Role of Redox-Sensitive Calcium/Calmodulin-Dependent Protein Kinase Ila in Angiotensin II Intra-neuronal Signaling and Angiotensin II-Mediated Hypertension

Urmi Basu
University of Nebraska Medical Center

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ROLE OF REDOX-SENSITIVE CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE IIα IN ANGIOTENSIN II INTRA-NEURONAL SIGNALING AND ANGIOTENSIN II-MEDIATED HYPERTENSION

By

Urmī Basu

A DISSERTATION

Presented to the Faculty of

The University of Nebraska Medical Center Graduate College

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Department of Cellular and Integrative Physiology

Under the supervision of Associate Professor Dr. Matthew C. Zimmerman
University of Nebraska Medical Center
Omaha, Nebraska
August, 2015

Supervisory Committee:

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ABSTRACT

ROLE OF REDOX-SENSITIVE CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE IIα IN ANGIOTENSIN II INTRA-NEURONAL SIGNALING AND ANGIOTENSIN II-MEDIATED HYPERTENSION

Urmi Basu, Ph.D.

University of Nebraska Medical Center, 2015

Advisor: Matthew C. Zimmerman, Ph.D.

Activation of renin-angiotensin system and elevated levels of circulating and brain angiotensin II (AngII) has been implicated in the pathogenesis of neuro-cardiovascular diseases, such as hypertension and heart failure. In central neurons AngII increases generation of reactive oxygen species (ROS), which in turn regulates neuronal ion channels. Previous studies have demonstrated that ion channels can also be regulated by protein kinases, such as calcium/calmodulin-dependent protein kinase II (CaMKII) as a downstream mediator of AngII signaling. In the heart, CaMKIIδ undergoes oxidation upon AngII stimulation and increased pro-oxidant conditions. However, the exact mechanism by which AngII and ROS regulate CaMKIIα, the major neuronal isoform, in central neurons is unclear. We hypothesized that overexpression of wild-type CaMKIIα in neurons exacerbates the AngII-mediated inhibition of neuronal ion channels and the AngII-induced hypertensive response. Indeed, in mouse catecholaminergic
(CATH.a) neurons overexpression of wt-CaMKIIα significantly enhanced the AngII-mediated reduction in $K^+$ current as compared to non-transduced and adenoviral control vector-transduced neurons. Overexpression of wt-CaMKIIα in brain subfornical organ sensitized mice to an immediate increase in blood pressure at an initial subpressor dose of chronic subcutaneous AngII infusion. We further hypothesized that mutation of redox-sensitive cysteine and methionine residues at positions 280 and 281 respectively of CaMKIIα will inhibit the potentiated AngII-signaling in neurons and the rise in blood pressure. Adenovrius-mediated expression of mut-CaMKIIα in CATH.a neurons resulted in a significant restoration of the potentiated AngII-mediated $K^+$ current inhibition observed with wt-CaMKIIα overexpression. Further, mice with mut-CaMKIIα in the brain were prevented from the initial sensitization mechanism following subcutaneous AngII infusion and the peak AngII-induced blood pressure was also significantly attenuated as compared to mice with wt-CaMKIIα overexpression. Together, these studies indicate that redox-regulation of CaMKIIα is an important mechanism in AngII-downstream signaling in neurons and in mediating AngII-induced neurogenic hypertension.
ACKNOWLEDGEMENT

I am forever grateful to several people who helped me in the endeavor of earning my PhD. I feel myself very fortunate to join the laboratory of Dr. Matthew C. Zimmerman, my advisor. His excellent supervision and mentorship has helped me to develop my abilities and build a foundation for my future career in science. I am thankful to Dr. Zimmerman for his guidance on developing critical skills, such as planning scientific experiments, applying for external fellowships and submitting manuscripts. An important aspect of Dr. Zimmerman’s interactions with students is his ability to provide just the right amount of structure yet allow the student some independence. He also provided me with every opportunity to present my research work in regional and national meetings which have truly developed my communicating skills and also gave me a chance to meet some of the experts in the field. These trainings have helped me to grow immensely and have prepared me to establish my own career in scientific research.

I must also thank the other members of my Supervisory Committee, Dr. Kaushik Patel, Dr. George Rozanski, and Dr. Keshore Bidasee, all of whom have played important roles in shaping my dissertation in many ways. It has been an honor to have such highly respected scientific experts on my committee. The committee always provided thoughtful suggestions, constructive feedback and challenged my intellect to think outside the box and generate my own ideas. In addition, my committee has been instrumental in writing letters of support,
advising me on research training plan and keeping me on track to graduate. I am extremely grateful for their guidance and support.

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expertise in mouse surgical procedures is commendable. She implanted telemetry in mice for my animal experiments and trained me on multiple surgical procedures and animal care in general. Jun is such a wonderful person to work with and I will miss her motherly concern and delicious homemade Chinese food. Jocelyn Jones, the past lab technician must also be thanked for keeping the Zimmerman lab organized and stocked up with supplies and helping everyone with their ordering needs. Importantly, I am thankful for the groundwork that a former postdoctoral fellow and good friend, Dr. Saleena Alikunju, put forth on my dissertation project. Former graduate students, Dr. Erin Rosenbaugh and Dr. Krupa Savalia, have been great friends and source of encouragement and together we have had some great moments in the lab.

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<tr>
<td>AdCuZnSOD</td>
<td>adenoviral copper zinc superoxide dismutase</td>
</tr>
<tr>
<td>AGT</td>
<td>angiotensinogen</td>
</tr>
<tr>
<td>AT1R</td>
<td>angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACE2</td>
<td>angiotensin converting enzyme2</td>
</tr>
<tr>
<td>ACEi</td>
<td>angiotensin converting enzyme inhibitor</td>
</tr>
<tr>
<td>AngI</td>
<td>angiotensin I, angiotensin-(1-10)</td>
</tr>
<tr>
<td>AngII</td>
<td>angiotensin II, angiotensin-(1-8)</td>
</tr>
<tr>
<td>Ang-(1-7)</td>
<td>angiotensin-(1-7)</td>
</tr>
<tr>
<td>Ang-(1-9)</td>
<td>angiotensin-(1-9)</td>
</tr>
<tr>
<td>AngIII</td>
<td>angiotensin III, angiotensin-(2-8)</td>
</tr>
<tr>
<td>AngIV</td>
<td>angiotensin IV, angiotensin -(3-8)</td>
</tr>
<tr>
<td>APA</td>
<td>aminopeptidase A</td>
</tr>
<tr>
<td>APN</td>
<td>aminopeptidase N</td>
</tr>
<tr>
<td>ARB</td>
<td>angiotensin receptor blocker</td>
</tr>
<tr>
<td>AT2R</td>
<td>angiotensin II type 2 receptor</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>CaMKII</td>
<td>calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTB</td>
<td>cholera toxin subunit B</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>copper zinc superoxide dismutase</td>
</tr>
<tr>
<td>ecSOD</td>
<td>extracellular SOD</td>
</tr>
<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>ICV</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia reperfusion</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
<tr>
<td>MasR</td>
<td>mas receptor</td>
</tr>
<tr>
<td>MnSOD</td>
<td>manganese SOD (mitochondrial localized)</td>
</tr>
<tr>
<td>Msr</td>
<td>methionine sulfoxide reductase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
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<tr>
<td>O2·-</td>
<td>superoxide</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PRR</td>
<td>prorenin receptor</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
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<tr>
<td>RAS</td>
<td>renin angiotensin system</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RVLM</td>
<td>rostral ventrolateral medulla</td>
</tr>
<tr>
<td>SFO</td>
<td>subfornical organ</td>
</tr>
<tr>
<td>SON</td>
<td>supraoptic nucleus</td>
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<tr>
<td>SHR</td>
<td>spontaneously hypertensive rats</td>
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Chapter I: BACKGROUND

Cardiovascular disease and hypertension

Cardiovascular disease is the major cause of morbidity and mortality worldwide. The American Heart Association (AHA) reported in 2013 that cardiovascular disease and particularly heart disease is the leading cause of death specifically within the United States (1). Cardiovascular disorders include coronary heart disease (heart attacks), cerebrovascular disease (stroke), raised blood pressure (hypertension), peripheral arterial disease, congenital heart disease and heart failure. Hypertension is a key risk factor of cardiovascular disease and according to World Health Organization (WHO) approximately 17 million deaths each year are due to cardiovascular disease of which 9.4 million is attributable to complications of hypertension (2). The increasing occurrence of hypertension includes a growing geriatric population, and behavioral risk factors, such as unhealthy diet, lack of physical activity, poor stress management, and the use of tobacco and alcohol (2). Hypertension is considered as a “silent” invisible killer which impacts global public health concerns and is in constant progress due to the growing incidence of diabetes and obesity (2-4). Furthermore, uncontrolled hypertension is a risk for several cardiovascular disorders, including stroke, heart failure, coronary artery disease, kidney failure, and peripheral vascular disease (5).
Blood pressure regulation and hypertension

Blood pressure is the mean arterial pressure that is monitored and regulated by physiological mechanisms in the body. It is the force of blood that pushes against the arterial walls of the arteries as the heart pumps blood to the systemic tissues of the body. Blood pressure is commonly measured by a stethoscope and a sphygmomanometer (also referred to as a sphygmometer). It is attached to an inflatable air-bladder cuff to temporarily restrict blood flow and a mercury or mechanical manometer measures the pressure. Blood pressure is recorded as two numbers written as a ratio where the top number is the systolic blood pressure (SBP) and the bottom is the diastolic blood pressure (DBP). Systolic blood pressure is recorded when the first heartbeat is detected after releasing the cuff and represents the pressure in the arteries when the heart muscle contracts. Diastolic blood pressure is the number recorded when the last heartbeat is detected after releasing the cuff and represents pressure in the arteries when the heart relaxes. As per the AHA, the recommendation is for adults to maintain blood pressure less than 120/80 mmHg (systolic/diastolic) to avoid other complications associated with hypertension (6-8). Clinically, hypertension is diagnosed as blood pressure which is consistently greater than 140 mmHg systolic pressure and 90 mmHg diastolic pressure. Blood pressure between the healthy and hypertensive range is considered to be prehypertension.

There are two major determinants of blood pressure: the heart’s cardiac output (CO) and the total peripheral resistance (TPR). Cardiac output is defined as the direct product of heart rate (HR) and cardiac stroke volume (SV). When
either HR or SV is high, CO increases. Stroke volume is determined by the contractility of the heart and how much blood returns to the heart (venous return). Total peripheral resistance is the resistance on the arteries when the blood flows and can be regulated by a number of circulating factors, including angiotensin II (AngII), catecholamines, prostaglandins, direct innervation of specific receptors ($\alpha_1$ and $\beta_2$), endothelin, adenosine, nitric oxide and also blood viscosity. Blood pressure is regulated by both divisions of the autonomic nervous system: the sympathetic and the parasympathetic nervous system.

**Renin-angiotensin System**

It has long been established that the renin-angiotensin system (RAS) plays a critical role in cardiovascular and renal homeostasis and has a central regulatory role on blood pressure maintenance (9). It is classically described as an endocrine system and regulates extracellular fluid and electrolyte balance thereby maintaining cardiovascular homeostasis in the body. The RAS comprises of several effector peptides that are formed through a series of enzymatic cleavages. The cascade is initiated when aspartyl-protease renin, secreted from the juxtaglomerular cells of the kidney cleaves the precursor globulin angiotensinogen (AGT) to form the decapeptide angiotensin 1-10 (commonly known as Ang I). Ang I is physiologically inactive but can be cleaved by the membrane-bound dipeptidyl carboxypeptidase, angiotensin converting enzyme (ACE), resulting in the formation of the octapeptide, AngII, the primary effector peptide of the RAS (10). Additionally, AngI can be cleaved by angiotensin converting enzyme 2 (ACE2) to produce Ang-(1-9), which can subsequently be
cleaved by ACE to form Ang-(1-7). Alternatively, ACE2 can also act upon AngII to generate Ang-(1-7).

AngII carries out its function by acting on two major G protein-coupled AngII receptor subtypes – AngII type 1 receptor (AT1R) and AngII type 2 receptor (AT2R). The AT1R has a wide tissue distribution with high expression in vasculature, kidney, adrenal gland, heart and some of the cardiovascular control brain regions (10-12). It is well recognized that the AT1R subtype mediates most of the well-known actions of AngII, including increased sympathetic output, vasoconstriction, release of vasopressin, norepinephrine (NE) and, aldosterone (13, 14). In addition the AngII-AT1R is also involved in sodium and water reabsorption in the kidneys, increase in heart rate, and stimulation of thirst and salt appetite(15) (Figure 1.1). Furthermore, recent studies have also demonstrated AngII-mediated activation of the immune system, particularly T cell and cytokine activation, which may act as a positive feedback loop in the development and maintenance of cardiovascular disease (16). The effects of AngII via the AT1R are mediated by complex signaling pathways involving phospholipids (phospholipase C (PLC), PLD, PLA2), induction of gene transcription (c-fos, c-jun, c-myc), activation of redox mechanisms such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and production of reactive oxygen species (ROS), and also activation of tyrosine kinases (Src, JAK-STAT, PI3K)(13, 17-19). In contrast, the AT2R which is highly expressed in fetal tissue, including fetal aorta, brain, adrenal medulla, kidney, and mesenchymal layer of the gastrointestinal tract, is involved more in counteracting
the actions of AT1R and does not have direct regulation on cardiovascular physiology (10, 20, 21). Furthermore, the AT2R can dimerize with the AT1R and interrupt the AngII-AT1R-mediated signaling mechanisms (21, 22). Most of the studies investigating the role of the different AngII receptor subtypes have been done by using specific receptor agonists and antagonists – for example, a selective agonist of the AT2R, compound 21, has shown beneficial effects on cardiovascular function and baroreflex sensitivity (23-25). On the other hand systemic administration of AT1R antagonist, such as losartan and irbesartan, lowers blood pressure and improves impaired baroreflex sensitivity in genetically hypertensive rats (26-29).

In contrast to the “hypertensive axis” of the RAS comprising ACE-AngII-AT1R signaling, Ang-(1-7) binds to Mas receptor (MasR) to induce opposing effects to AngII-AT1R mechanisms. MasR is primarily expressed in brain and testis but also in kidney, heart and vessels, and its activation leads to vasodilation, decreased inflammation, reduction in sympathetic activity, antifibrotic, antihypertrophic and antithrombotic effects (12, 30, 31). The MasR can also heterodimerize with the AT1R and thereby antagonize it (32-34). The ACE2-Ang-(1-7)-MasR is thereby considered as the “anti-hypertensive axis” of the RAS. Indeed, studies in the recent past have demonstrated the potential therapeutic effect of the ACE2-MasR axis on cardiovascular diseases including heart failure and hypertension (35). Moreover, the nonpeptide orally active MasR agonist AVE 0991 has shown to preserve cardiac function and prevent end-organ damage in animal models of hypertension (36).
Although AngII is considered as the primary effector peptide of the RAS, recent studies have identified some of the AngII metabolites as other active components of the system. Aminopeptidase A (APA) cleaves the N-terminal aspartyl residue of AngII resulting in the formation of Ang-(2-8), commonly referred to as Ang III. Ang III can be further metabolized into Ang-(3-8) (Ang IV) by aminopeptidase N (APN). Ang III displays comparable affinity to the AT1R and exerts similar actions as AngII such as aldosterone release and vasoconstriction (37, 38). APA inhibitors have also shown to attenuate central effects of AngII in AngII-mediated hypertension model (39).

Numerous studies done over the decades have illustrated the importance of different RAS components in blood pressure regulation and cardiovascular homeostasis by using specific pharmacological inhibitors as mentioned above. The pharmacological blockers were used either to inhibit the formation or actions of the key mediators of the RAS. In addition to β-blockers, some of the most widely used drugs in the treatment of hypertension and other cardiovascular diseases are the ones that inhibit ACE (ACEi) thereby limiting the formation of AngII or that block the actions of the AngII receptors (ARBs)(40).
Figure 1.1 - Renin-angiotensin system cascade. Abbreviations: ACE - angiotensin converting enzyme; ADH – anti-diuretic hormone (also known as vasopressin); AGT – angiotensinogen; ALD – aldosterone; Ang I – angiotensin I; AngII – angiotensin II; AT1R – angiotensin type 1 receptor; AT2R – angiotensin type 2 receptor; CAGE – chymostatin-sensitive angiotensin II-generating enzyme; MasR – mas receptor; NE – norepinephrine; NEP – neutral endopeptidase; PEP – prolylendopeptidase; PCP – prolylcarboxypeptidase; tPA – tissue plasminogen activator.
Brain-angiotensinergic systems and hypertension

The first identification of renin in the brain was made by Ganten et al. and Fisher-Ferraro et al. (41, 42) and this was subsequently followed by identification of the existence of AngII-processing pathways in the central nervous system (CNS). The fact that AngII immunoreactivity is detected in the brain after nephrectomy (43, 44) suggests that the brain RAS is modulated independently of the peripheral RAS. Central injections of AngII in specific brain regions elicit profound cardiovascular and drinking response indicating the pivotal role of brain angiotensinergic mechanisms in cardiovascular control (45). Almost all of the RAS components have been identified in the CNS, including the enzymes that are involved in the generation of these effector molecules. For example, AGT, the primary precursor of AngII is widely distributed in the brain with the highest expression found in the hypothalamus, mainly the supraoptic nucleus (SON), paraventricular nucleus (PVN), and preoptic nucleus (46, 47). Interestingly, levels of renin, the enzyme that cleaves AGT into Ang I, is low in the brain and is detected in regions similar to AGT in addition to brain stem and cortical regions (48). However, recent studies have demonstrated the existence of prorenin and prorenin receptor (PRR) in important cardiovascular control brain nuclei (49). Non-proteolytic activation of prorenin by binding to the PRR can lead to increased generation of AngII independent of the renin pathway and neuron-specific knock out of this receptor can attenuate blood pressure in a salt-sensitive hypertension model where brain AngII levels are increased (50, 51). Several
biochemical and immunohistochemical studies have found ACE, the enzyme responsible for the conversion of Ang I to AngII in the basal ganglia, SON, PVN, nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (52, 53). These evidences thus suggest that AngII is produced in local tissues in the brain which is independent of the peripheral RAS.

In addition, systemic AngII can also act upon certain blood brain barrier (BBB) deficient brain nuclei known as circumventricular organs (CVOs) (54). These CVOs are present mainly bordering the third and fourth ventricles and are densely populated with fenestrated capillaries for high permeability (55). Among the CVOs, the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT) and area postrema (AP) (Figure 1.2) are the key cardiovascular control brain regions and express high levels of the AT1R (55, 56). The CVOs receive inputs from systemic baroreceptors and chemoreceptors and in turn send out neural projections to some of the downstream extensive neural network involved in maintaining various cardiovascular responses, vasopressin release, and autonomic responses (54, 57). More specifically, anatomical studies with anterograde tracing markers have identified projections from SFO in the forebrain to SON and PVN (54, 57, 58). Additionally, neurons from the parvocellular region of the PVN send axons to centers in the rostral ventrolateral medulla (RVLM) and intermediolateral cell column of the spinal cord, which are also important centers of sympathetic outflow and baroreceptor reflex regulation (54, 57, 58) The importance of CVOs in brain angiotensinergic signaling have been demonstrated in studies whereby the AngII-induced activation of PVN
neurons is greatly attenuated by electrolytic lesion of the SFO in rats (59-61). Lesioning the SFO also attenuates mean arterial pressure in an AngII-salt sensitive hypertension model (62). In addition, central injection of AngII directly into the SFO increases pressor, bradycardic and dipsogenic response (62, 63). Electrophysiological studies show that intravenous administration of AngII increases excitability of SFO neurons via an AT1R-mediated mechanism which inhibited transient outward potassium current and increased intracellular calcium (63-65). Furthermore, intracerebroventricular (ICV) infusion of losartan reduces AT1R mRNA and immunoreactivity in the SFO of heart failure animals (66). ICV infusion of losartan also reduces elevations in metabolic rate in a deoxycorticosterone acetate-salt (DOCA-Salt) hypertension model indicating the importance of brain angiotensinergic signaling in various animal models of hypertension (67). In addition to the SFO, destruction of the AP eliminates the pressor effect of AngII infusion into the vertebral arteries (68). Similar to the SFO, the central AngII-mediated dipsogenic and pressor effects can be blocked by lesion of the OVLT (68).

Selective overexpression of other components of the RAS, such as ACE2, in the brain can attenuate neurogenic hypertension by preventing decrease in spontaneous baroreflex sensitivity and parasympathetic tone (69, 70). ACE2 overexpression in the brain also causes upregulation of the AT2 and MasR (69) thus shifting the balance between the “hypertensive” and the “anti-hypertensive” axis of the RAS. Ang III, another bioactive peptide of the RAS has similar affinity as AngII for AT1R in the brain and is involved in blood pressure control and body
fluid and electrolyte balance (38, 71). Both AngII and Ang III in the brain are involved in increasing sympathetic nerve activity, inhibition of baroreflex sensitivity in the NTS and release arginine vasopressin (AVP) from the posterior pituitary. As such, studies by Marc et al. has identified inhibitors of APA, enzyme responsible for the generation of Ang III from AngII, to therapeutically target the brain RAS in hypertension (39). Together, these studies clearly demonstrate the significance of the brain angiotensinergic mechanisms in the regulation of neurocardiovascular function and dysregulation in their signaling pathways can lead to diseases, including hypertension and heart failure.
Figure 1.2. Cardiovascular regulatory nuclei involved in brain angiotensinergic signaling mechanisms. Modified from Davisson and Zimmerman (72)
Angiotensin II intra-neuronal signaling mechanisms

The AngII intra-neuronal signaling cascade involves a number of key mediators, including reactive oxygen species (ROS), protein kinases and phosphatases, ion channels, transcription factors, and matrix metalloproteinases (MMPs). The localization of the AngII receptor subtypes in the brain is quite different. For example, most of the AT1Rs are concentrated in the SFO, PVN, SON, and in the nuclei of the brainstem, whereas the AT2Rs are found in the thalamus, cerebellum, locus coeruleus and particularly in the inferior olivary nucleus reflecting the different functional role of each of these receptor subtypes (13, 73). Numerous studies so far have well documented the AngII intracellular signaling mechanisms in the periphery (13, 74). In contrast, the neuronal signaling mechanisms mediated by AngII in the brain are still not fully understood. In the peripheral tissues and cells, AngII initiates a number of diverse signaling pathways via the AT1R, such as stimulation of phosphoinositide (PI) hydrolysis by coupling the AT1R to Gq or Gq-like protein. This is followed by generation of inositol (1,4,5)-triphosphatase (IP3), with subsequent increase in intracellular Ca^{2+} and diacylglycerol (DAG)-mediated increase in protein kinase C (PKC) activity (17, 74). Interestingly, AT1R coupling to the Gi protein can inhibit adenylyl cyclase depending on the cell and type of tissue. AT1R activation by AngII can also stimulate Ras/Raf/mitogen activated protein (MAP) kinase and JAK (Janus/Kinase) STAT (signal transducers and activators of transcription) signaling pathways (17, 74). Importantly, studies using cultured neurons from rat
brain have demonstrated that a number of these AT1R-mediated AngII signaling occurs in the CNS. Activation of AT1Rs in the median eminence as well as in cultured neurons leads to a stimulation of PI hydrolysis causing IP3 generation and increases in [Ca\textsubscript{i}] and activation of PKC (13, 14, 75, 76). Furthermore increased concentration of [Ca\textsubscript{i}] activates downstream signaling molecules, such as calcium/calmodulin-dependent protein kinase II (CaMKII). The AngII-AT1R mediated PI hydrolysis is also observed in a neuroblastoma glial cell line (NG 108-15). The Ras/Raf pathway and activation of MAP kinases by AngII by acting on the AT1R is also documented in cultured neurons. Increased MAP kinase activity mediates the known stimulatory effects of AngII on neuronal NE synthesis via the induction of Fos and Jun proteins (14, 77, 78). Furthermore, studies by Sumners and Raizada have demonstrated major short term effects of AngII on neuronal ion channel current and neuronal firing that is also mediated via the AT1R. For example, AT1R stimulation leads to an increase in total neuronal Ca\textsuperscript{2+} current (I\textsubscript{Ca}) and also causes inhibition of delayed rectifier K\textsuperscript{+} current (I\textsubscript{KV}) and the A type transient K\textsuperscript{+} (I\textsubscript{A}) current in primary neurons from rat hypothalamus and brain stem and also in a mouse catecholaminergic neuronal cell line (CATH.a neurons) (75, 79). These currents are the basis of neuronal action potentials and therefore, changes in Ca\textsuperscript{2+} and K\textsuperscript{+} ion channel current leads to an increase in neuronal firing frequency ultimately causing neuronal excitation. Involvement of signaling molecules, such as PKC and CaMKII have been investigated in the regulation of these ion channel currents by AngII by using specific inhibitors, such as calphostin C to inhibit PKC and KN-93 to block CaMKII activity (80, 81).
Inhibition of either PKC or CaMKII attenuates the AngII-mediated reduction in \( I_{KV} \) and the increase in neuronal firing frequency (80, 81). However, the precise mechanism by which AngII regulates PKC and CaMKII thereby modulating neuronal ion channels is not yet fully elucidated.

