ANGIOTENSIN (1-7) ATTENUATES THE CHRONOTROPIC RESPONSE TO

ANGIOTENSIN II VIA STIMULATION OF PTEN IN SPONTANEOUSLY HYPERTENSIVE

RAT BRAIN

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The Supervisory Committee certifies that this *disquisition* complies with North Dakota State
University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

The pathogenesis of hypertension and its mode of progression are complex, multifactorial and incompletely understood. Several studies have focused on the beneficial effects of peripheral Ang (1-7) in the regulation of cardiovascular functions, showing the counter-regulatory effects of Ang (1-7) against the actions of Ang II in the periphery. However, its actions in the central nervous system are not completely understood. In the present study, our main goal was to determine the central action of Ang (1-7) and its interaction with Ang II in the blood pressure control.

Previous studies reported that Ang II produces a greater degree of activation of neuronal cells from brainstem/hypothalamus cultures of SHR versus WKY rats. Our present findings showed that this enhanced action of Ang II was attenuated in co-presence of either Ang (1-7) or PI3-kinase inhibitor. These counter-regulatory effects of Ang (1-7) on Ang II action in SHR neurons were abolished by co-treatment with either A-779, a Mas-R antagonist, or bisperoxovanadium (BPV), a PTEN inhibitor. In addition, incubation of WKY and SHR neurons with Ang (1-7) significantly increased PTEN activity.

Chronic treatment with Ang (1-7) or chronic inhibition of PI3K using the lentiviral vector significantly abolished the enhanced chronotropic response to Ang II in SHR neurons and significantly enhanced PTEN protein and mRNA expression levels in both WKY and SHR neuronal cultures.

To further check the functional implications of our in vitro data, we further studied the interaction between Ang II and Ang (1-7) in the central control of cardiovascular functions. RVLM microinjection of Ang (1-7) or LY-294002 alone did not alter MAP, but reduced the pressor response to Ang II in SHR. Moreover, in compliance with our in vitro data, the

inhibitory effect of Ang (1-7) on the pressor response to Ang II in SHR was abolished when co-administered together with A-779 or BPV.

The data demonstrated that Ang (1-7) induce PTEN activity and expression via Mas-R, and depresses PI3-kinase-PKB/Akt signal transduction pathway, which lead to the counter-regulatory effect of Ang (1-7) on Ang II induced chronotropic and pressor effect on neuronal activity and cardiovascular functions including MAP and HR in SHR.

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DEDICATION

This dissertation is dedicated to my parents Avinash & Rekha for their constant support and encouragement throughout my life. I owe them everything and wish I could show them just how much I love and appreciate them. I would also like to dedicate this work to my lost relatives including Grandfather Mani Ram Modgil, Grandmother Panna Devi and my uncle Brigadier Dr. R. K. Modgil, who left us too soon. I hope that this work makes you proud.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACE..Angiotensin converting enzyme

AVP..Arginine vasopressin

AP..Action potential

ARC..Cytosine-β-d-arabinofuranoside

AT1R or AT2R..Angiotensin type-1or 2 receptor

Ang..Angiotensin

BP..Blood pressure

bpm...Beats per minute

BK...Bradykinin

BPV...Bisperoxovanadium

CaMKII..Calcium/calmodulin-dependent protein kinase II

CVS...Cardiovascular system

CNS...Central nervous system

DMEM..Dulbecco's modified eagle medium

DNase 1..Deoxyribonuclease 1

DN...Dominant negative construct

GPCR...G-protein coupled receptor

HR...Heart rate

I_A...transient A-type K^+ current

ICV..Intracerebroventricular

I_{Kv}..delayed rectifier K^+ current

LV..Lenti-virus

MAP..Mean atrial pressure

NTS...Nucleus tractus solitarii

PRR...Pro renin receptor

PVN...Paraventricular nucleus

PIP..Phosphatidy inositol mono phosphate

PI3-K..Phosphatidylinositol-3-kinase

PKC...Protein kinase C

PLC...Protein Lipase C

PTEN...Phosphatase and tensin homologue deleted on chromosome ten

PDHS...Plasma-derived horse serum

RAS...Renin angiotensin system

RVLM..Rostral ventrolateral medulla

SD...Sprague Dawly

SHR...Spontaneously hypertensive rats

SNA...Sympathetic nerve activity

WKY..Wister Kyoto rats

CHAPTER I. INTRODUCTION

Cardiovascular diseases will be the greatest health care burden of the twenty-first century

(Yusuf *et al*., 2001). A major risk factor for these deadly heart diseases is high blood pressure

(hypertension) (Carretero *et al*., 2000). Hypertension is defined as a chronic increase of blood

pressure (BP), and it is called neurogenic if the probable cause of the disease is an abnormality

of an autonomic nervous system rather than a vascular or renal defect. Despite, the extensive use

of traditional anti-hypertensive strategies in clinical practice and progress made by

pharmacotherapy in the management and control of hypertension, there are still more than ~74

million Americans affected by this deadly disease. By far, the most disappointing statistic is that

these numbers are growing continuously and its global occurrence is projected to exceed 1.5

billion in 2025, which implicate that one in three people worldwide are likely to suffer from this

morbid disease (Kearney *et al*., 2005).

1.1. Etiology of Neurogenic Hypertension

Hypertension is one of the most prevalent cardiovascular disorders with devastating

economic and financial impact to society and is the major risk factor for other cardiovascular

diseases such as stroke, arteriosclerosis, myocardial infarction, kidney failure etc (Figure 2). The

etiology of this disease is multifactorial, resulting from the interaction of a number of genetic

and environmental factors (Figure 1) (Jacob *et al*., 1999). Despite the intensive research efforts

in this area from last two decades, the specific mechanisms involved in mediating increase in BP

that characterize hypertension are poorly understood.
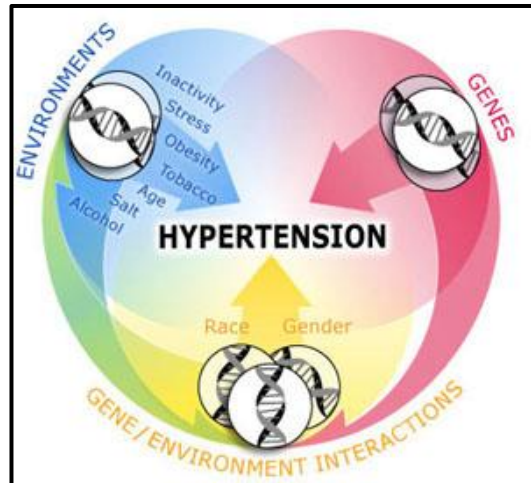
Figure 1: **M**ultifactorial nature of the Hypertension. a disease resulting from the interaction of a number of genetic and environmental factors (Source unknown: Internet)

The most distinctive characteristic of the neurogenic hypertension is an alteration in neural cardiovascular (vagal/sympathetic) control mechanisms. Studies demonstrating a significant enhancement of the vasomotor and cardiac sympathetic drive, as well as a reduction in the parasympathetic drive in borderline hypertensive patients, support this premise (Grassi *et al*., 2010; Grassi *et al*., 2007; Grassi *et al*., 2009). In addition, hypertensive patients show a greater plasma norepinephrine level and greater norepinephrine spillover in both the peripheral circulation and blood draining from the brain, as well as raised sympathetic postganglionic activity targeting the skeletal muscle vascular bed (Anderson *et al.,* 1989*;* Grassi *et al*., 1998; Esler *et al*., 1989; Ferrier *et al*., 1992). Studies on animal models corroborate the human data. Recent evidence shows that even the monogenic forms of hypertension, such as the apparent mineralocorticoid excess, which greatly depends on salt-absorptive mechanisms of the kidney, have also been attributed to an elevated sympathetic drive, indicating a very close association between renal dysfunction and sympathetic overdrive (Bailey *et al*., 2008*;* Krum *et al*., 2009).

Equally, the Goldblatt model partially depends on signaling within the rostral ventrolateral medulla for the maintenance of hypertension (Goldblatt *et al*., 1934).
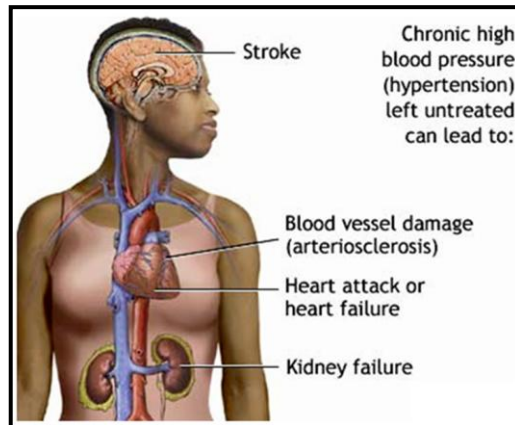
Figure 2: Consequences of chronic high blood pressure. such as stroke, arteriosclerosis, heart attack, kidney failure etc. (Source: www.adamimages.com)

1.2. Sympathetic Nervous System in Neurogenic Hypertension

The pathogenesis of hypertension and its mode of progression are complex, multifactoral and incompletely understood. There are accumulating evidences from humans and animal models of hypertension indicating that excessive central sympathetic nerve activity (SNA) plays a pathogenic role in triggering and sustaining the essential hypertensive state (Grassi *et al.,* 2010*;* Fisher & Fadel, 2010). BP is a function of two variables controlled by autonomic nervous system that is vascular resistance and cardiac output, which is a collection of afferent and efferent neurons that connect the central nervous system with visceral effectors. BP is regulated by sympathetic and parasympathetic nerve activity- the efferent arms of autonomic nervous system, circulating hormones and local autoregulatory mechanisms that interact to control cardiac output and vascular resistance. In addition, BP is modulated by the central integration of afferent neural and hormonal inputs from the periphery. Many studies have demonstrated a vital role for the

3

central nervous system in the development and maintenance of hypertension (Figure 3).

