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Review

Redox signaling in central neural regulation of cardiovascular function

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Abstract

One of the most prominent concepts to emerge in cardiovascular research over the past decade, especially in areas focused on angiotensin II (AngII), is that reactive oxygen species (ROS) are critical signaling molecules in a wide range of cellular processes. Many of the physiological effects of AngII are mediated by ROS, and alterations in AngII-mediated redox mechanisms are implicated in cardiovascular diseases such as hypertension and atherosclerosis. Although most investigations to date have focused on the vasculature as a key player, the nervous system has recently begun to gain attention in this field. Accumulating evidence suggests that ROS have important effects on central neural mechanisms involved in blood pressure regulation, volume homeostasis, and autonomic function, particularly those that involve AngII signaling. Furthermore, oxidant stress in the central nervous system is implicated in the neuro-dysregulation associated with some forms of hypertension and heart failure. The main objective of this review is to discuss the recent progress and prospects for this new field of central redox signaling in cardiovascular regulation, while also addressing the molecular tools that have spurred it forward.

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Keywords: Renin–angiotensin system; Free radicals; CNS; NAD(P)H oxidase; Superoxide dismutase

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1. Introduction

Neural mechanisms play a key role in the maintenance of cardiovascular and volume homeostasis (Chapleau and Abboud, 2001; reviewed in Johnson and Thunhorst, 1997). The central nervous system (CNS), working in concert with a number of neural reflexes and neurohumoral factors, is critical in regulating arterial pressure, fluid balance, and cardiovascular function. It receives neural, humoral or chemical signals that reflect changes in blood pressure and body fluid status, and engages appropriate effector systems that control cardiac, vascular, renal and behavioral responses for restoration of balance. Alterations in neuro-cardiovascular regulation have long been implicated in pathological states such as hypertension, heart failure, diabetes and obesity (Chapleau and Abboud, 2001).

One of the major circulating factors that signals central effector systems to restore cardiovascular balance is angiotensin II (AngII). Although many brain regions are sensitive to AngII, unique blood–brain-barrier-deficient regions, called circumventricular organs (CVOs), are primary sensors for this blood-borne signal (Simpson, 1981). Neural pathways originating from sensory CVOs, which also receive inputs from systemic baroreceptors and chemoreceptors, project into an extensive neural network that is responsible for mobilizing the various systems responsible for maintaining homeostasis, i.e. vasopressin release, autonomic responses, and ingestive behaviors (reviewed in Ganong, 2000; Johnson and Gross, 1993). In addition to circulating AngII acting at CVOs, locally derived AngII, generated inside the blood–brain barrier by the intrinsic brain renin–angiotensin system, is also thought to function as an important neurotransmitter/modulator in these cardiovascular regulatory networks (reviewed in Phillips and Summers, 1998). It is now well recognized that these brain angiotensinergic systems are major

contributors to normal neurocardiovascular function, and dysregulation of them leads to diseases such as hypertension and heart failure (reviewed in: Francis, 1989; Phillips and Sumners, 1998).

Over the last decade, one of the most prominent concepts to emerge at the forefront of cardiovascular research, especially in areas focused on AngII mechanisms, is that reactive oxygen species (ROS) are critical intracellular signaling molecules in a wide range of cellular processes (reviewed in Griendling and Ushio-Fukai, 2000; Thannickal and Fanburg, 2000). It has become clear that many of the physiological effects of AngII on the vasculature, kidney and heart are mediated by ROS such as superoxide and hydrogen peroxide (Griendling et al., 1994; Hannken et al., 1998; Nakamura et al., 1998; Wilcox, 2002). Furthermore, alterations in AngII-mediated redox mechanisms in peripheral tissues are implicated in cardiovascular diseases such as hypertension (Hanna et al., 2003; Laursen et al., 1997) and atherosclerosis (Harrison et al., 2003a; reviewed in Harrison et al., 2003b).

Although most investigations of ROS in cardiovascular function and disease to date have focused on the vasculature as a key player, other potentially important tissues and mechanisms have begun to emerge. One system that has received little attention until very recently is the nervous system. Accumulating evidence suggests that ROS have important effects on central and peripheral neural mechanisms that regulate cardiovascular function, particularly those that involve AngII signaling. The main objective of this review is to discuss recent progress and prospects for this new field of central redox signaling in cardiovascular regulation, while also addressing the molecular tools that have spurred it forward.

2. ROS in mammalian cells

ROS encompass a variety of diverse chemical species that are by-products of cellular metabolic processes designed to reduce molecular oxygen. Following a one-, two- or three-electron reduction, superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), or hydroxyl radicals (HO^{\bullet}), respectively, are formed. This occurs through enzyme-catalyzed reactions, as with the production of $O_2^{\bullet-}$ and H_2O_2 , via reactions with each other and/or transition metals, as with the reaction between H_2O_2 and $O_2^{\bullet-}$ or H_2O_2 and Fe^{2+} to form the highly toxic HO^{\bullet} , or by way of the mitochondrial electron transport system. Hypochlorous acid ($HOCl$), another ROS, is formed by the reaction of H_2O_2 with chloride ion in the presence of myeloperoxidase. Nitric oxide (NO^{\bullet}) and its oxidized form peroxynitrite ($ONOO^-$), the result of a reaction between NO^{\bullet} with either $O_2^{\bullet-}$ or HO^{\bullet} , also represent ROS, but are often referred to as reactive nitrogen species (RNS).

There are several enzymatic sources of ROS in mammalian cells, including xanthine oxidase, cyclooxygenase, the cytochrome p450, nitric oxide synthase (NOS) and the NAD(P)H oxidase (reviewed in Cohen, 1994). Although each of these appear to be important under certain conditions, considerable evidence now indicates that the NAD(P)H oxidases are particularly prominent sources of ROS in the cardiovascular system (reviewed in Griendling et al., 2000a, b). Catalyzing the reduction of O_2 to $O_2^{\bullet-}$ via the transfer of a single electron from NAD(P)H, the phagocytic form of this enzyme is best known for its role in host defense against invading microorganisms (Satriano et al., 1993). It consists of two transmembrane subunits, the catalytic subunit gp91^{phox} and p22^{phox}, along with the cytosolic components p67^{phox}, p47^{phox}, and the small GTP-binding protein Rac. Activation of the oxidase and $O_2^{\bullet-}$ generation occurs following

conversion of Rac from the GDP- to the GTP-bound form, with subsequent translocation of the cytosolic components to the membrane-bound subunits to form an active enzyme complex (Rinckel et al., 1999).