Similar to the AT1R, the AT2R-mediated intracellular signaling also modulates a variety of signaling molecules. In the heart, kidney, and cultured neurons, AT2R activation causes stimulation of phospholipase A2 (PLA2) activity (13). In neurons, this increase in PLA2 activity generates arachidonic acid which can then lead to regulation of neuronal ion channels (13). Several studies have also shown that in neurons, AngII by acting on the AT2R can activate serine/threonine phosphatase type 2A (PP2A) and in turn inhibit MAP kinase activity (78). Selective activation of the AT2R causes potentiation of neuronal \( I_{KV} \) and \( I_A \), an effect opposing to the AT1R. AT2R does not seem to have any regulatory role on \( I_{Ca} \) in cultured neurons, but inhibits T-type \( Ca^{2+} \) current in undifferentiated NG108-15 neuroblastoma x glioma cells (82). AngII-mediated AT2R activation thereby leads to a decrease in neuronal excitability.

To better understand AngII-intraneuronal signaling in the pathogenesis of neuro-cardiovascular disorders, several \textit{in vivo} and \textit{in vitro} experimental models have been established. Numerous studies have shown that acute stimulation of cultured neurons with AngII results in rapid increase in intracellular calcium, inhibition of outward potassium current (\( I_{KV} \)), and an increase in neuronal firing (80, 81, 83, 84). In addition, chronic stimulation (i.e. hours to days) of AngII-sensitive cultured neurons leads to alterations in the expression of angiotensin
receptors, potassium channel proteins, and transcription factors (85, 86).

Studies from these chronic AngII stimulation studies concluded that these events mimic the AngII neuronal signaling occurring \emph{in vivo} in case of disease models, such as hypertension and heart failure where AngII levels are chronically elevated (87-89). However, one limitation of these \emph{in vitro} studies has been the lack of evidence of chronic AngII stability in neuronal cell culture medium following a single administration of AngII.
Figure 1.3. Angiotensin II-intraneuronal signaling mechanisms in central neurons. Abbreviations: AT1R – Angiotensin II type 1 receptor; ROS – reactive oxygen species; CaMKII – calcium/calmodulin-dependent protein kinase II; PKC – protein kinase C; SNA – sympathetic nerve activity; MAP – mean arterial pressure
Reactive oxygen species in the central nervous system

Excessive activation of brain RAS or overproduction of AngII induces cardiovascular malfunctions (87). One of the most promising concepts that emerged during the last decade is AngII-mediated generation of reactive oxygen species (ROS) and activation of redox-dependent signaling cascades in the brain (84, 90). ROS are species of oxygen produced by all aerobic cells and are in a highly reactive state than molecular oxygen. Although they are best known for their role in host defense mechanisms (91, 92), ample evidences demonstrate ROS, primarily superoxide (O$_2^•$) and hydrogen peroxide (H$_2$O$_2$) as key signaling molecules following stimulation of various plasma membrane receptors (93-95). Imbalance between ROS production and the ability of different scavenging systems to detoxify the reactive intermediates plays a critical role in neural mechanism of hypertension and heart failure (89, 96, 97). In the cells, ROS are mainly generated from incomplete reduction of molecular oxygen through enzymatic reactions involving NADPH oxidase (NOX) (98, 99) and xanthine oxidase (95, 100, 101), mitochondrial electron transport chain (ETC) (102-104), uncoupling of nitric oxide synthases (105), and through cyclo-oxygenases, lipoxygenases, or cytochrome P450 reductases (95, 106). In the vasculature, Griendling and colleagues were the first to demonstrate that AngII activates NOX via the AT1R and this activation causes increase in intracellular O$_2^•$ formation which can lead to endothelial dysfunction and the pathogenesis of AngII-induced hypertension (107, 108). In the CNS, AngII generates ROS, particularly O$_2^•$ and
H$_2$O$_2$ from two major sources – NOX and the mitochondrial ETC. Additionally, activated proinflammatory cytokines can also mediate AngII-induced ROS production in the brain (56, 109). Accumulating evidence suggests that AngII activates AT1R in neurons of major cardiovascular control brain regions, such as the SFO, PVN and RVLM resulting in increased ROS levels which contribute to the increase in neuronal activation and subsequent stimulation of the sympathetic nervous system (97, 109). In addition, AngII reduces activities of Complexes I, II and III of the mitochondrial ETC, causing a suppression of electron transfer capacity between the complexes thus leading to electron leakage and generating increased O$_2^*$ and H$_2$O$_2$ (103). Importantly, the vasopressor, bradycardic and dipsogenic responses by intracerebroventricular (ICV) injection of AngII is mediated, at least in part, by O$_2^*$ in CVOs, such as the SFO (110). In the SFO, AngII leads to the increased production of O$_2^*$ and removing O$_2^*$ by specific antioxidants in the SFO abolishes the cardiovascular and dipsogenic responses to central AngII (90). The primary scavenger of O$_2^*$ is the superoxide dismutase (SOD). SOD catalyzes the dismutation of O$_2^*$ to H$_2$O$_2$ and oxygen and 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 and nucleus; 2) manganese SOD (MnSOD or SOD2), which is strictly localized in mitochondrial matrix; and 3) extracellular SOD (ecSOD or SOD3), which is found extracellular. Adenovirus-mediated overexpression of CuZnSOD in the SFO also prevents hypertension in chronic peripheral AngII-infused mice (90). The importance of NOX enzymes in
mediating the full vasopressor effects of brain AngII in the SFO has been illustrated (111, 112) by using adenoviral vectors encoding small interfering RNA to selectively silence NOX2 and NOX4, the two NOX isoforms in the brain. Interestingly, silencing NOX2 alone and not NOX4, significantly attenuates the central AngII-induced drinking responses. The differential role of these two NOX enzymes in mediating the central AngII responses can be due to their different subcellular localizations in neurons. For example, NOX2 resides primarily in the plasma membrane, although it has not been fully elucidated in central neurons (113, 114). In contrast, recent study from our lab has demonstrated NOX4 to be localized in the mitochondria in CATH.a neurons (115). Furthermore, an important downstream cellular mechanism induced by O$_2^•$ is the influx of intracellular Ca$^{2+}$ as seen in neuroblastoma Neuro 2A cells, which can be inhibited by adenoviral-mediated expression of a dominant −negative isoform of Ras-related C3 botulinum toxin substrate 1 (Rac1) (AdN17Rac1), a major component of NOX complex formation and activation (84). In addition, few studies suggest that ROS derived from NOX enhances nerve traffic in the SFO-PVN axis (116). Notably, Erdos et al have shown that ICV injection of AngII increases O$_2^•*$ production both in the SFO and PVN and this increase in O$_2^•*$ is attenuated by a NADPH oxidase inhibitor, apocynin (117). Importantly, the increase in AT1R and ACE mRNA in the PVN by ICV infusion of aldosterone is blocked by concomitant infusion of tempol, an SOD mimetic (118). Furthermore, in a model of 2-kidney-1-clip (2K1C) hypertension, the large increases in cardiac sympathetic activity, baseline renal sympathetic nerve activity and mean arterial pressure due to microinjection of
AngII into the PVN is abolished by pretreatment with tempol or apocynin (119). Microinjection of tempol into the PVN also reduces high blood pressure and sympathetic activity of renovascular hypertensive rats (120, 121). Gene expression of NOX subunits, such as p47phox and gp91phox as well as AT1Rs is also increased in the PVN of those rats (121-123). Recent studies by Braga and colleagues have shown that overexpression of CuZnSOD into the PVN prevents the elevation of $O_2^{* -}$ and the hypertensive response in the 2K1C hypertension model (122). Similar to the SFO and the PVN, the RVLM has also been recognized as one of the important brain sites for the accumulation of AngII-induced $O_2^{* -}$ and its impact on hypertension (124, 125). The RVLM receives inputs from the SFO and PVN forming the so-called SFO-PVN-RVLM pathway and contains bulbospinal neurons that are major inputs to the preganglionic neurons of the sympathetic nervous system (116, 126-128). It is densely populated with the AT1R (129) and pharmacological blockade of the AT1R attenuates the pressor response induced by AngII-microinjected into the RVLM of rabbits (130). In a slow pressor AngII hypertension model as well as in a 2K1C model, increased levels of $O_2^{* -}$ have been demonstrated in the RVLM along with an increase in sympathetic nerve activity and overexpression of CuZnSOD specifically in the RVLM attenuates both the accumulation of $O_2^{* -}$ and the sympathoexcitation (120, 121, 124). In SHRs the MnSOD levels in the RVLM are found to be decreased (96). The AT1R mRNA expression as well as the NADPH oxidase subunits is increased in the RVLM of 2K1C rats (120, 124). Injection of tempol into the RVLM has been documented to decrease blood pressure and renal sympathetic activity.
in 2K1C hypertensive rats (120). Additionally, overexpression of MnSOD in the RVLM inhibits the cardiovascular responses to AngII microinjected into the RVLM and also reduces sympathoexcitation and hypertension in SHRs. These studies therefore implicate the importance of ROS in the brain in different models of hypertension (131).

Several studies suggesting the role of brain ROS in mediating the pathogenesis of cardiovascular disorders has been done by using specific ROS scavengers, such as SOD. In addition to neurons in the brain, AngII can also cause $O_2^{-}$ generation from NOX enzymes in microglial cells (132, 133). Recent studies have also put forward a feed forward mechanism between NOX and mitochondria and the idea of a ROS-induced-ROS mechanism (134, 135). These studies suggest that AngII activates NOX leading to increases in intracellular $Ca^{2+}$ concentration which can then be taken up by the mitochondria thus generating mitochondrial ROS. Indeed studies showing that knockdown of the p22phox subunit of NOX attenuates the AngII-induced $O_2^{-}$ and $H_2O_2$ production in the mitochondrial fractions isolated from the RVLM (103).

One of the major targets of ROS are ion channels and numerous evidences support the hypothesis that AngII-induced sympathoexcitation is mediated, atleast in part, by redox modulation of ion channels. The two important ion channels that play crucial role in action potential generation and neuronal excitability and can also be regulated by ROS, either directly or indirectly, are the $Ca^{2+}$ and $K^+$ channels (79). Evidence from both in vivo and in vitro studies suggest that the AngII-mediated modulation of $Ca^{2+}$ and $K^+$ channels involves
ROS derived from NOX enzymes (84, 136) as well as mitochondrial sources (83). These studies also suggest a potential role of PKC and CaMKII in the regulation of these channels. Importantly inhibiting PKC and CaMKII by calphostin C and KN-93 respectively (80) in primary neurons cultured from rat hypothalamus and brainstem and scavenging superoxide by either CuZnSOD or MnSOD abolishes the AngII-mediated reduction in neuronal K⁺ current (83). Another class of protein kinases that are known to be redox-regulated and important in cardiovascular pathogenesis is the MAPK family of proteins. In the brain, redox activation of MAPK is involved in the AngII-mediated hypertension and heart failure (137, 138). It is well recognized that in the RVLM, AngII via the AT1R-dependent activation of PKC-NOX-ROS signaling cascade triggers the activation of MAPKs, particularly p38MAPK and extracellular signal-regulated kinase (ERK) (139, 140). The activation of p38MAPK pathway in turn mediates the short-term pressor response of AngII by potentiating the glutamatergic neurotransmission in the RVLM (139). On the other hand, activation of ERK results in the long-term pressor response to AngII by upregulating the AT1R mRNA expression (141). The AngII-ROS-p38MAPK/ERK pathway in the RVLM is also implicated in the hypertensive phenotype in stroke-prone SHRs (142). In rats with CHF, AngII triggers the activation of ERK and c-Jun N terminal kinase (JNK) via ROS which contributes to the upregulation of AT1R and sympathoexcitation (143, 144). Furthermore, it has been reported in mouse CATH.a neurons, the AngII-mediated inhibition of voltage-gated K⁺ channel expression is dependent on the AT1R-ROS-p38MAPK signaling pathway (13, 14). In addition, serine/threonine
protein kinases, such as Akt and Rho are also thought to be redox-sensitive and are active players in the pathogenesis of cardiovascular disorders (145-147). These studies confirm the role of ROS as key intermediates in AngII intraneuronal signaling and establish O$_2^{ullet-}$ as a sympatho-excitatory molecule in the brain involved in the cardiovascular complications association with hypertension and heart failure.

However, although O$_2^{ullet-}$ seem to be the major ROS molecule in AngII-redox signaling mechanisms, evidences also point out a potential role of H$_2$O$_2$ in some of the cardiovascular effects mediated by AngII. For example, in stroke-prone SHR, administration of catalase, the H$_2$O$_2$ scavenging enzyme, into the RVLM reduces the elevated levels of ROS (131). Catalase expression is also found to be low in the RVLM of SHRs and overexpression of catalase results in prolonged hypotension (96). In contrast, the H$_2$O$_2$ generated by dismutation of O$_2^{ullet-}$ by CuZnSOD in the PVN does not play a role in the manifestation of sympathoexcitation associated with myocardial infarction-induced heart failure (148). Since catalase is primarily present in the peroxisomes and is not responsible for reducing H$_2$O$_2$ levels in different subcellular compartments, therefore, additional studies with other H$_2$O$_2$ removing enzymes, such as glutathione peroxidase (GPx) and peroxiredoxins (Prx) are required to confirm the role of H$_2$O$_2$ in mediating AngII-induced neurocardiovascular disorders.
Protein thiol oxidation in signaling and pathophysiological responses

Elevated levels of ROS contribute to the adverse outcomes of many cardiovascular pathogenesis, including atherosclerosis, hypertension, heart failure, diabetes, cardiac hypertrophy and myocardial infarction (106, 149, 150). Increased production of oxidants, reduced nitric oxide (NO) bioavailability and decreased antioxidant defense mechanisms in specific organs are involved in these diseases. In many of these pathological models, in addition to ROS, excessive generation of reactive nitrogen species (RNS) is also involved thus leading to increased oxidative and nitrosative stress (151, 152). These excess oxidants not only affect cellular components, such as membrane lipids and nucleic acids (153, 154) but can also act as second messengers leading to post-translational modifications (PTMs) of proteins thus altering cellular homeostasis. Different amino acids that make up a protein differ greatly in their susceptibility to get oxidized; however, the two most common PTMs of proteins occur in their sulfur-containing amino acids – cysteine and methionine (155, 156). Inside the cell, the cytoplasm is in a highly reducing environment and the protein cysteines are maintained in their thiol (-SH) or thiolate (-S\(^{-}\)) state. This reducing environment is mainly due to the presence of the most abundant low-molecular-weight thiol buffer glutathione (GSH). GSH is a cysteine-containing tripeptide (glutamate-cysteine-glycine) and is present in millimolar concentrations inside the cell (156-158). In addition to GSH, there are also a variety of reductive enzymatic
pathways to remove any disulfide bond formation. These include the ubiquitous disulfide reductase, thioredoxin (Trx), which reduces oxidized protein substrates by using electrons derived from NADPH via its reactivating enzyme, thioredoxin reductase (TR), and glutaredoxin (Grx), which is reduced by GSH, which in turn is reduced by NADPH-dependent glutathione reductase (GR) (157, 159).

However, under oxidative and/or nitrosative stress conditions the cysteine thiol groups of proteins are particularly susceptible to oxidation by ROS/RNS and other electrophilic molecules. The protein thiols not only function in normal cellular signaling but can also undergo irreversible oxidation in aging and disease conditions thus changing protein function. The reactivity of most protein thiols is determined by its pKa (the acid dissociation constant) and at intracellular pH protein thiols have a pKa of approximately 8.5 which makes them less reactive (156, 157, 160). However, under certain local charge environments protein thiols can have a lower pKa and may exist as thiolate anions (cysteine-S⁻) at physiological pH thus making them more susceptible to oxidation to ROS/RNS.

One such product of \( \text{H}_2\text{O}_2 \) oxidation is sulfenic acid (RSOH), which is unstable and can undergo further oxidation to generate more stable derivatives, such as sulfinic acid (RSO₂H) or sulfonic acid (RSO₃H), or react with another thiol to yield inter- or intra-protein disulfides (PrSSPr, PrSSPr') (157). Importantly, protein disulfides, nitrosothiols, sulfenic acid and, in some cases sulfinic acid are reversible thus providing a mechanism of cell signaling regulation by reversible changes in protein function (160, 161). On the other hand, oxidation of sulfinic acid to sulfonic acid is irreversible and therefore the modified protein should either be
degraded or re-synthesized to restore their normal cellular levels and protect against oxidative damage (161). Additionally, cysteine residues can also be modified by RNS, such as NO to yield an S-nitrosothiol (R-SNO), and by peroxynitrite (ONOO⁻) to form S-nitrothiol (R-SNO₂) (158, 162). As compelling evidences suggest the importance of regulation of protein function by thiol modifications, there have been tremendous efforts to develop methods to determine which proteins, and which amino acid residues within them are most reactive and also detect the specific thiol modifications. So far, mass spectrometry and proteomics approaches along with other free thiol labeling techniques are being utilized to accurately identify thiol modifications (163, 164).

The other sulfur-containing naturally occurring amino acid which is sensitive to oxidation is methionine. Due to the chiral nature of the sulfur atom in methionine, both free and protein-based methionine residues can undergo oxidation by ROS to form a mixture of two diastereomers, methionine-S-sulfoxide and methionine-R-sulfoxide (165). In the presence of a strong oxidant meth sulfoxide can be further oxidized to methionine sulfone (MetO2) which can lead to many pathophysiological consequences (166). Similar to cysteine, oxidation of methionine can also lead to significant alterations of protein structure and function. As a means to counteract the methionine oxidation, methionine sulfoxide reductases (Msrs) have evolved as a reductase system in most organisms, including anaerobic organisms (167). Interestingly, in order to have a complete reduction of both methionine-(R,S)-sulfoxide residues in proteins two specific Msr isoforms have evolved – MsrA and MsrB. MsrA is specific for the
reduction of met-S-sulfoxide while MsrB acts only on met-R-sulfoxide (167-169). MsrA reduces both free and protein-based met sulfoxide and can also reduce other methyl sulfoxide compounds, such as dimethyl sulfoxide and sulindac (170). In contrast, MsrB has a very low activity towards free met sulfoxide and only acts efficiently on peptidyl met sulfoxide (170). In mammals, a single MsrA gene encodes for the MsrA protein and contains a mitochondrial signal peptide at the N-terminus. However, subcellular localization studies in rat and mouse have shown MsrAs are also present in the cytosol. The MsrB proteins are found in different cellular compartments and is encoded by three MsrB genes (B1-B3). The different subcellular localizations of MsrA and MsrB suggest that the met sulfoxide reduction system is maintained in different compartments of the mammalian cell to protect against met oxidation (165, 167).

Several studies have investigated the role of cysteine and met oxidation in target molecules in different experimental systems. For example, reversible oxidation of a specific cysteine residue in the amino-terminus of the voltage-dependent K+ channel (Kv1.4) channel (171) or its associated β subunits (172) has shown to modulate the N-type inactivation of this channel. In the brain, glutamate and H+ sensitivity of the NMDA receptors is also regulated by oxidation of two cysteine residues in NR1 subunits (173). In addition to ion channels, a number of protein kinases, phosphatases and transcription factors are also modulated by ROS and/or RNS. One such example is the redox regulation of different receptor tyrosine kinases (RTKs) that play an important role in signal transduction pathways by the recognition and response to stimulant
binding. Once activated by ligand binding, the RTKs can relay its signal through downstream non-receptor kinases to mediate several biological processes (174). Finkel and colleagues in their landmark study have showed that the platelet-derived growth factor (PDGF)-induced $\text{H}_2\text{O}_2$ production has downstream effects on global tyrosine phosphorylation, activation of MAPK pathways, DNA synthesis and chemotaxis (175). The other RTK, epidermal growth factor (EGF) also induced increase in ROS levels upon receptor binding (176). Further, inhibition of ROS production blocked the tyrosine signaling triggered by these RTKs stimulation. In addition, apoptosis signaling kinase 1, PKC, protein kinase A (PKA) has also been shown to be regulated by redox-based mechanisms in different signaling pathways (174, 177).