Specifically, elevation in sympathetic nerve activity and variation in arterial baroreflex function

contribute a major role in the pathogenesis and progression of this disease (reviewed by

Veerasingham *et al.*, 2003 and Guyenet et al., 2006).
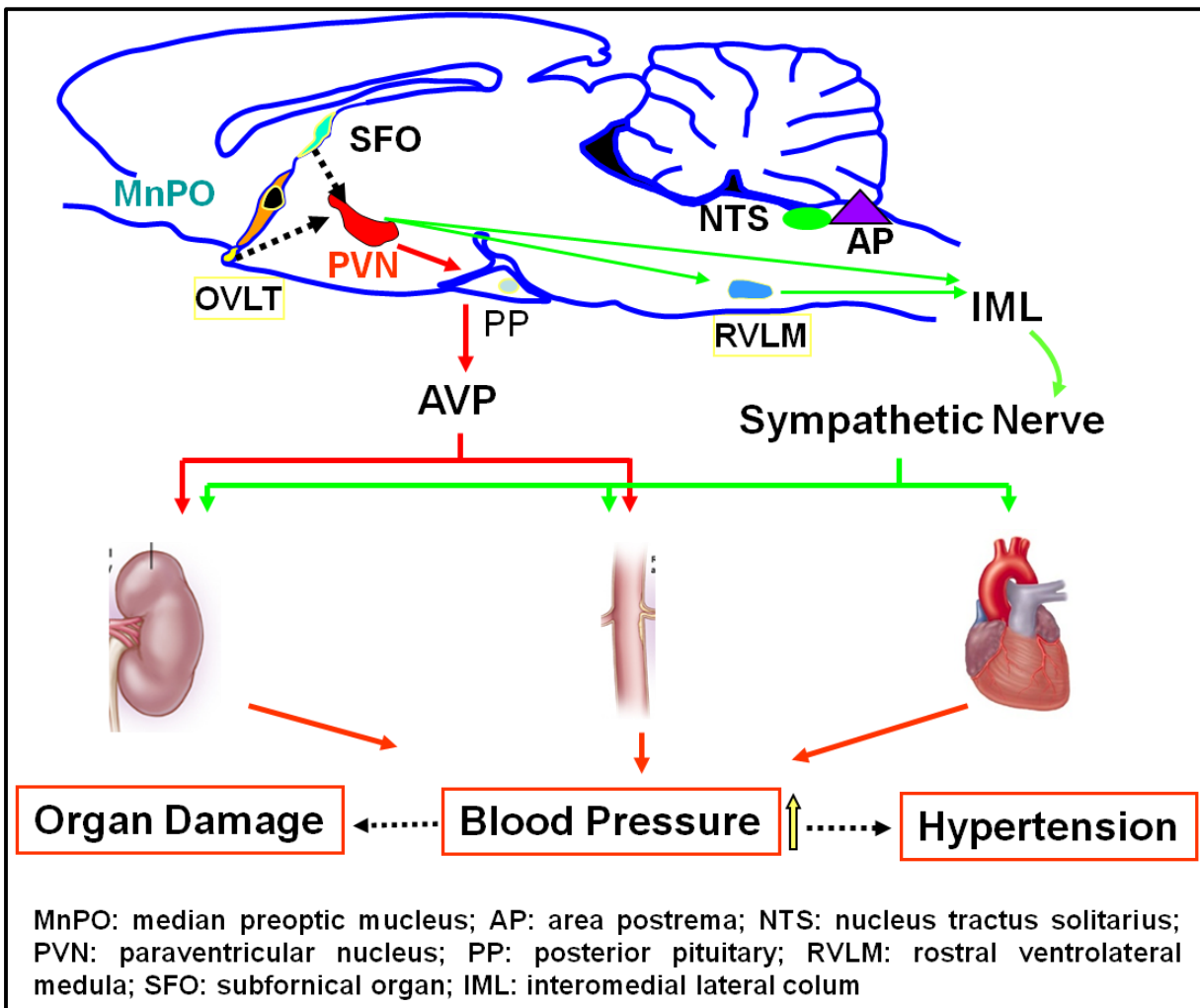
Figure 3: Central regulation and mechanisms of hypertension. In the brain, some areas, such as the subfornical organ (SFO), the organum vasculosum of the lamina terminals (OVLT), and the area postrema (AP), contain AT1 receptors that are accessible to circulating Ang II. Other areas, such as the supraoptic (SON) and the paraventricular nucleus (PVN) of the hypothalamus, the rostral (R) and caudal (C) ventrolateral medulla (VLM), and the nucleus tractus solitarii (NTS), also contain AT1 receptors that cannot be reached by systemic Ang II owing to the blood-brain barrier. These regions are only accessible to Ang II synthesized locally in the brain. The angiotensin-sensitive areas are influenced by outside effectors including plasma Ang II and interconnected by angiotensinergic pathways (red arrows) that elicit the indicated physiological effects.

Elevation in sympathetic drive is the major factor for the development and progression of hypertension in most of the animal models of hypertension, such as SHRs, the renin transgenic rat, the Dahl salt-sensitive rat and the deoxycorticosterone acetate salt rat (Takeda & Bunag, 1980; Arribas *et al*., 1996; Cabassi *et al*., 2002; Leenen *et al*., 2002). Increased sympathetic drive directly acts upon heart and amplifies the force and rate of heart contraction alongwith direct vasoconstriction and would lead to elevation in BP. Moreover, renal sympathetic nerve activity causes renin discharge that initiates a cascade of events via activating systemic RAS leading to more production of Ang II systemically which leads to Ang II-induced antinatriuresis and vasoconstriction. Sustained increases in sympathetic outflow would also contribute to elevation of BP by causing trophic effects on vascular smooth muscle, leading to increases in vascular resistance and enhanced responses to vasoconstrictor stimuli, and vascular hypertrophy. In addition to SNA, blunted arterial baroreflex function has also been implicated in the progression and development of hypertension. Any alteration in BP are detected by carotid sinus and aortic arch baroreceptors which further transmit the signals and modulate parasympathetic and sympathetic nerve activity and, hence, vascular tone and heart rate. This minimizes fluctuations in BP and maintains it close to a particular set point. In response to a static increase in BP, the baroreflex rapidly resets towards a higher pressure (Andresen & Yang, 1989). In hypertensive conditions, resetting of the operational point of the arterial baroreflex may therefore contribute to maintaining an increased BP rather than opposing it. In SHR, Lyon hypertensive rats, TGR mRen2 rats, DOCA-salt rats and Dahl salt-sensitive rats, the gain of the baroreflex, a measure of the baroreceptor reflex sensitivity, is decreased compared to normotensive controls resulting in blunted baroreflex control of heart rate and sympathetic nerve activity (Miyajima & Bunag, 1986; Hayashi *et al*., 1988; Nakamura *et al*., 1988; Lantelme *et al*., 1998; Borgonio *et*

5

al., 2001). In many of these models, the impairment in baroreflex function precedes the development of hypertension and may therefore contribute to the development and maintenance of hypertension. Similar to animal models of hypertension, essential human hypertension is also associated with increases in sympathetic drive and compromise in arterial baroreflex function. The antihypertensive efficacy of sympatholytic drugs such as α_1- and β- adrenergic receptor antagonists also implicates a central mechanism in primary human hypertension.

While these studies implicate central mechanisms of sympathetic activation in the pathogenesis of hypertension, particular areas/nuclei involved are yet to be identified. In most of hypertensive animal models, most of the functional changes within the CNS have been detected fundamentally in hypothalamic and medullary areas that modulate sympathetic outflow (Figure 3) (Colombari *et al*., 2001; De Wardener *et al*., 2001). A large number of neurotransmitters and neuromodulators contribute to regulating sympathetic outflow. Many studies have focused on a neuropeptide, Ang II, as increased expression and activity of components of the intrinsic brain RAS play a key role in many forms of experimental hypertension including the SHR, the TGR mRen2 rat, the Dahl salt-sensitive rat, the DOCA-salt rat, and renal hypertensive rats (De Wardener *et al*., 2001; Itaya *et al*., 1986*;* Berecek *et al*., 1987*;* Huang *et al*.,1998; Fontes *et al*., 2000; Kagiyama *et al*., 2001; Park *et al*., 2001). Some of these models, including the SHR, exhibit both a hyperactive endocrine RAS, characterized by elevated circulating Ang II, as well as a hyperactive tissue RAS, characterized by elevated tissue Ang II. Within the CNS, Ang II produces cardiovascular regulation *via* its direct action at various hypothalamic and medullary areas to elevate sympathetic outflow, compromise the sensitivity of the baroreflex and induce secretion of vasopressin (Culman *et al*., 1995 Averill *et al*., 2000; Mckinley *et al*., 2001; Dampney *et al*., 2002). More specifically, the contributions of Ang II to the central nervous

system control of BP are manifested via alterations in the electrical activity of neurons at specific

cardiovascular regulatory regions of the hypothalamus, which also receive many inputs from

brainstem sites such as the rostral ventrolateral medulla (RVLM) and nucleus tractus solitaries

(NTS), with subsequent activation of hypothalamic sites, such as the paraventricular nucleus

(PVN) (Figure 3).
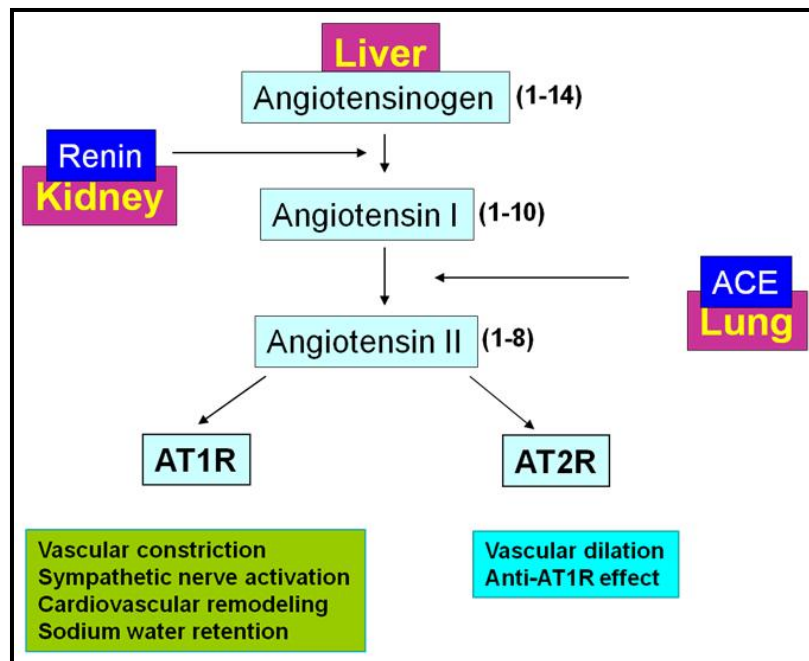
Figure 4: Classical Renin Angiotensin System. Angiotensinogen mainly expressed in the liver is cleaved by renin from the kidneys to release Ang I in plasma. ACE on Endothelial cells metabolizes Ang I further to Ang II. This peptide interacts with either AT1 receptor in numerous cardiovascular organs to elicit the indicated effects, all leading to sodium retention, hypertension, or organ damage. In the adrenal cortex, Ang II induces aldosterone secretion, which aggravates the effects of Ang II.

1.3. Overview of Renin Angiotensin System

The renin angiotensin system (RAS) is a central regulator of cardiovascular and renal

homeostasis and it is critically involved in the physiological regulation of blood pressure (BP),

7

body fluid homeostasis and several other cardiovascular functions (Veerasingham *et al.,* 2003*;* Averill *et al.,* 2000*;* de Gasparo *et al.,* 2000). Pharmacological inhibition of the RAS is a major therapeutic strategy currently used to manage hypertension and to reduce the risks of cardiovascular events. Since the discovery of renin in 1890s (Tigerstedt *et al*., 1898), the RAS is classically described as an endocrine system where the key hormone angiotensin II (Ang II) is synthesized in a series of coordinated enzymatic reactions (Figure 4). In the first step systemic kidney-derived renin, a rate limiting protease, cleaves the substrate angiotensinogen to generate the decapeptide, Angiotensin I (Ang 1-10) (Ang I). The Ang I produced from this reaction is physiologically inactive and acts as a substrate for Angiotensin converting enzyme (ACE). In a second step, the dicarboxypeptidase, ACE, a zinc metalloprotease ectoenzyme that hydrolyse two amino acids (His-Leu) from the carboxy terminal of Ang I to generate the physiologically active octapeptide Angiotensin II (Ang II) (Ang 1-8). This conversion can also be accomplished by the chymotrypsin-like serine protease, chymase (Unger, 2004). In addition to cleaving Ang I, ACE metabolizes bradykinin (BK), a vasodilator, to inactive BK-(1-7) (Carey & Siragy, 2003). Hence, ACE has a dual role in the vasculature in that it promotes the production of Ang II, a potent vasoconstrictor while degrading BK, a vasodilator. In physiological system, Ang II acts upon two main G-protein-coupled receptors with seven-transmembrane domains, angiotensin II type 1 (AT1) and angiotensin II type 2 (AT2) receptors. However, the major cardiovascular effects of Ang II are mediated via AT1 receptors (Figure 4).

