This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2003)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2003 Term

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Central Angiotensin II-Dependent Hypertension: A Role for Reactive Oxygen Species

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For 77:222, Spring 2003
8. May 2003

Abbreviations

ACE	angiotensin converting enzyme
Adenovirus	Ad
AGT	angiotensinogen
AngII	angiotensin II
AP	area postrema
BBB	blood brain barrier
BS	brainstem
$[Ca^{2+}]_i$	intracellular calcium concentration
Cu/ZnSOD	copper-zinc superoxide dismutase
CVO	circumventricular organs
DAG	diacylglycerol
DHE	dihydroethidium
ICV	intracerebroventricular
IP_3	inositol 1,4,5-triphosphate
MnSOD	manganese superoxide dismutase
NH	neurohypophysis
NTS	nucleus of solitarus tract
OVLT	organum vasculosum of the lamina terminalis
PKC	protein kinase C
PVN	paraventricular nucleus
RAS	renin-angiotensin system
ROS	reactive oxygen species
SFO	subfornical organ
SOD	superoxide dismutase
SON	supraoptic nucleus
VSMC	vascular smooth muscle cells
WHBP	working heart-brainstem preparation

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Abstract

The brain renin-angiotensin system (RAS), of which angiotensin II (AngII) is the primary effector peptide, plays a critical role in the neurohumoral regulation of cardiovascular and body fluid homeostasis. Dysregulation of central AngII production or activity is implicated in the pathogenesis of hypertension; therefore, understanding the mechanisms of AngII action in the central nervous system is an important area of investigation. We have recently shown, for the first time, that central AngII-induced cardiovascular effects involve the production of reactive oxygen species (ROS). By delivering adenovirus encoding the ROS scavenging enzymes copper-zinc superoxide dismutase (Cu/ZnSOD) or manganese superoxide dismutase (MnSOD) intracerebroventricularly (ICV) in mice, we have demonstrated that increased ROS scavenging markedly attenuates the characteristic pressor and bradycardic response of ICV administered AngII. Furthermore, our studies using the fluorogenic probe dihydroethidium (DHE) in primary neuronal cultures indicate that AngII increases superoxide ($O_2^{\bullet-}$) production. Although SOD-mediated inhibition of AngII-induced pressor and bradycardic responses infers a selective influence of ROS on AngII-mediated cardiovascular responses, the precise mechanism of AngII signaling in the brain remains unknown. It has been suggested that ROS can modulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in multiple cell types. In the brain, calcium-imaging and electrophysiological studies have demonstrated that AngII increases levels of $[Ca^{2+}]_i$. As such, it has been suggested that AngII depolarizes neurons as a result of the cationic influx of $[Ca^{2+}]_i$, which ultimately leads to the generation of pressor and dipsogenic responses. Taken together, these data have lead to the hypothesis that the central AngII signaling cascade involves ROS and $[Ca^{2+}]_i$ cross-talk and that dysregulation of AngII/ROS/Ca^{2+} signaling in the brain may result in hypertension.

Introduction

The renin-angiotensin system (RAS), an endocrine system also present in individual tissues, plays a critical role in the regulation of cardiovascular and body fluid homeostasis and is implicated in the pathogenesis of hypertension. The main effector peptide of the RAS, angiotensin-II (AngII), mediates its effects at peripheral and central sites of action via two pharmacological classes of receptors, At_1 and At_2. In the central nervous system, AngII alters cardiovascular, hormonal, and behavioral responses including an increase in blood pressure, secretion of hypothalamic and pituitary hormones, and stimulation of drinking responses. AngII acts primarily on areas of the brain called circumventricular organs (CVO), including the subfornical organ (SFO) and area postrema (AP), which are characterized by the lack of a blood-brain barrier (BBB), having a high density of fenestrated capillaries, and by their location near the brain ventricular system[1]. It is well known that intracerebroventricular (ICV) administration of AngII causes a characteristic pressor and bradycardic response[2]; however, the precise mechanism of AngII signaling in the brain remains unknown.

Recently, a novel signaling mechanism for AngII in the periphery has been shown to involve reactive oxygen species (ROS) including superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2)[2-4]. In vascular smooth muscle cells (VSMC) AngII has been shown to stimulate production of ROS through the activation of a NAD(P)H oxidase[2;3]. We have recently shown *in vivo* that the characteristic cardiovascular response to ICV administered AngII is markedly attenuated in mice pre-treated (ICV) with adenovirus encoding reactive oxygen species (ROS) scavenging enzymes, copper-zinc superoxide dismutase (CuZnSOD) or manganese superoxide dismutase (MnSOD)[5]. These data suggest that ROS, particularly $O_2^{\bullet-}$, play a critical role in the *central* AngII-induced response. In addition, reports from other investigators suggest that AngII

causes the depolarization of SFO and AP neurons by inducing an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)[6]. This document will review the brain RAS and the role of intracellular Ca^{2+} in central AngII signaling, while primarily focusing on the role ROS may play in central AngII signaling. Finally, we will propose a set of future experiments, which will address the hypothesis that ROS, including $O_2^{\bullet-}$, H_2O_2, and nitric oxide ($NO^{\bullet}$), play a role in the intracellular signaling mechanism of AngII in the brain under normal circumstances and that dysregulation of these ROS may lead to the pathogenesis of AngII-dependent hypertension.