Redox sensitivity of protein phosphatases, particularly the protein tyrosine phosphatases (PTPs) such as PTP1B, low molecular weight PTP (LMW-PTP), SH2 domain containing PTPs (SHP-1 and SHP-2) has been well described (178). The pKa of the catalytic site cysteine of many PTPs is around 6, which makes them extremely reactive and forms thiolate anions at physiological pH. This reactive thiol group not only makes them highly susceptible to oxidation but is also essential for the catalytic mechanism of these PTPs (178). The serine/threonine protein phosphatases, such as protein phosphatase 2A (PP2A), has also been shown to be regulated by $\text{H}_2\text{O}_2$ and glutathionylation (179, 180) and disulfide cross-linking of the catalytic subunit of PP2A with other proteins in the brain along with the formation of an intramolecular disulfide bond within PP2A leads to PP2A inhibition in the brain (180).
Figure 1.4. Thiol-based oxidation of cysteine residues: role of reactive oxygen species as signaling intermediates (157)
Redox regulation of CaMKII and its role in AngII signaling pathways

The family of multifunctional calcium/calmodulin (Ca$^{2+}$/CaM)-dependent protein kinases (CaMKs) including CaMKI, CaMKII and CaMKIV modulate many of the cellular responses to Ca$^{2+}$. These downstream effector molecules translate and co-ordinate the dynamic second messenger, Ca$^{2+}$, into appropriate cellular responses by a highly specific and reversible phosphorylation of a number of substrate proteins in their serine or threonine residues. This family of protein kinases has both common as well as unique features with respect to their structure, regulation and activation. The CaMKII is responsible for the coupling of Ca$^{2+}$ increase to ion channel activation (181), gene transcription (182), neurotransmitter synthesis and release, cytoskeletal organization and apoptosis (183). It should be noted that different functions of CaMKII are mediated by a family of CaMKII isoforms derived from four closely related yet distinct genes (α, β, γ and δ). CaMKII is highly enriched in the brain with the brain specific α (50 kDa) and β (60 kDa) isoform constituting upto 2% of the total protein in the hippocampus of rodents and upto 1% of the total protein in the forebrain itself. Furthermore, in forebrain homogenates 19-37 µM concentration of CaMKIIα has been estimated in the cytosol. CaMKIIδ and γ isoforms are abundantly expressed throughout the body with the δ isoform predominantly found in the heart. Purified brain CaMKII has a molecular weight of approximately 460000 – 654000 Da and is composed of 8-12 subunits. Both heteromultimers and homomers of CaMKIIα
have been identified from rat forebrain. The CaMKII is a multi-subunit holoenzyme containing three key domains: the association domain, which directs multimeric assembly, the regulatory domain, which controls enzyme activation and autoinhibition, and the catalytic domain, which is responsible for the kinase function of CaMKII. Under resting conditions CaMKII is maintained in an autoinhibited state by the autoregulatory domain that acts as a pseudosubstrate, preventing the binding of substrates to the catalytic domain. The binding of Ca\(^{2+}\)/CaM induces a conformational change in CaMKII that relieves the autoinhibitory effect of the regulatory domain and exposes the substrate binding site, thus activating the enzyme. One of the common features of all the CaMKII isoforms is the presence of autophosphorylation sites within the autoregulatory region, which is Thr286 in CaMKIIα (Thr 287 in β, γ and δ isoforms). In the sustained presence of Ca\(^{2+}\)/CaM, intersubunit autophosphorylation in these threonine residues results in Ca\(^{2+}\)/CaM-independent CaMKII activity by preventing the reassociation of the kinase domain with the autoinhibitory domain (181). The autophosphorylated CaMKII can then be dephosphorylated by either protein phosphatase 1 (PP1) or PP2A (184-186) to return to the inactive state. The molecular switch between the Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent CaMKII activation is critical for the transformation of a transient Ca\(^{2+}\) stimulus into sustained physiological or disease-causing activity.

Recent studies have shown that the AngII-mediated pathological responses in the heart involves a second Ca\(^{2+}\)/CaM independent activity of CaMKII under oxidant conditions. More specifically, studies by Erickson et al.
have shown that oxidation of a paired methionine residue (M281/282) in the cardiomyocyte isoform of CaMKII (CaMKIIδ) is a downstream signal for AngII and ischemic stress response in the heart and inhibition of CaMKII protects the heart against AngII-mediated apoptosis and other pathological myocardial responses (181, 187). They demonstrated that exposure of purified CaMKIIδ to H₂O₂ after pretreatment with Ca²⁺/CaM yielded persistent CaMKII activation even in the presence of EGTA suggesting that Ca²⁺/CaM binding is required for the initial exposure of the key segment of CaMKII for oxidation as seen with autophosphorylation and that oxidation is able to maintain sustained CaMKII activity even without Ca²⁺/CaM binding. Mutation of M281/282 to valine completely abolished the H₂O₂ dependent activation of CaMKIIδ. Since, the paired met residue is conserved in all three isoforms of CaMKII except CaMKIIα (188) (CaMKIIα has a cysteine residue for the first methionine of the pair in position 280), they also generated a M281C mutant of CaMKIIδ and also purified CaMKIIα. Both purified CaMKIIα and M281C mutant CaMKIIδ were activated by H₂O₂ indicating the importance of cysteine in ROS-dependent CaMKII activation. In addition, heart sections of mice treated with AngII showed increased CaMKII oxidation whereas mice lacking a critical subunit of NADPH oxidase (p47⁻/⁻) did not show the AngII-mediated increase in CaMKII oxidation (181, 187). Interestingly, mice knockout for Msr A (MsrA⁻/⁻) had increased CaMKII oxidation and apoptosis with AngII and ischemia along with increased mortality, greater left ventricular dilation, and reduced in vivo mechanical function after myocardial infarction (181, 187). More recently, a NO-mediated S-nitrosylation of CaMKIIα
has been implicated in excitotoxic neuronal cell death (189). The results from that study show that specific S-nitrosylation of C280/289 generated autonomous activation of CaMKII independent of Ca\(^{2+}\)/CaM binding and mutation of either site protected from NO-induced neuronal cell death. The redox sensitivity of CaMKII has also been illustrated in studies where large increases in cytosolic Ca\(^{2+}\) causes mitochondrial O\(_2\)\(^{\bullet-}\) generation and upregulation of CaMKII in hippocampal neurons (190). Activation of CaMKII by this mitochondrial-induced O\(_2\)\(^{\bullet-}\) is also mediated at least in part by oxidative suppression of protein phosphatases that dephosphorylate CaMKII. Studies by Yin et al. have also showed that in CATH.a neurons AngII increases CaMKII phosphorylation, causing CaMKII activation, with a significant increase observed after 5 minutes of AngII stimulation (83). However, neurons, which were transduced with AdMnSOD (adenovirus overexpressing manganese superoxide dismutase) had reduced CaMKII phosphorylation, indicating that superoxide is involved in mediating AngII-induced CaMKII activation in central neurons. These studies therefore illustrate the importance of redox regulation of CaMKII in physiological and pathological responses.

**Specific Aims and Hypotheses**

Based on the detailed discussion of the background, the following specific aims and hypotheses were developed to (1) determine the chronic stability of exogenous AngII in neuronal cell culture media and (2) examine the redox-sensitivity of neuronal CaMKII (CaMKII\(\alpha\)) in the regulation of neuronal ion
channels and increased blood pressure in AngII-dependent neurogenic hypertension.

The overall hypothesis is that mutation of specific redox-sensitive cysteine and methionine residues in CaMKIIα will prevent the AngII-mediated inhibition of neuronal K^+ current and also the hypertensive response induced by central or subcutaneous infusion of AngII. Additionally, a single exogenous administration of AngII is rapidly metabolized in neuronal cell culture media and in order to mimic chronic elevated levels of AngII in certain disease models repeated AngII administration in media is needed. The experiments proposed herein provide new information on how CaMKIIα can be modified by ROS and exaggerate the AngII signaling in neurons. More specifically, the results of the study provide important new insight on the specific residues of CaMKIIα that can be oxidatively modified and can thus be utilized for the treatment of neuro-cardiovascular pathologies such as hypertension.

**Specific Aim 1: Determine the stability of exogenous AngII in neuronal culture media.**

This aim tests the hypothesis that AngII is rapidly metabolized in neuronal cell culture media following exogenous AngII stimulation. The levels of AngII and its metabolites, Ang III, Ang IV and Ang-(1-7), were measured in the media from cultured CATH.a neurons by liquid chromatography-tandem mass spectrometry. A single administration of AngII (100 nM) was used to treat CATH.a neurons and media was collected after 15 minutes – 24 hours to measure AngII and its
metabolites. AngII levels were also measured after repeated administration of fresh exogenous AngII every 3 hours for up to 24 hours.

**Specific Aim 2: Determine the functional relevance of overexpressing wild-type CaMKIIα in the regulation of AngII-modulated neuronal K+ channel and the AngII-induced hypertensive response.**

This specific aim tests the hypothesis that adenovirus-mediated overexpression of wt-CaMKIIα (wt-AdCaMKIIα) in CATH.a neurons potentiates the AngII-mediated inhibition of K+ current. In addition, we hypothesize that wt-CaMKIIα overexpression in the brain exaggerates the AngII-induced increase in blood pressure in mice. A series of *in vivo* and *in vitro* experiments were performed to test the hypothesis. Molecular biology techniques, including polymerase chain reaction (PCR) and cloning was utilized to generate the major neuronal isoform of CaMKII (CaMKIIα) adenovirus. The overexpression of wt-CaMKIIα in CATH.a neurons was assessed by measuring the mRNA and protein expression using real time PCR and Western blot analysis respectively. Whole-cell patch clamp technique was utilized to measure changes in baseline K+ channel current and following AngII stimulation in non-infected, control adenoviral, AdEmpty and wt-AdCaMKIIα transduced CATH.a neurons. Furthermore, wt-AdCaMKIIα and adenovirus overexpressing green fluorescent protein (AdGFP) was injected intracerebroventricularly (ICV) in mice by ICV cannulas and blood pressure and heart rate responses were measured following either central acute or chronic subcutaneous AngII infusion. Blood pressure and heart rate responses were measured using radiotelemetry.
Specific Aim 3: Determine the functional relevance of oxidative post-translational modifications of CaMKIIα in regulating neuronal ion channel current and in mediating AngII hypertension in mice.

Based on the previous studies discussed, both cysteine and methionine residues present in the autoregulatory domain of CaMKII has been shown to be oxidatively modified. This aim will therefore test the hypothesis that mutation of specific redox-sensitive cysteine and methionine residues in CaMKIIα will attenuate the AngII-mediated inhibition of neuronal K⁺ current and also the AngII-induced rise in blood pressure. A mutant CaMKIIα adenovirus (mut-AdCaMKIIα) was generated with C280A and M281V substitutions and was transduced in CATH.a neurons to record K⁺ current at baseline and after 5 minutes of AngII superfusion. To investigate the effect of mut-CaMKIIα in the brain on the AngII-mediated hypertensive response, mice were injected with ICV mut-CaMKIIα adenovirus and blood pressure and heart rate responses were recorded after subcutaneous AngII infusion for 3 weeks.
Figure 1.5. Schematic representation of overall hypothesis. AngII increases superoxide which in turn leads to enhanced CaMKII oxidation and activation. Activated CaMKII subsequently decreases neuronal potassium current and increases neuronal firing frequency. Activation of central neurons leads to an increased sympathetic nerve activity and an increase in mean arterial pressure (hypertension).
Introduction

Activation of the renin-angiotensin system along with increased levels of AngII in the brain has been associated with the development and maintenance of various forms of experimental and genetic models of hypertension (87, 88, 191, 192). In the brain, AngII can act on specific nuclei that are important in autonomic control of cardiovascular function. These brain regions include the subfornical organ (SFO), paraventricular nucleus (PVN), rostral ventrolateral medulla (RVLM) and nucleus tractus solitaries (NTS). AngII has been shown to increase neuronal firing in these brain areas, thus leading to the deleterious sympathoexcitation commonly associated with neuro-cardiovascular diseases (193-196). In central neurons, AngII mediates most of its actions by acting primarily on its type 1 receptor (AT\textsubscript{1}R). For example, AngII stimulation of the AT\textsubscript{1}R in the above mentioned cardiovascular control brain regions and others contributes to vasopressin secretion, thirst and salt appetite, and baroreflex modulation (197-199). In addition, AngII can also activate its type 2 receptor (AT\textsubscript{2}R) on central neurons, which often results in opposing responses compared to AT\textsubscript{1}R activation, reflecting the different physiological and pathophysiological roles of AngII mediated via these receptors (13).
Considering the importance of AngII intra-neuronal signaling in the pathogenesis of neuro-cardiovascular diseases including hypertension and heart failure, numerous studies have been devoted to better understand the precise signaling pathways involved in an attempt to identify new therapeutic targets for these diseases. However, there are significant technical challenges in studying AngII-induced intra-neuronal signaling in vivo in animal models of hypertension and heart failure such as: 1) limited number of neurons within a specific cardiovascular control brain region of interest to perform a particular assay; 2) separating intra-neuronal signaling events from other pathways activated in neighboring cells including glia and endothelial cells; and 3) separating the signaling mechanisms of other peptides and hormones associated with these diseases from those directly induced by AngII. To circumvent these challenges, neuronal cell culture models have been frequently utilized to examine the specific intra-neuronal signaling pathways induced by AngII. Such studies have clearly identified important roles for calcium, reactive oxygen species, kinases, and transcription factors in the AngII intra-neuronal signaling pathway (80, 84, 86, 90).

Nevertheless, there are also limitations in using neuronal cell culture models to recapitulate the AngII intra-neuronal signaling events occurring in vivo such as: 1) neuronal cells are often immortalized and/or isolated from a tumor; 2) cells are usually cultured in a hyperoxic environment (i.e. 21% oxygen) as compared to their in vivo environment (1-4% oxygen); and 3) the lack of neighboring glia and endothelial cells may alter a particular response in the
cultured neurons that would normally occur in vivo. A fourth limitation in using neuronal cell culture models as it specifically relates to understanding AngII intra-neuronal signaling is the lack of evidence indicating the stability of exogenous AngII in neuronal cell culture media. Many studies have examined intra-neuronal responses, such as changes in mRNA levels and protein expression, 1-48 hours after a single administration of exogenous AngII into the neuronal cell culture media (200-202). Data from these studies are often interpreted to indicate that the observed changes are also occurring in neurons of various hypertensive and heart failure models in which circulating and/or brain levels of AngII are chronically elevated (203, 204). However, it remains unclear if a single treatment of exogenous AngII given to neurons in culture results in a chronic elevation of AngII levels in the media.

In the current study, we tested the hypothesis that exogenous AngII is rapidly metabolized in neuronal cell culture medium and thus fails to remain chronically elevated following a single exogenous administration. We utilized a mouse catecholaminergic neuronal cell line, CATH.a neurons, which express both AT1R and AT2R. CATH.a neurons have commonly been used by our group and others to study AngII intra-neuronal signaling pathways (80, 85, 115). Previous studies have shown that deletion of the AT1R from catecholaminergic neurons delays the onset of AngII-dependent hypertension and also reduces the maximal blood pressure response (205). In addition, catecholaminergic neurons, such as C1 neurons in the RVLM, play an important role in AngII-induced hypertension and blocking the AT1R specifically in these neurons can attenuate
the AngII-mediated rise in blood pressure (206). Using liquid chromatography-tandem mass spectrometry, we measured levels of AngII and its metabolites, Ang III, Ang IV, and Ang-1-7, in CATH.a neuronal cell culture media after administration of exogenous AngII. Herein, we report that exogenous AngII is rapidly metabolized in CATH.a neuron media with levels returning to near baseline after 3 hours of administration. Further, AngII levels in CATH.a neuron media can be significantly and chronically elevated for at least 24 hours with the addition of fresh exogenous AngII every 3 hours.

**Experimental Methods**

**Neuronal cell culture**

Mouse catecholaminergic CATH.a neurons (American Type Culture Collection (ATCC), Manassas, VA) were cultured in RPMI 1640 medium (Gibco/Invitrogen, Grand Island, NY) supplemented with 8% normal horse serum (NHS) (ATCC), 4% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin-streptomycin (Gibco) at 37 °C with 5% carbon dioxide (CO₂) as recommended by ATCC. CATH.a neurons were differentiated for 6 - 8 days before experimentation by adding \( N^6,2'\text{-O-dibutyryladenosine } 3',5'\text{- cyclic monophosphate sodium salt (dbcAMP, 1 mM, Sigma-Aldrich, St. Louis, MO) to the culture medium as previously described (207). Neurons were serum starved for 18-24 hours before treatment or experimentation. CATH.a neurons are derived from tyrosine hydroxylase (TH)-positive tumors in the locus ceruleus of transgenic mice. }
carrying the SV40 T-antigen oncogene. The CATH.a neuronal culture expresses tyrosine hydroxylase and dopamine β hydroxylase enzymes thereby producing norepinephrine and dopamine respectively. It should be noted that CATH.a neurons express both AT1 and AngII type 2 receptor (AT2R) and have been widely used by various groups to study AngII intra-neuronal signaling mechanisms (196, 207, 208).

**Liquid chromatography – tandem mass spectrometry (LC-MS/MS)**

CATH.a neuronal culture medium was collected after incubation (15 minutes – 24 hours) with AngII (100 nM). $^{13}$C- and $^{15}$N-labeled (Leu, + 7 amu) AngII (H-AngII, Anaspec, Fremont, CA) was added to the media samples at a concentration of 18.2 nM. Proteins were precipitated by addition of 6 volumes of cold neat acetone, typically 50 µl of sample and 300 µl of acetone and stored at -35 ºC for 1 hour. Then, the samples were centrifuged at 4 ºC for 15 minutes at 15,000 x g. Thereafter, the supernatants were removed and the acetone/water was removed by SpeedVac concentration for 2 - 3 hours at room temperature. The pellet was re-dissolved into 50 µl of 0.1% formic acid, vortexed, and centrifuged. The samples were loaded onto V-shaped polyethylene vials (Agilent, Santa Clara, CA) that were previously soaked with 0.1% w/v BSA and dried. The LC-MS/MS method was developed and used for the samples using an Agilent LC1200 HPLC system (Agilent, Santa Clara, CA) connected to an ABSciex QTrap4000 (ABSciex, Framingham, MA) operating in multiple reaction monitoring (MRM) mode with the electrospray operating in positive mode. Other ion-source
conditions were Temperature, 500 °C, ionization potential, 5500 V, GS1= 50, GS2= 25, curtain gas= 30. The transitions monitored and ionization parameters are shown in Table I. Transitions numbered 1,3,5,8 and 9 were used for quantitation while the remaining ones were used for confirmation of peak assignment. Column chromatography was performed via a 2.1 x 50 mm Kinetex C-18 300 Å (Phenomenex, Torrance, CA) at a flow rate of 250 µl/min with a mobile phase gradient from 98% A (0.1% formic acid in LC-water) to 98% B (0.1% formic acid in acetonitrile) over 20 min, with additional holding at 98% B for 2 mins and re-equilibration at 98% A for 10 min. Transitions were monitored with an acquisition time of 100 msec/MRM in non-scheduled mode. The data were analyzed using Analyst Ver 1.4.2. AngII levels were quantified using H-AngII as a standard. The other angiotensin peptides (Ang III, Ang IV, and Ang-1-7) were quantified by comparison to an external calibration curve of the unlabeled commercially available peptides, and H-AngII was used as a surrogate to correct for concentration loss of the peptides during sample preparation. The recoveries of the spiked H-AngII were equal to or higher than 80% for the analytical method. Individual samples were injected in triplicate and the average angiotensin peptide concentrations and standard error are reported. All reagents used for LC-MS/MS analysis were of Mass Spectrometry Grade and all unlabeled angiotensin peptide standards and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Statistical analysis**

All data are expressed as mean ± standard error of the mean (SEM) and were analyzed by Student’s t-test for two-group comparisons or by ANOVA followed by
Newman-Keuls correction for multiple comparisons. Statistical analyses were performed using GraphPad Prism 5.0 statistical and graphical software. Differences were considered significant at p<0.05.

Results

Utilizing liquid chromatography – tandem mass spectrometry to detect angiotensin peptides

AngII is generated from angiotensin converting enzyme (ACE) cleaving angiotensin I, which is produced by renin-induced cleavage of angiotensinogen. Although AngII is considered to be the primary effector peptide of the renin-angiotensin system, AngII can be further metabolized to other angiotensin peptides which have been shown to contribute to cardiovascular function (39, 207, 209). For example, AngII can be cleaved by angiotensin converting enzyme 2 (ACE2) (210-212) to form Ang-1-7 or by aminopeptidase A to form Ang III, which is further metabolized by aminopeptidase N to form Ang IV (Figure 2.1). Although the primary objective of this study was to examine the stability of exogenous AngII in neuronal cell culture media, it was also important to determine if exogenous AngII is metabolized to these other angiotensin peptides.
Figure 2.1. Schematic of the renin-angiotensin system. AngII is generated by angiotensin converting enzyme (ACE)-induced cleavage of angiotensin I. AngII is subsequently metabolized to Ang III and Ang-1-7 peptide via aminopeptidase A and angiotensin converting enzyme 2 (ACE 2), respectively. Ang III is further metabolized by aminopeptidase N to generate Ang IV.
As shown in the representative chromatogram (Figure 2.2), we were able to successfully separate and detect all four angiotensin peptides from a mixture containing the commercially available peptides ranging in concentration from 12-16 nM. AngII is an octapeptide which under the conditions of the chromatography should accumulate in the +3 charged state due to the positive charges at the terminal amino group as well as the Arg and His side chains. Upon loss of the C-terminus Phe, rendering Ang-1-7, the peptide is much less hydrophobic and thus binds less strongly to the C-18 side chains of the HPLC column particles. Since Phe is more hydrophobic than Asp, removal of Asp instead of Phe from AngII makes the Ang III peptide less hydrophobic than AngII but more than Ang-1-7. Therefore, Ang III elutes between Ang-1-7 and AngII (Figure 2.2). The hexapeptide Ang IV is generated from the removal of the N-terminus positive side chain Arg from Ang III, while keeping the hydrophobic residues Ile and Phe. As a result of Ang IV being the most hydrophobic, it has the strongest binding and thus appears as the last peak in the chromatogram (Figure 2.2). To accurately quantify the concentration of these peptides in our subsequent studies using media samples from the CATH.a neuronal culture, we utilized $^{13}$C- and $^{15}$N-labeled AngII (H-AngII).
Figure 2.2: Angiotensin peptides detected by liquid chromatography-tandem mass spectrometry. Representative chromatogram showing the detection of individual angiotensin peptides (AngII, Ang III, Ang IV, and Ang-1-7) in a mixture of the commercially available peptides in a concentration ranging from 12-16 nM. $^{13}$C- and $^{15}$N-labeled AngII (H-AngII) was also added to the mixture and used to quantify absolute amounts of the angiotensin peptides in the CATH.a neuronal cell culture media samples analyzed in subsequent experiments.
Exogenous AngII is rapidly metabolized in CATH.a neuronal cell culture media

Levels of AngII, Ang III, Ang IV, and Ang-1-7, were measured in CATH.a neuronal cell culture media 15 min – 24 hours after a single administration of exogenous AngII (100 nM) into the media. As expected, AngII was significantly elevated in the media 15 minutes after administration as compared to media collected immediately prior to treatment (Figure 2.3A). Levels of AngII rapidly decreased over time until 3 hours-post administration when levels of AngII in the media were significantly lower compared to the 15 min time-point and not significantly different than baseline levels. AngII remained low, that is, it was undetectable 6 and 24 hours after administration. Interestingly, levels of Ang III (Figure 2.3B) and Ang-1-7 (Figure 2.3D) modestly, but significantly, increased 15-60 minutes after exogenous AngII administration. In contrast, levels of Ang IV remained unchanged compared to baseline levels (Figure 2.3C). It should be noted that we specifically selected the AngII concentration of 100 nM for these studies because we wanted to replicate, as best we could, the experimental conditions used in other published studies(80, 83) designed to examine the AngII-induced acute and chronic responses in cultured neurons. For example, we previously used 100 nM AngII to study AngII-mediated intra-neuronal signaling in CATH.a neurons and have shown that at this concentration AngII-induced Nox4-generated mitochondrial superoxide production contributes to the rapid inhibition of outward
K⁺ current (83, 115). Furthermore, Sun et al. have reported that 100 nM AngII rapidly inhibits the delayed rectifier K⁺ current and increases neuronal firing rate in primary neurons isolated from the brain (80, 81). Additional studies examining more long-term changes (i.e. hours to days) in the expression of various proteins, including AngII receptors, potassium channels, and transcription factors, also used 100 nM AngII (85, 86, 201, 213).
Figure 2.3: Exogenous AngII levels rapidly decrease within 3 hours in CATH.a neuronal cell culture media. Levels of AngII (A), Ang III (B), Ang IV (C), and Ang-1-7 (D) in CATH.a neuronal cell culture media following a single administration of exogenous AngII (100 nM) as measured by liquid chromatography-tandem mass spectrometry. Absolute concentrations were calculated against the standard $^{13}$C- and $^{15}$N-labeled AngII peptide. n = 3 separate experiments performed in triplicate. *p < 0.05 vs. 0min AngII; #p < 0.05 vs. 15 min AngII.
**Exogenous AngII is stable in neuronal cell culture media in the absence of CATH.a neurons**