Ang II elicits an increase in blood volume and blood pressure by stimulating vasoconstriction, sodium retention, thirst, the sympathetic nervous system, and aldosterone synthesis and secretion from the adrenal cortex. In turn, the steroid hormone aldosterone interacts with the mineralocorticoid receptor (MR) in the renal collecting ducts and enhances the

sodium retaining effect (Thomas *et al.*, 2008). The effects of AT2 receptors are in most cases opposite to that of AT1R and are partly mediated through activation of the kallikrein-kinin system (KKS) (Steckelings *et al.*, 2005). The links between the RAS and the KKS are numerous (Bader *et al.*, 2001). ACE also cleaves BK, a vasodilator and thus produce additional hypertensive effects. Furthermore, a direct physical and functional interaction between the AT1 receptor and the kinin B2 receptor has been described (AbdAlla *et al.*, 2000), however, these data have been challenged recently (Hansen *et al.*, 2009).

Until recently this system was accepted as the conventional RAS. In addition to systemic RAS, local RAS exist ubiquitously in all the major organs of the body such as heart, lungs, kidneys, brain, testis, ovary, intestine etc and plays an important physiological and pathophysiological role. The local RAS in various tissues can operate autonomously of the systemic RAS and may be stimulated even in the situations when the systemic RAS is suppressed or normal. The local RAS acts in an autocrine/intracrine, paracrine and endocrine fashion. Elevated tissue levels of RAS components occur in cardiovascular diseases independently of BP elevation, such as, atherosclerosis, myocardial infarction, heart failure, diabetes and kidney disease (Raizada *et al.*, 2007). The best characterized tissue RAS include those in the brain, kidney and heart (Yang *et al.* 2010; Zhuo *et al.*, 2011; De Mello *et al.*, 2011; Bader *et al.*, 2010). Moreover, it is now clear that the kidney is not the only source of renin production; and the angiotensin peptides may be formed in tissues and organs that have a blood pressure-independent function. In addition, the production of renin is still in question that whether it is produced locally or whether it is taken up from the systemic circulation, possibly *via* the (pro)-renin receptor. Moreover, the presence of low levels of renin in the brain has been a

consistent enigma in establishing an independent role of the brain RAS (Dzau *et al.,* 1986; Baltatu *et al.,* 1998).

1.4. Brain Renin Angiotensin System

The existence of brain renin-angiotensin system (RAS) independent from the peripheral system was first claimed by Ganten *et al* (1971a; 1971b). These results were further supported by pressor and dispogenic effects of intracranial injected angiotensin II (Ang II) (Johnson and Epstein, 1975; Buggy and Johnson, 1978; Phillips and Felix, 1976; Phillips *et al*., 1977). These initial findings motivated the number of investigators to focus particularly on the brain RAS.

A complete RAS, comprising all necessary precursors and enzymes required for formation and metabolism of its components have been identified in the brain and play important functions in various cardiovascular diseases and BP regulation (Lenkei *et al*., 1997; Phillips and summers, 1998). Also, numerous brain manipulations including lesions and brain specific expression of various components of the RAS produces long term change in BP. All these studies suggest that the brain RAS exerts regulatory influences in the central control of cardiovascular functions and plays a key role in the development and establishment of hypertension.

The major difference between the brain RAS and systemic RAS is that the brain predominantly express the renin transcript containing an alternate first exon (Lee-Kirsch *et al*., 1999; Clausmeyer *et al*., 2000; Sinn & Sigmund, 2000). This transcript encodes a truncated renin isoform that is predicted to be intracellular as it lacks the prefragment that targets to the secretory pathway. Recently, a receptor for prorenin/renin that appears to increase the efficiency of angiotensinogen cleavage to Ang I has been identified (Nguyen *et al*., 2002). It is not yet clear whether renin is produced locally or taken up from systemic circulation via (pro)renin receptors. In addition, this is specifically true in brain RAS where renin expression is particularly very low.

In addition, it was discovered that renin and its precursor prorenin can bind to (pro)renin receptors (Nguyen *et al*., 2002), (P)RR, which retain renin activity in tissues and boost the Ang-II generation in tissues and thus considered to be powerful amplifier of tissue RAS.

Various studies have suggested that the progressive dysfunction of the arterial baroreflex may contribute to sustained increases in sympathetic nervous system activity and subsequent RAS overactivity during the development of hypertension (Veerasingham *et al*., 2003; Guyenet, 2006). Accumulating evidences indicate that the baroreceptor reflex plays a crucial role in chronic regulation of arterial pressure. The arterial baroreceptor controls the barosenstive sympathetic efferents. This large group of efferents has a dominant role in both short-term and long-term BP control. This background activity is set by a core network of neurons that reside in the RVLM, the spinal cord, the hypothalamus and the nucleus of solitary tract (NTS). Thus, the three central control regions-the RVLM, NTS and hypothalamus (including PVN) regulate the barosenstive sympathetic efferents, and consequently BP (Guyenet, 2006). The RVLM is a key integrative site within the medulla that participates in the tonic and baroreflex regulation of BP via sympathetic nerve activity. Pacemaker cells within this area provide the major excitatory input to sympathetic preganglionic neurons in the spinal cord that innervate sympathetic ganglia and adrenal medulla (Sun *et al.,* 1995*;* Dampney *et al.,* 2003). In addition, RVLM receives extensive inputs from other cardiovascular nuclei, including tonic excitatory input from the PVN and NTS and inhibitory input from caudal ventrolateral medulla (Dampney *et al.,* 2002*;* Schreihofer *et al.,* 2002). Thus, the RVLM is considered as major relay point for the transmission of SNA. In addition both altered RVLM function and elevated SNA have been implicated in the pathogenesis of hypertension in several hypertensive animal models (Colombari *et al.,* 2001*;* Esler *et al*., 2000*;* Mancia *et al*., 2000*;* de Wardener *et al.,* 2001).

1.5. Dysfunction of the Brain RAS in Pathogenesis of Hypertension: Central Role of Ang II

A hyperactive brain RAS plays a critical role in the development and maintenance of hypertension. Brain Ang II is found to be the most important regulator of this system and its actions on target cells are mediated through G-protein coupled angiotensin type-1 receptors (AT1R) and type-2 receptors (AT2R). Activation of AT1R at various hypothalamic and medullary areas of the brain result in elevation in BP, augmented drinking behavior, attenuation of baroreflex, enhancement of sympathetic outflow and augmented vasopressin release (Averill *et al.*, 2000; Casto and Phillips, 1986; Ferguson *et al.*, 2001; McKinley *et al.*, 1996; Muratani *et al.*, 1996; Paton *et al.*, 2001). Thus, hyperactivity of this hormone system is linked to numerous cardiovascular complications. This concept is further underscored by evidences that in hypertensive rat models, the classical RAS components (such as AT1R, ACE, Ang II) are found to be overexpressed specifically in cardiovascular regulatory brain regions of the hypothalamus and the brainstem regions such as PVN, median preoptic nucleus (MnPO), subfornical organ (SFO), NTS, and dorsal motor nucleus of the vagus (Gehlert *et al.*, 1986; Gutkind *et al.*, 1988; Han and Sim, 1998; Lebrun *et al.*, 1996; Lu *et al.*, 1994; Raizada *et al.*, 1999; Song *et al.*, 1994). Major drugs are developed as therapeutic strategy using this axis for treatment of hypertension and other cardiovascular diseases. These effects are mediated via complex interacting signaling pathways involving phospholipids (PLC, PLD, PLA2), stimulation of NAD(P)H oxidase and reactive oxygen species (i.e. O^{2-}, H_2O_2), induction of gene transcription (i.e. proto-oncogenes: c-fos, c-jun, c-myc), and activation of tyrosine kinases (Src, JAK/STAT, FAK, Pyk2, p130Cas, PI3-kinase). Some of these actions may be mediated directly or indirectly via transactivation of tyrosine kinase receptors including PDGFR, EGFR and IGFR. Following its formation, Ang II is rapidly degraded by circulating or tissue peptidases collectively termed angiotensinases.

12

Although, Angiotensin (Ang) II is the major effector of this system, several other peptides are now recognized as being biologically important.

The SHR has been the most extensively used model for investigating the dysfunction in brain RAS in neurogenic hypertension. Increases in brain angiotensinogen expression precede the development of hypertension in SHR (Tamura *et al*., 1996), especially in the preoptic area where increases in angiotensinogen were apparent in 4-week-old SHR and increased with age (Shibata *et al*., 1993). In addition, renin-like activity in the anterior hypothalamus and NTS was higher in SHR compared to WKY rats during the development of hypertension (Ruiz *et al*., 1990). Ang II content and turnover within the hypothalamus, and Ang II immunoreactivity within the PVN and supraoptic nucleus, is also increased in adult SHR compared to their normotensive WKY control rats (Weyhenmeyer & Phillips, 1982; Ganten *et al*., 1983; Hermann *et al*., 1984; Phillips & Kimura, 1988). SHR also exhibit increased density of Ang II binding sites within the MnPO, SFO, PVN and NTS, and $AT1_A$ receptor mRNA within the preoptic area compared to WKY rats (Gutkind *et al*., 1988; Komatus *et al*., 1996). Furthermore, a recent study demonstrated increased cellular AT1 receptor density within the RVLM in SHR *versus* WKY rats (Hu *et al*., 2002). Pressor responses to intracerebroventricular (i.c.v.) injection of Ang II, or microinjections of Ang II within the preoptic area, NTS and RVLM, and depressor effects within the CVLM, are also correspondingly greater in SHR *versus* WKY rats, indicating increased receptor density and/or sensitivity in the SHR (Casto & Phillips, 1985; Matsuda *et al*., 1987; Wright *et al*., 1987; Muratani *et al*., 1991; Zhu *et al*., 1998). Thus, it can be concluded from these studies that a hyperactivity of the brain RAS precedes and/or parallels the development of hypertension in the SHR.

Many studies utilizing pharmacological agents have shown previously that the hyperactivity of the brain RAS mediates hypertension. Attenuation of brain RAS using classical ACE inhibitors such as captopril, or AT1 receptor antagonist (losartan) into the cardiovascular regulatory regions of the brain (such as: lateral ventricle area, anterior hypothalamic area) decrease BP in SHR and other hypertensive rat models but does not affect BP in the normotensive controls (Hutchinson *et al*., 1980; Faber & Brody, 1984; Itaya *et al*., 1986; Berecek *et al*., 1987; Teruya *et al*., 1995; Szczepanska-Sadowska *et al*., 1998; Park & Leenen, 2001; Yang *et al*., 1992). This decrease in BP by blockade of brain RAS appears to be mainly due to decrease in sympathetic activity (Berecek *et al*., 1987; Huang & Leenen, 1998). Moreover, antisense gene targeting of components of RAS utilizing adeno-assocoiated viruses within the brain of SHR and studies employing transgenic techniques to overexpress RAS components in normotensive rats or mice further support for the view that a hyperactive brain RAS mediates hypertension. These studies indicate that a hyperactivity of the brain RAS mediates hypertension in SHR and other hypertensive models.

