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Angiotensin II and Angiotensin-1-7 Redox Signaling in the Central Nervous System

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Abstract

Reactive oxygen species (ROS) are important intra-neuronal signaling intermediates in angiotensin II (AngII)-related neuro-cardiovascular diseases associated with excessive sympathoexcitation, including hypertension and heart failure. ROS-sensitive effector mechanisms, such as modulation of ion channel activity, indicate that elevated levels of ROS increase neuronal activity. Nitric oxide, which may work to counter the effects of ROS, particularly superoxide, has been identified as a signaling molecule in angiotensin-1-7 (Ang-(1-7)) stimulated neurons. This review focuses on recent studies that have revealed details on the AngII-activated sources of ROS, the downstream redox-sensitive effectors, Ang-(1-7)-stimulated increase in nitric oxide, and the neuro-cardiovascular (patho)physiological responses modulated by these reactive species. Understanding these intra-neuronal signaling mechanisms should provide insight for the development of new redox-based therapeutics for the improved treatment of angiotensin-dependent neuro-cardiovascular diseases.

Introduction

Traditionally, reactive oxygen species (ROS) including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical are thought of as toxic by-products of cellular respiration. However, it is now well-accepted that these reactive species, primarily $O_2^{\bullet-}$ and H_2O_2 , act as important intracellular signaling intermediates following stimulation of various plasma membrane receptors [1;2]. A collection of kinases, phosphatases, ion channels, and transcription factors among other cellular proteins have all been identified as redox-sensitive proteins [3]. Activation or inhibition of these signaling proteins by ROS, which can occur rapidly and transiently, suggests that cells and tissues can respond to small changes in the oxidative environment and do not need to be under “oxidative stress” for (patho)physiological responses to occur.

The renin-angiotensin system (RAS), by acting on numerous organ systems, works to maintain cardiovascular and body fluid homeostasis. In central neurons, angiotensin II

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(AngII) activates the angiotensin II type 1 receptor (AT1R) to induce a cascade of intra-neuronal signaling events that ultimately leads to changes in membrane potential and an increase in neuronal firing [4]. Activation of neurons in cardiovascular control brain regions, such as the subfornical organ (SFO), paraventricular nucleus (PVN) and rostral ventral lateral medulla (RVLM), results in stimulation of the sympathetic nervous system, which mediates, at least in part, the cardiovascular complications associated with hypertension and heart failure [5;6]. Accumulating evidence over the past 8–10 years has established that AngII increases levels of ROS, particularly $O_2^{\bullet-}$, in neurons which contribute to the increase in neuronal activation [7–13]; thus, identifying $O_2^{\bullet-}$ as a sympatho-excitatory molecule in the brain.

Although AngII is considered to be the primary effector peptide of the RAS, it is now appreciated that angiotensin-1-7 (Ang-(1-7)), which is generated by angiotensin converting enzyme 2 (ACE2) cleaving the carboxyl terminus phenylalanine residue from AngII, plays an important role in controlling cardiovascular function [14]. In the brain, Ang-(1-7) increases nitric oxide (NO^*) levels via activation of the Mas receptor (MasR) and the angiotensin II type 2 receptor (AT2R) [15;16]. NO^* in the central nervous system (CNS) acts as a sympatho-inhibitory molecule [16;17]. Considering the reaction between $O_2^{\bullet-}$ and NO^* is diffusion limited it is plausible that the balance between these two radicals is critical in the maintenance of sympathetic output. This review will focus on recent studies that have clarified the sources of $O_2^{\bullet-}$ in AngII-stimulated neurons, the downstream signaling mechanisms modulated by ROS, the counter-balance between the Ang-(1-7)- NO^* and AngII- $O_2^{\bullet-}$ signaling pathways, and the neuro-cardiovascular responses mediated by these reactive species. In addition, the development of new redox-based therapeutics will be discussed.