Interestingly, over the past 10 years, functional NAD(P)H oxidases have been identified in a number of non-phagocytic cells in the cardiovascular system and other tissues, although the subunit composition and kinetics differ widely compared to the phagocytic oxidase (Fukui et al., 1997; Gorlach et al., 2000; Griendling et al., 1994). For example, in vascular smooth muscle cells, gp91^{phox} is replaced with the recently discovered homologue Nox-1 (Lassegue et al., 2001; Suh et al., 1999), and O₂^{•-} is produced at a low level constantly compared to the inactivity of the oxidase at baseline in neutrophils. Endothelial cells express all the subunits of the phagocyte oxidase, including gp91^{phox}, but they also contain the Nox-1 and Nox-4 catalytic subunit homologues (Gorlach et al., 2000; Jones et al., 1996; Lassegue et al., 2001). Cells of the kidney were among the first non-phagocytic cells identified to contain the oxidase subunits, with the Nox-4 homologue being discovered in this tissue (Chabrashvili et al., 2002; Geiszt et al., 2000). Relatively less is known about the structure of the NAD(P)H oxidase complex in the heart, although there is evidence implicating its role in O₂^{•-} production in cardiac cells (Nakagami et al., 2003; Xiao et al., 2002). It should be noted that while each of these recent studies have identified the NAD(P)H oxidase in cells and tissues of the cardiovascular system, detailed structure, function and biological significance of this non-phagocytic NAD(P)H oxidase have yet to be conclusively determined.

The generation of ROS in normal cells is under tight homeostatic regulation since excessive production of oxygen radicals can lead to DNA mutation, lipid peroxidation, protein damage, and ultimately cell death via apoptosis or necrosis (reviewed in Carmody and Cotter, 2001; Ueda et al., 2002). As such, an exquisitely complex system for defending against changes in the cellular redox environment has evolved, including the antioxidant enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase. Like ROS, which are generated within specific intracellular compartments, antioxidant molecules are also selectively distributed within particular subcellular regions. For example, three types of SOD are localized either to the mitochondria (MnSOD, SOD2), the cytoplasm (CuZnSOD, SOD1) or extracellular spaces (ecSOD, SOD3) (reviewed in Beyer et al., 1991). Each of these mediates the rapid dismutation of O₂^{•-} to form O₂ and H₂O₂. Catalase and glutathione peroxidases, residing in peroxisomes and cytoplasm, detoxify H₂O₂ by converting it to O₂ and H₂O. However, when ROS levels outstrip the antioxidant defense capacity of a cell, due to mitochondrial disruption, overactivation of ROS-generating enzymes, or dysregulation of ROS scavenging system(s), a deleterious condition known as 'oxidant stress' occurs. It is now well established that many pathophysiological conditions, including hypertension, heart failure, hypercholesterolemia and atherosclerosis, are associated with oxidant stress in the cardiovascular system (reviewed in Cai and Harrison, 2000; Landmesser and Harrison, 2001; Sorescu and Griendling, 2002; Wilcox, 2002).

3. Peripheral angiotensin II-induced oxidant stress and hypertension

There is now a large literature linking oxidant stress and hypertension, especially AngII-dependent forms of the disorder. It has been nearly a decade since Griendling and colleagues first

discovered that AngII activates the vascular smooth muscle NAD(P)H oxidase (Griendling et al., 1994). Subsequently, they along with others showed that AngII increases vascular $O_2^{\bullet-}$ levels *in vivo*, and SOD mimetics or membrane-targeted forms of SOD dramatically lowers blood pressure in AngII- but not norepinephrine-induced hypertension (Laursen et al., 1997; Schnackenberg et al., 1998). More recent studies from this group have demonstrated a role for NAD(P)H oxidase and H_2O_2 in AngII-stimulated vascular smooth muscle cell hypertrophy, as overexpression of catalase (Zafari et al., 1998) or antisense inhibition of p22^{phox} (Ushio-Fukai et al., 1996) or Nox-1 (Lassegue et al., 2001) attenuates the growth-promoting effects of AngII. Furthermore, the $O_2^{\bullet-}$ -generating and hypertensive effects of AngII involve activation of the NAD(P)H oxidase since mice lacking one of the key cytosolic subunits, p47^{phox}, exhibit reduced vascular $O_2^{\bullet-}$ levels and develop milder hypertension than wild types when chronically treated with AngII (Landmesser et al., 2002). This, together with findings that AngII infusion causes markedly exacerbated hypertension in mice lacking ecSOD (Fukai et al., 1999), unequivocally identify ROS as key players in the development of AngII-dependent hypertension. These animal experiments have recently been supported by human studies showing that the vasoconstrictor effect of AngII infusion on the brachial artery is reduced by treatment with the antioxidant vitamin C (Wada et al., 2002).

4. Oxidant signaling in the nervous system and blood pressure regulation

Despite pioneering work over the past decade that has indisputably established ROS as key regulators of cardiovascular function and disease, particularly that which involves AngII, the precise mechanisms and physiological systems involved still remain largely undefined. Although the vasculature and other peripheral targets are clearly implicated in AngII-induced ROS generation and hypertension (see above), one potentially important site of redox signaling and oxidant stress by AngII that has received little attention is the nervous system. A few studies have suggested the importance of oxidant mechanisms in the peripheral nervous system (Li et al., 1996; Xu et al., 2001), but only recently has the role of ROS in central neuro-cardiovascular regulation been investigated. The following sections summarize the rationale, critical tools and emerging evidence for this new area of focus.

4.1. Brain angiotensinergic systems

There is now an enormous literature establishing that key aspects of AngII-mediated effects on cardiovascular and volume homeostasis occur via the CNS. Through stimulation of the AngII type 1 receptor subtype (AT₁) in the brain, AngII increases sympathetic nerve activity, vasopressin release, and drinking behavior (Mangiapane and Simpson, 1980; reviewed in Phillips and Summers, 1998). The blood–brain-barrier-deficient CVOs, and in particular the subfornical organ (SFO), are rich in AT₁ receptors (Allen et al., 2000; Lenkei et al., 1995, 1997), and are known to be pivotal in mediating the increases in blood pressure and water intake elicited by AngII in the systemic circulation or ventricular system of the brain (Mangiapane and Simpson, 1980; Simpson, 1981). Extracellular recording studies show that AngII increases firing rates of SFO neurons (Li and Ferguson, 1993), and AngII infusion activates Fos expression in the SFO and several of its

downstream targets (McKinley et al., 1995). Tract-tracing studies demonstrate that SFO has efferent contacts with the vasopressin-synthesizing magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei, which in turn descend to the neurohypophysis where vasopressin is stored for release into the circulation (Jhamandas et al., 1989; Lind et al., 1982; Miselis, 1981). The SFO also innervates parvocellular neurons of the PVN, which project to sympathetic outflow centers in the rostral ventrolateral medulla (RVLM) and intermediolateral cell column of the spinal cord (Dampney, 1994; Lind, 1986). AngII has long been implicated as an important signaling molecule in these circuitries, both under normal conditions and in the pathogenesis of hypertension and heart failure (reviewed in Francis, 1989; Phillips and Summers, 1998).