1.6. RAS Revisited: The ACE2/Ang (1-7)/Mas axis in the Brain

Several peptides have been identified in the RAS as of being biologically important. One of the most interesting members of the RAS is the heptapeptide Ang (1–7). Ang (1-7) was shown to be present as an endogenous constituent of the brain, in areas including the hypothalamus, medulla oblongata, and amygdala, as well as in adrenal glands and plasma of normal rats (Chappell *et al*., 1989). However, the enzymatic cascades leading to the generation of this peptide were learned later (Figure 5). We now know of three different pathways to produce Ang (1-7), as reviewed in details by Karamyan and Speth (Karamyan and Speth, 2007). First, directly from Ang I by prolyl-endopeptidase or neutral endopeptidase (neprilysin), which cleave the bond

14

at residues Pro^7-Phe^8 (Wright at al., 1997); second, directly from Ang II by angiotensin converting enzyme 2 (ACE2), prolyl-carboxypeptidase or prolyl-endopeptidase; and third, indirectly, ACE2 converts Ang I to Ang -(1–9). Ang (1-7) is then produced by ACE cleavage of the dipeptide phenylalanine- histamine from Ang-(1–9) (Vauquelin *et al*., 2002) or by neprilysin (Rice *et al*., 2004). Several other enzymes can also participate in each of these three pathways (Karamyan and Speth*, 2007). However, it has recently been shown that, in the hypertensive rat heart, the majority of the Ang (1–7) formed results from the degradation of Ang II by ACE2 (Trask *et al*., 2007). It is conceivable that the other pathways might be activated or inhibited in specific pathological conditions. It is likely that the synthesis of Ang (1-7) is taking place mostly in the extracellular space since ACE2 is a transmembrane protein with its catalytic site located outside the cell (Guy *et al*., 2005). However, because ACE2 conserves its activity following shedding by ADAM17, one can speculate that endocytosis of the secreted enzyme could lead to formation of the heptapeptide inside the cell. After synthesis, Ang (1-7) can be metabolized into Ang -(1–5) by ACE (Chappell *et al*., 1998) or Ang -(1–4) by neprilysin (Allred *et al*., 2000). In the classical RAS, Ang (1–7) was considered to be an inactive metabolic breakdown product of Ang II. This view has been challenged with the discovery of angiotensin-converting enzyme 2 (ACE2), which cleaves Ang II to Ang (1-7), and the G-protein-coupled receptor, Mas, which has been recognized as the first binding site for Ang (1-7) (Figure 5). In addition, a large body of evidence has proven several beneficial effects of this peptide in the cardiovascular system, which is often opposite to the effects elicited by Ang II. This discovery provides a new axis to the brain RAS, comprising ACE2, Mas receptors, and Ang (1–7), counterregulating the classical ACE/AT1-receptor/Ang II axis through generation of Ang (1-7) (Figure 5). The latest working model of the brain RAS includes this axis, therefore forming a new arm for this system. Thus,

ACE2 and its product Ang (1-7) appear to set the balance of pressor/depressor tone of the RAS and have the potential to be cardioprotective. Through this additional axis, ACE2 shift the balance of production away from Ang II and towards the Ang (1-7). The dramatic beneficial effects of Ang (1-7) in the peripheral cardiovascular system, which occur via counterregulating Ang II actions, have been well studied. Meanwhile, Ang (1-7) and its Mas receptor are widely expressed in hypothalamic region. The role of this peptide in central control of BP and in the pathogenesis of neurogenic hypertension has been studied by several research groups using different techniques.
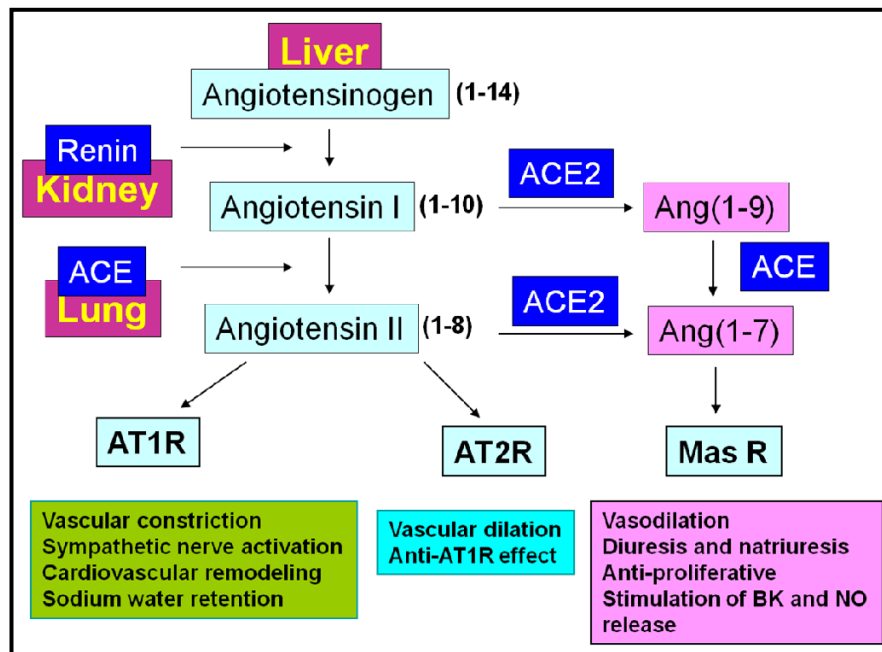
Figure 5: RAS revisited: Introduction of ACE2/Mas-R/Ang (1-7) axis. Besides AT1 receptor, Ang II can be metabolized by ACE2 to Ang-(1-7), which is the ligand for the Mas-receptor. Both pathways mostly counteract the effects elicited by the AT1 receptor. Ang (1-7) has been proven to have several beneficial effects in the cardiovascular system, which are often opposite to the effects elicited by ANG II such as vasodilation, dieresis and natriuresis, anti-proliferation, stimulation of BK and NO release.

ACE2, a new active player in RAS: The Angiotensin peptides produced from degradation of Ang II already existed from decades and their importance and function has been debated. Despite the numerous observations showing the benefits on baroreceptor reflex, cardiac, and vascular functions, the role of Ang (1-7) remained underappreciated until the discovery in 2000 of a new carboxypeptidase responsible for the conversion of Ang II into Ang (1-7), a heptapeptide (Donoghue *et al*., 2000: Tipnis *et al*., 2000). The discovery of this new enzyme, named ACE2, due to its homology with ACE (40% identity and 61% similarity), from human heart failure ventricle (Donoghue *et al*., 2000) and human lymphoma cDNA libraries (Tipnis *et al*., 2000), was followed in 2003 by the identification of a specific receptor (i.e., Mas) for Ang (1-7) (Santos *et al*., 2003). Together, these critical findings gave a new impetus for the understanding of the role of this new arm of the RAS, which is now known as the ACE2/Ang (1-7) /Mas receptor axis.

ACE2 is a glycoprotein of 120 kDa, expressed as a transmembrane protein but also exists in a soluble, truncated form, lacking the transmembrane and cytosolic domains, but conserving its activity (Tipnis *et al*., 2000). This metalloprotease contains a single zinc-binding domain and conserves other critical residues typical of the ACE family. The protein sequence consists of 805 amino acids, including a potential 17-amino acid NH_2-terminal signal peptide sequence and a putative COOH-terminal membrane anchor. Functionally, ACE2 acts as a monocarboxypeptidase and possesses one active site domain at its C-terminus portion. It shares 40% homology with ACE, but differs greatly in substrate specificity (function exclusively as carboxypeptidase), and its activity is not altered by classical ACE inhibitors such as captopril. It is mainly responsible for the cleavage of octapeptide Ang-II to heptapeptide Ang (1-7). Though it also converts Ang -I to Ang (1-9); Ang (1-7) is the main product because of 400 times high

affinity of Ang II for ACE2 as compared to Ang-I (Vickers *et al*., 2002). The specificity of ACE2 is not limited to peptides belonging to the RAS. In addition to its role in the formation of Ang (1–7), ACE2 acts with high catalytic efficiency on various vasoactive peptides from other systems such as apelin-36 and apelin-13, kinin metabolites [des-Arg10]kallidin and [des-Arg9]BK but not BK, neurotensin and the related peptide kinetensin, as well as opioid peptides such as dynorphin A (1–13) (Vickers *et al*., 2002). Taken together, these findings support our view that ACE2 is an enzyme with multifunctional regulatory roles in inflammation, neurotransmission and cardiovascular functions. In spite of this multifunctional nature, the role of ACE2 in the production of Ang (1–7) is possibly the most relevant in cardiovascular control and thus the most frequently studied function of the enzyme. By cleaving Ang-II into Ang (1-7), ACE2 may play a pivotal role in counter-regulating the actions of the well documented ACE/Ang-II/AT1R axis and be beneficial for the cardiovascular system.

Originally in year 2000, ACE2 was identified in testis, kidneys and heart by two independent groups (Donoghue *et al*., 2000; Tipnis *et al*., 2000). Later studies showed expression of ACE2 mRNA in brain (Igase *et al*., 20005; Sakima *et al*., 2005; Doobay *et al*., 2007), vasculature, colon, small intestine, ovary, testis, prostate, heart, placenta, liver, skeletal muscle, and pancreas with the highest levels of expression in lung and kidney (Tipnis *et al*., 2000), and it is now evident that the distribution of the protein is ubiquitous.

In the brain, ACE2 was reported to be widely distributed, in the cytoplasm of neuronal cell bodies but not in glial cells (Kar *et al*., 2010; Doobay *et al*., 2007). This is actually surprising since it is thought to be a transmembrane protein with most of its structure on the extracellular side. This observation suggests that a significant pool of ACE2 might be stored inside cytoplasmic vesicles. Whether the cytoplasmic ACE2 might play a role within the intracellular

RAS remains to be determined. *In vitro*, other groups have also observed ACE2 expression in astroglial cells (Gallagher *et al.*, 2006). Brain ACE2 activity was also reported to be the highest in the hypothalamus of C57BL/6 mice (Elased *et al.*, 2008).

In peripheral system, it is well accepted that disruption of the balance between ACE and ACE2 would result in abnormal BP control. Several evidences from various laboratories have shown the beneficial effects of peripheral ACE2 in the regulation of cardiovascular hypertrophy and BP control (Crackower *et al.*, 2002; Oudit *et al.*, 2007; Yamamoto *et al.*, 2006). In the CNS, using a lentivirus coding for ACE2, Yamazato *et al* (2007) previously showed that ACE2 overexpression in the rostral ventrolateral medulla, could reverse hypertension in spontaneously hypertensive rats (SHR). Kar *et al.* (2010) showed that in chronic heart failure rabbits, ACE2 expression was reduced in the RVLM, a situation that could be reversed by exercise training, suggesting that brain ACE2 may contribute the protective effect induced by exercise in heart failure. Recently, Feng *et al* (2010) reported that brain-targeted ACE2 overexpression in the subfornical organ (SFO) prevents the acute Ang II–mediated pressor and drinking responses. More recently, feng *et al* (2010), using long term and high level expression further clarified the role of central ACE2 using a new transgenic mouse model with human (h)ACE2 overexpression attenuates the development of neurogenic hypertension. These studies provide the further evidence for the central role of ACE2 in regulation of cardiovascular functions. There could be two possible reasons for attenuation of neurogenic hypertension with central overexpression of ACE2. It could be either mediated through breaking down of Ang II or production of Ang (1-7) or both. Unfortunately, the role of Ang (1-7) in the brain is not completely understood which is the major focus of the present study.