Reactive oxygen species and AngII intra-neuronal signaling

The first evidence suggesting that ROS signaling in the brain contributes to the maintenance of sympathetic output came from studies where superoxide dismutase (SOD) protein microinjected into the RVLM of anesthetized pigs moderately decreased baseline sympathetic nerve activity (SNA), mean arterial pressure (MAP), and heart rate (HR) [18]. SOD, which catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and oxygen, is endogenously expressed as three different proteins: 1) copper/zinc SOD (CuZnSOD or SOD1), which is primarily localized in the cytoplasm, but also present in the mitochondria [19]; 2) manganese SOD (MnSOD or SOD2), which is strictly expressed in mitochondria matrix; 3) extracellular SOD (ecSOD or SOD3), which, as its name suggests, is found extracellular. Previous studies have demonstrated that CuZnSOD or MnSOD overexpression in the SFO via adenoviral-mediated gene transfer virtually abolishes the central AngII-induced increase in MAP [11]. In addition, the characteristic dipsogenic response mediated by central AngII administration is significantly attenuated by overexpression of CuZnSOD or MnSOD [11]. These data indicate that intracellular $O_2^{\bullet-}$, perhaps generated in both the cytosol and mitochondria, is a critical signaling molecule mediating the physiological responses of central AngII. Further studies using chronic hypertensive animal models, including slow-pressor AngII infusion [12] and spontaneously hypertensive rats (SHR) [20], and SOD overexpression in different cardiovascular control regions of the brain, including the SFO and RVLM, corroborate these initial findings. In addition, studies using models of chronic heart failure (CHF) where AngII signaling in the brain is elevated have shown that increased $O_2^{\bullet-}$ scavenging in the SFO, RVLM, and PVN attenuates the deleterious sympathoexcitation associated with this disease [21–24]. Suggesting a role for extracellular $O_2^{\bullet-}$, Lob et al recently reported that deletion of ecSOD in the SFO augments the hypertensive response to peripheral AngII infusion [25]. Together, these studies indicate that

$O_2^{\bullet-}$ signaling in the CNS mediates the AngII-induced increase in SNA and contributes to the pathogenesis of AngII-dependent neuro-cardiovascular disorders.

The obvious next question in this line of research was: What is/are the AngII-activated source(s) of $O_2^{\bullet-}$ in the CNS? Initial studies using non-specific inhibitors of NADPH oxidase, such as apocynin, diphenylene iodonium (DPI), and dominant-negative Rac1, indicated that, similar to peripheral cell types, NADPH oxidase is a primary source of $O_2^{\bullet-}$ in AngII-stimulated neurons [8;26;27]. A more recent study using siRNA technology and the silencing of specific NADPH oxidase (Nox) homologs not only confirmed the initial studies, but uncovered divergent roles for two of the Nox homologs – Nox2 and Nox4. Silencing Nox2 or Nox4 alone in the SFO significantly attenuates the central AngII-induced pressor response, while silencing both Nox2 and Nox4 together abolishes this response [28]. In contrast, silencing Nox2, but not Nox4 significantly attenuates the central AngII-induced dipsogenic response [28]. Although the reason for the differential role of Nox2 versus Nox4 in mediating central AngII-induced physiological responses remains unclear, one possible explanation may be the different subcellular locations of these Nox homologs. Nox2 is traditionally thought to reside in the plasma membrane, although in central neurons this has not been conclusively demonstrated, while recent studies using peripheral cell types report Nox4 is localized to mitochondria [29;30]. In a central catecholaminergic neuronal cell line (CATH.a neurons), Nox4 also appears to be mitochondrial localized, as we recently reported (Zimmerman M.C., *Hypertension* 2010, 56:e58, abstract 27, American Heart Association High Blood Pressure Research 2010 Scientific Session, Washington DC, October 2010). Considering overexpression of the mitochondrial-targeted MnSOD virtually abolishes the AngII-induced increase in intra-neuronal $O_2^{\bullet-}$ as measured by electron paramagnetic resonance (EPR) spectroscopy (Figure 1) and in the brain MnSOD overexpression inhibits the central AngII-induced physiological responses to a similar extent as the primarily cytoplasmic localized CuZnSOD [11], it seems likely that mitochondria are also an important source of $O_2^{\bullet-}$ in AngII stimulated neurons. In fact, Yin et al, recently reported that AngII increases mitochondrial $O_2^{\bullet-}$ levels in CATH.a neurons and that this increase in mitochondrial $O_2^{\bullet-}$ mediates the AngII-induced activation of a redox-sensitive kinase, calcium/calmodulin kinase II (CaMKII), and the AngII-induced inhibition of neuronal potassium current (I_K) [10]. These studies and others [7;8;31] clearly demonstrate the both NADPH oxidase and mitochondria are important sources of $O_2^{\bullet-}$ in AngII-stimulated neurons. Additional studies are needed to determine the potentially important link between these two sources of $O_2^{\bullet-}$ in mediating the (patho)physiological responses of AngII in the CNS.