The rapid decrease in exogenous AngII levels in media collected from CATH.a neurons may suggest that the peptide is degraded by the media itself. To examine this possibility, AngII (100 nM) was added to the media in the absence of CATH.a neurons and samples were collected 15 min – 24 hours later (Figure 2.4). AngII levels remained significantly elevated at all time-points with concentrations near the starting concentration of 100 nM (Figure 4A). In contrast, levels of Ang III, Ang IV, and Ang-1-7 were not significantly different than baseline levels (Figure 2.4B-D).
Figure 2.4: AngII is stable in neuronal cell culture media in the absence of CATH.a neurons. Levels of AngII (A), Ang III (B), Ang IV (C) and Ang-1-7 (D) in cell culture media without CATH.a neurons following a single administration of exogenous AngII (100 nM) as detected by liquid chromatography and tandem mass spectrometry. Absolute concentrations were calculated against the standard $^{13}$C- and $^{15}$N-labeled AngII peptide. n = 3 separate experiments performed in triplicate. *p < 0.05 vs. 0 min.
Replenishing CATH.a neuronal cell culture media every 3 hours with fresh exogenous AngII results in chronic elevated levels

Finally, to determine if levels of AngII could be chronically elevated in CATH.a neuronal cell culture media, we administered fresh exogenous AngII (100 nM) every 3 hours and measured AngII for up to 24 hours. As shown in Figure 2.5, AngII levels remained similarly and significantly elevated (63-72 nM) at each time-point compared to baseline levels.
Figure 2.5: Repeated administration of exogenous AngII every 3 hours maintains a chronic elevated level of AngII in CATH.a neuronal cell culture media. Levels of AngII in CATH.a neuronal cell culture media following repeated administration of exogenous AngII (100 nM) into the media every 3 hours for 6, 9, 12, 15, 21, and 24 hours. Absolute concentrations were calculated against the standard $^{13}$C- and $^{15}$N-labeled AngII peptide. n = 3 separate experiments performed in triplicate. *p < 0.05 vs. 0 min.
Discussion

The pathogenesis of various cardiovascular disorders, such as hypertension and chronic heart failure, involves dysregulation of the brain angiotensinergic system (73, 89, 214). Understanding the precise intra-neuronal signaling mechanism(s) driving this dysregulation may lead to the identification of new targets for which novel therapeutics can be developed. As such, numerous studies have focused on examining the intra-neuronal signaling molecules and proteins mediating the physiological and pathophysiological responses of AngII (14, 97, 109). Many of these studies have relied on neuronal cell culture models to demonstrate a role for signaling intermediates, such as calcium, ROS, kinases, and transcription factors in mediating the AngII-induced response (80, 84, 86, 90). However, relating these in vitro observations to in vivo AngII-dependent cardiovascular diseases, such as hypertension and chronic heart failure, where circulating and central levels of AngII are chronically elevated (203, 204) is problematic as the stability of exogenously administered AngII in neuronal cell culture media is unknown. In the current study, we examined the stability of exogenous AngII in the culture media of an AngII-sensitive neuronal cell culture model, CATH.a neurons, that has commonly been utilized to study AngII intra-neuronal signaling (80, 85, 115). Herein, we report that levels of exogenous AngII are diminished to near baseline levels within 3 hours of treatment, and that media should be replenished with fresh AngII every 3 hours to maintain chronically elevated levels of the peptide in this neuronal cell culture model.

Acute stimulation (i.e. minutes) of cultured neurons with AngII results in a
rapid increase in intracellular calcium (84), inhibition of outward potassium current (I\textsubscript{\textit{K}}) (80, 83), and an increase in neuronal firing (81). Chronic stimulation (i.e. hours to days) of AngII-sensitive cultured neurons leads to alterations in the expression of angiotensin receptors (200, 202), potassium channel proteins (85), and transcription factors (201). However, in most, if not all, of these chronic stimulation studies a single administration of exogenous AngII was given to neurons in culture. This begs the questions of whether AngII remains stable in the culture media for hours or days to continuously activate its receptors to mediate these changes in protein expression or if these changes are a result of the immediate stimulation of AngII receptors followed by prolonged intra-neuronal signaling events. The more long term changes might be a result of downstream intermediate signaling events and not a direct effect of AngII binding to its receptors on these neurons. With these questions unanswered, it is difficult to compare results obtained from these types of \textit{in vitro} studies with data obtained from \textit{in vivo} cardiovascular disease models in which AngII receptors are likely constantly activated due to the chronically elevated levels of AngII. Our new data presented herein indicates that levels of exogenously administered AngII in neuronal cell culture media are decreased to near baseline levels within 3 hours; thus, suggesting that changes in protein expression at later time points are due to prolonged intra-neuronal signaling events rather than continuous activation of AngII receptors. In fact, we observed that within 15 minutes of treating cells with 100 nM AngII only 53±4 nM AngII remained in the media, and by 3 hours post-treatment less than 5 nM AngII remained. As we report, some of the exogenous
AngII is metabolized in the media to Ang III and Ang-1-7, as the concentration of these two peptides increased 15 minutes after AngII treatment. We speculate that most of the AngII lost within the first 15 minutes of treatment is bound to the AngII receptors and internalized via receptor-mediated endocytosis. In support of this hypothesis, previous studies using aortic smooth muscle cells have demonstrated that after AngII binds to its receptor the complex is internalized with a half-time of less than 2 minutes (215). In addition, previous reports indicate a functional role for internalized AngII after it has bound to the AT$_1$R and the complex has been endocytosed (216). For example, the complete AT$_1$R-induced activation of mitogen-activated protein kinase (MAPK) is believed to be dependent on internalization (217). Furthermore, nuclear membrane-associated angiotensin receptors have been identified (208, 218) and may be stimulated by internalized AngII and/or by AngII generated via an intracellular renin-angiotensin system (67).

After observing the rapid decrease in exogenously administered AngII in the media of our neuronal cell culture model, we questioned whether this was cell-mediated or non-specific degradation of AngII in the media. To address this question, we measured levels of AngII in culture media in the absence of CATH.a neurons after a single exogenous administration of 100 nM AngII, and found that AngII is relatively stable in the media. More specifically, the concentration of AngII at all time-points measured (15 minutes to 24 hours) was approximately 80 nM or greater. Taken together with our results in the presence of CATH.a neurons, these data suggest that that rapid loss of exogenous AngII in the media of
CATH.a neurons is mostly cell-dependent with the AngII being metabolized to Ang III and Ang-1-7, or binding to its receptors, along with some non-specific degradation of the peptide. As such, it is likely that the stability of exogenous AngII in cultured media will be drastically different between various cell types. That is, the expression levels of aminopeptidase A, which cleaves AngII to Ang III, ACE2, which cleaves AngII to Ang-1-7, and/or AngII receptors in a particular cell type will influence the stability of exogenous AngII in the respective media. Although we did not observe a change in Ang IV in the current study with CATH.a neurons, it is tempting to speculate that the amount of aminopeptidase N, which cleaves Ang III to Ang IV, expressed by a particular cell line will also contribute to the levels of these angiotensin peptides in culture media. Furthermore, it is possible that the stability of AngII in culture media may be experimentally manipulated by the exogenous administration of aminopeptidase inhibitors, ACE2 inhibitors, and/or AngII receptor antagonists.

In conclusion, our results demonstrate that a single exogenous administration of AngII to CATH.a neurons in culture does not result in chronically elevated levels of AngII in the media. As such, we sought to determine if multiple and sequential administration would keep AngII levels elevated chronically. Our data show that giving fresh AngII (100 nM) every 3 hours to cultured CATH.a neurons does indeed keep levels of AngII in the media significantly elevated for at least 24 hours. We believe cell culture models with chronically elevated levels of AngII are the ideal in vitro models that one should utilize to study the precise AngII-dependent intracellular signaling events that occur in cardiovascular
diseases associated with systemic and/or local chronically elevated levels of AngII, including hypertension and chronic heart failure. We posit that keeping levels of AngII chronically elevated in cell culture models will change the cellular response induced by AngII compared to the response induced by a single administration. Alternatively, it is possible that AngII levels may not need to be chronically elevated in cell culture media to induce long-term effects as the initial stimulation of AngII receptors may activate intracellular signaling pathways that lead to chronic changes in the expression of AngII receptors, ion channel proteins, and transcription factors. To address these hypotheses, our laboratory is currently investigating changes in AngII-dependent intracellular signaling events in CATH.a neurons exposed to single versus repeated administration of AngII. More specifically, we are examining changes in AngII receptor expression, levels of ROS and antioxidants, intracellular calcium concentration, activation of transcription factors, and expression of ion channels. Importantly, we are comparing any changes in response to single or repeated administration of AngII to those that we observe in cardiovascular control brain nuclei (e.g. SFO, PVN, RVLM) collected from animal models in which AngII is known to be chronically elevated in the brain, including hypertensive and chronic heart failure models. We believe these, and other, future studies will provide further insight into the importance of establishing cell culture models with chronically elevated levels of AngII to improve our understanding of AngII intracellular signaling pathways.
Chapter III: Overexpression of wild-type calcium/calmodulin-dependent protein kinase IIα potentiates angiotensin II-mediated signaling in neurons

Introduction

As described in the previous chapter, we have shown that exogenous AngII added to neuronal cells in culture is metabolized within 3 hours; whereas, at 15 minutes post-treatment levels of AngII remain significantly elevated in the culture media. In the current chapter, my overall objective was to determine the contribution of CaMKII in AngII intra-neuronal signaling. Considering the observations discussed in the previous chapter, I decided to focus on the acute (i.e. 5-10 minutes) AngII-induced physiological response, which in cultured neurons is a change in ion channel activity. In fact, AngII has been shown to regulate neuronal ion channels, including the voltage-gated potassium and calcium channels (80, 84). However, the signaling mechanism(s) driving this response has yet to be precisely identified.

Several studies by Sun and colleagues have shown that AngII can regulate neuronal K⁺ current (I_{KV}) thereby increasing neuronal firing rate and causing neuronal activation (80, 81). More specifically, they showed that AngII inhibits I_{KV}, particularly the delayed rectifier K⁺ current and this results in an increased frequency of neuronal firing both in primary neurons isolated from rat hypothalamus and brain stem, and in a mouse catecholaminergic neuronal cell line (CATH.a neurons)(80, 81). Additionally, they showed that the AngII-induced increase in neuronal firing rate was significantly attenuated in the presence of the
AngII type 1 receptor (AT1R) antagonist losartan (79). Interestingly, these studies also demonstrated that the AngII-mediated reduction in $I_{KV}$ and increased neuronal firing rate involves signaling intermediates, such as phospholipase C (PLC), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase II (CaMKII) (80, 81, 219). These studies elucidated that inhibition of PLC, PKC or CaMKII using U73122, calphostin C or KN-93, respectively, attenuates AngII-mediated reduction of $I_{KV}$ and increased neuronal firing suggesting a critical role for these signaling proteins in AngII-induced neuronal responses. Although these data suggest a role for CaMKII in AngII signaling in central neurons, the non-specificity of the CaMKII inhibitor, KN-93, used in these previous studies brings into question the precise CaMKII isoform involved. As such, we set out to specifically investigate the contribution of the predominant neuronal isoform of CaMKII (CaMKIIα) in mediating the AngII-induced regulation of neuronal ion channels.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional enzyme which couples increases in intracellular Ca$^{2+}$ to regulation of ion channels. Under resting conditions CaMKII remains inactive, but upon binding with Ca$^{2+}$/CaM the autoinhibitory domain of CaMKII is relieved from the kinase domain and undergoes inter-subunit autophosphorylation of a threonine residue (T286 in neuronal CaMKIIα isoform). Autophosphorylation in the regulatory domain causes CaMKII activation enabling it to phosphorylate several target proteins. Several protein phosphatases, like protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) can then dephosphorylate CaMKII
leading to its inactive resting state (184-186). The switch between \( \text{Ca}^{2+}/\text{CaM} \)-dependent and \( \text{Ca}^{2+}/\text{CaM} \)-independent sustained CaMKII activation is therefore important in determining the role of CaMKII in physiological and pathological conditions. Interestingly, recent studies have shown that the AngII-mediated pathological responses in the heart involves a second \( \text{Ca}^{2+}/\text{CaM} \) independent activity of CaMKII under elevated ROS conditions. These studies have identified the CaMKII\( \delta \) isoform, which is primarily expressed in cardiomyocytes, as a downstream target of AngII in case of myocardial infarction and ischemic stress responses during heart diseases (181, 187, 220). Elevated levels of ROS in presence of AngII can cause oxidation of a paired methionine residue (M281/282) in CaMKII\( \delta \), which thereby leads to sustained CaMKII\( \delta \) activation independent of \( \text{Ca}^{2+}/\text{CaM} \) and mediates AngII-stimulated apoptosis in the heart. Additionally, the study also found that substituting the first methionine (M281) of CaMKII\( \delta \) with a cysteine residue, which is similar to the CaMKII\( \alpha \) isoform, also sustains CaMKII\( \delta \) activation under oxidant conditions. These data provide insight into the redox sensitivity of CaMKII\( \alpha \) which has a cysteine/methionine (C280/M281) instead of the paired methionine residues.

Studies in our lab have previously shown that in CATH.a neurons AngII rapidly activates CaMKII by increasing CaMKII phosphorylation with a significant increase observed after 5 minutes of AngII stimulation (83). However, in that study, neurons which were transduced with AdMnSOD (adenovirus overexpressing manganese superoxide dismutase) had reduced CaMKII phosphorylation, indicating the involvement of ROS in mediating AngII-induced
CaMKII phosphorylation. Additionally, scavenging superoxide in CATH.a neurons also attenuated the AngII-mediated reduction in $I_{KV}$. In an attempt to identify specific voltage-gated $K^+$ channels involved in AngII-mediated increased neuronal firing, Gao et al., have showed that expression of Kv4.3 is downregulated in presence of AngII stimulation in CATH.a neurons (85). However, the precise mechanism(s) by which AngII regulates CaMKIIα thereby modulating neuronal ion channels is not fully understood.

Furthermore, as discussed in Chapter I, AngII in the brain acts on specific nuclei that are important in autonomic control of cardiovascular function, including the paraventricular nucleus (PVN), rostral ventrolateral medulla (RVLM), and nucleus tractus solitarius (NTS) (193, 195, 196). AngII can also activate neurons of circumventricular organs, such as the subfornical organ (SFO) and lead to sympathoexcitation, which contributes to hypertensive symptoms, such as increased vasoconstriction, enhanced salt and water reabsorption, increased heart rate, and activation of T-lymphocytes and inflammatory cytokines (194, 197-199). Past studies have also demonstrated that scavenging superoxide with adenoviral-mediated overexpression of copper-zinc superoxide dismutase (AdCuZnSOD) in the SFO attenuates blood pressure in an AngII-infused hypertensive animal model (90). However, the role of downstream effector molecules, such as CaMKIIα in the brain in mediating the AngII-induced hypertensive response in animal model is unclear.

In the present study we tested the hypothesis that adenovirus-mediated overexpression of the wild-type predominant neuronal isoform of CaMKII (wt-
AdCaMKIIα) exacerbates the AngII-mediated inhibition of neuronal K⁺ current and potentiates the AngII-induced hypertensive response in mice. We show that wt-CaMKIIα overexpression indeed intensifies the reduction of I_{KV} in AngII-stimulated CATH.a neurons and also enhances the AngII-induced pressor response in mice. Overall, this study implicates CaMKIIα as a key mechanistic player in AngII-signaling in central neurons.

**Experimental Methods**

**CATH.a neuronal cell culture**
For detailed descriptions of mouse CATH.a neuronal cell culture and differentiation, see experimental methods in Chapter II. During adenovirus transduction cells were treated with serum free media with dbcAMP for 18-24 hours following which virus was removed and media replaced with serum full media with fresh dbcAMP.

**Adenoviral vector construction**
Replication-deficient recombinant adenovirus (Ad5-CMV) encoding mouse wild-type calcium/calmodulin-dependent protein kinase IIα (wt-AdCaMKIIα) was generated. CaMKIIα plasmid (Origene) was amplified by conventional Polymerase Chain Reaction (PCR) using the HotStart PCR Master Mix (Qiagen, Venlo, Limburg) and the following forward and reverse primers: forward 5’- GAA TTC ATG GCT ACC ATC ACC TGC ACC C – 3’; reverse 5’ – GGA TCC TCA ATG CGG CAG GAC GGA - 3’. The PCR product was then run on a 1% agarose gel, followed by gel extraction utilizing QIA quick gel-extraction kit (Qiagen). A pJet1.2
PCR clone jet kit (Thermo Scientific) was used to clone CaMKIIα insert in various vector to insert ratios to determine the optimum ratio for cloning. It was then transformed into DH5 alpha bacteria and grown overnight onto ampicillin treated plates at 37°C in a bacterial incubator. Bacterial colonies were picked and grown in luria broth (LB) media overnight by shaking. PureLink Quick Plasmid DNA Miniprep Kits (Invitrogen, Grand Island, NY) were used to isolate CaMKIIα plasmid DNA as per manufacturer’s instructions. Following quantification of the DNA using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) 1µg DNA was digested with EcoR1 and BaMH1 (New England Biolabs, Ipswich, MA) restriction enzymes and then run on a 1% agarose gel to confirm the presence of the correct insert. The plasmid samples were then sent off to the UNMC Gene Sequencing Core. Once the wt-CaMKIIα sequence was confirmed it was then cloned into an expression plasmid, transformed into DH5 alpha bacteria, DNA isolated, digested using restriction enzymes and again sent to the UNMC Gene sequencing core to confirm the presence of correct wt-CaMKIIα sequence in the expression plasmid. Adenoviral vectors were then constructed, purified and provided by the University of Iowa Gene Vector Core as previously described (83, 221).

**RNA extraction, cDNA and quantitative real-time RT-PCR**

To confirm the overexpression of wt-CaMKIIα at the transcriptional level and to evaluate the effect of wt-CaMKIIα overexpression on other CaMKII isoforms, total RNA was extracted from differentiated CATH.a neurons using the Trizol method (Life technologies, Carlsbad, CA) according to the manufacturer’s protocol. RNA
concentration was determined spectrophotometrically by using a Nanodrop 2000 Spectrophotometer (Thermo Scientific). 1 µg of each sample RNA was reverse transcribed and cDNA was generated by Taqman reverse transcription kit (Applied Biosystems, Foster City, CA). SYBR green (Applied Biosystems) quantitative real-time PCR was performed with generated cDNA with primers specific to the coding sequence of CaMKIIα, CaMKIIβ, CaMKIIδ, CaMKIIγ. Additional primers specific to voltage-dependent K⁺ channels such as Kv2.1, Kv2.2, Kv4.2 and Kv4.3 were also used. Primer sequences were as follows: CaMKIIα forward, 5’- TGA CAG CCT TTG AAC CAG AG - 3’; CaMKIIα reverse, 5’- CAA AAT AGA ATC GAT GAA AGT CCA - 3’; CaMKIIβ forward, 5’- GGA CAT CTG GGC ATG TGG - 3’; CaMKIIβ reverse, 5’- CAC TCA GGG GAT GGG AAA T -3’; CaMKIIδ forward, 5’- TCA ACC CTG CCA AAC GTA TC - 3’; CaMKIIδ reverse, 5’ – GAG GCA ACA GTA GAG CGT TGA - 3’; CaMKIIγ forward, 5’ – GCT CAA TGT CCA CTA TCA CTG C - 3’; CaMKIIγ reverse, 5’ – TCA CTC AGG CCC TCC AGA C – 3’; Kv2.1 forward, 5’ – GGA GAA GCC CAA CTC ATC G - 3’; Kv2.1 reverse, 5’ – TGT TGA GTG ACA GGG CAA TG – 3’; Kv2.2 forward, 5’ – CCG GAG AAA CGG AAG AAA C – 3’; Kv2.2 reverse, 5’ – ATA GAC ACG ATG GCC AGG AT – 3’; Kv4.2 forward, 5’ – GCT TTG AGA CAC AGC ACC AC – 3’; Kv4.2 reverse, 5’ – TGT TCA TCG ACA AAC TCA TGG – 3’; Kv4.3 forward, 5’ – TGC CAC TGT GAT GTT TTA TGC – 3’; Kv4.3 reverse, 5’ – TCT TAG GCA CCA TGT CTC C – 3’.

The PCR parameters were 95⁰C for 10 minutes, followed by 40 cycles of 95⁰C for 15 seconds and 60⁰C for 1 minute. Thermal dissociation was used to determine PCR product specificity. Briefly, a threshold in the linear range of PCR
amplification was selected and the cycle threshold (Ct) was determined. Levels of transcripts were then normalized to the 18S rRNA loading control and compared relative to the control sample using the $\Delta\Delta$ Ct method.

**Western blot analysis**

To determine the optimal overexpression of CaMKIIα, CATH.a neurons were transduced with wt-AdCaMKIIα on day 3 of differentiation and Western blot analysis was performed to confirm CaMKIIα overexpression. Protein expression of total CaMKIIα, phosphorylated CaMKIIα (P-CaMKIIα) and actin was determined using standard Western blot analysis in whole cell lysates prepared from differentiated CATH.a neurons either non-transduced or transduced with control adenoviral vector, AdEmpty (10, 25 and 50 multiplicity of infection (MOI)), or wt-AdCaMKIIα (10, 25 and 50 MOI). Briefly, cells were harvested in phosphate buffer saline (PBS) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor (Thermo Scientific) and then sonicated. Cell lysates (25-30 µg of protein per lane) were loaded onto 12.5% Bis-Tris gels, separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated at 4 ºC overnight with primary antibodies directed against CaMKIIα (1:1000 dilution, abcam, Cambridge, UK) P-CaMKIIα (1:1000 dilution, abcam) and actin (1:1000 dilution, Sigma-Aldrich). After incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000 dilution, Thermo Scientific) for two hours at room temperature, bands were visualized using the Pierce enhanced chemiluminescence detection system (Thermo Scientific, Rockford, IL). Densitometric analysis of band density was determined using National
Institutes of Health (NIH) ImageJ analysis software. Values were normalized to actin to correct for any variations in protein loading.