Discovery of GPCR Mas, a G protein-coupled receptor, was recently identified as a functional receptor for Ang (1-7). Mas was originally described as a protooncogene, due to its ability to induce tumorigenicity in nude mice (Young *et al*., 1986). The human Mas gene was mapped to chromosome 6 (6q24–6q27), within a region frequently rearranged in malignant cells (Rabin *et al*., 1987). The protein has seven hydrophobic transmembrane domains, while the NH_2- and COOH-terminal ends are hydrophilic. It shares a strong sequence similarity with the G protein-coupled receptor subfamily of hormone-receptor proteins (Probst *et al*., 1992). In 2003, Santos *et al* identified Ang (1-7) as a ligand for the Mas receptor, in experiments showing that genetic deletion of the Mas receptor abolishes not only binding of Ang (1-7) to mouse kidneys but also Ang (1-7) induced relaxation and antidiuretic responses. In addition, Mas knockout did not alter vasoprotein and Ang II function, suggesting that Ang (1-7) is the specific ligand for Mas receptor. This is supported by studies showing that the most of the known peripheral actions of Ang (1-7) are mediated by Mas, including antidiuresis, vasodilation, improvement of endothelial function and its antifibrotic effect. All of these evidences suggest that Mas is the functional receptor of Ang (1-7).

Mas expression in mice was found in the heart, kidney, lung, liver, spleen, tongue, and skeletal muscle (Villar *et al*., 1994; Metzger *et al*., 1995). In the heart, low levels of Mas transcripts were detected in cardiomyocytes and much more in the endothelium of coronaries. Similarly, Mas expression was also observed in brain endothelial cells derived from rat cerebral resistance vessels (Kumar *et al*., 1996). While, Mas mRNA expression was originally thought to be restricted to the hippocampus, cortex, and olfactory bulb (Metzger *et al*., 1995; Young *et al*., 1988), later development of specific antibodies extended these observations to other brain structures. A dense Mas immunoreactive staining was observed in cardiovascular regulatory

areas, from the medulla to the forebrain, such as the NTS, RVLM, CVLM, inferior olive, parvo-and magnocellular portions of the PVN, SFO, and lateral preoptic area, shown in several previous studies as sites for the action of Ang (1-7) in the brain (Santos at al., 2005). Moreover, at the cellular level, Mas was predominantly present in neurons, as evidenced by co-localization of immunostaining for the neuronal marker, Neu-N, and the Mas receptor antibody (Becker *et al*., 2007). However, it was recently reported that astrocytes located in the RVLM of wistar rats could respond to Ang (1-7) stimulation, leading to increases in intracellular Ca^{2+} (Guo at al., 2010). Interestingly, this response was prevented by administration of A-779, suggesting the participation of Mas receptors.

Previous studies have described high level of Mas mRNA in the forebrain areas, including hippocampus, cortex, and olfactory bulb of the rat and mouse. In the central nervous system, Ang (1-7) acts as an important neuromodulator, especially in those areas related to tonic and reflex control of arterial pressure, in the hypothalamus and in the dorsomedial and ventrolateral medulla. At these sites, the cardiovascular effects induced by Ang (1-7) are blocked by A-779, suggesting that in the brain, Ang (1-7) actions may be mediated by the interaction with GPCR Mas. In addition, Becker *et al* showed the presence of Mas in cardiovascular and hydroelectrolytic control areas of the rat brain supporting the role of the Ang (1–7) receptor in these processes (Becker *et al*., 2007). However, the action of brain Ang (1-7) in the hypertensive animal models (such as SH rats) is still not clear. This is another aim of current study.

1.7. Central Role of Ang (1-7) in BP Regulation and in the Pathogenesis of Hypertension

A large body of evidence suggests that the renin-angiotensin system (RAS) product, the heptapeptide angiotensin Ang (1–7), is a potent endogenous effector hormone of the RAS. Ang (1-7), the main product of Ang II cleaved by ACE2, has been found to counter-regulate the

actions of Ang II, mediating the beneficial effects in peripheral cardiovascular diseases. Accumulating evidences indicate that peripheral actions of Ang (1-7) have beneficial effects in the regulation cardiovascular diseases (Ferrario *et al*., 2005; Santos *et al*., 2000; Santos *et al*., 2003). By acting through GPCR-Mas, Ang (1-7) promotes vasodilation, antiproliferation and antihypertrophy in peripheral system (Ferrario *et al*., 2005; Santos *et al*., 2000; Santos *et al*., 2003). But very few studies are focused on the central role of this heptapeptide. Ang (1-7) is found to be involved in BP regulation and in brain it is mainly present in central nuclei related to BP regulation, such as brainstem areas and hypothalamus (Chappell *et al*., 1989). Brain Ang- (1–7) has been shown to act as an important neuromodulator of cardiac baroreflex mechanisms, leading to an increased sensitivity of cardiac baroreflex (Campagnole-Santos *et al.,* 1992, Santos *et al.,* 2003). In addition, central Ang (1-7) prevents norepinephrine release; induces depressor responses; and increases bradykinin levels in hypertensive rats. These effects are found to be mediated by Mas (Pörsti *et al*., 1994). Despite the dramatic beneficial effects of Ang (1-7) in the peripheral system and the observation that Ang (1-7) is present in various cardiovascular regulatory brain areas, the role of this peptide in neural control of blood pressure and in the pathogenesis of hypertension is poorly defined. Here, we compared the effect of Ang (1-7) and Ang II microinjected into the PVN on the BP regulation in SHR and WKY rats.

1.8. Ang II-Induced Chronotropic Effect in SHR Neurons

Elevated sympathetic nerve activity (SNA) is present in most forms of hypertension and a causal relationship is suggested by the well-documented anti-hypertensive efficacy of sympatholytic drugs (for eg. α and β adrenergic receptor antagonists) (Hoffman, Goodman and Gilman's, 2006). The specific AT1R mediated actions of Ang II at hypothalamic and brain stem neurons play important role in cardiovascular regulation and contribution to the pathogenesis of

hypertension (Ferguson *et al*., 2001; Saavedra *et al*., 2005). These contributions of Ang II to the central nervous system (CNS) control of blood pressure are manifested via alterations in the electrical activity of neurons at specific circumventricular organs, with subsequent activation of hypothalamic and brain stem sites, such as the PVN, RVLM, and NTS (Guyenet, 2006). The result of these CNS actions of Ang II is the enhancement of sympathetic outflow, a blunting of the sensitivity of the baroreflex, and increased vasopressin secretion (Averill & Diz, 2000; Guyenet, 2006). Because these actions of Ang II are amplified in hypertension (Toney *et al*., 1993; Veerasingham *et al*., 2003; Ito *et al*., 2002; Kubo *et al*., 2006), it is essential to fully understand the intracellular mechanisms that regulate the AT1R-mediated effects of Ang II on the neuronal activity in SHR and WKY rats.

The significance of the brain Ang II system in BP control and hypertension is further supported by studies with the spontaneously hypertensive rat (SHR). Previous studies have confirmed that the expression of the AT1R is 2- to 4-fold higher in the neurons from SHR compared with those from WKY rat (Veerasingham *et al*., 2003; Raizada *et al.,* 1993). This increase was consistent with elevated levels reported in the cardiovascular-relevant brain regions of the adult SHR (Veerasingham *et al*., 2003). In addition, increased expression of the AT1R in the SHR neuron was associated with a greater firing rate response and neuromodulatory actions of Ang II in these strains of rat neurons (Sun *et al.,* 2003; Sun *et al*., 2009). SHRs exhibit increased expression and activity of AT1R in a number of hypothalamic and brain stem regions (Song *et al.,* 1994*;* Ito *et al*., 2002), and interruption of brain AT1R function by pharmacological or genetic means lowers blood pressure in these animals (Ito *et al*., 2002; Wielbo *et al*., 1995). These data are supported by *in vitro* studies indicating that AT1R expression and the

chronotropic action of Ang II are enhanced in neurons cultured from the SHR hypothalamus and brain stem (Sun *et al*., 2003*;* Sumners *et al*., 2002*)*.

Alterations in action potential (AP) frequency or duration are determined by the activity of membrane K^+ and Ca^{2+} currents. Previous studies have shown that activation of AT1 receptors by Ang II in these neurons inhibits delayed rectifier K^+ current (I_{Kv}) and transient A-type K^+ current (I_A); and stimulates total Ca^{2+} current (Sumners *et al*., 1996; Raizada *et al*., 1999; Sumners *et al*., 2002). The changes in I_{Kv}, I_A, and total Ca^{2+} current are consistent with the chronotropic action of Ang II in these cells, mediated by AT1 receptors (Wang *et al*., 1997). These effects are also consistent with observation that Ang II elicits a positive chronotropic action involving phospholipase C (PLC) and Ca^{2+}-dependent signaling molecules including protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII) (Wang *et al*., 1997; Zhu *et al*., 1997; Zhu *et al*., 1999*;* Sumners *et al*., 1996). Moreover, it was also reported that specific knockdown of PKCα isoform and not the β or γ isoform, blocked the inhibitory effects of Ang II on K_v current indicating involvement of PKCα in the action of Ang II (Sheng-jun *et al*., 2000).

1.9. Enhanced Chronotropic Effects of Ang II in SHR neurons

It has been found that through AT1-R, Ang II-induced increase in neuronal firing is significantly greater in the neuronal cells from the brainstem/hypothalamus co-culture of spontaneously hypertensive rats (SHR), compared with normotensive wister kyoto (WKY) control rats. Blockade of PKC and CaMKII completely attenuated Ang II action in WKY rat neurons, while they caused only ~50% attenuation in SHR neurons. The residual increase in firing rate produced by Ang II in SHR neurons was blocked by inhibitors of phosphatidylinositol 3 kinase (PI3-kinase), either LY 294002 (10 µM) or wortmannin (100 nM). (Sun *et al*., 2003;

Sun *et al*., 2009). More interestingly, complete attenuation of Ang II response in neurons from

SHR is accomplished by co-administration of PKC and PI3-kinase inhibitors. Furthermore,

bilateral injection of a PKC inhibitor into the NTS of the WKY results in a complete attenuation

of the Ang II–induced depressant effect on baroreflex gain (Sun *et al*., 2009). However, this Ang

II effect on both components of the baroreflex was only partially attenuated in the SHR. This

suggests that an additional cellular pathway must exist in the cardiovascular regulatory regions

of SHR brain through which Ang II exerts its effects on the baroreflex.

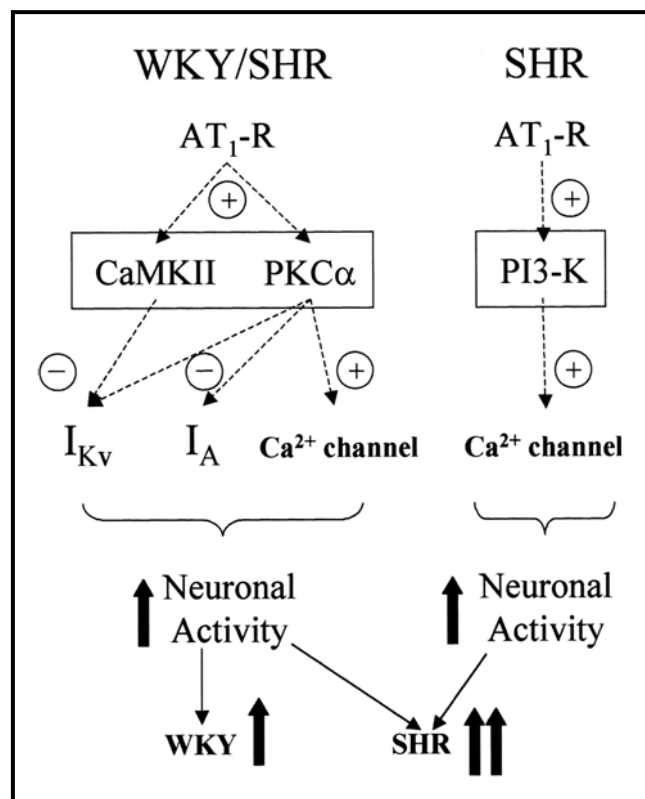Figure 6: Diagram summarizing proposed signaling pathways in the Ang-II-induced chronotropic responses in neurons. I_{Kv}, voltage-dependent potassium current; I_A, A-type potassium current (*Sun et al., 2003*)

There was elevation of Ang II–dependent PI3-kinase in the brainstem and hypothalamic

cardiovascular regions of the SHR as compared to the WKY rats (Veerasingham *et al*., 2005), as

well as finding that chronic blockade of PI3-kinase affects the spontaneous baroreflex gain and arterial pressure only in the SHR but, not the WKY rat (Zubcevic *et al*., 2009) suggested that the additional signaling pathway in SHR neurons may be linked to PI3-kinase pathway. This notion is supported by the observation that PI3-kinase blockade completely abolished the effect of exogenous Ang II in the NTS on the baroreceptor reflex in the SHR (ref) and that the absence of any effects of PI3-kinase blockade in the NTS on the cardiovascular action in the adult WKY (Zubcevic *et al*., 2009). In current study, we compared the role of PI3-kinase in the chronotropic action of Ang II in the brainstem/hypothalamic neurons of SHR versus WKY rats providing additional evidences that PI3-kinase pathway is a unique signaling pathway in SHR, and may be responsible for the enhanced action of Ang II in the *in vivo* and *in vitro* SHR models.