In addition to its importance in activating the CVOs via the systemic circulation, AngII generated within the CNS itself is also strongly implicated in cardiovascular function. Early evidence that direct injection of AngII into particular brain nuclei elicited profound cardiovascular and dipsogenic effects suggested the existence of AngII-processing system(s) inside the blood–brain barrier (Mangiapane and Simpson, 1980). Subsequent identification of all components necessary for AngII production and action in the brain (Bunnemann et al., 1993), along with evidence for AngII-positive nerve cell bodies and projecting fibers in the PVN, SON, neurohypophyseal tracts, and RVLM (Imboden et al., 1989; Lind et al., 1985; Swanson and Lind, 1986) has led to the notion that locally generated AngII functions as a neurotransmitter/modulator in central cardiovascular control networks. Upregulation of renin–angiotensin system gene expression in the CNS of experimental and genetic models of hypertension (Saavedra et al., 1986), and findings that transgenic overactivation of the renin–angiotensin system selectively in the brain leads to hypertension and disordered volume homeostasis (Davisson et al., 1998; Lazartigues et al., 2002; Morimoto et al., 2001) lend further support to the concept that brain angiotensinergic systems are critical in the maintenance of cardiovascular homeostasis.

4.2. ROS in the CNS

In the brain, ROS are perhaps best known for their association with neurological disorders. Primary neurodegenerative diseases, ischemic conditions such as stroke, and aging are known to be associated with increases in ROS formation and markers of oxidant stress in the brain (reviewed in Klein and Ackerman, 2003). For example, DNA oxidation, protein oxidation and lipid peroxidation have been observed in brain regions containing neurofibrillary tangles from Alzheimer disease patients (reviewed in Sayre et al., 2001). In agreement, β -amyloid fragments, the main constituent of senile plaques, have been shown to generate free radicals, which are in turn linked to some of the molecular changes observed in Alzheimer disease brains (Hensley et al., 1994; Jang and Surh, 2002). Dopaminergic neurons of brains from Parkinson's disease patients also exhibit markers of oxidant stress (reviewed in Jenner and Olanow, 1996). Furthermore, free radicals are implicated in the formation of inclusions in the substantia nigra of these patients (reviewed in Giasson et al., 2002) and in brains of patients with age-related neurodegenerative diseases such as Huntington's disease and amyotrophic lateral sclerosis (ALS) (reviewed in Butterfield and Kanski, 2001).

Although the precise cellular mechanisms by which oxidative stress induces neurodegeneration remain unclear, recent studies suggest that dysregulation of antioxidant genes and overloading of

the CNS with ROS lead to neuronal death in these disorders. For example, *Sod2*^{-/-} mice exhibit gait abnormalities that are associated with vacuolization in certain brain regions (Melov et al., 1998), and mice with targeted deletions of a gene that regulates α -tocopherol (the antioxidant vitamin E) show behavioral and pathological changes due to degeneration of the posterior column of the spinal cord (Yokota et al., 2001). In patients with familial ALS, a dysfunction in CuZnSOD is thought to play a causal role in the motoneuron loss seen in this disease (Deng et al., 1993; reviewed in Rowland, 1995).

The notion that antioxidant systems may be dysregulated in CNS diseases has prompted investigators to begin examining central ROS-generating and scavenging systems under physiological conditions. Recently, subunits of the NAD(P)H oxidase complex were found to be expressed in cells derived from CNS tissue. For example, Noh and Koh (2000) demonstrated that both cortical neurons and astrocytes express p47^{phox}, p67^{phox}, gp91^{phox} and Rac1 under basal conditions, and that treatment with zinc causes induction and activation of the complex. NAD(P)H-dependent production of ROS in rat microglia has been demonstrated *in vitro* (Bianca et al., 1999), and increased activation of the complex, as indicated by translocation of cytosolic p47^{phox} and p67^{phox} to the membrane, was demonstrated in brain tissue from Alzheimer disease patients (Shimohama et al., 2000). Lavigne et al. (2001) provided direct evidence of p47^{phox}-dependent production of O₂^{•-} in murine microglia, and Rac1-mediated generation of ROS in response to nerve growth factor was demonstrated in the pheochromocytoma neuronal cell line PC12 (Suzukawa et al., 2000). Rac1 has also been identified in axons and dendrites of hippocampal neurons (Kumanogoh et al., 2001).

The normal localization and cellular distribution of ROS-scavenging enzymes in the brain of some species has also been examined recently. For example, Lindenau et al. (2000) showed that CuZnSOD and MnSOD are differentially distributed in neuronal and glial cell types in the rat brain. CuZnSOD immunoreactivity was localized predominantly to the cytoplasm of astrocytes in neocortex, striatum, hippocampus (CA3 and gyrus dentatus) and cerebellum, although neuronal distribution of the enzyme was detected in some regions such as the spinal cord. In contrast, MnSOD was detected primarily in neurons and their processes in these brain regions and in the spinal cord, with limited localization to astroglial cells. This latter finding is supported by other investigations focusing on a subset of these CNS regions (Inagaki et al., 1991; Kato et al., 1995; Liu et al., 1993); however, there is considerable controversy about the cellular distribution of CuZnSOD. For example, Pardo et al. (1995) report abundant levels of this enzyme in cell bodies, dendrites and axons of motor neurons, but not in astrocytes. Similarly, Moreno et al. (1997) detected CuZnSOD immunoreactivity in neurons in a number of CNS sites, but do not report astrocytic localization. Interestingly, astrocytes and microglia possess high levels of glutathione peroxidase (Lindenau et al., 1998).

Because of the links between oxidative stress and neurodegenerative disorders, most studies to date have focused on relevant regions of the CNS for these diseases, such as those described above. The distribution of ROS-modulating enzymes to components of the nervous system involved in cardiovascular regulation has received little attention, although a few recent findings hint at the potential importance of these sites in redox mechanisms. Tammariello et al. (2000) demonstrated that all of the subunits of NAD(P)H oxidase, including the catalytic subunit gp91^{phox}, are expressed in primary rat sympathetic ganglion neurons. A functional role for the oxidase in these cells was confirmed in studies showing that sympathetic neurons derived from

gp91^{phox} knockout mice are protected from cell death induced by nerve growth factor deprivation (Tammariello et al., 2000). Another recent finding that is provocative in its implications is the particularly prominent localization of ecSOD to the CVOs in adult mouse brain (Oury et al., 1999). Although the activity of ecSOD is relatively low in whole brain homogenates, regions lacking a blood-brain barrier contain particularly high levels of this isoform of SOD (Oury et al., 1999). In these regions, the enzyme is distributed both in neurons and in tanycytes, specialized ependymal cells whose tail processes traverse around and terminate on fenestrated capillaries and neurons. The role of ecSOD in this and other sites in the brain remain undefined. Furthermore, the distribution and levels of the other SOD isoforms, as well as the ROS-generating enzymes, to CVOs and other cardiovascular control regions of the nervous system also remain unknown at this time. It should be noted that the enzymes responsible for NO[•] generation, the NO[•] synthases (NOS), have been detected throughout the central and peripheral nervous system sites involved in cardiovascular regulation (reviewed in Chowdhary and Townend, 1999).

