensive and pathologic ranges, and also demonstrates the complexity of the neuronal signaling pathways involved in the pathogenesis of hypertension. AT_1R—angiotensin type 1 receptor; CaMKII—calcium-calmodulin kinase II; DAG—diacyglycerol; IKK—IκB kinase complex; IP_3—inositol 1,4,5-triphosphate; PLC—phospholipase C; PKC—protein kinase C; TF—transcription factors; TH—tyrosine hydroxylase.

neurons [41]. Additionally MIF-mediated increases in I_{Kv} also depend on its thiol-oxidoreductase activity [42]. Therefore, the ability of MIF to limit Ang II signaling by increasing potassium currents depends on its ROS-scavenging ability. Although studies are needed to further define the role of MIF in the regulation of blood pressure, these intriguing studies hint at a possible role for MIF in the pathogenesis of Ang II–dependent hypertension.

In the periphery, Ang II signal transduction occurs through numerous distinct pathways, many of which are redox-sensitive [19,21]. Recent research has explored the possibility that these pathways are also present in neurons, and has implicated the mitogen-activated protein (MAP) kinase pathway in neuronal Ang II signaling (Fig. 1). Importantly, the MAP kinase pathway has been shown to be redox-sensitive, as various steps in the regulatory pathways upstream of MAP kinases are activated by ROS [19,21,43,44]. An important series of experiments by Chan et al. [14••] implicates NAD(P)H-oxidase–derived $O_2^{\bullet-}$ in Ang II–induced MAP kinase activation in neurons of the RVLM. Ang II injection into the RVLM of rats elicits phosphorylation of p38 MAPK and ERK1/2, which is inhibited either by scavenging $O_2^{\bullet-}$ with tempol or by inhibiting NAD(P)H oxidase with DPI or with $p22^{phox}$ or $p47^{phox}$ antisense oligonucleotides [14••]. This pathway is also implicated in the functional activation of RVLM neurons, as inhibition of p38 MAPK in the RVLM prevented Ang II–induced increases in presynaptic transmitter release and the pressor response to RVLM Ang II injections [14••]. The precise downstream effects of MAPK activation that ultimately result in these functional outcomes are as yet unknown, but one idea is that these kinases may be involved in further phosphorylation events that result in ion channel modulation. Additionally, MAP kinases activate a number of transcription factors, most notably activator protein-1 (AP-1) [43,44].

Studies summarized earlier suggest that Ang II increases neuronal activation through at least two separate intracellular signaling cascades, both of which are regulated by ROS. A recent study using the Ang II analog SII has demonstrated that these pathways may play distinct roles in the behavioral effects of central Ang II [45]. Binding of SII to the AT_1 receptor in neurons results in activation of the MAPK but not the PLC/PKC/IP_3 pathway. Interestingly, ICV administration of SII in rats results in increased salt appetite but no immediate increase in water intake, whereas ICV Ang II results in increases in both salt appetite and water intake. Because Ang II, and not SII, activates the PLC/PKC/IP_3 pathway and increases water intake, and because both Ang II and SII activate the MAPK pathway and increase salt appetite, this study suggests that the PLC/PKC/IP_3 pathway may be independently responsible for water intake, and that salt appetite requires either the MAPK or PLC/PKC/IP_3 pathway, or both [45]. This finding is substantiated by earlier reports that the dipsogenic response to ICV Ang II is prevented by inhibitors of PKC and CaMKII [46]. A possible role for ROS in the activation of one cascade over another is unknown, although it is likely that the response is encoded at the level of the AT_1 receptor and may be dependent on such factors as receptor location, Ang II concentration, or cytoplasmic levels of signaling molecules. Additionally, two separate AT_1-receptor isoforms are present in rats and mice, and are differentially expressed and regulated in various locations in the brain [47]. These two isoforms play unique roles in the physiologic responses to central Ang II, where the AT_{1A} isoform mediates the pressor effects, while the AT_{1B} isoform mediates the dipsogenic response [47]. Although it is not known whether these receptors use separate or overlapping signaling pathways, it is tempting to consider the possibility that distinct intracellular signaling cascades may be responsible for the divergent functions of the AT_{1A} and AT_{1B} receptor isoforms. Nevertheless, these results suggest many possible scenarios in which discrete Ang II signaling pathways, activated to different extents in specific neuronal networks, may regulate the actions of Ang II in both health and disease. For example, it is possible that lower levels of Ang II in the normotensive state can activate intracellular signaling cascades in the absence of increased ROS production, but that heightened Ang II signaling in pathologic states recruits ROS-producing enzymes to enhance the effects of Ang II on neuronal activation.