**Electrophysiological record of voltage-gated K⁺ currents**

Differentiated CATH.a neurons either non-transduced or transduced with AdEmpty (25MOI), or wt-AdCaMKIIα (25MOI) were used to measure K⁺ currents (I\textsubscript{KV}). I\textsubscript{KV} was recorded by the whole-cell configuration of the patch-clamp technique using an Axopatch 200-B patch-clamp amplifier (Axon Instruments, Inc, Union City, CA). Briefly, in the voltage-clamp experiments, resistance of the patch pipette was 4-6 M\(\Omega\) when filled with the following solution (in mM): 130 KCl, 2 MgCl\(_2\), 0.25 CaCl\(_2\), 5 EGTA, 1 Mg-ATP, 0.1 Tris-GTP, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 8 glucose, pH 7.2. The extracellular solution consisted of (in mM): 137 NaCl, 5.4 KCl, 1.35 CaCl\(_2\), 2 MgCl\(_2\), 0.3 NaH\(_2\)PO\(_4\), 10 HEPES, 10 sucrose, pH 7.4. To selectively record I\textsubscript{KV}, Na\(^+\) and Ca\(^{2+}\) channels were blocked by 0.5µM tetrodotoxin (TTX, Sigma-Aldrich) and 0.3 mM CdCl\(_2\) (Sigma Aldrich), respectively. Current traces were sampled at 10 kHz and filtered at 5 kHz. Holding potential was -80 mV and current-voltage (I-V) relationship was elicited by 10 mV step increments to potentials between -80 and +80 mV for 400 ms. The change of I\textsubscript{KV} with AngII was tested by superfusing CATH.a neurons with AngII (100 nM) for 5 minutes and repeating the voltage pulse regimen. To confirm the observed changes with AngII, I\textsubscript{KV} was recorded again after 15 minutes of washout. Cells that responded to AngII stimulation and then recovered with the washout were considered for data analysis. Current density of I\textsubscript{KV} was calculated by dividing the respective current by cell membrane
capacitance ($C_m$). The $C_m$ of CATH.a neurons utilized in the study ranged from 10-60 pF and was not significantly different between the groups. The pClamp 10.2 program (Axon Instruments) was used for data acquisition and analysis. All experiments were carried out at room temperature.

**In vivo mouse studies**

All animal procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee, and were performed in accordance with the Guidelines for the Care and Use of Experimental Animals of the American Physiological Society and the National Institutes of Health. Adult C57BL/6 male mice 7-8 weeks of age (20 - 26 g body weight) were acquired from Harlan laboratories (Indianapolis, IN) for the *in vivo* experiments. Mice were individually housed in temperature and humidity-controlled cages at the University of Nebraska Medical Center animal facilities with a 12 hour light-dark cycle. Mice were provided standard chow and water ad libitum. At the end of each experiment mice were euthanized with intraperitoneal injection of pentobarbital (150 mg/kg).

**Implantation of radiotelemeters in mice**

For blood pressure recordings anesthetized mice were surgically instrumented with left carotid radiotelemeters (TA11PA-C10 catheter, Data Sciences International, St. Paul, MN). Mice were anesthetized with 0.5-2.0% isoflurane and a ventral midline skin incision was made from the jaw to the sternum to carefully isolate the common carotid artery. A tiny incision was made in the
carotid artery and the telemeter catheter was advanced so that the tip was positioned inside the thoracic aorta. The transmitter of the radiotelemeter was placed in a subcutaneous pouch in the right abdominal flank of each mouse. The neck incision was then closed with an interrupted suture using a prolene monofilament suture material (Ethicon, Inc). Topical bupivacaine (AAP Pharmaceuticals, LLC, India) was applied on the surgical site. For real-time measure of cardiovascular parameters, such as mean arterial pressure, systolic pressure, diastolic pressure, heart rate and pulse pressure, mice were placed on individual telemeter receivers after magnetically activating the radiotelemeters. Data were viewed using Dataquest Advanced Research Technology version 4.1 Data Acquisition and Analysis software and analyzed with Microsoft Excel.

![Image](www.ddah.org.uk/phenotyping-telemetry.htm)

**Figure 3.1: Placement of radiotelemeter catheter in the intracarotid artery.** Image from: [www.ddah.org.uk/phenotyping-telemetry.htm](http://www.ddah.org.uk/phenotyping-telemetry.htm)

**Intracerebroventricular (ICV) cannula implantation in mice**

Anesthetized mice were strategically placed on a stereotaxic apparatus (World Precision Instruments) with the help of ear bars. A ventral midline incision was made on the scalp and the head was leveled so as the lambda and bregma was
in the same horizontal plane. An anchoring screw was inserted into the right frontal bone slightly anterior to the coronal suture. The cannula (25 gauge, Plastic One, Roanoke, Virginia) was then placed 0.3 mm posterior and 1 mm lateral to the bregma and a hole was dilled following which the cannula was inserted into the hole 3.3 mm below the bregma and affixed to the skull with acrylic dental cement (Stoelting, Wood Dale, Illinois). A stylus, specifically fitted to the length of the cannula, was inserted into the cannula to maintain an unobstructed opening into the ICV system. Note that mice which received both radiotelemeter implantation and ICV cannula had their cannulas placed on the same day immediately following radiotelemetry surgery.

Intracerebroventricular administration of AngII and AdGFP and wt-AdCaMKIIα adenoviruses

All ICV injections were made through a 33-gauge stainless steel injector connected to a Hamilton microsyringe via PE-10 tubing. For the acute studies, following 1 week of recovery from ICV cannula and radiotelemetry implantation surgery, the mice received a single acute ICV AngII (350 ng) injection. Blood pressure and heart rate responses were recorded in conscious freely, moving mice for 20 minutes after injection. 3 days of acute AngII response was recorded prior to injecting adenoviruses. Mice were injected with either ICV AdGFP or wt-AdCaMKIIα (5x10⁷ plaque forming units). Following adenovirus injection, baseline blood pressure was recorded for 3 days after which acute physiological experiments were performed again in presence of ICV AngII. For
immunohistochemistry studies, 7 days after adenovirus injection, mice were euthanized with intraperitoneal injection of fatal plus (40 mg/kg, Henry-Schrein, Inc., Melville, NY). Brains were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) and then stored in 30% sucrose in PB overnight prior to sectioning. For the chronic AngII-induced hypertension studies ICV cannula-instrumented mice received either AdGFP or wt-AdCaMKIIα adenovirus (5x10^7 plaque forming units) 3 days prior to osmotic minipump implantation.

Figure 3.2: Intracerebroventricular cannula implantation for AngII and adenovirus injection to the subfornical organ. Modified from: Peterson et al. Hypertension. 2009 (111).

Subcutaneous implantation of osmotic minipumps in mice

For chronic AngII infusion studies mice were implanted 3 days post adenovirus injection with osmotic minipumps (Alzet, Durect Corporation, Cupertino, CA) subcutaneously to deliver either Ang II (400 ng·kg⁻¹·min⁻¹) or saline for approximately 3 weeks. It is to be noted that the dose of AngII was selected based on previous studies demonstrating that this slow pressor model of AngII
hypertension induces a gradual increase in blood pressure that mimics human essential hypertension (222, 223). The osmotic minipumps were primed at least for 12 hours in saline at 37\(^\circ\)C prior to implantation. On the day of surgery, mice were anesthetized (0.5 – 2.0\% isoflurane) and the left flank was shaved. A small horizontal incision was made in the left flank and a subcutaneous pocket was created to insert the osmotic minipumps. The incision was closed with an interrupted suture using a prolene monofilament suture (Ethicon, Inc.). Topical bupivacaine (AAP Pharmaceuticals) was then applied to the incision site.
Figure 3.3: Timeline for acute central AngII-mediated pressor response prior to and following central administration of wt-CaMKIIα adenovirus.

Figure 3.4: Timeline for chronic subcutaneous AngII-mediated pressor response following central administration of wt-CaMKIIα adenovirus.
**Immunohistochemistry**

To verify the overexpression of CaMKIIα in mouse brain tissues immunohistochemical staining was performed in brain sections 7 days post ICV adenovirus injections. 20 µm sections of the mouse brains were prepared using a cryostat (Leica CM3050S) and immunohistochemistry was carried out. Sections were first incubated for 1 hour with blocking buffer (10% NHS in 0.1 M PB) and then incubated at 4 ºC overnight with a rabbit primary antibody against CaMKIIα (1:200, abcam) and a rabbit P-CaMKIIα (1:200, abcam) prepared in 0.1 M PB containing 2% NHS and 0.3% Triton X-100. The sections were then washed in 0.1 M PB and incubated with Alexa Fluor 598 secondary antibody (1:200, Invitrogen) at room temperature for 2 hours. Fluorescent images of the subfornical organ and ventricles were detected by confocal laser scanning microscopy (Zeiss LSM 510 Meta Confocal Microscope) at 20X and 40X magnification.

**Statistical Analysis**

All data are presented as means ± standard error of the mean (SEM). Data were analyzed by Student's t-test for two group comparisons or by ANOVA with Newman Keuls post-hoc test. P-value less than 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.0 statistical and graphical software.
Results

Adenoviral-mediated transduction of wt-CaMKIIα increases total and phosphorylated CaMKIIα protein in CATH.a neurons

To confirm the adenovirus efficiency and overexpression of CaMKIIα, mouse CATH.a neurons were either non-transduced or transduced with control adenovirus, AdEmpty, or wt-AdCaMKIIα at 10, 25 and 50MOI on day 3 of differentiation. As compared to non-transduced or AdEmpty-transduced neurons, CATH.a neurons transduced with wt-AdCaMKIIα had a significant increase in total CaMKIIα protein levels at 25 and 50 MOI as detected by Western blot analysis (Figure 3.5A). This was accompanied by an increase in phosphorylated levels of CaMKIIα suggesting an increase in active CaMKIIα in CATH.a neurons at 25 and 50 MOI (Figure 3.5B). CaMKIIα mRNA overexpression was also confirmed by real-time quantitative PCR in differentiated CATH.a neurons. While transduction with wt-AdCaMKIIα resulted in a significant dose-dependent increase in CaMKIIα mRNA in CATH.a neurons, the other isoforms of CaMKII, such as CaMKIIβ, CaMKIIδ and CaMKIIγ did not change in between groups (Figure 3.5C). Kinase activity assay was utilized to measure CaMKII activity in AdEmpty (25MOI) and wt-AdCaMKIIα (25MOI)-transduced CATH.a neurons. Neurons with wt-CaMKIIα overexpression had 3-fold higher activity as compared to AdEmpty-transduced neurons (n = 2).
Figure A: Western blot analysis of CaMKIIα, P-CaMKIIα, and Actin expression in cells transduced with AdEmpty or wt-AdCaMKIIα at different MOIs (10, 25, 50).

Figure B and C: Quantitative analysis of CaMKIIα and P-CaMKIIα expression, showing significant increases at 50 MOI compared to non-transduced and AdEmpty groups.
Figure 3.5. Transduction of wt-CaMKIIα adenovirus causes significant upregulation of CaMKIIα expression and activity in CATH.a neurons. (A) Representative Western blot analysis of CATH.a neuronal cell lysates probed for primary antibody against total and phosphorylated CaMKIIα; Summary densitometry data of Western blot analysis showing levels of (B) CaMKIIα and (C) P-CaMKIIα in non-transduced, AdEmpty (10, 25 and 50 MOI)- and wt-AdCaMKIIα (10, 25 and 50 MOI)-transduced neurons; Real-time RT-PCR data with primers specific for CaMKIIα (D), CaMKIIβ (E), and CaMKIIδ (F) genes normalized to 18s rRNA and expressed as fold change; (G) CaMKII activity in AdEmpty (25MOI) and wt-AdCaMKIIα (25MOI)-transduced neurons as measured by kinase activity assay and reported as fold change from control. *P<0.05 vs. non-transduced and AdEmpty.
Wild-type CaMKIIα overexpression potentiates AngII-mediated inhibition of K⁺ channel current in CATH.a neurons

To investigate the effect of wt-CaMKIIα overexpression on the AngII-dependent changes in electrophysiological properties, voltage-gated K⁺ channel current (I_{KV}) was measured in differentiated non-transduced, either AdEmpty or wt-AdCaMKIIα-transduced CATH.a neurons. Whole-cell patch-clamp technique was used to record baseline I_{KV} and I_{KV} after 5 minutes of AngII (100 nM) superfusion. Compared to baseline, 5 minutes of AngII superfusion inhibited I_{KV} by 20% in non-transduced and AdEmpty-transduced CATH.a neurons and this effect of AngII could be restored after AngII washout (Figure 3.6). Interestingly, AngII superfusion caused a significant reduction in I_{KV} by approximately 37% in wt-AdCaMKIIα-transduced CATH.a neurons. It is to be noted that AngII attenuated both transient K⁺ current (I_{peak}) and the steady-state K⁺ current (I_{ss}) to a similar extent in CATH.a neurons as previously described (241). To confirm our finding that the AngII-induced reduction in I_{KV} was mediated by CaMKII, CATH.a neurons were pretreated for 30 minutes with the CaMKII inhibitor, KN-93 (10 µM) and baseline I_{KV} recordings were measured followed by 5 minutes of AngII superfusion and wash out. The AngII-mediated reduction in I_{KV} was significantly abolished in presence of KN-93. These data indicate that wt-CaMKIIα overexpression enhances the AngII-induced inhibition of neuronal K⁺ channel current in CATH.a neurons.
Figure 3.6. Wild-type CaMKIIα overexpression potentiates the AngII-mediated inhibition of outward K⁺ current in CATH.a neurons. Representative traces of evoked K⁺ currents in CATH.a neurons superfused with vehicle or 100 nM AngII (A) and percent inhibition of outward K⁺ current following 5 minutes of AngII (100 nM) superfusion pretreated for 30 minutes in the absence or presence of 10 µM KN 93 (B) in non-transduced, AdEmpty (25MOI) or wt-AdCaMKIIα (25 MOI)-transduced CATH.a neurons (n = 7 biological replicates for all experimental groups). *P < 0.05 vs. non-infected. #P < 0.05 vs. no KN 93.
Wild-type CaMKIIα overexpression upregulates mRNA expression of Kv channels in CATH.a neurons

To determine the causal relationship between overexpression of wt-CaMKIIα and significant reduction in K⁺ current in CATH.a neurons, we sought to detect mRNA expression of specific voltage-dependent K⁺ channels which have previously been shown to be present in important autonomic regions of the brain and also involved in AngII-mediated signaling in neurons (85, 224). Quantitative real-time PCR was utilized with specific primers for individual Kv channel transcript to detect mRNA levels of Kv2.1, Kv2.2, Kv4.2 and Kv4.3. Interestingly, as compared to AdEmpty-transduced CATH.a neurons, transduction of wt-AdCaMKIIα modestly increased mRNA expression of the above mentioned Kv channels in CATH.a neurons at baseline (Figure 3.7). These data indicate that overexpression of wt-CaMKIIα leads to transcriptional upregulation of these voltage-dependent K⁺ channels which may act as a compensatory mechanism to counteract the decrease in channel current.
Figure 3.7. Overexpression of wt-CaMKIIα modestly increase mRNA expression of voltage-dependent K⁺ channels in CATH.a neurons. Quantitative real-time PCR analysis showing (A) Kv2.1, (B) Kv2.2, (C) Kv4.2 and (D) Kv4.3 mRNA levels in AdEmpty (25MOI) and wt-AdCaMKIIα (25MOI) transduced CATH.a neurons. n=3 biological replicates in all experimental groups.
Overexpression of wt-CaMKIIα potentiates the central acute AngII-induced increase in mean arterial pressure

Our *in vitro* studies suggest that overexpressing CaMKIIα in CATH.a neurons exacerbates the AngII-mediated reduction of neuronal K⁺ channel current. To determine the role of CaMKIIα overexpression in the brain on central actions of AngII *in vivo*, C57BL/6 male mice underwent radiotelemetry implantation and intracerebroventricular (ICV) cannula surgeries. Following a week’s recovery, blood pressure responses to ICV administered AngII (350 ng) were recorded in conscious mice. As seen in the representative recordings mice receiving acute ICV AngII had a characteristic significant increase in blood pressure as compared to their baseline blood pressure (Figure 3.8A). Interestingly, 3 days post adenovirus injection, the AngII-mediated rise in blood pressure was further potentiated in ICV AdCaMKIIα-injected (n = 9) mice as compared to AdGFP-injected (n = 9) mice. The peak changes in mean arterial pressure following acute ICV AngII injections in AdCaMKIIα and AdGFP mice group are summarized in Figure 3.8B. In addition, the duration of the AngII-induced pressor response was also longer in CaMKIIα-overexpressed mice as compared to mice receiving AdGFP (Figure 3.8C). The duration of the AngII-mediated response was calculated from the start of the peak AngII-induced rise in blood pressure until the time when blood pressures came back to half the maximal response.
Figure 3.8. Overexpression of wt-CaMKIIα in the brain potentiates the pressor response of central acute AngII. (A) Representative mean arterial pressure tracings and (B) summary data showing AngII-induced acute pressor response in mice injected with ICV AngII (350 ng) both pre- and 3 days post-ICV injection of AdGFP or wt-CaMKIIα adenovirus. (C) Duration (in minutes) of the AngII-mediated rise in blood pressure in AdGFP and wt-AdCaMKIIα injected mice. *P < 0.05 vs. AdGFP. n = 8-9 mice for each experimental group.
To confirm efficient adenovirus-mediated overexpression of wt-CaMKIIα in the subfornical organ (SFO), an important cardiovascular regulatory brain nucleus, immunohistochemistry was performed on AdGFP and wt-AdCaMKIIα-injected mice brain tissue sections 7 days post ICV adenovirus injections. As shown in the representative confocal microscopy images, the SFO of wt-AdCaMKIIα injected mouse brains possessed higher levels of CaMKIIα fluorescence in comparison to AdGFP brain sections (Figure 3.9). Together, the data presented here strongly indicate that CaMKIIα in the brain is an important player in central AngII-mediated cardiovascular effects.
Figure 3.9. Adenoviral-mediated overexpression of wt-CaMKIIα in the subfornical organ (SFO). Representative confocal microscopy images showing expression of (A) GFP, CaMKIIα and (B) GFP, P-CaMKIIα in SFO of mice 1 week after intracerebroventricular (ICV) injection of AdGFP or wt-AdCaMKIIα. Coronal brain sections (20 µm) were stained for GFP (green), total and phosphorylated CaMKIIα (red). Double staining is shown in the merged (yellow) images. Scale bar = 100 µm
Adenovirus-mediated wt-CaMKIIα overexpression in the brain sensitizes mice to an immediate increase in blood pressure following subcutaneous AngII infusion

We next evaluated the effect of ICV administered wt-CaMKIIα on the slow pressor AngII-induced hypertensive model. Similar to the acute AngII studies, mice were implanted with radiotelemeters and ICV cannulas for adenovirus injections. Following 1 week of recovery from surgery ICV AdGFP and wt-AdCaMKIIα adenovirus was injected. Blood pressure and heart rate responses were recorded in conscious mice after subcutaneous AngII (400 ng/kg/min) or saline osmotic minipump implantation 3 days post adenovirus injection. Interestingly, blood pressure was significantly elevated following 2 days of AngII infusion in wt-AdCaMKIIα-injected mice group and continued to increase gradually until day 23 when the minipumps emptied and blood pressure came back to basal levels (Figure 3.10A). In contrast, blood pressure in AdGFP-injected mice increased gradually with AngII infusion with a peak at around day 16 and then came back to baseline levels at day 23-24 when the osmotic minipumps were empty. It should be noted that the blood pressure response following subcutaneous AngII infusion in case of AdGFP-injected mice is characteristic of a slow pressor AngII-induced hypertensive model. Systolic and diastolic blood pressures followed similar pattern as the mean arterial pressure (Figure 3.10B and C). Heart rate was not significantly different in between AdGFP and wt-AdCaMKIIα groups infused with AngII. Blood pressure and heart rate
responses did not differ in case of saline infused animals. Overall, these studies indicate that CaMKIIα overexpression in the brain sensitizes mice to an immediate increase in blood pressure at an initial subpressor dose of subcutaneous AngII.
Figure 3.10. Overexpression of wt-CaMKIIα in the brain sensitizes mice to an immediate increase in blood pressure following chronic subcutaneous AngII infusion. Representative line graphs showing (A) mean arterial pressure, (B) systolic blood pressure, (C) diastolic blood pressure and (D) heart rate recorded via radiotelemetry in C57BL/6 mice at baseline, following intracerebroventricular injection of AdGFP or wt-AdCaMKIIα, and after subcutaneous implantation of osmotic minipumps set to release saline or AngII (400 ng/kg/min). *P < 0.05 vs. AdGFP mice with AngII infusion. n = 6-9 mice for each experimental group.
Discussion

Considering the importance of AngII signaling in central neurons and its contribution to neuro-cardiovascular diseases, such as hypertension and heart failure, it is necessary to investigate the intra-neuronal signaling mechanisms mediated by AngII and the role of secondary intermediates in AngII-neurogenic hypertension. In central neurons AngII increases intracellular Ca\(^{2+}\), activates redox mechanisms including NADPH oxidases, signaling intermediates, and transcription factors leading to a variety of physiological and pathophysiological responses (13, 84, 86, 115, 213). However, the underlying mechanism by which specific redox-sensitive downstream signaling molecules, more specifically neuronal calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) modulates AngII-signaling in neurons is unclear. We report here that adenoviral-mediated overexpression of wt-CaMKIIα in central neurons potentiates the AngII-regulated intra-neuronal signaling and the AngII-induced hypertensive response. The main findings from these set of studies can be summarized as follows: (1) wt-CaMKIIα overexpression in mouse catecholaminergic neurons (CATH.a neurons) exacerbates the AngII-mediated inhibition of neuronal K\(^+\) channel current (I\(_{kv}\)); (2) intracerebroventricular (ICV) injection of wt-CaMKIIα in the brain potentiates the central acute AngII-induced pressor response; and (3) ICV injection of wt-CaMKIIα sensitizes mice to an immediate increase in blood pressure at an initial subpressor dose of subcutaneous chronic AngII infusion.

Previous studies have identified protein kinases, such as CaMKII and protein kinase C (PKC) as signaling intermediates in AngII-stimulated neurons
(80, 81, 219). For example, two previous studies have shown a clear contribution of CaMKII and PKC in mediating the AngII-induced inhibition of neuronal ion channels and subsequent increase in neuronal firing both in primary neuronal cultures as well as in neuronal cell lines (80, 136). One previous study from our lab has also demonstrated activation of CaMKII by inducing CaMKII phosphorylation in AngII-stimulated CATH.a neurons and this CaMKII phosphorylation can be blocked by overexpressing mitochondrial-targeted manganese superoxide dismutase (83). These studies therefore suggest CaMKII as a key player in AngII-mediated signaling in neurons. Additionally, Erickson et al. have shown that AngII can induce CaMKIIδ oxidation in cardiomyocytes in a paired methionine residue and sustain CaMKIIδ activity independent of Ca²⁺/CaM binding (181). This sustained activity of CaMKIIδ leads to AngII-mediated apoptosis in ischemic heart and under myocardial infarction conditions.

Although these studies indicated CaMKII as an important downstream molecule, in the present study we wanted to specifically investigate the role of major neuronal isoform of CaMKII (CaMKIIα) in mediating AngII intra-neuronal signaling and the AngII-induced hypertension.