1.10. Central ACE2/Ang (1-7)/Mas-R axis in the BP regulation and in the Pathogenesis of Hypertension

A recent study indicates that ACE2 expression is reduced in the cardiovascular regulatory areas of SHR as compared with WKY normotensive rats and that over-expression of ACE2 in this brain region decreases high blood pressure exclusively in SHR. This effect of ACE2 could be two possible mechanisms: (1) reduced Ang II levels caused by increased degradation of Ang II through ACE2; (2) Elevated Ang (1-7) production. Unfortunately, the actions of Ang (1-7) in the brain are not completely understood. Thus, the objectives of the present study were two folds: (1) to determine whether Ang (1-7) counter-regulate the chronotropic actions of Ang II in SHR versus WKY rat neurons; (2) to elucidate the underlying signaling pathways involved in the action of Ang (1-7) and Ang II in SHR versus WKY rats.

1.11. Hypothesis

Several studies have focused on the beneficial effects of peripheral Ang (1-7) in the regulation of cardiovascular function, showing the counter-regulatory effect of Ang (1-7) against the actions of Ang II in the periphery. However, its actions in the central nervous system are not completely understood. In the present study, our main goal was to determine the central action of Ang (1-7) and its interaction with Ang II in the BP control, using *in vitro* and *in vivo* models. Previous studies demonstrated that the chronotropic action of Ang II is enhanced in neurons cultured from SHR and this action is mediated by a PI3-kinase pathway. Our preliminary data has showed that this enhanced action of Ang II in cultured neurons is attenuated by Ang (1-7). In addition, our preliminary studies also indicated that a phosphatase, PTEN, is involved in the action of Ang (1-7) in neurons cultured from SHR rats. Based upon literature and our preliminary studies, we hypothesized that Ang (1-7) acting through Mas-R, stimulate PTEN, which dephosphorylates the phosphatidylinositol-3,4,5-trisphosphate (PIP3), thus, inactivating the PI3-kinase mediated signaling pathway, leading to attenuation of Ang II response and lowering BP in SHR. Thus, such a mechanism might be involved in the pathogenesis of hypertension. This hypothesis was broadly tested via the following two aims:

(1)   Unraveling the intracellular mechanism involved in the counter regulatory effect of Ang (1-7) against Ang II action in cultured neurons from SHR vs. Wistar Kyoto (WKY) rats.

(2)   Investigating the *in vivo* effects of Ang (1-7) microinjected into the rostral ventrolateral medulla (RVLM) on the cardiovascular functions and on the pressor action of Ang II in this brain area of SHR versus WKY rats.

Thus, our major objective was to provide evidences to support/or refute the above hypothesis. The combinations of *in vitro* and *in vivo* techniques with cellular, molecular and physiological approaches were used to accomplish the objective. We believed that efforts of our group in this study raised the possibility to find new therapeutic targets based on the recently identified antihypertensive arm in Renin Angiotensin System (RAS), ACE2-Ang (1-7) -Mas axis. Most hypertensive research has been focused on increase in prohypertensive system activity and much less on the antihypertensive counter-regulatory pathways. Thus, identification of counter-regulatory system may open venues for development of new therapies.

CHAPTER II. ROLE OF ANGIOTENSIN (1-7) IN THE BRAIN AND ITS INTERACTION WITH ANG II

2.1. Introduction

It is well established that the brain renin Angiotensin system (RAS) exerts regulatory influences in the control of blood pressure (BP) and plays an important role in the development and establishment of hypertension (Averill *et al*., 2000; de Gasparo *et al*., 2000; Muratani *et al*., 1996). Brain Angiotensin (Ang) II is one of the well-studied peptides and represents the major effector hormone of this system. Hyperactivity of this hormone system is linked to hypertension and other cardiovascular diseases (Zucker *et al*., 2006). The contributions of Ang II to the central nervous system control of BP are manifested via alterations in the electrical activity of neurons at specific cardiovascular regulatory regions of the hypothalamus, which also receive many inputs from brainstem sites such as the rostral ventrolateral medulla (RVLM) and nucleus tractus solitarius, with neuronal body located at several nuclei including paraventricular nucleus (PVN, Guyenet *et al*., 2006). Previous studies (Ito *et al*., 2002) demonstrated that these actions of Ang II are amplified in the cardiovascular regulatory regions of spontaneously hypertensive rats (SHRs) and that interruption of brain AT1-receptor function by pharmacological or genetic means lowers BP in these hypertensive animals. Our previous *in vitro* studies (Sun *et al*., 2003; Sun *et al*., 2009) also demonstrate that the chronotropic action of Ang II is enhanced in neurons cultured from the SHR brainstem/hypothalamus as compared to that of WKY rat. The enhanced chronotropic action of Ang II in SHR neurons is mediated by phosphatidylinositol 3-kinase (PI3-kinase; Sun *et al*., 2003; Sun *et al*., 2009). Thus it is essential to identify the neuronal factors that regulate the actions of Ang II or target intracellular signaling molecules of the Ang II pathway in SHR neurons.

Several peptides have been identified in the RAS. One of the most interesting members of the RAS is the heptapeptide Ang (1-7), which is produced by ACE2, and endogenous enzyme located in the brain. The action of Ang (1-7) is mediated by its Mas receptor, a G-protein-coupled receptor (Santos *et al*., 2003). A large body of evidences has proven several beneficial effects of this peptide in the cardiovascular system, which is often opposite to the effects elicited by Ang II (Santos *et al*., 2003; Santos *et al*., 2000; Ferrario *et al*., 2005). This discovery provides a new axis to the brain RAS, comprising ACE2, Mas receptor, and Ang (1-7), counterregulating the classical ACE/AT1-receptor/Ang II axis through generation of Ang (1-7). The dramatic beneficial effects of Ang (1-7) in the peripheral cardiovascular system, which occur via counterregulating Ang II actions, have been well studied (Santos *et al*., 2003; Santos *et al*., 2000; Ferrario *et al*., 2005). Meanwhile, Ang (1-7) and its Mas receptor are widely expressed in hypothalamic region (Chappell *et al*., 1989; Becker *et al*., 2007). The role of this peptide in central control of BP and in the pathogenesis of neurogenic hypertension has been studied by several research groups using different techniques.

It has been reported that Ang (1-7) acts as an important neuromodulator, increases sensitivity of baroreflexes, and prevents norepinephrine release in SHRs (Campagnole-santos *et al*., 1992; Santos *et al*., 2003). More interestingly, viral vector-mediated overexpression of ACE2, an enzyme responsible for converting Ang II to Ang (1-7), in the RVLM prevents the development of hypertension in SHRs (Yamazato *et al*., 2007). In addition, central-specifically overexpression of ACE2 significantly prevents Ang II-induced hypertension in mice (Feng *et al*., 2010). The cellular and molecular mechanism underlying the beneficial effect of overexpression of ACE2 against hypertension is still unknown. Two possible mechanisms may be involved: 1) reduction of Ang II levels and 2) elevation of Ang (1-7) levels. Thus, the objects of current study were to

examine the action of Ang (1-7) on the neuronal activity in SHR and WKY rats and to determine whether Ang (1-7) counter-regulates Ang II action, as it does in peripheral cardiovascular system.

2.2. Materials and Methods

2.2.1. Animals:

Twelve-week-old male SHR and WKY rats were obtained from Charles River Farms (Wilmington, MA). These rats were used as breeders to produce a constant supply of newborn SHR and WKY rat pups to prepare neuronal cultures. Rats were housed at $25 \pm 2°C$ on a 12:12-h light-dark cycle and provided with food and water ad libitum. All animal protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee.

2.2.2. Preparation of neuronal cultures:

Neuronal co-cultures will be prepared from the brainstem/hypothalamus and brain stem of newborn SHR and WKY rats exactly as described previously (Sumners *et al.,* 1990). Briefly, trypsin (375 U/ml) and DNase I (496 U/ml) dissociated cells will be resuspended in DMEM containing 10% PDHS and will be plated on 35-mm Nunc plastic tissue culture dishes precoated with poly-L-lysine at 3.0×10^6 cells/dish. After the cells will be grown for 3 days at $37°C$ in a humidified incubator with 95% O_2 and 5% CO_2, they will be exposed to 1 µM ARC for 2 days in fresh DMEM containing 10% PDHS. ARC will be then removed and the cells will be incubated with DMEM (plus 10% PDHS) for an additional 11–14 days before use. This protocol will allow the neurons to recover from the isolation procedure, develop extensive network of neuritis, and express neuron specific properties comparable to those seen in the *in vivo* situation. At the time of use, cultures will consist of 90% neurons and 10% astrocyte glia cells, as determined by

immunofluorescent staining with antibodies against neurofilament proteins and glial fibrillary acidic protein (Sumners *et al.*, 1990).

2.2.3. Electrophysiological recording:

Spontaneous action potentials (APs) will be recorded from WKY and SHR neurons at room temperature using the whole cell patch-clamp technique in current-clamp mode as described previously. Recordings will be taken from a morphologically distinct population of multipolar neurons. The recordings will be made using an Axon Digidata 1200 B interface (Axon Instruments, Burlingame, CA) as described previously (Zhu *et al.,* 2001). Briefly, cells will be bathed in Tyrode's solution containing (in mM) 140 NaCl, 5.4 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 0.3 NaH_2PO_4, 10 dextrose, and 10 HEPES, pH 7.4. Neurons will be superfused at a rate of 2–4 ml/min. The patch electrodes will have resistance of 2–4 MΩ when filled with an internal pipette solution containing (in mM) 140 KCl, 4 $MgCl_2$, 10 dextrose, 10 HEPES, 4 ATP, and 0.1 GTP, pH 7.2. The whole cell configuration will be obtained by applying negative pressure to the patch electrode. The resting membrane potential (RMP) will be defined as the potential within a 1-s time period during which there will be no spontaneous APs. The neuronal firing rate will be measured as the number of fully developed APs per second (Hz) essentially as described previously (Zhu *et al.,* 2001). In individual experiments, test agents will be added sequentially in the superfusate.