ROS-mediated activation of transcription factors

The pathways discussed thus far, resulting in the modification of ion channel properties and neuronal activation, are mostly responsible for the immediate, short-term effects of Ang II. Many of these same pathways and/or others, however, may also result in the transcription of specific genes that alter the structural and functional properties of neurons, thereby mediating the long-term effects of Ang II. Because many of these signaling pathways involve redox-sensitive molecules, increased ROS production may ultimately initiate gene transcription by activating transcription factors via these pathways (Fig. 1). One such redox-sensitive transcription factor is AP-1. In mammals, AP-1 is a dimer of proteins in the jun and fos families, also considered inducible transcription factors. The isoforms comprising the dimer confer DNA-binding specificity such that different isoform combinations control the expression of different genes [43]. Activation of AP-1 is regulated at the transcriptional, posttranscriptional, and posttranslational levels by intracellular calcium and protein kinases, such as PKC and members of the MAP kinase family [44]. Phosphorylation of both Jun and Fos proteins by JNK and Fos-regulating kinase (FRK) is required for activation. Ang II–induced AP-1 activation is redox-sensitive, mainly through its regulation by the MAP kinase pathway [43,44]. In the brain, AP-1 is a ubiquitous transcription factor, and, in fact, *c-fos* expression is often used as a marker of neuronal activation. Peripheral and central Ang II induce different patterns of *c-fos* activation in numerous cardiovascular-regulating nuclei, including the SFO, and this Ang II–induced *c-fos* expression in neurons is enhanced and exhibits a unique pattern in SHR [32]. Recent studies have begun to outline the molecular mechanisms involved in Ang II–mediated AP-1 activation in neurons. Ang II induces activation of FRK through a PKC/Ca^{2+}-dependent pathway, while activation of JNK requires phosphorylation by phosphatidylinositol 3-kinase [38,48]. Interestingly, AP-1 in neurons promotes transcription of the genes for the AT_1 receptor and tyrosine hydroxylase, as well as a number of genes involved in the regulation of synapse morphology [48,49]. Thus, the activation of AP-1 by Ang II can result in long-term changes in the neuron through receptor upregulation, increased transmitter synthesis, and possibly by modulating neuronal plasticity. Although a recent study has implicated NAD(P)H oxidase–derived $O_2^{\bullet-}$ in Ang II–induced AP-1 activation in rat cardiomyocytes [50], a precise role for ROS in neuronal AP-1 activation awaits investigation. We have previously shown that adenoviral-mediated overexpression of Cu/ZnSOD causes a decrease in number of Fos-positive neurons in the paraventricular and supraoptic nuclei in a mouse model of heart failure [51]. Ultimately, central oxidant scavenging resulted in decreased sympathetic drive in these mice [51].

Although there is considerable evidence that Ang II is linked to increased expression of Fos and Jun family members in neuronal cultures and in the CNS in vivo, there is limited evidence for Ang II causing *trans*-activation of AP-1—that is, increased potential for gene expression—in cultured neurons or the brain. Current work in our laboratory involves longitudinal tracking of brain AP-1 activity in response to Ang II infusions by utilizing in vivo bioluminescence technology [52]. In combination with viral gene transfer of a luciferase reporter gene,

which confirms functionally activated AP-1, this strategy is providing new insights into the role of AP-1 in central Ang II responses.

Ang II–induced ROS production may also mediate gene transcription through activation of nuclear factor-κB (NF-κB), another redox-sensitive transcription factor. Like AP-1, NF-κB is a heterodimer whose DNA-binding specificity is isoform-dependent [53]. The most common isoform consists of the p50 and p65 subunits [43,53]. The inactive form of NF-κB is sequestered in the cytosol by the binding of p65 to the inhibitor protein IκB. Activation requires phosphorylation of the inhibitory protein IκB by the IκB kinase complex (IKK), resulting in ubiquitination and proteosomal degradation of IκB to allow translocation of p50/p65 to the nucleus. Direct phosphorylation of p65 also results in NF-κB DNA binding [54]. NF-κB activity is known to be redox-regulated [43,44], with redox-sensitive MAP kinase pathways, especially those regulating JNK and p38 MAPK, being involved in NF-κB activation. Furthermore, increased levels of oxidized thioredoxin activate NF-κB [43]. NF-κB is a known mediator of Ang II actions in the periphery, and initiates transcription of angiotensinogen in the liver [55]. In VSMCs, Ang II activation of NF-κB is known to be redox-regulated, as Ang II stimulation results in IKK phosphorylation, IκB degradation, and functional NF-κB activation that is inhibited by catalase [56,57]. Furthermore, a recent study suggests that Ang II–induced NF-κB activation in VSMCs may involve direct phosphorylation of p65, either by IKK or by an MAPK/Ras-activated ribosomal kinase.