Although it is generally well-accepted that AngII intra-neuronal signaling is mediated, at least in part, by ROS, the downstream redox-sensitive effector mechanisms are yet to be fully elucidated. Few studies both *in vivo* and *in vitro* have demonstrated that the AngII-induced increase in $O_2^{\bullet-}$ modulates intracellular Ca^{2+} signaling by inducing the influx of extracellular Ca^{2+} [8;13]. Additional evidence obtained from neurons in culture indicate that AngII-induced increase in $O_2^{\bullet-}$ controls activity of K^+ channels [7;10]. By regulating these ion channels and controlling membrane potential, $O_2^{\bullet-}$ is believed to play a critical role in increasing the neuronal firing rate of AngII-stimulated neurons. In fact, Raizada and colleagues using NADPH oxidase inhibitors and $O_2^{\bullet-}$ scavengers have shown this to be the case [7]. However, it remains unclear if $O_2^{\bullet-}$ acts directly on an ion channel protein to control ion flux or if it acts on a signaling protein which in turn controls channel activity. Indicating the latter may be true, at least in regards to K^+ channel activity, and as mentioned earlier, increased scavenging of mitochondrial $O_2^{\bullet-}$ via MnSOD overexpression in cultured neurons significantly attenuates the AngII-induced activation of CaMKII [10]. Interestingly, CaMKII has recently been identified as a redox-sensitive protein [32] and previous studies have shown that CaMKII is required for the AngII-induced inhibition of neuronal I_K [33;34].

Additional studies are needed to identify other AngII modulated redox-sensitive proteins that are either ion channel subunits or upstream signaling proteins that control channel activity. Once identified as redox-sensitive it will be ideal to determine which amino acids, likely cysteine and/or methionine residues, in the protein are being oxidized/reduced to modulate activity.