2.2.4. Data analysis:

Results are expressed as means ± SE. Statistical significance was evaluated with the use of a one-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at $P < 0.05$.
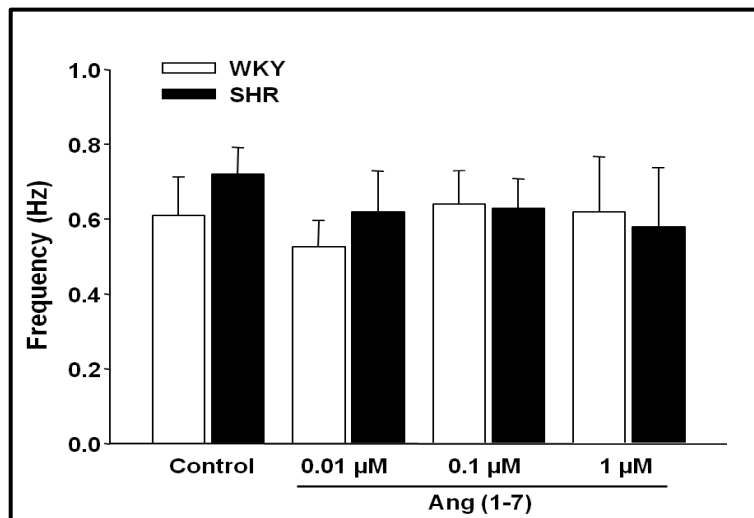
2.3. Results and Data Analysis

Figure 7: Dose dependent effect of Ang (1-7) on neuronal activity. Ang (1-7) effect was measured in neurons cultured from hypothalamus and ventral brainstem of SHR and WKY rats brains. Bar graph summarizing the average neuronal activity/frequency (Hz) (n=6 cells) at various concentrations of Ang (1-7) indicated in figure (from 0.01 µM-1 µM) using whole cell patch clamp in current clamp mode . Values are mean±SEM. *$P<0.05$ indicates a significant difference from the corresponding control value. #$P<0.05$ as compared between SHR and WKY neurons under the same treatment condition.

2.3.1. Effect of Ang (1-7) on the neuronal activity of the neurons cultured from brainstem/hypothalamus of SHR and WKY rats:

Despite the dramatic beneficial effects of Ang (1-7) in the peripheral system (Santos *et al.* 2000, 2003, Ferrario *et al.,* 2001, 2005, Grobe *et al.,* 2007, Mercure *et al.,* 2008), the role of this peptide in neural control of blood pressure and in the pathogenesis of hypertension is poorly defined. Our objective in the first series of experiments was to investigate the effect of the Ang (1-7) on neuronal firing rate in neurons cultured from brainstem/hypothalamus of WKY and SH rats using the whole cell configuration of patch clamp technique in current clamp mode. Primary

neuronal cultures were prepared from the brainstem/hypothalamus of new-born rats as described

in Methods (Section 2.2.2). Neuronal activity of both WKY and SHR neurons was recorded

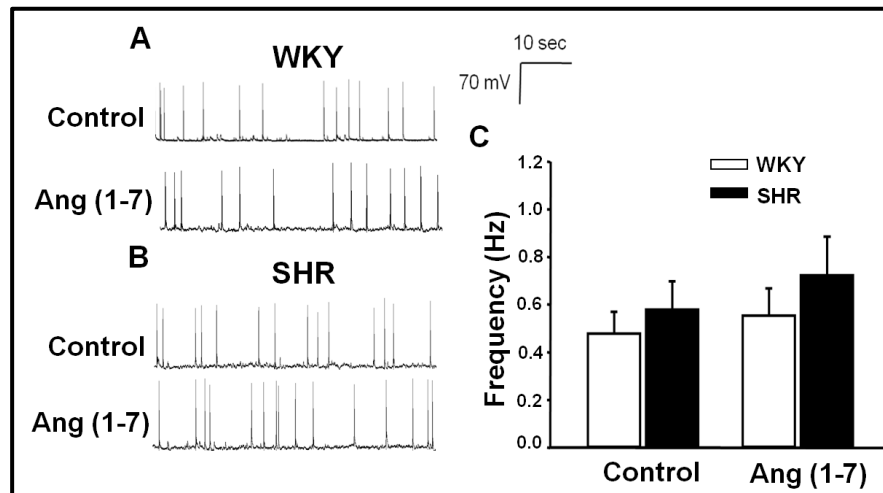before and after superfusion of neurons with Ang (1-7) (100 nM).

Figure 8: Effect of Ang (1–7) on the neuronal firing rate. Firing rate was recorded from neurons under each treatment condition. *A*: representative tracings showing the action potentials (APs) recorded from a single WKY rat neuron under the following sequential treatment conditions: superfusion with control (Con) solution (PBS), ANG (1-7) (100 nM) *B*: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in *A*. *C*: bar graphs summarizing the effect of ANG-(1–7) on the neuronal activity in SHR and WKY rat neurons. Data are means ± SE from 6 neurons for each group. *$P < 0.05$, compared with respective control recordings.

The results are shown in Figure 7 & 8, indicating that the basal neuronal firing rate between

the two rat strains were comparable. It was observed that superfusion of neurons with Ang (1-7)

(100 nM, 5 min) did not significantly alter the basal neuronal firing rate in neurons cultured from

either SHR (0.58±0.12 to 0.72±0.16 Hz) or WKY rats (0.48±0.09 to 0.55±0.11 Hz) before and

after administration of Ang (1-7) (n=6 cells, p>0.05) (Figure 8). To confirm the proper dose for

the effect of Ang (1-7) on neuronal activity, dose dependent (Figure 7) and time dependent

studies were performed. It was observed that Ang (1-7) did not alter the neuronal activity in

either SHR or WKY rate neurons at 0.01 μM, 0.1 μM and 1 μM doses (n=6 cells, p>0.05 Figure 7). Furthermore, the effect of Ang (1-7) at different doses (0.01 μM, 0.1 μM and 1 μM) was measured in a time dependent manner at 0, 5, 10, 15 and 30 minutes. The data demonstrated that the Ang (1-7) did not alter neuronal activity at above mentioned doses   and times. These results demonstrated that Ang (1-7) did not significantly alter the basal neuronal activity in either dose dependent or time dependent manner in either SHR or WKY rat neurons.

2.3.2. Effect of Ang II on the neuronal activity in the neurons cultured from brainstem/hypothalamus of SHR and WKY rats:

Our previous *in vitro* studies (Sun *et al*., 2003; Sun *et al*., 2009) also demonstrate that the chronotropic action of Ang II is enhanced in neurons cultured from the SHR brainstem/hypothalamus as compared to that of WKY rat. The enhanced chronotropic action of Ang II in SHR neurons is mediated by PI3-kinase (Sun *et al*., 2003; Sun *et al*., 2009). In the present studies, we confirmed the effects of Ang II (Figure 10) as well as measured the dose dependent effect of Ang II (Figure 9) on neuronal activity in neurons cultured from brainstem/hypothalamus of SHR and WKY rats using the whole cell configuration of patch clamp technique in a current clamp mode. Primary neuronal cultures were prepared from the brainstem/hypothalamus of new-born rats as described in Methods (Section 2.2.2). Neuronal activity of both WKY and SHR neurons was recorded before and after superfusion of neurons with Ang II using different concentration of 10 nM, 100 nM, 1000 nM and 10,000 nM. The results are shown in Figure 9, indicating that the basal neuronal firing rate between the two rat strains were comparable. Ang II produced significant effect at 100 nM concentration and this effect of Ang II on neuronal activity in both SHR and WKY rat neurons get saturated or decrease at higher concentration than 100 nM as shown in the figure 9. The data demonstrate that the

similar to previous study, Ang II significantly increase the neuronal activity in both SHR and WKY rat neurons (Figure 10). However, the chronotropic effect of Ang II was significantly increased in SHR neurons (from 0.52±0.07 to 2.97±0.17 Hz) as compared to WKY neurons (from 0.50±0.11 to 1.23±0.19 Hz) (Figure 10). These results demonstrate that Ang II significantly increases the neuronal activity in both SHR and WKY rat neurons. However the chronotropic action of Ang II was significantly enhanced in SHR neurons, as compared to WKY neurons. These results are consistent to the previous observation.
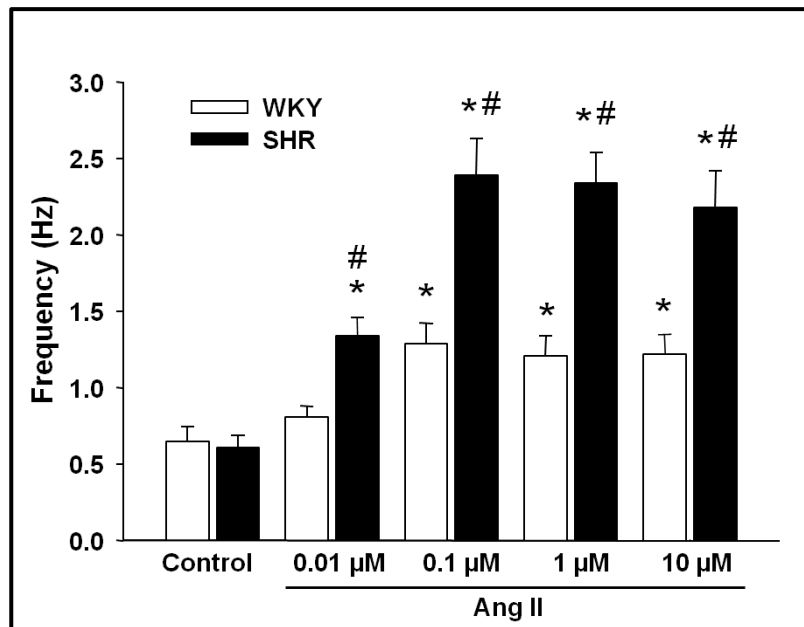
Figure 9: Dose dependent effect of Ang II on neuronal activity. Ang II effect was measured in neurons cultured from hypothalamus and brainstem of SHR and WKY rats brains. Bar graph summarizing the average neuronal activity/frequency (Hz) (n=6 cells) at various concentrations of Ang II indicated (from 0.01 μM-10 μM) using whole cell patch clamp in current clamp mode . Values are mean±SEM. *P<0.05 indicates a significant difference from the corresponding control value. #P<0.05 as compared between SHR and WKY neurons under the same treatment condition.