The Brain Renin-Angiotensin System

It is well established that the RAS is one of the most important regulators of arterial blood pressure and body fluid homeostasis. The RAS was classically considered to be an endocrine system, in which the effector peptide AngII is enzymatically generated (Figure 1) to mediate its effects through At_1 and At_2 receptors localized throughout the organism. However, recent studies on the RAS have been focused on local tissue specific systems in the vessels, heart and kidney, in which AngII exerts paracrine and autocrine effects in the local vicinity of its site of formation[7]. In 1961, Bickerton and Buckley reported the first evidence that the brain may mediate effects of blood-borne AngII[8].

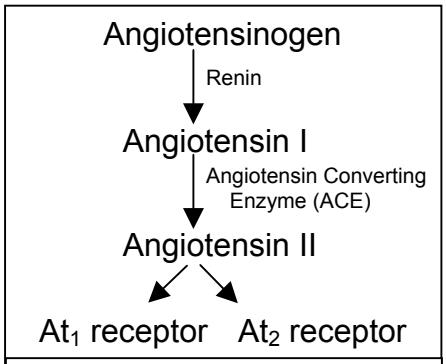

Figure 1. Schematic of angiotensin II enzymatic generation.

Fisher-Ferraro *et al*. made the first demonstration of renin in the brain a decade after Bickerton and Buckely's observation[9]. Subsequent molecular, anatomical, and functional studies have provided the well-accepted evidence of an endogenous central RAS. All components for a functional brain RAS including substrates, enzymes, and receptors have been identified.

Investigators have focused their studies of the brain RAS on the circumventricular organs (CVO), which are characterized by the lack of a blood-brain barrier (BBB), having a high density of fenestrated capillaries for high permeability, and by their location around the third and fourth ventricles[1]. Angiotensinogen (AGT), the only known precursor of AngII, has been identified by immunoreactive assays in neuronal and glial cells and is widely distributed in the brain[10], with the highest levels being found in the hypothalamus, particularly the supraoptic nucleus (SON), paraventicular nucleus (PVN) and preoptic nucleus. Renin-like immunoreactivity and low levels of renin mRNA have been detected in similar areas, in addition to brain stem and cortical regions[11]. Immunohistochemical and biochemical studies have identified angiotensin-converting enzyme (ACE) at its highest levels in the basal ganglia, SON, PVN, nucleus of solitarus tract (NTS) and dorsal motor nucleus of the vagus[12]. AngII-like immunoreactivity has been reported in neuron fibers and terminals whose cell bodies lie in the PVN, SON, and NTS, among others[13]. Similarly, numerous reports have localized AngII receptors to neurons in key central cardiovascular control regions, namely the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), median eminence and area postrema (AP)[14].

The importance of the SFO lies in evidence implicating the SFO in the maintenance of body fluid homeostasis, blood pressure, and the regulation of vasopressin and oxytocin release from the neurohypophysis[15]. The SFO, a highly vascularized circumventricular organ lacking the BBB is one of several portals whereby circulating AngII can exert its effects. Injection of AngII directly into the SFO, which contains At_1 receptors, produces the characteristic pressor, bradycardic, and dipsogenic actions[2]. Anatomical studies using anterograde transport trace molecules have demonstrated that the SFO sends direct projections to magnocellular

neurosecretory cells of the SON and PVN (Figure 2)[16]. The SON and PVN are sites for vasopressin and oxytocin synthesis and fibers from these two regions project to the neurohypophysis (NH) where the hormones are released into the circulation to exert their peripheral effects (Figure 2). In addition, neurons from the PVN send axons to autonomic centers in the brainstem (BS) and spinal cord involved in the regulation of sympathetic activity and may play a role in the barorececptor reflex (Figure 2)[17].