To determine the role of CaMKIIα in mediating the AngII-induced reduction in neuronal I_{kv}, we used adenoviral-mediated overexpression of wt-CaMKIIα in CATH.a neurons and measured changes in outward K⁺ current. Consistent with previous findings, AngII stimulation inhibited I_{kv} by almost 16% both in non-transduced as well as in AdEmpty-transduced neurons. Interestingly, this effect was significantly exaggerated in wt-AdCaMKIIα-transduced neurons. In
addition to enhancing the AngII effects wt-CaMKIIα overexpression by itself had a lower baseline $I_{kv}$ in CATH.a neurons. To correlate changes in baseline electrophysiological properties with ion channel expression we also investigated the basal expression levels of different voltage-gated $K^+$ channels that could potentially play a role in CaMKIIα-mediated $I_{kv}$ reduction. Previously Gao et al. have shown that in neurons isolated from the rostral ventrolateral medulla of chronic heart failure animals as well as in AngII-stimulated CATH.a neurons expression of $K_{v4.3}$, one of the important voltage-gated $K^+$ channels, is downregulated (85). In the present study we found an upregulation in the basal gene expression of four of the voltage-gated $K^+$ channels, $K_{v2.1}$, $K_{v2.2}$, $K_{v4.2}$ and $K_{v4.3}$ in CATH.a neurons with wt-CaMKIIα overexpression. We speculate that this increase in mRNA expression of these channels could be a potential compensatory mechanism as a result of wt-CaMKIIα overexpression and subsequent inhibition of $I_{kv}$. However, further studies are needed to determine the protein expression and the cellular localization of these voltage-gated channels in wt-CaMKIIα-overexpressed and control neurons.

In the central nervous system AngII acts on its type 1 receptor (AT1R) to promote vasopressin secretion, thirst and salt appetite, sympathetic outflow and also modulates baroreflex sensitivity (73, 199, 212). Brain AngII is known as a potent regulator of arterial blood pressure and when administered acutely in an ICV route it elicits a classic transient systemic and bradycardic response. As seen in Figure 3.8, acute ICV injection of AngII caused a characteristic rapid and relatively short-lived increase in mean arterial pressure in AdGFP-treated mice.
In contrast the AngII-induced increase in blood pressure was significantly potentiated in mice injected with ICV wt-AdCaMKIIα. We did not observe changes in baseline blood pressure or heart rate with ICV wt-AdCaMKIIα overexpression as opposed to our *in vitro* patch-clamp data. This could be due to a variety of reasons: firstly, our *in vitro* studies are carried out in a homogenous CATH.a neuronal cell population where as in the brain different cell types including astrocytes, glial cells and endothelial cells have their own signaling pathways but can also interact with neuronal signaling. Secondly, although we clearly demonstrate CaMKIIα overexpression in the subfornical organ when given ICV, we cannot rule out the possibility that the adenovirus is being taken up by other cardiovascular brain nuclei or even some non-cardiovascular brain regions. Furthermore, other downstream mechanisms may be involved *in vivo* to counteract any mild change in baseline blood pressure or heart rate that may be occurring with wt-CaMKIIα overexpression.

The slow pressor chronic AngII-induced hypertensive model has been extensively used by our lab and others to investigate intraneuronal signaling mechanisms in AngII-mediated neurogenic hypertension (115, 222, 223). We therefore wanted to evaluate the role of ICV wt-CaMKIIα injection on chronic subcutaneous AngII infusion and the AngII-induced hypertensive response. Consistent with the acute AngII data, ICV wt-CaMKIIα did not alter baseline blood pressure (baseline blood pressure ~ 95 mmHg). Intriguingly, we observed a significant rise in blood pressure in wt-AdCaMKIIα-injected mice immediately after the start of AngII infusion as compared to a more gradual rise in AdGFP-
injected mice and this was maintained up to 2-3 days post minipump implantation. We speculate that mice receiving wt-CaMKIIα injection in the brain are being sensitized to an immediate increase in blood pressure at an early subpressor dose of chronic subcutaneous AngII infusion. Further studies are needed to determine the mechanism of action by which wt-CaMKIIα in the brain is sensitizing mice to this immediate rise in blood pressure with subcutaneous AngII. Previous studies have shown that CaMKII can activate upstream molecules, such as NADPH oxidase and in turn lead to increased generation of reactive oxygen species which can then cause CaMKII oxidation thereby sustaining CaMKII activity in vascular smooth muscle cells (225). In addition, it is also possible that wt-CaMKIIα overexpression in the brain disrupts the blood brain barrier integrity as a result of which AngII, immediately following the start of infusion, can rapidly access cardiovascular control brain regions which are otherwise protected by the blood brain barrier.

In summary, the experimental data presented herein provide new evidence supporting neuronal CaMKIIα as an important downstream intermediate in AngII-intraneuronal signaling and subsequent AngII-dependent hypertension. More specifically, we showed that overexpression of wt-CaMKIIα exacerbates the AngII-mediated inhibition of outward K⁺ current. Additionally, wt-CaMKIIα overexpression in the brain subfornical organ potentiates both the acute central and the chronic subcutaneous AngII-induced hypertension in mice. These set of data led us to examine the effect of mutating redox-sensitive residues in CaMKIIα on the AngII-mediated inhibition of neuronal K⁺ current and AngII-dependent
hypertensive response as discussed in the next chapter.
Chapter IV: Mutation of CaMKIIα at Cys280-Met281 attenuates the potentiated Angiotensin II intra-neuronal signaling mechanisms mediated by wild-type CaMKIIα overexpression

Introduction

Our wild-type CaMKIIα (wt-CaMKIIα) studies (Chapter III) indicated that overexpression of wt-CaMKIIα exacerbated the AngII-mediated inhibition of K⁺ current in CATH.a neurons. Overexpressing wt-CaMKIIα in the brain subfornical organ (SFO), an important cardiovascular regulatory brain nuclei, also potentiated the acute AngII-mediated increase in blood pressure response (Figure 3.8 Chapter III). Interestingly, in an AngII-infusion hypertensive model, wt-CaMKIIα overexpression sensitized mice to an immediate increase in blood pressure at an initial subpressor dose of subcutaneous AngII. Previous studies have shown the importance of the SFO in AngII-mediated hypertension and overexpression of copper zinc superoxide dismutase (CuZnSOD) in the SFO attenuates the AngII-mediated increase in blood pressure in mice (90). These studies therefore indicate the importance of CaMKIIα in mediating AngII-signaling in neurons and the AngII-hypertensive response. However, the exact mechanism by which AngII-mediated increase in ROS regulate CaMKIIα in neurons is still unknown.

Past studies have extensively investigated AngII intra-neuronal signaling mechanisms both in primary neurons from rat hypothalamus and brain, and also in cultured neurons. Their studies showed that AngII stimulation inhibits K⁺
current thus increasing neuronal firing frequency (80, 81, 226). However, when they used inhibitors to inhibit signaling proteins, such as CaMKII and protein kinase C (PKC) the AngII-mediated effects could be significantly blunted thereby suggesting a role of CaMKII and PKC in AngII-signaling in central neurons (80, 81, 219). In addition to neurons, CaMKII in the cradiomyocytes (CaMKIIδ) has also been illustrated in AngII-mediated ischemic stress response and apoptosis in the heart. Importantly, Erickson et al. showed that in presence of AngII stimulation and increased ROS CaMKIIδ undergoes oxidation in a paired methionine residue (M281/282) (181, 220). Although Ca$$^{2+}$$/CaM is required for the initial activation of CaMKII yet oxidation in methionine 281/282 sustains CaMKIIδ activity when the Ca$$^{2+}$$ stimulus has subsided and the Ca$$^{2+}$$/CaM has dissociated. Whereas CaMKIIδ along with CaMKIIB and CaMKIIγ have the paired methionine residues at positions 281 and 282, the predominant brain isoform of CaMKII (CaMKIIα) instead has a cysteine and methionine at 280 and 281 respectively. In order to demonstrate that CaMKIIα is also susceptible to oxidation Erickson and colleagues purified CaMKIIα and exposed to pro-oxidant conditions. They further mutated the first methionine of CaMKIIδ with a cysteine to mimic CaMKIIα. Interestingly, in presence of H$_2$O$_2$ purified CaMKIIα had increased oxidation thereby suggesting the redox-sensitivity of CaMKIIα. Redox-regulation of CaMKIIα has also been demonstrated by a nitric oxide (NO)-induced S-nitrosylation of CaMKIIα in excitotoxic neuronal cell death mechanisms (189). In this study, Coultrap et al. showed that NO generates autonomous CaMKIIα activity by promoting S-nitrosylation of cysteine 289 in addition to cysteine 280.
Inhibition of S-nitrosylation in either of those residues abolished CaMKIIα autonomy and prevented the NO-induced excitotoxic neuronal cell death. Additionally, in hippocampal neurons large increases in cytosolic Ca\(^{2+}\) has been associated with mitochondrial O\(_2^{-}\) generation which in turn activates protein kinases, such as CaMKII (190). Activation of CaMKII by mitochondrial O\(_2^{-}\) is mediated at least in part by inhibition of inactivating protein phosphatases, such as protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1). Taken together, these studies suggest the importance of redox regulation of different CaMKII isoforms in a variety of pathological conditions (227).

In the present study we tested the hypothesis that mutation of redox-sensitive cysteine and methionine residues in CaMKIIα (C280A/M281V) attenuates the AngII-mediated inhibition of neuronal K\(^{+}\) channel current and the AngII-induced hypertensive response in mice. We show that mut-CaMKIIα attenuates the potentiated AngII response following wt-CaMKIIα overexpression in CATH.a neurons. In addition, mut-CaMKIIα in the brain prevents the sensitization mechanism following AngII infusion as observed with wt-CaMKIIα overexpression.

**Experimental Methods**

**CATH.a neuronal cell culture**

For detailed descriptions of mouse CATH.a neuronal cell culture and differentiation, see experimental methods in Chapter II.
Site-directed mutagenesis and mutant CaMKIIα adenovirus generation

Replication-deficient recombinant adenovirus (Ad5-CMV) encoding mouse mutant calcium/calmodulin-dependent protein kinase IIα (mut-AdCaMKIIα) was generated. Primer sequences were made specifically to mutate cysteine 280 to alanine and methionine 281 to valine with overlapped extension polymerase chain reaction (PCR). Briefly two sections of the wt-CaMKIIα plasmid (Origene) were amplified by conventional PCR using HotStart PCR Master Mix (Qiagen, Venlo, Limburg) with the following two set of primers: Section 1 forward 5’- GAA TTC ATG GCT ACC ATC ACC TGC ACC C – 3’; reverse 5’ – CCA CGG TCT CCT GTC TGT GCA - 3’. Section 2 forward 5’- GTG CAC AGA CAG GAC GGA ACC GTG – 3’; reverse 5’ – GGA TCC TCA ATG CGG CAG GAC GGA - 3’. For description on cloning using pJet1.2 PCR clone jet kit (Thermo Scientific) and bacterial colony culture see experimental methods in Chapter III. Adenoviral vectors encoding mut-CaMKIIα were constructed, purified and provided by the University of Iowa Gene Vector Core as previously described (83, 221).

RNA extraction, cDNA and quantitative real-time RT-PCR

CaMKII mRNA levels were measured in CATH.a neurons following adenovirus-mediated transduction of mut-CaMKIIα. Real-time RT-PCR was utilized as previously described in experimental methods of Chapter III. mRNA levels of voltage-dependent K⁺ channels, such as $K_{v2.1}$, $K_{v2.2}$, $K_{v4.2}$ and $K_{v4.3}$ were also detected in CATH.a neurons following adenoviral transduction. To confirm the overexpression of CaMKIIα in mouse brain following intracerebroventricular (ICV)
injections, micro punches of the SFO along with ventricular tissues were collected and then RNA was extracted in Trizol reagent.

**Western blot analysis**

Total and phosphorylated CaMKIIα protein expression was detected in CATH.a neurons following adenoviral transduction at different multiplicity of infection. For description of Western blotting protocol, see experimental methods in Chapter III.

**Electrophysiological record of voltage-gated K\(^+\) currents**

For description of whole-cell patch-clamp technique to record voltage-gated K\(^+\) current, see experimental methods in Chapter III. Voltage-gated K\(^+\) currents were recorded in non-transduced, AdEmpty (25MOI), wt-AdCaMKIIα (25MOI) and mut-AdCaMKIIα (25MOI) at baseline and following 5 minutes of AngII (100 nM) superfusion. To confirm the AngII-mediated response on K\(^+\) channel currents, AngII was washed out and K\(^+\) current was recorded for 15 minutes.

**In vivo mouse studies – radiotelemetry implantation, intracerebroventricular cannula placement and osmotic minipump implantation**

All animal procedures were performed as described in experimental methods of Chapter III, in accordance with Guidelines for the Care and Use of Experimental Animals of the American Physiological Society and the National Institutes of Health, with specific experimental protocols being reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use
Committee. Mice received left carotid artery radiotelemeters to measure cardiovascular parameters such as mean arterial pressure, systolic pressure, diastolic pressure, heart rate and pulse pressure. Cannulas were implanted intracerebroventricularly to inject control adenovirus (AdGFP) and mut-CaMKIIα adenovirus. Both the adenoviruses were injected at $5 \times 10^7$ plaque forming units. It is to be noted that mice for these set of in vivo studies did not receive central acute AngII injections. ICV adenovirus injections were followed by saline and AngII subcutaneous osmotic minipump implantation to establish the AngII-infused mouse model of hypertension. The slow pressor dose infusion rate of 400 ng/kg/min was selected, as previously detailed in Chapter III Methods.

**Immunohistochemistry**

Mouse brains were collected 7 days post-ICV AdGFP or mut-AdCaMKIIα injections and 20 µm sections of the brains were prepared using a cryostat (Leica CM3050S). The sections were incubated at 4 °C overnight with a rabbit primary antibody against CaMKIIα (1:200, abcam) and a rabbit P-CaMKIIα (1:200, abcam) prepared in 0.1 M PB containing 2% NHS and 0.3% Triton X-100. Alexa Fluor 598 secondary antibody (1:200, Invitrogen) was then used to incubate the brain sections at room temperature for 2 hours. Fluorescent images of the subfornical organ and ventricles were detected by confocal laser scanning microscopy (Zeiss LSM 510 Meta Confocal Microscope) at 20X and 40X magnification. For detailed immunohistochemical staining procedure, see experimental methods in Chapter III.
Statistical Analysis

All data are presented as means ± standard error of the mean (SEM). Data were analyzed by Student’s t-test for two group comparisons or by ANOVA with Newman Keuls post-hoc test. P-value less than 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.0 statistical and graphical software.

Results

Adenoviral-mediated transduction of mut-CaMKIIα increases total and phosphorylated CaMKIIα protein in CATH.a neurons

To check if the mut-CaMKIIα adenovirus expresses CaMKIIα protein, we first detected total CaMKIIα protein expression in non-transduced CATH.a neurons and neurons transduced with AdEmpty and mut-AdCaMKIIα at 10, 25 and 50MOI. CaMKIIα protein was significantly overexpressed in all the mut-AdCaMKIIα transduced groups (Figure 4.1A,B); however, in order to be consistent with our wt-CaMKIIα in vitro studies we selected the 25MOI of the adenovirus for the next set of cell culture experiments. Phosphorylated CaMKIIα levels were also increased in CATH.a neurons transduced with mut-CaMKIIα adenovirus as compared to the non-transduced and AdEmpty-transduced neurons (Figure 4.1A,C). To determine mRNA levels of CaMKIIα we utilized real-time RT-PCR in CATH.a neurons following 25MOI mut-AdCaMKIIα transduction.
Primers specific for CaMKIIα revealed a significant increase in CaMKIIα mRNA in CATH.a neurons transduced with mut-CaMKIIα adenovirus (Figure 4.1D). The other CaMKII isoforms, such as CaMKIIβ and CaMKIIδ did not change significantly in between the non-transduced, AdEmpty, and mut-AdCaMKIIα groups (Figure 4.1 E, F). Kinase activity assay was utilized to measure CaMKII activity in AdEmpty (25MOI) and wt-AdCaMKIIα (25MOI) and mut-AdCaMKIIα-transduced CATH.a neurons. Neurons with wt-CaMKIIα overexpression had 3-fold higher activity as compared to AdEmpty-transduced neurons. CaMKII activity in mut-AdCaMKIIα group was lower than wt-CaMKIIα transduced neurons but higher than AdEmpty-transduced neurons (Figure 4.1G) (n = 2).
A

Non-transduced AdEmpty Mut-CaMKIIα

CaMKIIα

P-CaMKIIα

Actin

B

C

Non-infected AdEmpty 10MOI AdEmpty 25MOI AdEmpty 50MOI mut-AdCaMKIIα 10MOI mut-AdCaMKIIα 25MOI mut-AdCaMKIIα 50MOI

CaMKIIα/Actin

P-CaMKIIα/Actin

*
Figure 4.1. Transduction of mut-AdCaMKIIα causes significant upregulation of CaMKIIα expression in CATH.a neurons. (A) Representative Western blot analysis of CATH.a neuronal cell lysates probed for primary antibody against total and phosphorylated CaMKIIα; Summary densitometry data of Western blot analysis showing levels of (B) CaMKIIα and (C) P-CaMKIIα in non-transduced, AdEmpty (10, 25 and 50 MOI)- and mut-AdCaMKIIα (10, 25 and 50 MOI)-transduced neurons; Real-time RT-PCR data with primers specific for CaMKIIα (D), CaMKIIβ (E), and CaMKIIδ (F) genes normalized to 18s rRNA and expressed as fold control. (G) CaMKII activity in AdEmpty (25MOI) and wt-AdCaMKIIα (25MOI) and mut-AdCaMKIIα-transduced neurons as measured by kinase activity assay and reported as fold change from control. *P<0.05 vs. non-transduced and AdEmpty.
AngII-mediated increased inhibition of K⁺ current following wt-CaMKIIα overexpression is rescued in mut-CaMKIIα-transduced CATH.a neurons

Whole-cell configuration of the patch-clamp technique was utilized to record voltage-gated K⁺ current (I_{kv}) in non-infected and adenovirus transduced CATH.a neurons at baseline and following 5 minutes of AngII (100 nM) superfusion. Neurons transduced with mut-CaMKIIα adenovirus showed similar percent inhibition (13%) in I_{kv} when compared to non-transduced (16%) and AdEmpty (16%)-transduced CATH.a neurons (Figure 4.2). Interestingly, when compared to wt-CaMKIIα data as presented in Chapter III (37% inhibition of outward K⁺ current) the restoration in the AngII-mediated reduction in I_{kv} with mut-CaMKIIα was significant. Both transient and steady-state K⁺ current followed a similar pattern. To note, the I_{kv} data in the non-transduced, AdEmpty and wt-AdCaMKIIα groups are similar to those in Chapter III results.
Figure 4.2. Mut-CaMKIIα transduction restores the potentiated AngII-mediated inhibition of outward K⁺ current in CATH.a neurons. Percent inhibition of outward K⁺ current following 5 minutes of AngII (100 nM) superfusion in non-transduced, AdEmpty (25MOI) or mut-AdCaMKIIα (25 MOI)-transduced CATH.a neurons (n = 4-7 biological replicates for all experimental groups). *P < 0.05 vs. non-transduced. #P < 0.05 vs. wt-AdCaMKIIα.
Mutant CaMKIIα transduction does not change mRNA levels of voltage-dependent K⁺ channels in CATH.a neurons

To determine the outcome of mut-CaMKIIα expression on voltage-dependent K⁺ channels, real-time RT-PCR was utilized to measure mRNA levels of Kv2.1, Kv2.2, Kv4.2 and Kv4.3 in CATH.a neurons. Baseline expression of the channels was detected in AdEmpty (25MOI) and mut-AdCaMKIIα transduced neurons. Initial experiments showed a modest decrease in Kv2.1, and Kv4.2 and Kv4.3 mRNA expression in mut-CaMKIIα transduced neurons as compared to AdEmpty; however, the data did not reach statistical significance. Surprisingly Kv2.2 mRNA levels were modestly increased with mut-CaMKIIα expression (Figure 4.3).
Figure 4.3. Mut-CaMKIIα modestly decrease mRNA expression of voltage-dependent K⁺ channels in CATH. neurons. Quantitative real-time PCR analysis showing (A) Kv2.1, (B) Kv2.2, (C) Kv4.2 and (D) Kv4.3 mRNA levels in AdEmpty (25MOI) and mut-AdCaMKIIα (25MOI) transduced CATH.a neurons. n=2 biological replicates in all experimental groups.
Intracerebroventricular injections of mut-CaMKIIα adenovirus increases CaMKIIα expression in brain subfornical organ of mice

Mice were implanted with ICV cannulas to receive central injections of AdGFP and mut-AdCaMKIIα adenovirus. The expression of CaMKIIα in the brain SFO was confirmed by immunohistochemistry 7 days post ICV adenovirus injections. CaMKIIα was greatly increased in mice receiving mut-CaMKIIα adenovirus as compared to AdGFP group (Figure 4.4A). Interestingly, levels of P-CaMKIIα did not increase as seen in our previous studies with wt-CaMKIIα overexpression in the SFO (Figure 4.4A). CaMKII mRNA levels were also detected by real-time RT-PCR in brain SFO micropunches following ICV adenovirus injections. The mRNA level for CaMKIIα was significantly increased by ~12 fold in mice receiving mut-CaMKIIα injections (Figure 4.4B). Interestingly, another CaMKII isoform (CaMKIIβ), which has a wide tissue distribution and is also present in the brain, was increased by 3 fold. The mRNA levels of other CaMKII isoforms (CaMKIIδ and CaMKIIγ) did not change between the groups.
Figure 4.4. Adenoviral-mediated overexpression of CaMKIIα in the subfornical organ (SFO). (A) Representative confocal microscopy images showing expression of GFP, CaMKIIα and P-CaMKIIα in SFO of mice 1 week after intracerebroventricular (ICV) injection of AdGFP or mut-AdCaMKIIα. Coronal brain sections (20 µm) were stained for GFP (green), total and phosphorylated CaMKIIα (red). (B) Real-time PCR data showing relative mRNA expression of CaMKIIα, CaMKIIβ, CaMKIIδ and CaMKIIγ genes in brain micropunches. Values are normalized to 18S rRNA and expressed as relative to control. Scale bar = 100 µm
Mutant CaMKIIα expression in the brain subfornical organ prevents the sensitization mechanism following AngII infusion

Mice received ICV injections of AdGFP or mut-CaMKIIα adenovirus prior to minipump implantation. Baseline blood pressure was recorded with radiotelemetry for 3 days following which subcutaneous saline and AngII osmotic minipumps were implanted. Interestingly, mice which received ICV mut-AdCaMKIIα had a modest increase in blood pressure on days 1-2 after AngII infusion but declined to near baseline levels on day 3. Blood pressure then gradually increased in mice receiving mut-AdCaMKIIα with AngII infusion (Figure 4.5A). This increase in mean arterial pressure (MAP) was similar to the AdGFP mice with AngII infusion and is characteristic of the slow-pressor AngII-hypertensive model (222, 223). The peak AngII response on days 15-17 was lower in mut-AdCaMKIIα group (115 mmHg) as compared to the AdGFP group (125 mmHg). Importantly, when compared to the wt-CaMKIIα data (peak AngII response – 133 mmHg), as presented in results of Chapter III, the peak AngII response is significantly attenuated in the mut-AdCaMKIIα group and the sensitization mechanism that is seen immediately following AngII infusion in the wt-CaMKIIα overexpression mice is prevented with mut-CaMKIIα expression. Blood pressures came back to baseline levels on day 22-23 for each group when the minipumps were empty. Systolic and diastolic blood pressures followed a similar pattern as the MAP (Figure 4.5C, D). Heart rate did not differ between the groups (Figure 4.5B).
C

Systolic Blood Pressure (mmHg)

ICV Adenovirus Injection

Days of AngII infusion

D

Diastolic Blood Pressure (mmHg)

ICV Adenovirus Injection

Days of AngII Infusion
Figure 4.5. Intracerebroventricular injection of mut-CaMKIIα in the brain and chronic infusion of subcutaneous AngII via osmotic minipumps. Representative line graphs showing (A) mean arterial pressure, (B) heart rate, (C) systolic blood pressure, and (D) diastolic blood pressure and via radiotelemetry in C57BL/6 mice at baseline, following intracerebroventricular injection of AdGFP or mut-AdCaMKIIα, and after subcutaneous implantation of osmotic minipumps set to release saline or AngII (400 ng/kg/min). *P < 0.05 vs. wt-AdCaMKIIα mice with AngII infusion and #P < 0.05 vs. AdGFP mice with AngII infusion. n = 6-9 mice for each experimental group.
Discussion

Calcium/calmodulin-dependent protein kinase II (CaMKII) belongs to a multifunctional family of serine/threonine protein kinases. Different isoforms of CaMKII have diverse roles and distribution and their function is often determined by the tissues in which they are expressed. For examples, numerous studies have shown that CaMKII in neuronal tissues play important role in memory and synaptic plasticity (228). On the other hand, in T lymphocytes, CaMKII have a major role in regulation of CD8 T cell proliferation, cytotoxic effector function and response to re-stimulation (228). In central neurons, the AngII-mediated regulation of ion channels has elucidated the involvement of CaMKII as a downstream signaling molecule in AngII-intraneuronal signaling pathways (80, 219). Additionally, increased reactive oxygen species (ROS) can also act as signaling intermediates in AngII signaling pathway in neurons. However, the exact mechanism by which AngII and increased ROS regulates CaMKII in neurons, specifically the major neuronal isoform of CaMKII (CaMKIIα) is unclear.