Recent in vivo studies have examined the role of NF-κB in the pathogenesis of hypertension. Increased expression of NF-κB subunits has been demonstrated in the thoracic aorta and cardiac tissue of SHR, and these levels are normalized by treatments targeting the renin-angiotensin system [58,59]. Importantly, early inhibition of NF-κB prevents hypertension in SHR [60], and also prevents cardiac hypertrophy independent of its pressure-reducing effects [59]. Given that the etiology of hypertension in SHR may involve Ang II–induced oxidative stress in the brain [31,34], one intriguing possibility is that the hypertension in these animals also involves redox-dependent activation of NF-κB within neurons of cardiovascular nuclei. In the brain, NF-κB is known to play a role in a number of inflammatory processes, including ischemia, seizures, and acute trauma, as well as in a number of neurodegenerative diseases, especially Alzheimer's disease [61]. Additionally, neuronal firing activates NF-κB via a calcium-dependent pathway and/or phosphorylation by CaMKII, and NF-κB in the hippocampus mediates long-term potentiation [62]. In addition to the production of cytokines, NF-κB initiates the transcription of a number of genes in neurons that might play a role in the regulation of blood pressure, including inducible nitric oxide (NO) synthase (iNOS), CaMKII, and MnSOD [61,62]. However, to date, no studies have examined the role of NF-κB in Ang II-mediated neuronal signaling. Because many aspects of Ang II signaling in the periphery are mirrored in neurons, it is possible that activation of NF-κB by Ang II in central cardiovascular nuclei may be involved in neuronal changes underlying sympathoexcitation, neurohumoral synthesis/release, and, ultimately, sustained elevations in blood pressure.

Brain ROS and nitric oxide

Increased brain ROS may also influence neurocardiovascular regulation through interactions with NO. In the peripheral vasculature, NO mediates vasodilation, and Ang II–dependent vasoconstriction results in part from the oxidative inactivation of NO by $O_2^{\bullet-}$ [63]. In the brain, NO serves as a neurotransmitter, and NO synthases (NOS) are expressed in various brainstem nuclei [64]. Peroxynitrite, the product of the oxidation of NO by $O_2^{\bullet-}$, may interact with neuronal membrane ion channels [27••]. Although a definite interaction exists between NO, Ang II, and sympathetic outflow [63,64], there are conflicting reports about the precise mechanisms involved. It is generally accepted that NO in the brain is sympathoinhibitory, and that Ang II impairs NO availability [63,64]. Recent work has shown that ICV Ang II downregulates expression of neuronal NOS (nNOS) in various cardiovascular control regions, including the posterior hypothalamus, paraventricular nucleus, and locus coeruleus, and pretreatment with tempol or polyethylene glycol-superoxide dismutase (PEG-SOD) prevents this effect [16]. Thus, in addition to the inactivation of NO, Ang II–induced ROS may directly impair NO availability in these cardiovascular-regulating nuclei by preventing transcription of nNOS. Increasing NO production by overexpression of endothelial NOS (eNOS) in the RVLM reduces blood pressure, heart rate, and sympathetic activity in both WKY and SHR rats [18]. The extent of this cardiovascular depression is less in SHR rats, probably because increased basal RVLM $O_2^{\bullet-}$ levels in these animals provides greater opportunity for NO inactivation. It should be noted that overexpression of iNOS in the RVLM did not result in sympathoinhibition, but rather increased blood pressure and urinary norepinephrine excretion [65]. This was a surprising finding, because overexpression of an NOS would be expected to increase NO production, inhibiting sympathetic outflow. However, in this study, overexpression of iNOS resulted in a marked increase in oxidative stress in the RVLM. The authors explain that because iNOS is a more efficient producer of NO, L-arginine substrate stores may have been depleted, in which case the enzyme would produce $O_2^{\bullet-}$ rather than NO. Furthermore, it is possible that high levels of NO directly induce $O_2^{\bullet-}$ production as a compensatory mechanism. Although the sympathoexcitation resulting from overexpression of iNOS in the RVLM is most likely a result of increased $O_2^{\bullet-}$ production, this study demonstrates the complexity of the interactions between ROS, NO, and the sympathetic nervous system. Together, these studies sug-

gest that Ang II–derived brain ROS increase sympathetic drive in part through impairing NO availability, but the effects of brain NO in cardiovascular regulation appear to be concentration-dependent and site-specific. Additional studies are needed to clearly define the role of the interaction between brain NO and ROS-mediated signaling in the pathogenesis of hypertension.

Conclusions

A recent acceleration in the study of redox mechanisms in central neural control of cardiovascular function strongly implicates ROS signaling in the brain as a crucial step in the pathogenesis of hypertension. Various cardiovascular nuclei have the capacity to enzymatically produce ROS in response to external signals, especially Ang II, and it is apparent that many of the signaling pathways used by neurons in these nuclei are sensitive to increased NAD(P)H oxidase–dependent ROS production. The role of ROS in the regulation of blood pressure in the normotensive state remains unclear, although it is likely that the regulated production of low levels of ROS is involved in the maintenance of baseline blood pressure. However, dysregulated ROS production results in pathologic elevations that hijack excitatory pathways in the brain to orchestrate and maintain a rise in blood pressure. Progress in this field continues to reveal the increasing complexity of the molecular mechanisms involved in the neural control of blood pressure, and it is becoming apparent that perturbations in brain ROS have a profound effect on cardiovascular function. The challenge remains in defining the precise mechanisms by which ROS participate in the neural regulation of blood pressure under acute and chronic conditions. A clear understanding of the molecular underpinnings of ROS-mediated long-term changes in neurocardiovascular regulation has the potential to fundamentally advance our understanding of the mechanisms linking the CNS with cardiovascular diseases, and will ultimately lead to the development of novel therapeutic strategies for the treatment of neurogenic hypertension.

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