4.3. Approaches to targeting oxidant systems in cardiovascular control regions of the CNS

Defining the role of ROS and RNS in physiological and pathological states is challenging, in part due to the complex chemistry and regulation of these molecules and limitations in experimental approaches. Studies have commonly relied on exogenously administered antioxidants, free radical scavengers and oxidant donors to evaluate functional roles of these molecules. However, these molecules are generally membrane impermeable, non-selective and often unstable under physiological conditions. The recent development of synthetic membrane-permeable SOD and catalase mimetics (Day et al., 1995; Doctrow et al., 1997; Melov et al., 2000), and the discovery and cloning of pro/antioxidant molecules with subsequent production of transgenic and knockout mice (Melov et al., 1998, 1999), have provided new approaches for examining functional roles of free radicals *in vivo*. Furthermore, replication-deficient viral vectors now enable selective, localized overexpression or deletion of key ROS-generating and -scavenging antioxidant molecules. This is particularly important for studies examining cardiovascular signaling mechanisms in the brain, since site-selective targeting of particular nuclei via transgenesis is limited due to the lack of specific promoters for these CNS regions. Indeed, virally mediated gene transfer to CVOs (Zimmerman et al., 2002), nucleus tractus solitarius (NTS) (Paton et al., 2001) and RVLN (Kishi et al., 2001) has been a critical technology in revealing novel redox mechanisms in central regulation of cardiovascular function (see below).

Recent studies suggest that adenovirus (Ad) and lentivirus such as feline immunodeficiency virus (FIV) are among the most promising vehicles for gene transfer to the brain (Blomer et al., 1997; Davidson et al., 1993). However, much of the work had been carried out *in vitro* (Meyrelles et al., 1997; Poeschla et al., 1998) or in the cerebellum, cerebral cortex or striatum *in vivo* (Alisky et al., 2000; Davidson et al., 1993; Kordower et al., 1999). We recently undertook a series of studies to evaluate the potential of these vectors for *in vivo* targeting of genes to a number of cardiovascular regulatory sites in mice. The mouse was utilized because of our long-term goal of combining virally mediated gene delivery in genetically engineered mouse models. Focusing initially on the SFO–hypothalamic axis and related brainstem nuclei (see above), first we demonstrated that tiny nuclei in the mouse brain such as the SFO and SON could be selectively and efficiently targeted *in vivo* using either viral vector (Sinnayah et al., 2002). By placing the

injector just dorsal to the structures and using small injection volumes, we were able to induce highly localized gene transfer to the individual sites without damaging them. These studies also confirmed what had been shown in other brain regions—that Ad and FIV differ markedly with regard to cell-type specificity (Sinnayah et al., 2002). FIV targeted transgene expression selectively to neurons in these nuclei, presumably by virtue of the pseudotype of this particular virus (Alisky et al., 2000), whereas Ad-mediated gene transfer resulted in both neurons and glial cells being transduced. Given the differential pattern of pro-oxidant and antioxidant gene expression in neurons and glia in the CNS (see above), this could be a feature of these viruses that would be important for molecular and cellular dissection of redox signaling mechanisms in the brain. Finally, our studies demonstrated different stabilities of the two viruses in nuclei along the SFO–hypothalamic axis. Whereas transgene activity was sustained over months when it was delivered by FIV, Ad-mediated gene transfer was more transient (2–3 weeks) (Sinnayah et al., 2002). The stability of FIV-induced gene transfer is likely due in part to the fact that lentiviruses integrate the message into the host genome, whereas Ad-delivered DNA remains primarily episomal (Kordower et al., 1999). These results suggest that FIV would be more desirable for studies addressing long-term regulation of cardiovascular function, i.e. over months, whereas Ad is suitable for examining questions of more short-term regulation.

4.4. Redox mechanisms in AngII-mediated actions in the CNS

Despite the evidence for ROS-generating and -scavenging systems in the brain under physiological conditions, and emerging data suggesting that free radicals may be involved in normal neuronal activity (Yermolaieva et al., 2000), our understanding of oxidant mechanisms in the CNS has still been mainly in the context of neurodegenerative diseases and neuronal death. Recently, a number of investigators have turned to exploring the role of ROS and/or RNS in CNS-mediated regulation of cardiovascular function. Given the importance of brain angiotensinergic systems in central neural control, and the importance of ROS in a wide range of AngII-regulated cellular processes, a logical hypothesis is that free radicals are key signaling molecules in AngII-mediated actions in the CNS. Furthermore, oxidant stress in the brain may be involved in AngII-dependent hypertension.

Studies by Paton and colleagues helped to pave the way for addressing these hypotheses, both with regard to a new focus on oxidant molecules in central cardiovascular control, and also the experimental methodology employed. These investigators have long been interested in the mechanisms by which AngII, acting in the NTS, depresses the baroreflex (Paton and Kasparov, 1999). It is now well established that a number of cardiovascular diseases associated with elevated AngII levels, including some forms of hypertension and congestive heart failure, are characterized by a baroreceptor reflex depression (DiBona et al., 1995). Using an Ad vector to deliver in vivo a dominant-negative inhibitor of endothelial NO[•] synthase (eNOS) to the NTS of a rat working heart-brainstem preparation, these investigators demonstrated that AngII-mediated depression of the cardiac baroreflex involves the activation of eNOS and release of NO[•] in this cardiovascular control region (Paton et al., 2001). This study was important since there had been considerable controversy regarding the actions of NO[•] in the NTS due to the use of non-selective chemical NO[•] inhibitors and donors in a variety of different models (Paton et al., 2002). By using a viral vector to deliver a highly specific gene product in a model that circumvents

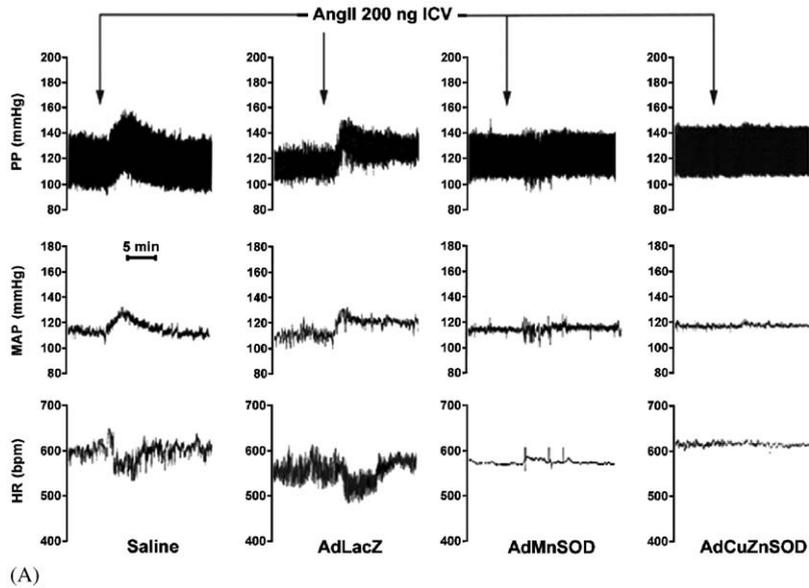
problems related to anesthesia, Paton et al. were the first to provide conclusive molecular evidence that eNOS plays a central role in the depressant effect of AngII on the baroreflex.