In the present study we report that: (1) mutation of redox-sensitive cysteine and methionine residues in CaMKIIα restores the potentiated AngII-mediated K⁺ current inhibition, (2) intracerebroventricular injection of mutant CaMKIIα adenovirus in brain subfornical organ of mice prevents the initial sensitization following AngII infusion, and (3) mutant CaMKIIα expression in the brain significantly attenuates the AngII-induced peak rise in blood pressure as compared to wt-CaMKIIα overexpressed mice.

Previous studies by Erickson et al. demonstrated that in the heart
CaMKIIδ undergoes oxidation in paired methionine residues (met 281/282) present in the regulatory domain (181). Oxidation in methionine residues sustains CaMKIIδ oxidation in absence of Ca²⁺/CaM. They further showed that AngII-induced CaMKIIδ oxidation leads to cardiomyocyte apoptosis. However, neuronal CaMKIIα differs from the other CaMKII isoforms because of the presence of a cysteine and methionine in positions 280 and 281 respectively. Importantly, in presence of increased mitochondrial superoxide, CaMKII in hippocampal neurons have increased kinase activity (190). This increase in CaMKII activity is mediated at least in part by inhibition of protein phosphatases that regulate CaMKII autophosphorylation state. Moreover, recent studies by Coultrap and Bayer demonstrated that in addition to oxidation, cysteine residues in CaMKIIα are also susceptible to nitric oxide-mediated S-nitrosylation (189). In their study they investigated the susceptibility of two cysteines at positions 280 and 289 to S-nitrosylation and showed that mutation of either cysteine prevents NO-induced excitotoxic neuronal cell death. Although these studies indicate the redox-sensitivity of CaMKIIα, we wanted to specifically determine the outcome of mutating C280/M281 of CaMKIIα on AngII-intraneuronal signaling mechanisms. Furthermore, we wanted to compare the effect of CaMKIIα mutation with our wt-CaMKIIα overexpression studies by looking at AngII-mediated K⁺ current inhibition and the AngII-induced hypertension.

First, we wanted to investigate the effect of mut-CaMKIIα on AngII-mediated inhibition of K⁺ current. Voltage-dependent K⁺ current (Iₖᵥ) was measured in mouse CATH.a neurons either non-infected or transduced with
AdEmpty and mut-CaMKIIα adenovirus at 25MOI. Interestingly, the percent inhibition of \( I_{kv} \) in all the three groups was approximately similar with a modest decrease observed in mut-CaMKIIα transduced neurons. However, when compared with the wt-CaMKIIα data from Chapter III, it is evident that the exacerbated AngII-mediated inhibition of \( I_{kv} \) with wt-CaMKIIα overexpression is restored with mutation of CaMKIIα. Importantly, as previously mentioned in Chapter III discussion, CATH.a neurons do not appear to have endogenous CaMKIIα and this may explain why CaMKIIα mutation does not completely abolish the AngII-mediated \( I_{kv} \) inhibition. Other protein kinases, such as PKC or CaMKIIβ may play a role in mediating AngII-downstream signaling in CATH.a neurons. These studies therefore confirm that redox-regulation of CaMKIIα plays an important role in AngII-signaling in central neurons.

Since wt-CaMKIIα overexpression elucidated a potential compensatory mechanism in the transcriptional upregulation of different voltage-dependent \( K^+ \) channels, we wanted to determine whether mut-CaMKIIα has any role in the regulation of these channels. To do that, we utilized real-time RT-PCR and measured mRNA levels of \( K_{v2.1} \), \( K_{v2.2} \), \( K_{v4.2} \) and \( K_{v4.3} \) in CATH.a neurons transduced with either AdEmpty or mut-AdCaMKIIα. From these initial studies we observed that there was no significant different in mRNA expression of any of the channels between the two groups. This data once again confirms the compensatory mechanism that overexpression of wt-CaMKIIα lowers baseline \( I_{kv} \) which then leads to the transcriptional upregulation of these channels.

We next wanted to evaluate the role of ICV mut-CaMKIIα injection on
chronic subcutaneous AngII infusion and the AngII-induced hypertensive response in mice. Consistent to wt-CaMKIIα overexpression ICV mut-CaMKIIα did not alter baseline blood pressure and heart rate. Intriguingly we observed a similar rise in blood pressure in mut-AdCaMKIIα injected mice for 2 days immediately after the start of AngII infusion as observed with wt-CaMKIIα mice. However, blood pressure came back to baseline levels after 2 days of AngII infusion and continued to rise gradually as AdGFP mice infused with AngII. This is the characteristic slow pressor chronic AngII-induced hypertensive model that has been extensively used by our lab and others to study AngII-mediated neurogenic hypertension. We speculate that the immediate increase in blood pressure in mice with mut-CaMKIIα occurs as a result of some forms of active CaMKIIα already present in the brain other than oxidized form. This can happen as a result of autophosphorylation of CaMKIIα in threonine 286 residue and can prolong CaMKIIα activity in absence of Ca^{2+}/CaM. However, with increase in AngII levels in the brain and the increase in reactive oxygen species, mut-CaMKIIα is prevented from being oxidized as opposed to wt-CaMKIIα. This in turn prevents the sustained CaMKIIα activation and the rise in blood pressure follows the characteristic pattern of slow-pressor AngII-induced hypertension. Since recent studies have indicated potential S-nitrosylation of CaMKIIα in cysteine 280/289 (199), it would be interesting to see if mutation of cysteine 289 in addition to the C280/M281 can further attenuate the AngII-induced peak increase in blood pressure and whether it would have an effect on the initial rise in blood pressure with AngII infusion.
In summary, the experimental data presented herein provide important insight on the role of redox-sensitive CaMKIIα in AngII-intraneuronal signaling and subsequent AngII-dependent hypertension. More specifically, we showed that mutation of CaMKIIα at cysteine 280 and methionine 281 restores the exacerbated AngII-mediated inhibition of outward K⁺ current observed with wt-CaMKIIα overexpression. Additionally, mut-CaMKIIα in the brain subfornical organ prevents the early sensitization mechanism and lowers the peak AngII-response in a slow-pressor AngII-hypertensive model. In conclusion these studies may help to identify potential therapeutic targets, such as CaMKIIα, in the treatment of neuro-cardiovascular diseases, such as hypertension and heart failure.
Chapter V: Conclusion

Overall summary of findings and discussion

Dysregulation of angiotensin II (AngII) signaling in the central nervous system (CNS) is involved in the pathogenesis of cardiovascular disorders, including hypertension and heart failure (73, 89, 214, 229). Several components of the renin-angiotensin system (RAS) including AngII have been shown to be elevated both in periphery as well as in the brain during these diseases (87, 88, 191). Numerous studies so far have investigated the AngII-intraneuronal signaling mechanisms associated with the pathogenesis of hypertension and heart failure. One such mediator of AngII signaling in neurons is reactive oxygen species (ROS) (56, 90, 112, 139, 230). Imbalance between the ROS producing components and the antioxidant defense mechanisms can lead to downstream pathological responses of AngII (96, 110). By acting on the type 1 receptor (AT1R) AngII leads to physiological responses such as vasoconstriction, sympathetic nervous system activation, sodium and water reabsorption, vascular and cardiac remodeling, inflammation, fibrosis, thrombosis, and release of aldosterone (ALD), and vasopressin (AVP) (10, 22). Clinically, the common therapeutics used for the treatment of diseases, like hypertension, consist of RAS blockers, such as angiotensin converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARBs) in addition to β-adrenergic receptor blockers (β-blockers) (231). However, according to a report released by the Center for Disease Control and Prevention (CDC) in 2012, despite of taking the above mentioned medications, approximately 34% of the hypertensive patients in the United States are unable to have their
blood pressures controlled (7). Since enhanced activation of RAS and elevated AngII levels in the brain are implicated in hypertension therefore it is of utmost importance to understand the intracellular signaling mechanisms mediated by AngII in neurons in order to identify novel therapeutic targets to treat neurocardiovascular diseases.

In AngII-stimulated central neurons, the two major sources of ROS production are the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) and the mitochondria (99, 103, 139). Both the sources primarily lead to an increase in superoxide ($O_2^\cdot$) and hydrogen peroxide ($H_2O_2$). In addition to electron leakage from the mitochondrial electron transport chain (ETC) leading to excessive ROS production (102, 103), recent studies from our lab have shown that NOX localized in neuronal mitochondria can also increase mitochondrial ROS levels (115). In addition, proinflammatory cytokines activated by AngII signaling also generate ROS in the brain (109, 232). The increased $O_2^\cdot$ can then regulate ion channels thereby modulating the channel currents to increase neuronal firing rate and neuronal activation. For example, studies by Sumners and colleagues have shown that in central neurons, AngII inhibits $K^+$ current and this in turn leads to an increase in neuronal firing frequency (79-81). Furthermore, our lab has showed that this reduction in $K^+$ current mediated by AngII can be attenuated by scavenging $O_2^\cdot$ with specific scavenging enzymes, such as copper zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD) (83). Neuronal firing can also be increased by the increase in $Ca^{2+}$ current in AngII-stimulated neurons. AngII has indeed been
shown to increase intracellular Ca\(^{2+}\) levels in a neuronal cell line (84). Additionally, Sumners and Raizada have shown that the AngII-mediated reduction in K\(^{+}\) current and the increased neuronal firing frequency can be attenuated by inhibiting some of the Ca\(^{2+}\) dependent enzymes, such as protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII) (80, 81).

In the brain AngII can activate neurons in the circumventricular organs (CVOs), which abundantly express the AT1R (64, 75, 233). The CVOs are devoid of an intact blood brain barrier (BBB) and is therefore exposed to circulating peptides and hormones. One such CVO is the subfornical organ (SFO), which lies in the roof of the third ventricle and is an important cardiovascular regulatory brain nucleus (90, 110). The importance of SFO in AngII-mediated signaling was shown by lesioning the SFO that abrogated the hypertensive response induced by circulating AngII (45, 60). Furthermore, adenoviral-mediated overexpression of CuZnSOD in the SFO scavenges the AngII-induced O\(_2^*\) and attenuates sympathetic drive and blood pressure in numerous animal models of hypertension (90).

The family of multifunctional calcium/calmodulin (Ca\(^{2+}/\text{CaM}\))-dependent protein kinases (CaMKs) including CaMKI, CaMKII and CaMKIV modulate many of the cellular responses to Ca\(^{2+}\). These downstream effector molecules translate and co-ordinate the dynamic second messenger, Ca\(^{2+}\), into appropriate cellular responses by a highly specific and reversible phosphorylation of a number of substrate proteins in their serine or threonine residues. This family of protein
kinases has both common as well as unique features with respect to their structure, regulation and activation. The CaMKII is responsible for the coupling of Ca\(^{2+}\) increase to ion channel activation, gene transcription, neurotransmitter synthesis and release, cytoskeletal organization and apoptosis (181-183). Classically, CaMKII is activated by the binding of Ca\(^{2+}/\text{CaM}\) followed by autophosphorylation in the regulatory domain. Autophosphorylation confers the kinase activity of CaMKII which can then phosphorylate specific serine and threonine residues of target proteins. Active CaMKII is switched back to the inactive resting form by dephosphorylation by specific phosphatases such as protein phosphatase 1 (PP1) or protein phosphatase 2A (PP2A) (184-186). Interestingly, recent studies have shown that the AngII-mediated pathological responses in the heart involve a second mechanism of sustained CaMKII activation under oxidant conditions which is independent of Ca\(^{2+}/\text{CaM}\). More specifically, studies by Erickson et al. have demonstrated that oxidation of a paired methionine residue (M281/282) in the cardiomyocyte isoform of CaMKII (CaMKII\(\delta\)) is a downstream signal for AngII and ischemic stress response in the heart and inhibition of CaMKII\(\delta\) protects the heart against AngII-mediated apoptosis and other pathological myocardial responses (181, 187). Since, the paired methionine residue is conserved in all three isoforms of CaMKII except the predominant neuronal isoform of CaMKII (188) (CaMKII\(\alpha\)) (CaMKII\(\alpha\) has a cysteine residue in position 280 instead of the first methionine of the pair), they also generated a M281C mutant of CaMKII\(\delta\) and also purified CaMKII\(\alpha\). Both purified CaMKII\(\alpha\) and M281C mutant CaMKII\(\delta\) were activated by H\(_2\)O\(_2\) indicating...
the importance of cysteine in ROS-dependent CaMKII activation. More recently, a nitric oxide (NO)-mediated S-nitrosylation of CaMKIIα has been implicated in excitotoxic neuronal cell death. The results from that study show that specific S-nitrosylation of C280/289 generated autonomous activation of CaMKIIα independent of Ca$^{2+}$/CaM binding and mutation of either site protected from NO-induced neuronal cell death (189). The redox sensitivity of CaMKII has also been illustrated in studies where large increases in cytosolic Ca$^{2+}$ causes mitochondrial O$_{2}^{-}$ generation and upregulation of CaMKII in hippocampal neurons (190).

Activation of CaMKII by this mitochondrial-induced O$_{2}^{-}$ is also mediated at least in part by oxidative suppression of protein phosphatases that dephosphorylate CaMKII. Studies by Yin et al. have also showed that in CATH.a neurons AngII increases CaMKII phosphorylation, causing CaMKII activation, with a significant increase observed after 5 minutes of AngII stimulation. However, neurons, which were transduced with AdMnSOD (adenovirus overexpressing manganese superoxide dismutase) had reduced CaMKII phosphorylation, indicating that ROS is involved at least in past in mediating AngII-induced CaMKII activation in central neurons (83). Although these studies convincingly demonstrate the redox sensitivity of CaMKII in physiological and pathological responses, the specific role of redox regulation of neuronal CaMKIIα in AngII-intraneuronal signaling and the AngII-induced hypertensive response is unknown.

Considering the importance of AngII intra-neuronal signaling in the pathogenesis of diseases including hypertension and heart failure, numerous investigations are being carried out to date to better understand the precise
signaling mechanisms both in vivo and in vitro. However, due to the technical challenges in studying AngII-induced intra-neuronal signaling in vivo in animal models of hypertension and heart failure as discussed in Chapter II, neuronal cell culture models have been frequently utilized to examine the specific intra-neuronal signaling pathways induced by AngII (80, 86). Nevertheless, there are also limitations in using neuronal cell culture models to recapitulate the AngII intra-neuronal signaling events occurring in vivo such as: 1) neuronal cells are often immortalized and/or isolated from a tumor; 2) cells are usually cultured in a hyperoxic environment (i.e. 21% oxygen) as compared to their in vivo environment (1-4% oxygen); and 3) the lack of neighboring glia and endothelial cells may alter a particular response in the cultured neurons that would normally occur in vivo. A fourth limitation in using neuronal cell culture models as it specifically relates to understanding AngII intra-neuronal signaling is the lack of evidence indicating the stability of exogenous AngII in neuronal cell culture media. Many studies have examined intra-neuronal responses, such as changes in mRNA levels and protein expression, 1-48 hours after a single administration of exogenous AngII into the neuronal cell culture media (200-202). Data from these studies are often interpreted to indicate that the observed changes are also occurring in neurons of various hypertensive and heart failure models in which circulating and/or brain levels of AngII are chronically elevated (203, 204). However, it remains unclear if a single treatment of exogenous AngII given to neurons in culture results in a chronic elevation of AngII levels in the media. Therefore, considering our ultimate goal of studies to understand AngII-
intraneuronal signaling, we began by measuring levels of AngII in our CATH.a neuronal cell culture media following a single AngII (100 nM) administration. Using liquid chromatography and tandem mass spectrometry, we were able to detect AngII levels in culture media. Importantly, AngII declined to near basal levels 3 hours post the single exogenous AngII administration. Levels of two of the AngII metabolites, AngIII and Ang1-7 modestly, but significantly increased in the media after 15-60 minutes of AngII administration. Furthermore, replenishing the neuronal media with fresh AngII every 3 hours resulted in chronically elevated levels of AngII. It should be noted that as a result of differential expression of AngII receptors and other components of the RAS, the stability of exogenous AngII in cultured media will be drastically different in various cell types. In addition, enzymes involved in AngII metabolism, such as aminopeptidase A (APA) and angiotensin converting enzyme 2 (ACE2) may also have different expression levels depending on the cell type thereby influencing AngII stability in the media.

Next, to determine the redox-regulation of CaMKIIα and its role in AngII-signaling in neurons, we compared adenoviral-mediated overexpression of wild-type CaMKIIα (wt-CaMKIIα) to an oxidation-resistant mutant CaMKIIα (mut-CaMKIIα) in modulating AngII-regulated neuronal K⁺ channel current and the AngII-induced neurogenic hypertension in mice. In an attempt to determine the functional relevance of overexpressing wt-CaMKIIα on AngII-signaling in neurons, we cloned the wt-CaMKIIα gene and an adenovirus (wt-AdCaMKIIα) was generated in the University of Iowa Viral Vector Core Facility. To begin our
studies, we confirmed the adenovirus-mediated overexpression of total and active wt-CaMKIIα protein with increasing multiplicity of infection (MOI) in CATH.a neurons. Both 25MOI and 50MOI of the adenovirus transduction yielded significant overexpression of total and phosphorylated (active) wt-CaMKIIα protein as detected by Western blot analysis. It should be noted that in order to avoid any cytotoxicity with a higher MOI of adenovirus, all the subsequent *in vitro* cell culture experiments were done in the presence of 25MOI of wt-AdCaMKIIα. Wt-CaMKIIα was also transcriptionally upregulated as evident by the mRNA levels measured by RT-PCR. We also looked for transcriptional upregulation of other CaMKII isoforms following overexpression of wt-CaMKIIα. However, mRNA levels of the other CaMKII isoforms did not change confirming the specificity of the adenovirus for CaMKIIα. To note, although CaMKIIα is predominantly present in neurons; however, CATH.a neurons have very little to non-detectable levels of basal CaMKIIα as compared to the other CaMKII isoforms as evident by the very high cycle number in the RT-PCR analysis (Chapter III). Thus, CATH.a neurons serve as an appropriate cell culture model to study the role of CaMKIIα in mediating the downstream signaling effects of AngII in neurons. However, the presence of other CaMKII isoforms should also be considered while analyzing some of the AngII data in CATH.a neurons.

We next set out to examine the effect of wt-CaMKIIα overexpression on the AngII-mediated inhibition of K⁺ current. To perform these set of experiments we collaborated with Dr. Yulong Li and his lab in the department of Cellular and Integrative Physiology at UNMC. Whole-cell configuration of the patch-clamp
technique was used to record voltage-gated $K^+$ current in differentiated mouse CATH.a neurons at baseline and post 5 minutes of AngII superfusion. $K^+$ current was also measured after AngII washout for 15 minutes in order to confirm the changes observed with AngII superfusion. The percent inhibition in peak and steady-state $K^+$ current following AngII stimulation was approximately 16% in both non-transduced and AdEmpty-transduced CATH.a neurons. However, in neurons where wt-CaMKIIα was overexpressed, the AngII-mediated percent inhibition in $K^+$ current was almost 37%, which is significantly higher than the non-transduced and AdEmpty group. Baseline $K^+$ current was also modestly lower in the wt-AdCaMKIIα-transduced CATH.a neurons as compared to the other two groups. Simultaneous experiments were performed where $K^+$ current was recorded in the three groups in the presence of a CaMKII inhibitor, KN-93. Neurons were pretreated with KN-93 for 30 minutes and then baseline current was measured followed by AngII superfusion and washout. Interestingly, the AngII-mediated percent inhibition in $K^+$ current was completely abolished in all the three groups when pretreated with KN-93.

It is important to consider that the presence of other CaMKII isoforms in CATH.a neurons, as demonstrated by the mRNA data, may account for the AngII-mediated reduction in $K^+$ current in the non-transduced and AdEmpty-transduced neurons. Moreover, as illustrated by previous studies that PKC is also involved in the AngII-intraneuronal signaling (80, 81), we speculate that CATH.a neurons may have basal PKC levels which contribute to the AngII-regulation of neuronal ion channels. Furthermore, since KN-93 is a non-specific inhibitor of
CaMKII, it can inhibit different CaMKII isoforms in CATH.a neurons thereby completely attenuating the AngII response in all the three groups. Taken together, these studies identified CaMKIIα as one of the mediators of AngII signaling and its involvement in the exacerbated reduction of K⁺ current in AngII-stimulated neurons.

To determine the causal relationship between overexpression of wt-CaMKIIα and the significant reduction in K⁺ current in CATH.a neurons, we sought to detect the mRNA expression of specific voltage-dependent K⁺ channels which have previously been shown to be present in important autonomic regions of the brain and also involved in AngII signaling in neurons (85, 224, 234). Surprisingly, wt-CaMKIIα overexpression modestly but not significantly increased the basal mRNA expression of Kv2.1, Kv2.2, Kv4.2 and Kv4.3 as compared to non-transduced or AdEmpty-transduced groups. We speculate that transcriptional increase in Kv channels may act as a compensatory mechanism to counterbalance the reduction in K⁺ current following wt-CaMKIIα overexpression in CATH.a neurons. It is also important to consider that the protein expression of these voltage-dependent K⁺ channels was not examined. Moreover, the cellular localization of these voltage-gated channels is crucial for their function and transcriptional up regulation does not necessarily indicate increased membrane localization.