As described above, most of the *in vivo* and *in vitro* studies to date implicating $O_2^{\bullet-}$ as a key intra-neuronal signaling intermediate in AngII-stimulated neurons have utilized SOD overexpression or SOD mimetics. Although concluding that $O_2^{\bullet-}$ is an important signaling intermediate when SOD overexpression inhibits the AngII-mediated response is a reasonable interpretation, it must be considered that the H_2O_2 produced by the efficient scavenging of $O_2^{\bullet-}$ by SOD may have an inhibitory effect on AngII signaling. In fact, H_2O_2 is considered by many to be more of a signaling molecule as compared to $O_2^{\bullet-}$ because it is more diffusible and it has been demonstrated to oxidize cysteine and methionine residues [3;32], which in turn can be reduced by appropriate reductases. Few studies have addressed the hypothesis that AngII increases H_2O_2 levels in cells overexpressing SOD; however, a recent study showed that CuZnSOD inhibition decreases H_2O_2 levels and attenuates growth factor-induced oxidation of protein tyrosine phosphatases (PTP) [35]; thus, suggesting that CuZnSOD-produced H_2O_2 does act as a signaling molecule to modulate activity of downstream effectors. In contrast, in a myocardial infarct-induced heart failure model the improved cardiac performance observed in mice overexpressing CuZnSOD in the PVN was not affected when both CuZnSOD and the H_2O_2 scavenging enzyme catalase were overexpressed together [22]. This study suggests that CuZnSOD-produced H_2O_2 , at least in the PVN, is not involved in inhibiting the intra-neuronal signaling mechanism(s) that drives the deleterious sympathoexcitation in this model of heart failure. However, it should be noted that although catalase does efficiently scavenge H_2O_2 , it is primarily localized to peroxisomes and thus it is possible that catalase overexpression may not scavenge H_2O_2 produced in other subcellular compartments. As such, additional studies using other H_2O_2 scavenging enzymes, like glutathione peroxidases and peroxiredoxins, that are present in other subcellular locations are needed to distinguish the importance, or lack thereof, of H_2O_2 in central AngII-mediated cardiovascular responses.

Nitric oxide and Ang-(1-7) intra-neuronal signaling

In contrast to the hypertensive actions of AngII via activation of the AT1 receptor, including vasoconstriction and sympathoexcitation, Ang-(1-7) acts through the MasR to induce vasodilation and sympathoinhibition [15;17;36]. In the vasculature, the Ang-(1-7) vasodilatory action is believed to be dependent on an increase in $NO^{\bullet}$ [37;38]. In the brain, it has also been shown that Ang-(1-7) increases $NO^{\bullet}$ levels [39]. In addition, overexpression of ACE2, which cleaves AngII to Ang-(1-7), in the brain prevents the development of hypertension induced by peripheral AngII infusion by, at least in part, increasing nitric oxide synthase (NOS) and $NO^{\bullet}$ [15]. In cultured neurons, Ang-(1-7) increases $NO^{\bullet}$ levels, which mediates activation of K^+ current [40]. Ang-(1-7)-induced increase in neuronal K^+ current is directly opposite to the inhibition of K^+ current induced by AngII (discussed above); thus, suggesting that $O_2^{\bullet-}$ -dependent signaling induced by AngII directly opposes that of $NO^{\bullet}$ -dependent signaling induced by Ang-(1-7) (Figure 2). Interestingly, in SHR the central AngII-induced pressor response is attenuated when Ang-(1-7) and AngII are administered simultaneously [41]. Although the downstream effectors of $O_2^{\bullet-}$ and $NO^{\bullet}$ modulated by AngII and Ang-(1-7), respectively, may work to counteract each other, it is tempting to speculate that $O_2^{\bullet-}$ and $NO^{\bullet}$ counter-balance each other directly, especially considering the reaction of these two radicals is diffusion limited. However, if this reaction is indeed the mechanism by which AngII and Ang-(1-7) counteract each other, the effects of the reaction product, peroxynitrite ($ONOO^-$), must be investigated. To date, we are unaware of any

studies linking AngII and Ang-(1-7) signaling in neurons to changes in ONOO^- and/or the downstream signaling targets, if any, of ONOO^- .

Antioxidant therapeutic strategies for angiotensin-related neuro-cardiovascular diseases