In subsequent complementary studies, Kishi et al. (2002) demonstrated an important role for eNOS in the RVLM of rats. Using Ad-mediated gene transfer of wildtype eNOS to this cardiovascular regulatory site, in conjunction with *in vivo* testing in conscious animals, they demonstrated that overproduction of NO in the RVLM causes a decrease in blood pressure, heart rate, and sympathetic nerve activity, and these effects were enhanced in stroke-prone spontaneously hypertensive rats (SHRSP) (Kishi et al., 2002). Overexpression of eNOS in the RVLM was also shown to improve baroreflex function in this model (Kishi et al., 2003). Although a role for AngII was not directly addressed in these studies, it is known that there is an overactivation of the brain RAS in brainstem sites of spontaneously hypertensive rat strains (Hu et al., 2002; Matsuura et al., 2002; Muratani et al., 1993). It is possible that mechanisms involving links between AngII and NO in this model are similar to that which was reported by Paton and colleagues (Paton et al., 2001).

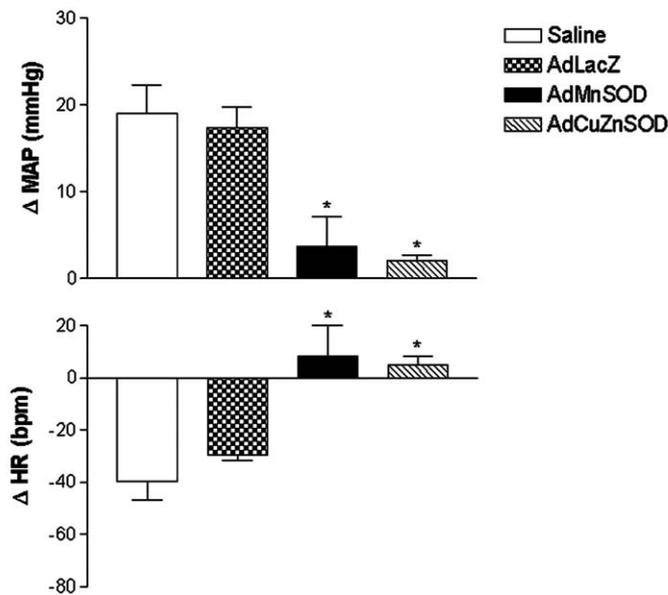
Our interest in the mechanisms of central AngII-mediated effects on cardiovascular and volume homeostasis prompted us to investigate the role of ROS in AngII-elicited increases in blood pressure and water intake. Given the studies such as that by Oury et al. (1999) demonstrating prominent localization of endogenous SOD enzymes to CVOs, along with the evidence that CVOs are critical in the hypertensive and dipsogenic effects of central AngII (reviewed in Johnson and Gross, 1993; Simpson, 1981), we have focused our studies on these regions of the brain. Ad-mediated gene transfer of intracellular SOD to CVOs of mice, through intracerebroventricular (ICV) injections of viruses encoding either MnSOD or CuZnSOD, significantly attenuates the pressor, bradycardic and dipsogenic responses of conscious mice to acute administration of AngII into the ventricles (Fig. 1) (Zimmerman et al., 2002). Moreover, isolated CVO neurons generate $O_2^{\bullet-}$ in response to AngII *in vitro*, and this is blocked by either the AT_1 receptor antagonist losartan or infection with the SOD viruses (Zimmerman et al., 2002). These results suggest that $O_2^{\bullet-}$ plays a key role in the central neuronal and functional effects of AngII, and that the CVOs are important in this process.

This is not to say that the CVOs are the only sites involved in the SOD-mediated loss of central AngII effects. While our studies do demonstrate robust SOD transgene expression in CVOs such as the SFO and OVLT, including some co-localization of SOD and AT_1 receptors in these sites (Lindley et al., 2003b; Zimmerman et al., 2002), it is possible that the effects of the transgene are through an indirect impact on downstream networks that receive inputs from regions such as the SFO and OVLT. For example, angiotensinergic pathways projecting from the SFO to hypothalamic nuclei (see above) could potentially be affected by modulation of the upstream redox state. Unraveling the relative role of the CVOs versus other CNS sites (as well as the various CVOs themselves) in ROS-mediated modulation of blood pressure and drinking behavior is the subject of ongoing investigations. Our parallel studies establishing the optimal protocols and viral vectors for long-term site-selective gene transfer to various cardiovascular regulatory nuclei in mouse brain should facilitate these efforts (see above) (Sinnayah et al., 2002).

With the now well-established role of NAD(P)H oxidase in AngII-induced $O_2^{\bullet-}$ production in vascular smooth muscle and other cell types (Griendling et al., 1994), we next turned to investigating its role in this central AngII redox mechanism. Using similar experimental protocols as described above for SOD, we compared the central AngII-elicited blood pressure, heart rate



(A)



(B)

Fig. 1. Superoxide mediates the cardiovascular responses of central AngII. (A) Typical blood pressure and heart rate recordings of conscious mice in response to ICV administered AngII (200 ng, 200 nl) 3-days after ICV injection of adenoviruses encoding either mitochondrial- (AdMnSOD) or cytoplasmic-targeted (AdCuZnSOD) SOD, control vector (AdLacZ), or saline. Arrows indicate time of AngII injection; PP, pulsatile pressure; MAP, mean arterial pressure; HR, heart rate in beats per minute (bpm). (B) Summary data of the peak change in MAP (Δ MAP) and HR (Δ HR) elicited by ICV AngII in mice ICV-administered saline ($n=7$), AdLacZ ($n=7$), AdMnSOD ($n=7$), or AdCuZnSOD ($n=5$) 3 days earlier. * $P < 0.05$ vs. saline and AdLacZ.

and drinking responses in conscious mice 3 days after the CVOs had been transduced with Ad vectors encoding either a dominant-negative inhibitor (N17Rac1) or a wildtype form of Rac1, a pivotal molecule for activating the NAD(P)H oxidase. As seen in Fig. 2, the AngII-stimulated pressor and bradycardic responses were virtually abolished in the N17Rac1-treated mice, whereas these responses were enhanced in mice overexpressing wildtype Rac1 in CVOs (Zimmerman et al., 2003a). Similar effects of the viruses were observed with the dipsogenic actions of AngII in these mice (Zimmerman et al., 2003a). Furthermore, direct confirmation of the link to NAD(P)H oxidase was achieved by lucigenin-enhanced chemiluminescence assays in cultured CVO neurons. In these studies, AngII elicited a three-fold increase in NAD(P)H oxidase-dependent $O_2^{\bullet-}$ generation. This response was abolished by the dominant-negative inhibitor of Rac1, whereas the wildtype vector caused an even greater increase in $O_2^{\bullet-}$ generation upon AngII stimulation (Zimmerman et al., 2003a). Taken together, these results strongly suggest that a Rac1-dependent NAD(P)H oxidase is activated by AngII in CVO neurons and is a key source of $O_2^{\bullet-}$ in this central oxidant signaling cascade.