We next investigated the outcome of wt-CaMKIIα overexpression in mouse brain on the acute central AngII-induced pressor response. To compare the acute central AngII response pre and post-adenovirus injection we initially
injected AngII (350 ng) intracerebroventricularly (ICV) to specifically target the SFO. Central AngII injections elicited the characteristic rapid transient increase in blood pressure. Interestingly, 3 days post ICV adenovirus injections the central acute AngII-induced rise in blood pressure was significantly potentiated in wt-CaMKIIα overexpressed mice as compared to AdGFP injected mice. In addition to the increased peak changes in mean arterial pressure (MAP), the duration of the AngII-response was also greater in wt-CaMKIIα overexpressed group. To confirm CaMKIIα overexpression in the SFO, we stained mouse brain sections for total and phosphorylated CaMKIIα protein and found a prominent increase in CaMKIIα expression in the SFO. These data indicate that overexpression of wt-CaMKIIα in the SFO greatly potentiates the central acute AngII-mediated changes in blood pressure. We speculate that since CaMKIIα acts as a downstream molecule of AngII signaling in neurons, therefore increasing wt-CaMKIIα expression will enhance the effects mediated by AngII on neurons, including reduction in $K^+$ current and an increase in neuronal firing thereby leading to the rapid increase in blood pressure.

Whereas the central AngII studies implicate the importance of wt-CaMKIIα in the brain, to correlate it clinically with human essential hypertension, the functional relevance of wt-CaMKIIα overexpression in the brain was evaluated following chronic subcutaneous AngII infusion via osmotic minipumps. For these experiments mice received ICV injections of GFP and wt-CaMKIIα adenovirus following which osmotic minipumps were implanted to subcutaneously infuse chronic AngII (400 ng/kg/min). Mice which received
AdGFP injections showed the characteristic chronic AngII-induced increase in mean arterial pressure with a peak change of 31 mmHg. This gradual increase in blood pressure resembles the slow-pressor AngII-induced hypertensive model and closely correlates with human hypertension. Interestingly, mice with wt-CaMKIIα overexpression had a significant increase in blood pressure immediately following AngII minipump implantation and continued to rise over the course of AngII infusion with a peak change of 39 mmHg. We posit that overexpression of wt-CaMKIIα in the brain sensitized these mice to an immediate increase in blood pressure at an initial subpressor dose of subcutaneous AngII. There could be several possible explanations for this phenomenon. As discussed with the acute AngII data, overexpression of a downstream signaling molecule, such as wt-CaMKIIα, in the central AngII pathway, can simply potentiate AngII response following minipump implantation. Additionally, although CaMKII is thought to be downstream of the redox mechanisms, few studies have shown that CaMKII can also activate NOX and increase ROS generation via a positive feedback mechanism (225). Further studies are also needed to determine if wt-CaMKIIα overexpression has any effect on the integrity of the blood brain barrier which may cause a rapid access of AngII, following subcutaneous infusion, to certain brain regions that are normally protected by the blood brain barrier.

Based on previous studies demonstrating the Ca\(^{2+}\)/CaM independent sustained CaMKII oxidation in the cardiomyocyte CaMKIIδ isoform (181), we next generated an adenovirus overexpressing CaMKIIα (mut-AdCaMKIIα) with mutations in cysteine and methionine at residues 280 and 281 respectively. All
the CaMKII isoforms except CaMKIIα has paired methionine residues at 281/282 position where as CaMKIIα has a cysteine at the 280 position. Since both cysteine and methionine are highly susceptible to oxidation because of their low pKa values, we mutated cysteine to an alanine and the methionine to a valine in order for CaMKIIα to become resistant to oxidation. To check whether mutation in these residues interfere with CaMKIIα expression we used Western blot analysis in CATH.a neurons that were transduced with mut-AdCaMKIIα at different MOIs. As seen with the wt-CaMKIIα adenovirus, transduction of the mut adenovirus had a significant overexpression of both total and phosphorylated CaMKIIα protein at 10, 25 and 50 MOI. It should be noted that in order to be consistent with our wt-CaMKIIα studies in vitro, we selected the 25MOI of the mutant adenovirus for our next set of cell culture studies. CaMKIIα gene expression was also detected using RT-PCR in CATH.a neurons following adenovirus transduction. Similar to the wild-type adenovirus data, mut-CaMKIIα did not interfere with transcription of the other CaMKII isoforms. These studies suggest that the mutant CaMKIIα gene is able to make a functional protein and does not act as a dominant negative mutation to inhibit the endogenous protein in CATH.a neurons.

We next investigated the effect of mut-CaMKIIα on the AngII-mediated reduction in K⁺ current in CATH.a neurons. While our studies with the wt-CaMKIIα adenovirus clearly demonstrated that the AngII-mediated inhibition of K⁺ current was greatly exacerbated in wt-CaMKIIα overexpressing neurons, CATH.a neurons that were transduced with mut-AdCaMKIIα had similar change in K⁺ current as the non-transduced and AdEmpty-transduced neurons. These studies
once again indicate that while in CATH.a neurons CaMKIIα may not be the major mediator of AngII downstream signaling, however, mutation of the oxidation susceptible residues of CaMKIIα attenuate the potentiated effects of wt-CaMKIIα on AngII-regulation of K+ current indicating the importance of CaMKIIα in AngII intraneuronal signaling.

Our *in vivo* studies with wt-CaMKIIα suggested that overexpression of wt-CaMKIIα in the SFO sensitizes mice to an immediate increase in blood pressure at an initial subpressor dose of subcutaneous AngII. To investigate the role of mut-CaMKIIα on the chronic AngII-induced hypertensive response, we injected either ICV AdGFP or mut-AdCaMKIIα prior to AngII minipump implantation. Interestingly, blood pressure in mice with mut-CaMKIIα went up immediately after minipump implantation as seen with wt-CaMKIIα overexpression; however, after 2 days the blood pressure declined and followed the characteristic AngII-mediated gradual rise in blood pressure as seen in AdGFP group. The peak change (28 mmHg) in mut-AdCaMKIIα group was modestly decreased than the AdGFP injected mice. However, the peak increase in blood pressure was significantly lower in the mut-CaMKIIα mice as compared to the wt-CaMKIIα overexpressed group. Heart rate did not change between the groups. The initial rise in blood pressure in the mutant group 1-2 days after AngII infusion may be explained as a result of the presence of activated (phosphorylated) CaMKIIα to some extent. It should be noted that although mutation of the two residues will inhibit oxidation in those specific residues thereby preventing sustained CaMKIIα activation; however, CaMKIIα can still be
autophosphorylated in its threonine residue and get activated. Thus, our data confirm that mutation of the redox-sensitive residues in CaMKIIα leads to the characteristic gradual increase in blood pressure with slow pressor AngII infusion with a modest decrease in the peak AngII response as compared with AdGFP group. Furthermore, mut-CaMKIIα prevents the initial sensitization in mice as seen with wt-CaMKIIα overexpression.
Significance of Research

Increasing prevalence of hypertension associated with a high morbidity and mortality rate is a worldwide epidemic that is persistently rising. As per the World Health Organization (WHO) an estimated 17 million deaths worldwide each year are caused by cardiovascular disease, and complications of hypertension account for 9.4 million of these deaths. In 2012, a report released by the Center for Disease Control (CDC) stated that within the United States an estimated 67 million Americans have hypertension and of the 47 million people who are receiving current treatment for hypertension, only 31 million people have their blood pressure controlled by prescribed medications and/or lifestyle changes. This alarming statistics indicate that 16 million hypertension patients (34%) are non-responsive to the current hypertensive therapeutics.

Chronic hypertension results in a myriad of pathologies such as end-organ damage, including vasculature remodeling and dysfunction, cardiac hypertrophy, thrombosis, fibrosis, nephrosclerosis, atherosclerosis, neurocognitive disorders, retinopathy, and aneurysm. Since high blood pressure often has no symptoms, it is left untreated and uncontrolled hypertension is a risk for several cardiovascular diseases, such as heart failure, stroke, coronary artery disease, kidney disease, and peripheral vascular disease (5, 235). Currently, the available antihypertensive prescribed medications are the ones that regulate renin-angiotensin-aldosterone activity, sympathetic/parasympathetic tone, salt and water excretion by the kidneys, and the ion channels. One of the common therapies for cardiovascular diseases is systemic treatment with β-blockers to
reduce heart rate and blood pressure by blocking the effects of norepinephrine and epinephrine. However, many of these drugs can cause adverse side effects including dizziness, headache, insomnia, weakness and fatigue (231). In addition, a significant population of hypertensive patients is not able to control their high blood pressure in spite of taking a single or a combination of the above mentioned drugs.

Numerous studies indicate that cardiovascular diseases, such as hypertension and heart failure involves activation of the brain renin-angiotensin system and elevated levels of AngII, the primary effector peptide of the RAS (203, 204). AngII-intraneuronal signaling involves increased generation of $O_2^{•-}$ which is involved in the regulation of neuronal ion channels, increased neuronal activation thereby leading to sympathoexcitation and increased blood pressure (83, 110). We therefore wanted to closely examine the AngII signaling mechanisms in the neurons in order to identify novel therapeutic targets for hypertension. Since, CaMKII has been identified previously as an important mediator of AngII downstream signaling in neurons (80, 81), we propose that AngII stimulation of neurons induce redox-modification of specific residues in CaMKIIα which leads to inhibition of K⁺ channel current and an increase in blood pressure following AngII infusion in mice.

Although CaMKII has been shown to be redox-sensitive and susceptible to oxidation in the cardiomyocytes in presence of AngII (181, 187, 220), the role of AngII in regulation of predominant neuronal isoform of CaMKII (CaMKIIα) is unknown. To closely examine the role of CaMKIIα in AngII signaling
in neurons we generated adenoviruses to overexpress both a wild-type form of CaMKIIα (wt-CaMKIIα) and a mutant CaMKIIα (mut-CaMKIIα) in which specific redox-sensitive cysteine and methionine residues were mutated. Our studies suggest that overexpressing the wt-CaMKIIα in neurons exacerbated the AngII-mediated inhibition of neuronal K⁺ current. Additionally, wt-CaMKIIα when overexpressed in the brain sensitized mice to a significant increase in blood pressure immediately following AngII infusion. In comparison, mut-CaMKIIα attenuated the AngII-mediation reduction in K⁺ current as compared to the wt-CaMKIIα and overexpression of mut-CaMKIIα in the brain attenuated the peak AngII response in a mouse model of AngII hypertension.

Furthermore, our mass spectrometry studies using CATH.a neuronal cell culture media indicated that a single exogenous administration of AngII (100 nM) is rapidly metabolized within 3 hours in the culture media and should be replenished every 3 hours to in order to maintain chronically elevated AngII levels. This is relevant for studies that examine AngII neuronal signaling in vitro as an attempt to identify mechanisms occurring in patients with hypertension or heart failure in which levels of AngII is chronically elevated.

In summary, our studies have identified CaMKIIα as a downstream target of AngII signaling in neurons. More specifically we demonstrated that mutation of redox-sensitive residues in CaMKIIα can attenuate the potentiated AngII-response in wt-CaMKIIα overexpressed CATH.a neurons. The AngII-induced hypertensive response was also attenuated in mice with mut-CaMKIIα overexpression. Taken together, these studies provide an important insight in the
redox modifications of CaMKIIα as seen in previous studies with a different CaMKII isoform. Targeting these residues in CaMKIIα or preventing their oxidation may therefore identify novel therapeutic targets to treat hypertension or other diseases in which CaMKIIα is an important player and is susceptible to oxidation.

Limitations of the study

Although we believe that our studies have advanced the understanding of AngII signaling in neurons associated with AngII-mediated neurogenic hypertension, there are certain limitations of this study that we need to consider. One obvious limitation to our studies is the use of a single in vitro cell culture model. It is important to highlight that the catecholaminergic CATH.a neuronal cell line used in this study have been widely identified in the literature as exhibiting similar AngII-intraneuronal signaling mechanisms as primary neurons isolated from the hypothalamus and brain stem (14, 79). However, further investigation is needed with primary neurons isolated from the brains of AngII-infused hypertensive mice to determine the ROS-mediated CaMKIIα oxidation levels. In addition, neurons cultured from a variety of cardiovascular brain centers, including the area postrema, paraventricular nucleus and the rostral ventrolateral medulla, in addition to the subfornical organ, will also provide important insight into the role of CaMKIIα in AngII-downstream signaling in those brain regions.

It is also important to consider that the CATH.a neuronal cells were
cultured in a 21% hyperoxic environment. This is important particularly for our studies as reactive oxygen species are generated by electron reduction of molecular oxygen. In addition, neuronal cells in the brain receive about 2-4% oxygen which is significantly less than the culture environment for CATH.a cells. An ideal in vitro neuronal cell culture environment for conducting these experiments would have been in an incubator with 2-4% oxygen and 5% CO₂.

A major limitation of the study is that we were not able to differentiate between the different isoforms of CaMKII in mediating AngII intra-neuronal signaling. Although we are able to show that CaMKIIα is involved in AngII signaling and mutating oxidation-sensitive residues of CaMKIIα blunts the potentiated AngII-mediated blood pressure response and the AngII-induced decrease in K⁺ current; however, the involvement of other CaMKII isoforms, such as CaMKIIβ cannot be overruled. Infact, CATH.a neurons has greater levels of CaMKIIβ as compared to CaMKIIα as detected by RT-PCR. Future studies are needed to investigate role of different CaMKII isoforms in mediating AngII signaling in central neurons.

Another limitation of the study is that we did not perform central acute AngII injections with ICV mut-CaMKIIα in mice brain. Our wt-CaMKIIα adenovirus studies indicated that mice with wt-AdCaMKIIα had a greater increase in mean arterial pressure with central acute AngII injections. It would be interesting to see if mut-CaMKIIα prevents the increased rise in blood pressure following AngII injection. If there is still an increase in blood pressure in the mut-AdCaMKIIα group, that may further confirm our chronic AngII infusion data that the initial rise
in blood pressure is a result of active phosphorylated CaMKIIα where as the chronic sustained blood pressure increase is because of CaMKIIα oxidation.

**Future Studies**

Although we believe our studies have provided important insight in AngII signaling in central neurons, future studies are required to better understand the redox regulation of CaMKIIα and its effect on AngII-stimulated central neurons. One limitation of our studies that needs to be addressed is the identification of specific residues in CaMKIIα that are important in mediating AngII responses in neurons. Based on previous studies in a cardiomyocyte isoform of CaMKII (181), we mutated the cysteine and methionine residues of CaMKIIα at positions 280 and 281 respectively; however, mass spectrometry and proteomics approaches should be utilized to confirm our findings. Moreover, it would be interesting to see whether a single residue mutation of either the cysteine or methionine is as effective as the double mutant. Furthermore, one study has suggested that CaMKIIα, involved in nitric oxide-induced excitotoxic neuronal cell death, undergoes S-nitrosylation in two cysteine residues at positions 280 and 289 (189). The study also suggested that mutation of either cysteine abolished the autonomous activity of CaMKIIα thereby indicating the importance of those two residues in CaMKIIα activation. It is therefore reasonable to believe that mutating C289 in addition to our double mutation model may have a greater attenuation of the AngII-induced increased blood pressure.
Another important question that needs to be addressed is the role of specific oxidants in mediating post-translational modifications of CaMKIIα. Anderson et al. showed that CaMKIIδ undergoes oxidation in the paired methionine residues in the presence of H₂O₂ (181). Studies from our lab show that AngII increased CaMKII phosphorylation in CATH.a neurons and overexpression of MnSOD attenuates the AngII-mediated CaMKII phosphorylation (83). Preliminary studies in our lab also suggest that CaMKII oxidation increases within 30 minutes in presence of AngII stimulation; however, the specific role of O₂⁻ or H₂O₂ in mediating CaMKIIα oxidation is still not clear. Further studies are needed to overexpress specific ROS scavenging enzyme in order to tease out their role in AngII-mediated CaMKIIα oxidation. More specifically, experiments should be done in CATH.a neurons with adenovirus overexpressing superoxide dismutase (SOD) to scavenge O₂⁻ to determine their role in mediating CaMKIIα oxidation. Since dismutation of O₂⁻ generates H₂O₂, it is important to use a specific H₂O₂ scavenger such as, catalase or glutathione peroxidase (GPx). Co-transfection of adenovirus overexpressing SOD and catalase should be utilized to determine whether the effect is due to scavenging O₂⁻ or producing high amounts of H₂O₂. Furthermore, previous reports indicate that AngII stimulation may increase H₂O₂ levels through NOX activity, monoamine oxidase activity, or from mitochondrial respiration coupled to endogenous SOD (236, 237). Additionally, since S-nitrosylation of CaMKIIα causes autonomous CaMKIIα activity (189), it would be interesting to see the functional relevance of overexpressing the different scavenging enzymes on the S-nitrosylation status. Since CaMKII is primarily
cytoplasmic, investigating the effect of scavenging oxidants in specific subcellular compartments on CaMKIIα oxidation is also important. Apart from targeting cytoplasmic ROS mitochondrial targeted SOD and catalase can be utilized to specifically scavenge mitochondrial O$_2^{•-}$ and H$_2$O$_2$ and CaMKIIα oxidation levels can then be measured.

Future studies should also aim at evaluating some of the mechanism regulating CaMKII oxidation. For example, oxidation of methionine residues leads to formation of methionine sulfoxide which can be reversed by methionine sulfoxide reductases (Msr) (176). Indeed, methionine sulfoxide reductase A knockout mouse (MsrA$^{-/-}$) show increased CaMKII δ oxidation with AngII and ischemic stress response (181). However, the role of Msr in AngII intra-neuronal signaling and AngII-induced hypertensive response is not clear. The expression levels and activity of these enzymes in cardiovascular regulatory brain nuclei, such as the subfornical organ in case of AngII-infused hypertensive model is not known. It would be interesting to see if overexpression of Msr in neurons is able to prevent the potentiated AngII-response and the increased blood pressure in mice.

Studies by Hongpaisan et al. have shown that in hippocampal neurons, increased mitochondrial O$_2^{•-}$ activates CaMKII and increases CaMKII kinase activity (190). They also demonstrated that the increased kinase activity is mediated at least in part, by suppressing protein phosphatases, such as PP1 and PP2A. Since CaMKII is classically activated by autophosphorylation in Thr 286 (Thr 287 in other CaMKII isoforms) protein phosphatases play a crucial role in
deactivating CaMKII by dephosphorylating the threonine residue. In our preliminary studies we found that AngII inhibits PP2A activity in CATH.a neurons without affecting PP2A protein expression. Western blot analysis for all three subunits of PP2A demonstrated that AngII does not change protein expression of PP2A subunits. However, overexpression of MnSOD to inhibit mitochondrial O$_2^{\cdot-}$ attenuates the AngII-mediated decrease in PP2A activity. Since, protein phosphatases are also known to be redox-sensitive (179, 180), future studies are needed to determine ROS-mediated PP2A modification which might explain the AngII-induced decrease in PP2A activity.

Our studies elucidated that overexpression of wt-CaMKIIα exacerbates the AngII-mediated inhibition of $K^+$ current in CATH.a neurons. In addition the mut-CaMKIIα is capable of attenuating the potentiated AngII response in wt-CaMKIIα transduced neurons. Although the RT-PCR data for the different voltage-gated $K^+$ channels show that wt-CaMKIIα overexpression increases the basal expression of these Kv channels, further studies must be done to determine their protein expression in addition to cellular localization. Among the several targets of CaMKII, one of the important target are the ion channels and so direct regulation of Kv channels by CaMKIIα could be assessed in CATH.a neurons. Co-immunoprecipitation experiments could ascertain if CaMKIIα directly interacts with Kv channels and phosphorylates them. It was previously reported that AngII downregulates $K_{v4.3}$ in CATH.a neurons and also in the RVLM of rats with chronic heart failure (85). It is therefore important to determine whether the AngII-mediated downregulation of $K_{v4.3}$ along with the
other Kv channels is attenuated with mut-CaMKIIα overexpression.

Additionally, one of the most exciting future experiments would be to identify other proteins that are modulated by \( \text{O}_2^\cdot \) in presence of AngII. ROS are known to regulate a number of proteins by modifying their amino acid residues within their active sites. An exciting future investigation would be to determine whether ROS can directly act on ion channels to post-translationally modify them and regulate the flow of ions. It is known that in neurons \( \text{H}_2\text{O}_2 \) accelerates the \( \text{Ca}^{2+} \) channel opening in the plasma membrane by oxidizing certain subunits within the channel complex. Studies by Zimmerman et al. have demonstrated that the AngII-mediated \( \text{O}_2^\cdot \) is involved in the increased influx of extracellular \( \text{Ca}^{2+} \) through voltage-gated \( \text{Ca}^{2+} \) channels (84). It is therefore important to know whether \( \text{O}_2^\cdot \) or \( \text{H}_2\text{O}_2 \) play a direct regulation of either the \( \text{Ca}^{2+} \) or the \( \text{K}^+ \) channels in neuronal cells. Furthermore, Sumners and colleagues have clearly identified PKC as a downstream mediator of AngII signaling in neurons (14, 79). Since PKCs are also \( \text{Ca}^{2+} \) dependent enzymes and have active site cysteines it is reasonable to believe that PKCs are susceptible to ROS-mediated oxidation. In fact reports have previously shown that in different cell types ROS can act either upstream or downstream of PKCs thereby amplifying the response. For example, PKC can activate NADPH oxidase thus increasing the generation of ROS which in turn activate and regulate PKC function (238). These are exciting and critical studies that will build upon the novel findings of our studies.

In addition to ROS, reactive nitrogen species (RNS) has also received considerable attention in the past years regarding their role on the cardiovascular
system. Nitric oxide (NO) plays an important role in cardiovascular regulation by causing vasodilation in the periphery. In the cardiovascular regulatory regions of the brain, NO acts through a GABA-dependent mechanism to induce sympatho-inhibition that decreases blood pressure and heart rate (239). In a diffusion-limited reaction $O_2^*$ has the ability to quench endogenous NO and form peroxynitrite (OONO$^-$) thereby reducing NO bioavailability. As opposed to NO, OONO$^-$ is considered to be a toxic and powerful oxidant with known pathological consequences (240, 241). ONOO$^-$ is also potent in causing irreversible protein nitrination thereby changing the biological function of the target protein. Hence, the role of ONOO$^-$ on CaMKII$\alpha$ or other downstream molecules of AngII-signaling could reveal other possible redox modifications of those proteins. Therefore unraveling these AngII-redox mechanisms within the CNS will be crucial studies to pursue.

Additional research is also needed to determine the role of reactive carbonyl species (RCS) on CaMKII$\alpha$ and other downstream targets of AngII-intraneuronal signaling and their subsequent involvement in neuro-cardiovascular diseases. Reactive carbonyl species, such as acrolein, methylglyoxyl, malondialdehyde, 3-deoxyglucosone, and 4-hydroxy-2-alkenals, are small diffusible aldehydes or ketones that are generated from oxidation of lipids, glucose, and amino acids. Although the importance of RCS in cardiovascular disease is unclear, it may be possible that the RCS act as the primary mediators of dysfunction and damage (242-245) whereas ROS are the secondary mediators. This might explain why many clinical trials with
antioxidants trying to target the ROS have failed. In future studies, we could overexpress glutathione S-transferases, aldose reductase, aldehyde dehydrogenase, and glyoxylases to degrade RCS and determine their functional relevance in mediating post-translational modifications of CaMKIIα or other downstream signaling molecule of AngII in central neurons.

In summary, we believe that our studies have demonstrated the importance of CaMKIIα in mediating AngII-signaling in neurons and the AngII-induced hypertension. Importantly, our studies have opened the door for an extensive series of both in vivo and in vitro future experiments designed to better understand the role of redox-sensitive proteins in central AngII signaling. In performing these additional studies, it is our hope that novel therapeutics will be developed to treat AngII-dependent cardiovascular diseases, including hypertension.
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