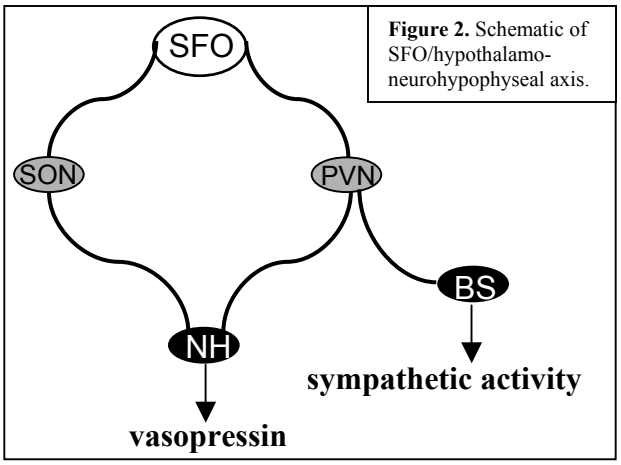

Figure 2. Schematic of SFO/hypothalamo-neurohypophyseal axis.

AngII Intra-neuronal Signaling: A Role for Calcium

Within the brain, AngII mediates its effects through both At_1 and At_2 receptors, however it is well accepted that At_1 is the classical AngII receptor that mediates most of the characteristic cardiovascular effects of the peptide. Pharmacological studies have indicated that central AngII mediates the pressor and dipsogenic responses through At_1 receptors as these actions are abolished by losartan, an At_1 receptor specific antagonist[18]. Using neuronal cell cultures, studies have shown that AngII stimulates phosphoinositide hydrolysis resulting in the production of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), which in turn increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and activate protein kinase C (PKC), respectively[19-21]. Recent accumulating evidence has shown that AngII elicits an At_1 receptor-mediated increase of neuronal firing rate in key cardiovascular regulatory brain regions discussed earlier, including the SFO, PVN, and SON[22]; however, the precise mechanism of this AngII-mediated action is not clearly understood. In general, nerve cell activity is dependent on action potentials and the ionic

currents associated with action potentials. Therefore, one can conclude that determining the mediation of neuronal membrane ionic currents will result in better understanding of the physiological changes that are controlled by a particular cell. It is postulated that AngII decreases the net sum of all inward and outward currents and more specifically AngII is implicated in inhibiting the delayed rectifier K^+ current (I_k)[6]. Inhibition of the delayed rectifier current leads to depolarization and rapid firing of the neuron. Interestingly, a current report suggests that AngII-induced inhibition of I_k encompasses the signaling cascade involving the stimulation of PI hydrolysis, an increase in $[Ca^{2+}]_i$ and activation of PKC[6].

Reactive Oxygen Species

Molecular oxygen (O_2) is essential for the survival of all aerobic organisms, as aerobic respiration is dependent on oxidative phosphorylation. In mitochondria, energy generated from the reduction of O_2 is converted to useable ATP energy for the cell through the mitochondria electron transport chain. During these reactions, highly reactive reduced metabolites of O_2, which may include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_20_2), or hydroxyl radical ($HO^{\bullet}$), are generated. Collectively these chemical species are referred to as reactive oxygen species (ROS). Traditionally, ROS have been regarded as toxic by-products of aerobic respiration and other cellular reactions. As such, the cell has developed numerous defense mechanisms such as superoxide dismutase (SOD), catalase, and glutithione peroxidase to keep the levels of ROS in balance and to maintain physiological homeostasis. Thus, the balance between ROS production and anti-oxidant defense mechanisms determines the degree of oxidative stress within the cell. Oxidative stress resulting in lipid peroxidation, protein modification, DNA cleavage, and/or damaged connective tissue matrices has been implicated in numerous human diseases including atherosclerosis, cancer, neurodegenerative diseases and aging[23-25].

Sources of ROS

In addition to the classical source of ROS production from the mitochondria electron transport chain, numerous additional cellular systems have the ability to generate ROS. In smooth endoplasmic reticulum, cytochrome P-450 and b_5 families of enzymes catalyze a series of reactions that oxidize unsaturated fatty acids and reduce O_2 to produce $O_2^{\bullet-}$ and H_2O_2[26]. Peroxisomes contain a collection of H_2O_2-generating enzymes and use H_2O_2 to oxidize other substrates of oxidative reactions[27]. Enzymatic reactions using xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, or flavoprotein dehydrogenase can also produce ROS[26]. Plasma membrane-associated oxidases are implicated in the generation of ROS with the best-characterized plasma membrane oxidase being the phagocytic NADPH oxidase. This oxidase is a multicomponent enzyme, which catalyzes the reduction of O_2 to $O_2^{\bullet-}$ and is best known for its role in host defense against invading microorganisms. NADPH serves as the electron donor for this one-electron reduction through the transmembrane protein cytochrome b_{558}, a heterodimeric complex of gp91phox and p22phox protein subunits. Recent evidence suggests that functional components of the phagocytic NADPH oxidase complex are present in various nonphagocytic cells[3;28]. In fact, accumulating evidence suggests NADPH oxidase derived $O_2^{\bullet-}$ plays a role in systemic angiotensin II-induced hypertension[28;29].