These latter findings of an important role for NAD(P)H oxidase are consistent with and support our earlier studies demonstrating that overexpression of CuZnSOD inhibits the actions of central AngII (Zimmerman et al., 2002). Targeted delivery of SOD to the cytoplasm would be expected to inhibit the effects of $O_2^{\bullet-}$ derived from activation of this cytosolic oxidase system. However, our observation that Ad-mediated overexpression of MnSOD was equally effective in blocking the responses to AngII is more provocative (Fig. 1) (Zimmerman et al., 2002). These findings suggest that AngII, working through its membrane-bound AT_1 receptor, stimulated the

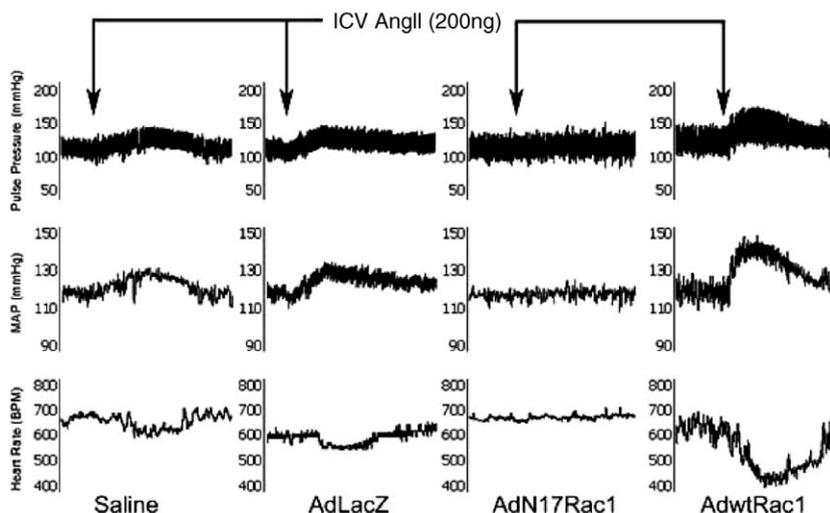


Fig. 2. Central AngII-mediated cardiovascular responses are mediated by Rac1. A representative recording of the acute effects of AngII (ICV, 200 nl, 200 ng) on blood pressure and heart rate in conscious normotensive mice that had received an adenovirus encoding dominant-negative Rac1 (AdN17Rac1), wild-type Rac1 (AdwtRac1), control vector AdLacZ, or saline 3 days earlier ICV (500 nl). Saline- and AdLacZ-treated animals exhibited the characteristic pressor and bradycardic responses to ICV AngII. These responses were abolished in AdN17Rac1-treated animals, whereas they were enhanced in mice overexpressing wild-type Rac1. Arrows indicate the time of AngII injection; PP, pulsatile pressure; MAP, mean arterial pressure; HR, heart rate.

production of $O_2^{\bullet-}$ in mitochondria of neurons in these central sites, and implicates a dual requirement for mitochondrial and cytosolic ROS in the AngII responses. Interestingly, there is precedent for stimulation of membrane-bound receptors leading to mitochondrial ROS production. For example, activation of tumor necrosis factor- α (TNF) receptors located in the plasma membrane causes an increase in mitochondria-derived ROS levels (Chandel et al., 2001; Schulze-Osthoff et al., 1992), and the mitochondrial electron chain inhibitor antimycin A attenuates TNF-stimulated intracellular free radical production (Rogers et al., 2001; Schulze-Osthoff et al., 1992). These findings are particularly interesting in the context of AngII responses since TNF-induced ROS generation from the mitochondria involves an increase of intracellular arachidonic acid (AA) (Rogers et al., 2001). It is well established that AngII can activate phospholipase A2 to generate AA in a number of cell types, including neurons in central cardiovascular control regions (reviewed in Piomelli, 1993; Zhu et al., 1998). In fact, cultured hypothalamic and brainstem neurons exhibit AT_1 receptor-dependent AA generation upon AngII stimulation (Zhu et al., 1998). Taken together, these findings are suggestive of an AngII-stimulated mitochondrial ROS mechanism, perhaps involving AA as an intermediate. This hypothesis, in addition to others such as the possibility that separate intracellular oxidant generators could amplify initiating free radical signals across subcellular domains (Schumacker, 2002), are under investigation.

Finally, while these studies strongly implicate ROS in the acute effects of central AngII, we were interested in the role of oxidant stress in the pathogenesis of AngII-dependent hypertension. The work by Harrison and colleagues, as well as that by Wilcox and colleagues clearly established that ROS, particularly $O_2^{\bullet-}$, are involved in mediating the hypertensive effects of peripheral AngII infusion (Fukai et al., 1999; Kawada et al., 2002); however, the precise mechanisms and physiological systems involved remain to be elucidated. In ongoing studies, we have begun to investigate whether oxidant stress in the CVOs plays a role in peripheral AngII-induced hypertension. Using a 'slow-pressor' model of AngII infusion because of its relevance to human hypertension (Edgley et al., 2001) and involvement of neurogenic mechanisms (Melaragno and Fink, 1995), we examined the effect of overexpressing CuZnSOD in the CVOs of mice infused chronically with a slow-pressor dose of AngII ($600 \text{ ng kg}^{-1} \text{ min}^{-1}$, subcutaneous). Preliminary results indicate that increased $O_2^{\bullet-}$ scavenging in these brain regions causes a significant blunting of the slow-pressor effects of AngII (Zimmerman et al., 2003b). These data implicate central redox mechanisms in the pathogenesis of hypertension produced by systemic AngII infusion.

5. Redox mechanisms in the CNS and heart failure

Congestive heart failure (CHF) is a leading cause of morbidity and mortality in our society, reaching epidemic proportions in recent years (reviewed in Miller and Missov, 2001). Coronary artery disease leading to myocardial infarction (MI) is a primary cause of CHF (reviewed in Gheorghade and Bonow, 1998), which is now well-established to be involved in autonomic dysregulation. Excessive activation of central neurohumoral systems, leading to increased sympathetic nerve activity, loss of cardiac parasympathetic tone, and decreased baroreflex sensitivity are hallmarks of the post-MI decline to CHF (La Rovere et al., 1998; Schrier and

Abraham, 1999; Kaye et al., 1994). The autonomic dysfunction correlates with, and predicts the occurrence of arrhythmias, sudden cardiac death, and mortality (La Rovere et al., 1998).

Nearly every humoral system linked to central cardiovascular regulation has been shown to be altered in humans and animal models with CHF, including vasopressin, endothelin-1 and various cytokines (reviewed in Felder et al., 2003). However, one of the most extensively investigated and strongly implicated factors in the neuro-dysregulation that accompanies CHF is AngII. During the development of CHF, plasma AngII levels become elevated and act both in the peripheral and central nervous systems to alter autonomic function. A number of studies using different animal models of CHF have demonstrated that central blockade of AT₁ receptors attenuates sympathetic hyperactivity, improves baroreflex function and ameliorates the development of CHF (DiBona et al., 1998; Leenen et al., 1999; Murakami et al., 1997). Clinical studies also implicate AngII as an important mediator of sympatho-excitation in CHF patients (reviewed in Francis, 1989; Turini et al., 1979). Circulating AngII acting at CVOs with subsequent stimulation of central autonomic centers is thought to be a critical mechanism in AngII-mediated neuro-dysregulation in CHF (reviewed in Felder et al., 2003; Liu et al., 1999).