Most of the clinical trials designed to test the therapeutic efficacy of antioxidant vitamins, such as Vitamin C and E, in cardiovascular diseases have failed [42–46]. Although the reasons for these failed trials are not completely understood, there are a few hypotheses that seem reasonable: 1) The antioxidant vitamins may not target the tissue, cells, or subcellular compartments where the ROS are being primarily generated; 2) The antioxidant vitamins may not scavenge the specific ROS driving the pathophysiological condition; 3) The antioxidant vitamins may not efficiently scavenge the ROS driving the pathophysiological condition and thus some of the ROS are still available to carry out their detrimental signaling. To overcome these potential pitfalls of antioxidant vitamin therapy, numerous groups have developed small molecule antioxidants that have SOD-like and/or catalase-like activity and in most cases these new redox-based therapies have been beneficial in various cardiovascular disease animal models [47–49]. In addition, recent advances in nanotechnology have lead to the development of new cell permeable delivery systems for endogenous antioxidant proteins, including SOD and catalase [50–52]. For example, we recently reported that CuZnSOD protein electrostatically wrapped with a synthetic poly(ethyleneimine)-poly(ethyleneglycol) (PEI-PEG) polymer is taken up by central neurons and inhibits AngII signaling *in vitro* and *in vivo* [53]. It is tempting to speculate that these new small molecule and nanomedicine-based antioxidants will be therapeutically efficacious because they possess specific ROS scavenging activities and may be designed to target specific tissues, cells, and even subcellular compartments.

Conclusions

A large collection of literature over the past decade has clearly identified $\text{O}_2^{\bullet-}$ as an important AngII intra-neuronal signaling intermediate which leads to an increase in neuronal activity and elevated sympathetic output. More recent studies suggest $\text{NO}^\bullet$ is a key signaling molecule in Ang-(1-7) stimulated neurons which may counteract the signaling events of AngII and $\text{O}_2^{\bullet-}$ (Figure 2). Future studies are needed to fully elucidate the cross-talk between AngII- $\text{O}_2^{\bullet-}$ and Ang-(1-7)- $\text{NO}^\bullet$ intra-neuronal signaling and the downstream redox-sensitive proteins (RSP) involved in controlling neuronal firing rate, sympathetic outflow, and the pathogenesis of neuro-cardiovascular diseases, like hypertension and heart failure. Perhaps more importantly, future studies are needed to identify new redox-based therapeutics that not only works in animal models, but also in patients suffering from these prevalent diseases.

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Figure 1.

Adenovirus-mediated overexpression of the mitochondrial-localized MnSOD (AdMnSOD) in cultured neurons inhibits the AngII-induced increase in $O_2^{\bullet-}$ levels, as measured by EPR spectroscopy. Non-infected (control) or AdMnSOD-infected catecholaminergic neurons (CATH.a neurons) were loaded with the $O_2^{\bullet-}$ sensitive, cell permeable spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) and stimulated with AngII (100 nM). AngII significantly increased the EPR amplitude in control neurons (red line) as compared to non-treated neurons (black line), indicating an increase in $O_2^{\bullet-}$ levels. This increase in $O_2^{\bullet-}$ was virtually abolished in neurons overexpressing MnSOD (blue line), suggesting mitochondria are a primary source of $O_2^{\bullet-}$ in AngII-stimulated neurons. A.U. = arbitrary units.

Figure 2.

Schematic illustration of AngII and Ang-(1-7) intra-neuronal signaling involving an increase in O₂^{•-} and NO[•], respectively. AngII, acting through the AT1 receptor (AT1R), has been shown to increase O₂^{•-} from both NADPH oxidase and mitochondria (mito), while Ang-(1-7) signaling through the MasR leads to nNOS activation and an increase in NO[•]. NO[•] and O₂^{•-} react in a diffusion-limited manner to produce peroxynitrite (ONOO⁻). Previous studies indicate that the AngII-induced increase in O₂^{•-} modulates ion channels to increase neuronal firing rate leading to an increase in sympathetic nerve activity (SNA) and mean arterial pressure (MAP). While other studies suggest that the Ang-(1-7)-induced increase in NO[•] leads to sympatho-inhibition. Future studies (indicated by ?) are needed to determine if O₂^{•-}, NO[•], and/or ONOO⁻ act on redox-sensitive proteins (RSP) that control ion channel activity or if these reactive species act directly on ion channel proteins to regulate ion flux and neuronal activity.