AngII-activated NADPH Oxidase

The phagocytic NADPH oxidase complex protein subunits include gp91phox and p22phox (plasma membrane bound b558), as well as cytosolic components p67phox, p47phox, and p40phox. The activation of the oxidase requires Rac1, a member of the Ras superfamily of small GTP-binding proteins. Upon binding GTP, Rac1 is activated and translocated to the plasma membrane to activate the formed NADPH oxidase complex. Components of this well

characterized phagocytic NADPH oxidase have been identified in numerous nonphagocytic cell types including vascular smooth muscle cells (VSMC), endothelial cells, and glial cells[3;4;30;31]. The components found in these cells are not always identical to their respective counterparts of the phagocytic NADPH oxidase; however, there seems to be a conserved homology between different cell types. Recently, a novel signaling mechanism for AngII has been shown to involve ROS including $O_2^{\bullet-}$ and H_2O_2. In VSMC, AngII has been shown to stimulate ROS production by activating a NADPH oxidase complex[3] that generates a 3-fold increase in $O_2^{\bullet-}$ production and a 5-fold increase in H_2O_2[29;32]. AngII-induced hypertension was found to be in concert with increased expression of p22phox mRNA and cytochrome b558 protein, both of which were corrected by an infusion of ROS scavenging enzyme, superoxide dismutase (SOD)[28;33]. In addition, the known components of this AngII-stimulated, nonphagocytic NADPH oxidase include p22phox, p67phox, p47phox and activated Rac1 protein[34;35]. Moreover, recent studies have identified numerous homologues of the phagocytic gp91phox subunit, referred to as Nox proteins, in various tissues[4;36]. Taken together, these studies clearly suggest a critical role for an NADPH oxidase in AngII signaling; however, the role of this novel mechanism remains to be investigated in the central AngII-dependent hypertension.

Superoxide and Nitric Oxide in Central AngII Signaling

ROS, including superoxide are now well appreciated as second messengers in numerous signaling cascades found in different cell types. A novel signaling cascade for AngII has been shown to involve ROS production in several cell types[3;35]; however, very little is known about the capacity of ROS in *central* AngII signaling. Therefore, we have recently performed experiments to determine if over-expressing genes encoding redox-scavenging enzymes attenuates the well-characterized central AngII-induced pressor and bradycardic response[5].

Genes for superoxide dismutase (SOD) localized both in the mitochondria (MnSOD) and cytoplasm (CuZnSOD) were delivered via recombinant E1-deleted adenoviral vectors. These adenoviral vectors are beneficial because of their ability to infect non-dividing cells, their capacity for large transgene inserts, and their highly efficient transgene expression. Previous work from Dr. John Engelhardt's group has demonstrated proper targeting of these adenoviral transgenes to their respective subcellular compartments[37]. Normal adult C57BL/6 mice were instrumented with left carotid arterial catheters for direct measurement of mean arterial pressure (MAP) and heart rate (HR). After arterial catheterization, animals were implanted with an intracerebroventricular (ICV) cannula for brain microinjections of AngII and adenovirus. Mice were ICV injected with saline, control vector (AdLacZ), AdMnSOD, or AdCuZnSOD and three days later we measured the acute effects of AngII injected ICV on MAP and HR. The well-established characteristic pressor and bradycardic response of ICV injected AngII was observed in saline and AdLacZ treated mice; however, these central AngII responses were virtually abolished in mice treated with AdMnSOD or AdCuZnSOD (Figure 3A)[5]. In summary, there was a significant difference in the mean change of MAP and HR after ICV injection of AngII

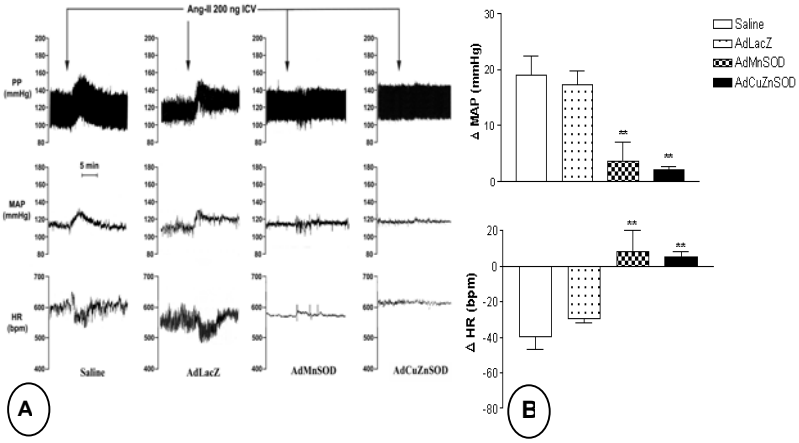

Figure 3. Overexpressing ROS scavenging enzymes attenuates the characteristic pressor and bradycardic response of ICV AngII. A) A typical blood pressure recording of the effects of AngII (200ng, 200nl) administered intracerebroventricularly (ICV) on cardiovascular responses in saline, AdLacZ, AdMnSOD or AdCuZnSOD treated mice. Arrows indicate time of AngII injection. MAP, mean arterial pressure; PP, pulsatile pressure; HR, heart rate. B) A summary of the effects of AngII administered ICV on MAP and HR in mice pretreated with saline (n=7), AdLacZ (n=6), AdMnSOD (n=7) or AdCuZnSOD (n=6), ***P*<0.01 vs. saline and AdLacZ.