Besides AngII, another mechanism now strongly implicated in the autonomic dysregulation of CHF is oxidant signaling, particularly NO[•]. Well established as a sympatho-inhibitory substance in the CNS (Sakuma et al., 1992; Toda et al., 1993), it has been shown to inhibit neuronal activity in the NTS (Ma et al., 1995), PVN (Bains and Ferguson, 1997), and SFO (Schmid et al., 1995). Studies from Zucker and Patel and colleagues, as well as others, have subsequently demonstrated that a downregulation of NOS and/or NO[•] mechanisms in the CNS plays a key role in the sympatho-excitation of CHF (reviewed in: Li and Patel, 2003; Patel et al., 1996; Zhang et al., 1997, 1998).

Interestingly, similar to what has been shown for blood pressure regulation (see above), an important role for AngII–NO[•] interactions has been demonstrated in CHF. For example, Zucker and colleagues showed that the NO[•] donor sodium nitroprusside only caused sympathoinhibition in rabbits with CHF if treatment was preceded by blockade of central AT₁ receptors (Liu and Zucker, 1999). Their recent studies using an Ad vector to overexpress neuronal NOS (nNOS) specifically in the RVLM showed that augmentation of NO[•] in this region enhanced baroreflex function in rats with MI-induced CHF (Wang et al., 2003). These findings implicate the RVLM as an important site for AngII–NO[•] mechanisms. In addition, the potentiating effects of NOS blockade on AngII-induced responses in the PVN have also been demonstrated (Bains and Ferguson, 1994).

Recently, evidence has begun to emerge that suggests a role for ROS in the central neurohumoral activation associated with CHF. In a recent clinical study, Piccirillo et al. (2003) demonstrated that chronic antioxidant treatment with vitamin C in CHF patients improves baroreflex function. Using microinjection of SOD protein into the RVLM of anesthetized swine, Zanzinger and Czachurski (2000) provided evidence for the involvement of ROS in sympathetic nerve activity. Since the SOD protein used in these studies would not be expected to cross the cell membrane, these findings implicate extracellular O₂^{•-} in this sympatho-regulatory effect.

Given the strong link between AngII signaling through the CVOs and central neuro-dysregulation in CHF, along with our recent studies that suggest a role for ROS in the CVOs in mediating the pressor effects of AngII administration (see above), we recently tested the hypothesis that oxidant stress in these central cardiovascular control regions is a key mechanism

in the post-MI sympathetic hyperactivity and decline to CHF. Targeting overexpression of cytoplasmic SOD to the CVOs with Ad-CuZnSOD (see above), we showed that $O_2^{\bullet-}$ plays a key role in the activation of downstream PVN and SON neurons in a mouse model of MI-induced CHF (Lindley et al., 2003a, b). Furthermore, oxidant signaling through these pathways is important in the sympatho-excitation that ensues post-MI (Lindley et al., 2003a, b). Ongoing studies suggest that amelioration of the central neuro-dysregulation with increased $O_2^{\bullet-}$ scavenging in the CVOs results in improved cardiac function at 4 weeks post-MI (Lindley et al., 2003c), although the underlying mechanisms of this cardioprotective effect will require further investigation. In addition, while AngII is implicated as an initiating signal for this central redox mechanism in CHF, other factors appear to be involved (Lindley et al., 2003b). This, too, is the subject of ongoing investigations in our laboratory.

6. Perspectives

6.1. *AngII–NO–ROS interactions*

As indicated above, common themes that have emerged from these recent studies of central redox mechanisms in neural control of cardiovascular function are the interactions between AngII-NO $^{\bullet}$, and AngII-ROS. Both blood pressure regulation and central autonomic control involve each of these AngII-oxidant mechanisms, with dysregulation of either resulting in hypertension or heart failure. Given the links between NO $^{\bullet}$ and ROS, with the ability of $O_2^{\bullet-}$ to quench endogenous NO $^{\bullet}$ and convert it to peroxynitrite (reviewed in Jeremy et al., 2002; Ronson et al., 1999), along with the universal role of AngII in neurohumoral activation, the hypothesis that AngII, NO $^{\bullet}$ and ROS are working in concert to impair autonomic function and contribute to cardiovascular diseases needs to be considered.

A few recent studies hint at the potential importance of this mechanism. For example, Bauersachs et al. (1999) demonstrated that the endothelial dysfunction in heart failure is in part caused by a reduction in NO $^{\bullet}$ bioavailability due to increased $O_2^{\bullet-}$ production and conversion of NO $^{\bullet}$ to peroxynitrite. Studies by Zanzinger and Czachurski (2000) showed that SOD injected into the RVLM of pigs caused sympathoinhibition to a greater extent when the animals were under conditions of chronic overproduction of ROS, and that these effects of SOD were blocked by a NOS inhibitor. This suggests that NO $^{\bullet}$ suppresses central sympathetic nerve activity and that $O_2^{\bullet-}$ inactivates endogenous NO $^{\bullet}$.

The involvement of AngII in this process, with its ability to cause neurohumoral activation and to promote oxidative stress, has yet to be examined directly. One possible scenario is that, with increased AngII generation in diseases such as hypertension and heart failure, a concomitant increase in $O_2^{\bullet-}$ levels in central cardiovascular networks, perhaps through activation of NAD(P)H oxidase, may not only contribute to increased sympathetic outflow, but may also diminish the bioavailability of NO $^{\bullet}$ and limit the antagonistic sympathoinhibitory influences of this free radical. This would exacerbate the neuro-dysregulation initiated by AngII and other factors, contributing to the pathogenesis of the diseases. The direct effects of peroxynitrite, formed by the reaction between NO $^{\bullet}$ and $O_2^{\bullet-}$, should also be considered in this scenario. Apart from the loss of NO $^{\bullet}$, increases in peroxynitrite formation in central cardiovascular neurons could

have significant neuro-cardiovascular effects in and of themselves. Unraveling these central AngII-redox mechanisms will be important studies to pursue.

6.2. Intracellular mechanisms of AngII/ROS signaling

In addition to the potential interactions between ROS, NO[•], AngII and other neuroexcitatory factors, additional important questions for this new field concern the intracellular signaling mechanisms utilized by AngII and ROS in these cardiovascular regulatory neurons. Although a full discussion of this topic is outside the scope of this review, a few potentially important mechanisms will be discussed briefly.