between mice treated with saline (Δ19±3 mmHg, Δ-40±7 bpm) or AdLacZ (Δ18±2 mmHg, Δ-28±3 bpm) versus mice treated with AdMnSOD (Δ4±3 mmHg, Δ7±10 bpm, $P < 0.01$) or

AdCuZnSOD (Δ2±1 mmHg, Δ5±3 bpm, $P < 0.01$) (Figure 3B)[5]. However, the mean change of MAP and HR after AngII injection was not significant in comparing saline treated mice with AdLacZ treated mice ($P > 0.05$). In addition to the cardiovascular response of ICV AngII, we also measured the characteristic drinking response to AngII administered ICV. The total time the animal spent drinking and the number of drinking episodes was significantly reduced in AdMnSOD and AdCuZnSOD treated mice in comparison to saline or AdLacZ treated animals[5]. Taken together, these results demonstrate that the central AngII-induced pressor, bradycardic, and dipsogenic responses are mediated by ROS, particularly superoxide.

To provide more direct evidence that AngII can stimulate $O_2^{\bullet-}$ in neurons, we established a primary neuronal cell culture derived from the lamina terminalis, which includes the SFO and other CVO. We utilized the fluorescent dye dihydroethidium (DHE) to monitor intracellular AngII-mediated $O_2^{\bullet-}$ production in cultured neurons. Lamina terminalis cultured neurons (identified with spherical somata as seen with light-field microscopy) treated with AngII and DHE showed increase fluorescence indicating an increase in $O_2^{\bullet-}$ production (Figure 4b,) compared to control (non-treated) neurons (Figure 4a) or cells treated with the At_1 receptor antagonist, losartan (Figure 4c)[5]. In addition, infection with AdMnSOD 24 hours before AngII stimulation also prevented an increase in fluorescence (data not shown), corroborating the fidelity of the assay. In support of our *in vivo* physiological experiments, these studies provide more direct evidence suggesting that

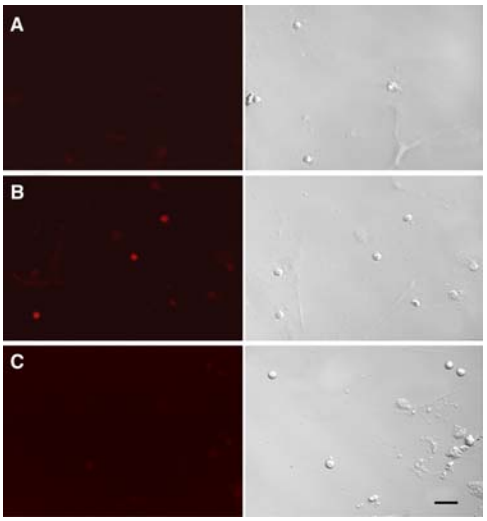

Figure 4. AngII increased intracellular superoxide in cultured cells of the lamina terminalis. AngII caused an increase in DHE)

as

$O_2^{\bullet-}$ may act as a second messenger in AngII signaling in cardiovascular control regions of the brain.

After demonstrating that $O_2^{\bullet-}$ is a critical second messenger in central AngII signaling, we turned our attention to identifying the source of AngII-mediated $O_2^{\bullet-}$ production. As discussed earlier, recent studies have shown that AngII stimulates a Rac1-dependent NADPH oxidase complex to produce $O_2^{\bullet-}$ in different peripheral cell types[3]; however, the role of this enzyme in central AngII signaling remains unknown. We performed studies similar to the one discussed above to address that hypothesis that a Rac1-dependent NADPH oxidase plays a role in the cardiovascular response to ICV AngII. We have demonstrated that mice treated ICV with adenovirus encoding dominant negative Rac1 (AdN17Rac1) have an attenuated pressor and bradycardic response to ICV AngII compared to control animals (unpublished observations). However, mice overexpressing wild-type Rac1 *via* adenovirus (AdwtRac1) had a more robust increase in blood pressure and greater bradycardia than control animals (unpublished observations). From these studies, we conclude that a Rac1-dependent NADPH oxidase may be the source of central AngII-induced $O_2^{\bullet-}$ production.

While our recent studies have been focused around the cardiovascular centers in the forebrain, others have used adenoviral-mediated gene transfer to cardiovascular control regions in the medulla and brainstem, primarily the nucleus of the solitary tract (NTS), to study central AngII signaling[38]. The NTS lies in the dorsal medulla and plays a key role in autonomic regulation, including the baroreceptor reflex[39]. Recently, extensive attention has been given to the role of nitric oxide ($NO^{\bullet}$) in AngII-mediated responses within the NTS. Since $NO^{\bullet}$ exerts a tonic inhibition of the sympathetic nervous system, it has been suggested that increased plasma AngII leading to an increase in blood pressure but lacking the bradycardic reflex, may increase

$NO^{\bullet}$ levels and activity in the NTS to depress the baroreceptor reflex[38]. Paton *et al*. used bilateral microinjections of adenovirus encoding dominant negative endothelial nitric oxide synthase (AdTeNOS) into rats to knock-out the activity of eNOS and repress the productions of $NO^{\bullet}$ by endothelial cells in the NTS[38]. After 5-6 days, a working brain-stem preparation (WHBP) was established to determine the physiological effects of AngII in the NTS. In control WHBP, NTS microinjections of AngII significantly attenuated the baroreceptor reflex gain. However, in AdTeNOS WHBP, AngII produced no change in the reflex, thus suggesting that AngII activates eNOS to produce $NO^{\bullet}$, which acts to depress the baroreflex[38]. Moreover, these findings provide an explanation to why increased plasma levels of AngII produce a pressor response without the bradycardic reflex.

Although the combination of our studies and the experiments on the NTS have been productive in our attempt to better understand the role of ROS in central AngII signaling, they also present more avenues that must be investigated. The remainder of this review will address where the field is heading and propose some experiments that will further test the hypothesis that ROS play a key role in the central AngII signaling and may provide new therapeutic targets in disease states associated with dysregulation of the brain RAS including hypertension and heart failure.

Determine the role of H_2O_2 on central AngII cardiovascular responses

Our recent findings that AdMnSOD and AdCuZnSOD are equally effective in inhibiting central AngII-induced cardiovascular and dipsogenic responses suggest that $O_2^{\bullet-}$ may be important in modulating these responses. However, we cannot rule out the possibility that the increased production of H_2O_2 from the dismutation of $O_2^{\bullet-}$ by overexpressing SOD (Reaction 1)

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad [1]$$

is having an inhibitory effect on neuronal activation, thus attenuating the cardiovascular responses of central AngII. In order to address this important issue, we propose to use adenoviral gene transfer of catalase (AdCat) and/or glutithione peroxidase (AdGPx), two antioxidants that remove H_2O_2, to overexpress these enzymes in the brains of normotensive mice followed by measuring the cardiovascular and dipsogenic response to ICV AngII. By administering AdCat and AdGPx independently or together, we will be able to address the role of H_2O_2 in central AngII signaling. Another important key experiment designed to confirm our early conclusions that $O_2^{\bullet-}$ is a key second messenger in central AngII signaling is to co-transfect the brains with AdCuZnSOD (or AdMnSOD) and AdCat. By overexpressing the combination of these antioxidants, we hypothesize that the $O_2^{\bullet-}$ generated from AngII stimulation will quickly be dismutated to H_2O_2 (by SOD, Reaction 1), which will in turn be rapidly removed by catalase to produce water and oxygen (Reaction 2). If the results of this experiment were similar to those

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad [2]$$

we have observed in transfecting AdSOD alone, then we would conclude that H_2O_2 production is not have an inhibitory effect on neuronal activation. Furthermore, this would substantiate our earlier conclusions.

Determine the link between central AngII-stimulated Ca^{2+} and ROS

As discussed earlier, previous studies have shown that AngII increases $[Ca^{2+}]_i$ in neurons within cardiovascular control regions accessed by ICV AngII[20]. In addition, in other cell types, it has been suggested that ROS can mediate AngII-stimulated $[Ca^{2+}]_i$ mobilization[40;41]. Furthermore, it has been suggested that AngII depolarizes neurons as a result of the cationic influx of $[Ca^{2+}]_i$ and this AngII-mediated neuronal excitability leads to the activation of neural pathways that ultimately cause the generation of pressor and dipsogenic responses[6]. We

hypothesize that $O_2^{\bullet-}$ stimulates an increase in $[Ca^{2+}]_i$ in a signaling cascade for central AngII-mediated neuronal excitation (Figure 5). The goal of this study will be to determine if the inhibition of AngII-stimulated $O_2^{\bullet-}$ will inhibit the AngII-mediated increase in $[Ca^{2+}]_i$. In order