There is now abundant evidence that ion channels and ion exchangers are modulated by ROS in a number of cell types, including neurons involved in cardiovascular regulation. For example, oxidizing agents such as H₂O₂ and dithiodipyridine were shown to inhibit voltage-dependent sodium currents in isolated nodose ganglion neurons (Li et al., 1997a). A-type K⁺ channels (Chapleau et al., 1993) and a Ca²⁺-activated K⁺ channel (Li et al., 1997b) have also been shown to be targets of ROS in baroreceptor neurons, leading to changes in membrane excitability and action potential discharge. As such, oxidation of ion channel proteins in response to AngII-induced O₂^{•-} production in CNS neurons should be considered as a potential mechanism.

Another mechanism that deserves attention involves intracellular calcium ([Ca²⁺]_i) signaling. Previous studies using neuronal cell cultures have provided evidence that AngII stimulates phosphoinositol hydrolysis, resulting in the production of IP₃ and diacylglycerol, which in turn increases [Ca²⁺]_i and activates protein kinase C (Pan et al., 2001; reviewed in Richards et al., 1999). The increase in [Ca²⁺]_i leads to inhibition of delayed rectifier K⁺ and transient A-type currents, which are believed to be pivotal in AngII-mediated neuronal activation (Pan et al., 2001). Intracellular Ca²⁺ mechanisms are also implicated in ROS signaling in neurons. For example, exposure of isolated CNS neurons to H₂O₂ leads to a dose-dependent increase in [Ca²⁺]_i (Oyama et al., 1996). On the other hand, a large influx of [Ca²⁺]_i into neurons has been shown to increase intracellular ROS production, establishing a feed-forward signaling loop (Jacobson and Duchon, 2002; Oyama et al., 1996). ROS have also been shown to potentiate Ca²⁺ signaling in rat cortical brain slices and the PC12 neuronal cell line (Yermolaieva et al., 2000). Taken together, the strong links between Ca²⁺ and both AngII and ROS suggest that neuronal Ca²⁺ may be a key component of the AngII-redox signaling pathways in central neural control of cardiovascular function. Studies designed to test this hypothesis are currently underway in our laboratory.

7. Concluding remarks

The importance of redox mechanisms in central neural control of cardiovascular function is a relatively new concept. Accumulating evidence presented above suggests that free radical species, including O₂^{•-} and NO[•], have important effects on neurohumoral mechanisms involved in blood pressure regulation, volume homeostasis, baroreflex function and sympathetic activity, particularly those that involve AngII signaling. Furthermore, oxidative stress in the CNS is implicated in the neuro-dysregulation associated with some forms of hypertension and heart failure. Summarized in Fig. 3, recent data suggest the importance of oxidant signaling in

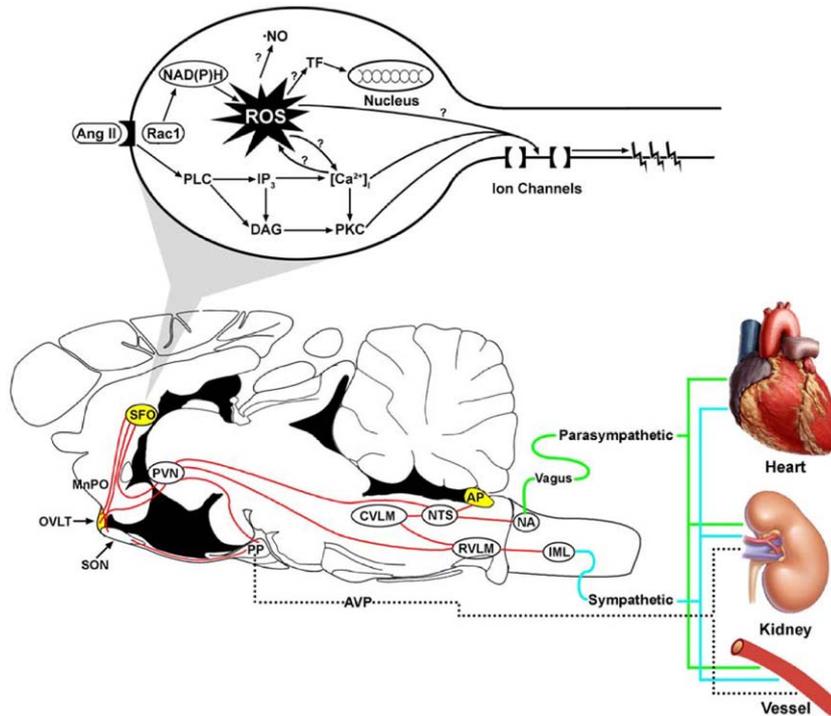


Fig. 3. Schematic summarizing neural pathways and intracellular signaling mechanisms that may play a role in central redox regulation of cardiovascular function. Accumulating data suggest that ROS play key signaling roles in central cardiovascular regulatory networks that control neurohumoral outputs to peripheral end-organ targets. Redox signaling in circuitries of the forebrain, hypothalamus and brainstem, including key sites such as the PVN, NTS and RVLM, may be important in the regulation of sympathetic and parasympathetic outflow and the release of AVP. The CVOs such as the SFO (highlighted in yellow), which project to these hypothalamic and brainstem sites either directly or indirectly, appear to be pivotal in mediating central oxidant mechanisms that involve AngII. As depicted in the enlarged schematic of a neuron, evidence suggests that isolated CVO neurons generate ROS in response to AngII in a Rac1/NAD(P)H oxidase-dependent manner. However, the role of ROS in the intracellular signaling mechanisms of these and other central cardiovascular neurons is unknown at this time. One intriguing possibility is that ROS may mediate interactions between AngII and NO[•]. Additionally, interactions between ROS and well-established AngII-stimulated pathways such as the PLC/IP₃-mediated mobilization of intracellular Ca²⁺ are also possible. The prospect that ROS stimulate transcription factors and/or act directly on ion channels to alter neuronal activity must also be considered. AP, area postrema; AVP, arginine vasopressin; CVLM, caudal ventrolateral medulla; DAG, diacylglycerol; IML, intermedial lateral cell column of the spinal cord; IP₃, inositol 1,4,5-triphosphate; MnPO, median preoptic nucleus; NA, nucleus ambiguus; NTS, nucleus tractus solitarius; OVLT, organum vasculosum of the lamina terminalis; PLC, phospholipase C; PP, posterior pituitary; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla; SFO, subfornical organ; SON, supraoptic nucleus; TF, transcription factors.

cardiovascular regulatory circuitry that includes brainstem sites such as the RVLM and NTS, as well as the hypothalamic PVN and SON. The CVOs, with their role as an interface between peripheral blood-borne signals and these regions as well as other important cardiovascular sites in the brain, appear to be particularly important in central redox mechanisms involving AngII. The possibility that the now well-characterized interactions between AngII and NO[•] in the CNS may be mediated by O₂⁻ is an intriguing one that future studies should address. Subcellular

compartmentalization of redox signaling in CNS neurons, as well as intracellular Ca^{2+} mechanisms are also interesting issues in the context of this new area of research. Finally, given the implications of these recent studies for the pathogenesis of hypertension and heart failure, central neuronal oxidative stress may be an important new target for therapeutic treatment of these diseases.

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