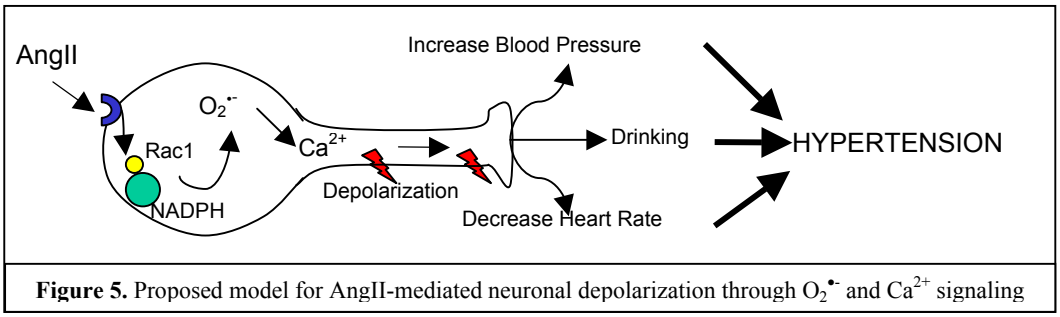

Figure 5. Proposed model for AngII-mediated neuronal depolarization through $O_2^{\bullet-}$ and Ca^{2+} signaling

to examine this hypothesis, we propose experiments using a primary neuronal cell culture from periventricular brain tissue in which cells will be infected with AdCuZnSOD or AdMnSOD 24 hrs prior to AngII treatment. Following AngII stimulation, intracellular Ca^{2+} will be measured using Fura-2 ratio fluorescence and a video microscopic image analysis system. We predict that cells pre-treated with AdCuZnSOD or AdMnSOD will show lower Fura-2 ratio fluorescence versus non-treated when stimulated with AngII, indicating an inhibition of AngII-mediated Ca^{2+} release. It is possible that inhibiting $O_2^{\bullet-}$ production with our adenoviral vectors will not attenuate Ca^{2+} influx in cells stimulated with AngII. This result would suggest two conclusions: 1) $O_2^{\bullet-}$ acts as a second messenger downstream from AngII-stimulated increases in $[Ca^{2+}]_i$, or 2) $O_2^{\bullet-}$ acts as a signaling molecule in a signaling cascade independent of Ca^{2+}. This outcome would be examined by performing DHE assay studies in a lamina terminalis cell culture. Cells would be treated with inhibitors of the IP_3/calcium system or incubated in Ca^{2+}-free media and the production of $O_2^{\bullet-}$ after AngII-stimulation would be determined by measuring DHE in our newly developed fast kinetic microscopy imaging system. If reducing the production of AngII-

stimulated Ca^{2+} inhibited DHE fluorescence, we would conclude that Ca^{2+} signals to increase $O_2^{\bullet-}$ production. Overall, these proposed experiments will determine the precise order of signaling (if any) between AngII-stimulated $O_2^{\bullet-}$ and Ca^{2+} in central neurons.

Determine the link between AngII-stimulated superoxide and nitric oxide in the brain

As discussed earlier in this document, Paton *et al.* have postulated that increased plasma levels of AngII stimulated the activation and/or production of $NO^{\bullet}$ from eNOS in the NTS[38]. This hypothesis is very intriguing in light of our recent studies demonstrating that AngII signaling in the forebrain, particularly the SFO, involves $O_2^{\bullet-}$. Nitric oxide has unique properties in that it can act as both a pro-oxidant and an antioxidant and thus may play a key role in numerous redox regulated signaling cascades. $NO^{\bullet}$ reacts rapidly with $O_2^{\bullet-}$ at a near diffusion limiting rate ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$) to produce the highly reactive molecule peroxynitrite ($ONOO^-$). In regards to central AngII signaling, one hypothesis is that AngII-stimulated $O_2^{\bullet-}$ reacts with $NO^{\bullet}$ to decrease the bioavailability of $NO^{\bullet}$, thus removing the tonic inhibition of the sympathetic nervous system. We propose to test this hypothesis by using NOS inhibitors and $NO^{\bullet}$ donors centrally while measuring AngII-induced $O_2^{\bullet-}$ production as well as recording the physiological cardiovascular response to central AngII. If the ICV administered AngII-induced cardiovascular responses are normal in mice that were treated with NOS inhibitors, we would conclude that $NO^{\bullet}$ does not play a role in AngII signaling in the forebrain. This result would refute Paton's work but this could be explained by the fact that his work focuses on brain regions in the brainstem and medulla, while we study AngII signaling in the forebrain. Addressing this problem will be very difficult and is beyond the scope of this document. Briefly, we suggest experiments that use Paton's WHBP and adenovirus encoding SOD to understand the role of $O_2^{\bullet-}$ in AngII signaling on sympathetic activation in the medulla.

Determine the role of $O_2^{\bullet-}$ in the pathogenesis of brain AngII-dependent hypertension

The previous discussion and proposed experiments have focused on the signaling cascade of central AngII; however, to truly determine the role of ROS, particularly $O_2^{\bullet-}$, in the pathogenesis of brain AngII-dependent hypertension it will be important to perform experiments in an *in vivo* model system that demonstrates brain AngII-dependent hypertension. One such model can be generated by chronic peripheral infusion of low doses of AngII. The slow-pressor response in this model is considered to recapitulate some forms of human hypertension. In addition, it is hypothesized that the slowly developing hypertension is due to the activation of the brain renin-angiotensin system[42]. Using this model, we propose to perform experiments in which mice are chronically infused with a slow-pressor dose of AngII after being centrally transfected with AdCuZnSOD or AdMnSOD. We anticipate that control animals will show the characteristic slow-pressor response with the low dose of AngII, whereas mice overexpressing SOD will have an attenuated increase in blood pressure. These results would demonstrate that $O_2^{\bullet-}$ in the brain plays a critical role in the slow-pressor response to increased peripheral AngII, and may provide more insight into the central mechanism of AngII-dependent cardiovascular diseases including hypertension and heart failure.

A second *in vivo* animal model we propose to use to better understand the role of $O_2^{\bullet-}$ in the pathogenesis of brain AngII-dependent hypertension is the R^+A^+ transgenic mouse. These transgenic mice are chronically hypertensive because they expresses both human renin (hREN) and human angiotensinogen (hAGT) (Figure 1) transgenes in appropriate tissues and cells, including expression in key cardiovascular control regions of the brain[43;44]. Earlier studies on R^+A^+ mice demonstrated that the brain renin-angiotensin system contributes to the chronic hypertensive state exhibited by these animals, as shown by reducing the blood pressure with ICV

administration of the At_1 receptor antagonist, losartan[43]. We hypothesize that R^+A^+ mice have increased $O_2^{\bullet-}$ levels in the brain due to an increase in AngII signaling compared to control mice. In addition, we postulate that overexpressing SOD in the brain will reduce the elevated blood pressure of R^+A^+ mice. We propose to measure basal levels of $O_2^{\bullet-}$ in the brains of R^+A^+ mice by measuring DHE fluorescence in freshly prepared brain sections. We expect that R^+A^+ mice would have greater DHE fluorescence in key cardiovascular control regions, including the SFO, compared to non-transgenic control mice. In order to address the physiological significance of the expected increase in $O_2^{\bullet-}$, we propose experiments in which SOD would be overexpressed *via* adenovirus in the brain of R^+A^+ mice followed by recording the blood pressure over 2-4 weeks. We anticipate that, similar to the response to losartan, overexpression of SOD would reduce the elevated blood pressure of R^+A^+ mice. Taken together, these results would support our hypothesis that $O_2^{\bullet-}$ plays a critical role in central AngII signaling, and in addition would provide the first known link between $O_2^{\bullet-}$ and the pathogenesis of brain renin-angiotensin system-dependent hypertension.

Summary

Angiotensin II is a highly conserved peptide to which the survival of most species is closely linked. Key aspects of AngII-mediated effects on cardiovascular and volume homeostasis occur in the central nervous system, where it promotes vasopressin secretion, a thirst response, sympathetic outflow, and modulation of blood pressure. Dysregulation of brain angiotensinergic systems is implicated in a number of cardiovascular diseases including hypertension and heart failure. Despite its importance in cardiovascular regulation, brain angiotensinergic signaling is incompletely understood.

We have recently shown, for the first time, that ROS, particularly $O_2^{\bullet-}$ are key mediators in central AngII-mediated effects. In addition, Paton *et al*. have demonstrated that $NO^{\bullet}$, another ROS, plays a role in AngII modulation of sympathetic outflow from the medulla and brainstem. As discussed above, further experiments should focus on the cross-talk between $O_2^{\bullet-}$ and $NO^{\bullet}$ to better understand the concept that AngII-stimulated $O_2^{\bullet-}$ removes $NO^{\bullet}$ to disinhibit sympathetic activity. In addition, future experiments are needed to determine the role (or lack thereof) other ROS, such as H_2O_2, in central AngII signaling. Furthermore, future studies should address the concept that in neurons ROS can increase intracellular Ca^{2+} (or vice versa) in response to AngII. Finally, *in vivo* studies using animal models that exhibit brain renin-angiotensin system-dependent hypertension are required to provide a link between central AngII and ROS in a chronic pathophysiological condition. Overall, recent studies and the proposed experiments will provide new information on the intracellular signaling mechanisms of AngII in the brain under physiological and pathophysiological circumstances, and may implicate oxygen radicals as important new targets for therapeutic treatment of hypertension.

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