2.3.3. Effect of Ang (1-7) on the chronotropic action of Ang II in the neurons cultured from SHR versus WKY rats:

It has been found that Ang II increases neuronal firing rate in neurons cultured from both SHR and WKY rats and this chronotropic effect of Ang II is enhanced in neurons of SHR, as compared with WKY rats (Sun *et al.,* 2003, Sun *et al.,* 2002,). Here, we examined the effect of Ang (1-7) on the chronotropic action of Ang II in neurons cultured from SHR and WKY rats (Figure 12). Neuronal firing was recorded using the whole cell configuration of patch clamp technique in current clamp mode on WKY and SHR neurons treated under the following sequential conditions: basal neuronal firing, superfusion with Ang II (100 nM), washout Ang II, superfusion with Ang (1-7) (100 nM), and Ang (1-7) + Ang II. The results are shown in Figure 12. In compliance with previous studies, Ang II significantly increased neuronal firing rate in neurons cultured from either SHR (from 0.49 ± 0.08 to 3.12 ± 0.20 Hz, n=6 cells, $p<0.05$) or WKY rats (from 0.42 ± 0.05 to 0.93 ± 0.19 Hz, n=6 cells, $p<0.05$) (Figure 12). This chronotropic action of Ang II was significantly enhanced in SHR neurons (526 %, n=6 cell, $p<0.05$) as compared to WKY neurons (123 %, n=6 cell, $p<0.05$). The results are presented in Figure 12. Ang (1-7) alone did not alter the basal neuronal activity in both neurons from SHR and WKY rats in compliance with the results from previous observations (Figure 12). Pretreatment of neurons with Ang (1-7) (100 nM, 5 min) had no effect on the chronotropic action of Ang II in WKY rat neurons. Ang II resulted in increases in neuronal activity by 123% and 112% before and after treatment with Ang 1-7 respectively (n=6 cell, $p>0.05$) in WKY neurons (Figure 12). However, in contrast to WKY neurons, Ang (1-7) significantly attenuated the chronotropic effect of Ang II in SHR neurons. Ang II resulted in increases in neuronal activity by 526 % and 162 % before and after treatment with Ang 1-7 respectively (n=6 cell, $p<0.05$) in SHR neurons (Figure 12). In summary, these

results demonstrate that Ang (1-7) alone did not alter the basal firing rate in both WKY & SHR

neurons. However, Ang (1-7) abolished the enhanced chronotropic effect of Ang II in SHR

neurons, as compared to the neurons from WKY rats. Moreover, to further confirm the

appropriate dose for Ang (1-7), three different concentrations (0.01 µM, 0.1 µM and 1µM) were

used in combination with Ang II (0.1 µM) in the neurons cultured from the

brainstem/hypothalamus of SHR and WKY rats (Figure 11).
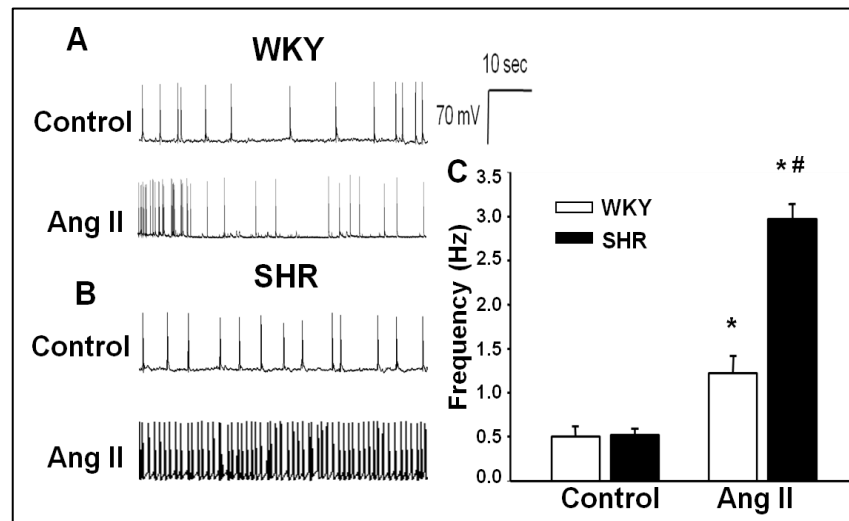
Figure 10: Effect of Ang II on the neuronal firing rate. Firing rate was recorded from neurons under each treatment condition. *A*: representative tracings showing the action potentials (APs) recorded from a single WKY rat neuron under the following sequential treatment conditions: superfusion with control (Con) solution (PBS), ANG II (100 nM) *B*: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in *A*. *C*: bar graphs summarizing the effect of ANG-(1–7) on the neuronal activity in SHR and WKY rat neurons. Data are means ± SE from 6 neurons for each group. *$P < 0.05$, compared with respective control recordings. #P<0.05 as compared between SHR and WKY neurons under the same treatment condition.

The results demonstrated that none of the Ang (1-7) dose alters Ang II induced neuronal

activity in WKY rat neurons. In contrast, the inhibitory effect of Ang (1-7) was found to be dose

dependent with maximum inhibitory effect at 0.1 µM concentration (Figure 11). The data

presented in Figure 11 demonstrate that the 0.01 µM concentration has lower inhibitory effect as

compared to 0.1µM concentration in SHR neurons, whereas, 1 µM concentration has lower or

saturated inhibitory effect as compared to 0.1 µM concentration in SHR neurons. The Ang (1-7)

produced maximum inhibitory effect at 0.1 µM concentration Figure 11. From this data as well

as from literature review, we selected 0.1 µM dose for Ang (1-7) for further experiments

designed to study its central role.
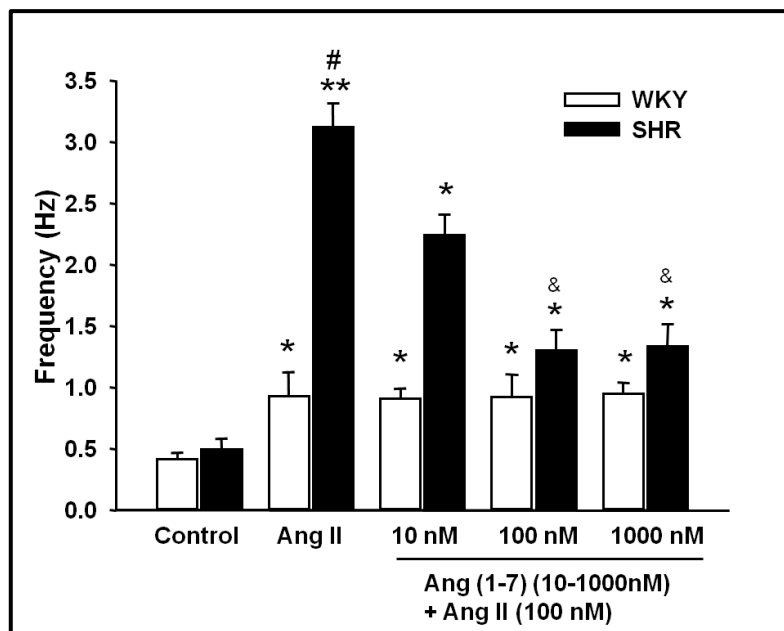
Figure 11: Effect of different concentrations of Ang (1-7) on the Ang II action. Firing rate was recorded from neurons under each treatment condition. Bar graphs summarizing the effect of different concentrations (10-1000 nM) of ANG-(1-7) on the chronotropic action of ANG II in SHR and WKY rat neurons. Data are means ± SE from 6 neurons for each group. *P < 0.05, compared with respective control recordings. **P < 0.01, compared with respective control. #P < 0.01, compared recordings between SHR and WKY rat neurons under the same condition. &P < 0.05, compared recording between ANG II and ANG II + ANG-(1–7) in the same strain rat neuron.
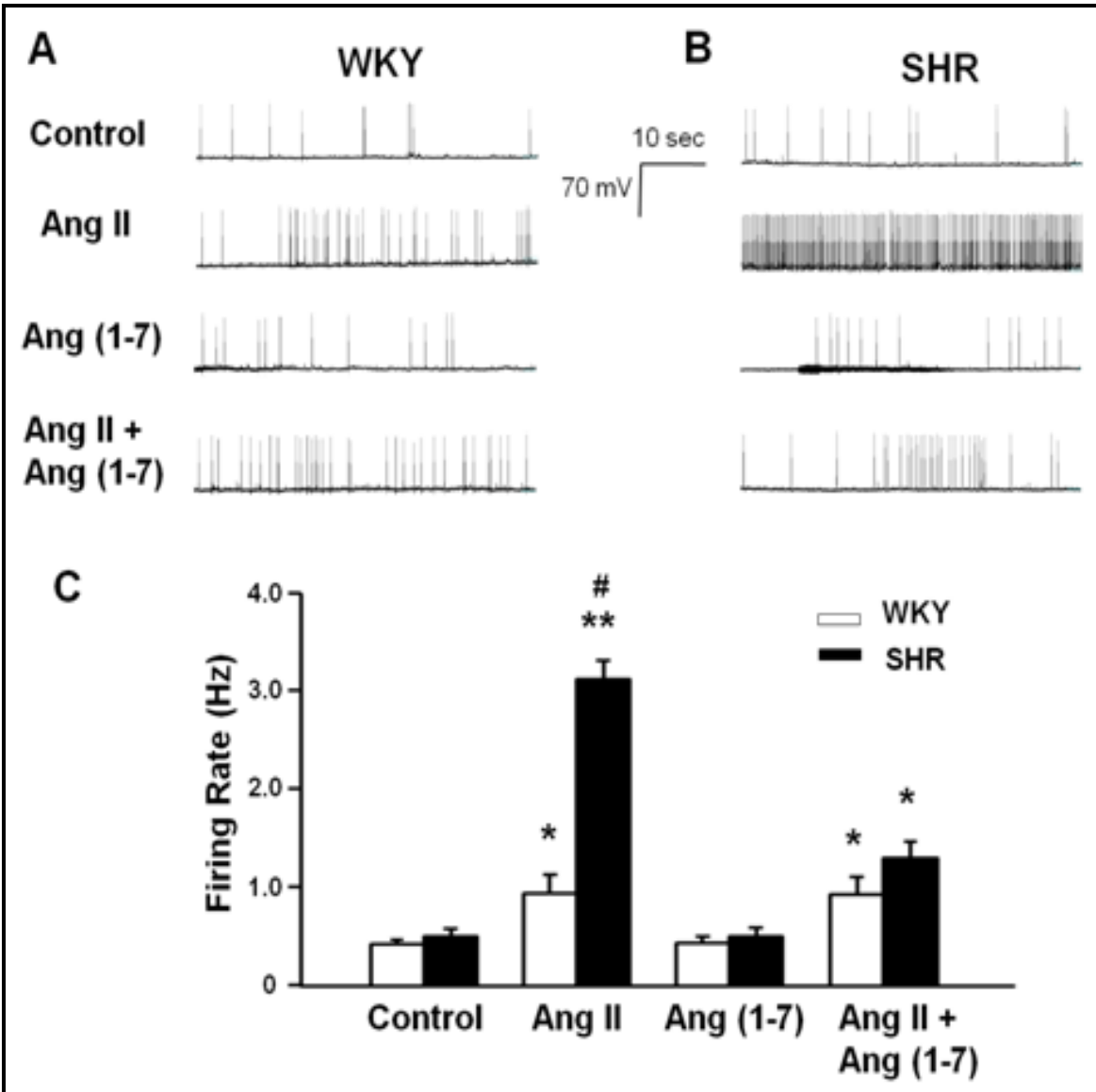
Figure 12: Effect of Ang (1-7) on the chronotropic action of Ang II. Firing rate was recorded from neurons under each treatment condition. *A*: representative tracings showing the action potentials (APs) recorded from a single WKY rat neuron under the following sequential treatment conditions: superfusion with control (Con) solution (PBS), ANG II (100 nM), and washout of ANG II, and then neurons were superfused with 100 nM ANG-(1–7); this was followed by superfusion of ANG II + ANG-(1–7). *B*: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in *A*. *C*: bar graphs summarizing the effect of ANG-(1–7) on the chronotropic action of ANG II in SHR and WKY rat neurons. Data are means ± SE from 6 neurons for each group. *$P < 0.05$, compared with respective control recordings. **$P < 0.01$, compared with respective control. #$P < 0.01$, compared recordings between SHR and WKY rat neurons under the same condition. &$P < 0.05$, compared recording between ANG II and ANG II + ANG-(1–7) in the same strain rat neuron.

40

To confirm the inhibitory effect of Ang (1-7) on the enhanced effect of Ang II in SHR neurons, the experiment was designed to check whether the inhibitory effect was due to Ang (1-7) or because of internalization of AT1-R during prior treatment of Ang II. To confirm the results, after the enhanced effect of Ang II in SHR neurons and washing step, in spite of Ang (1-7)+Ang II, only Ang II was administered. The results indicated that second time treatment of Ang II, there was no significant difference in the response produced as compared to the prior response produced during early treatment of Ang II before washing step. The data ruled out the possibility of AT1-R internalization and suggested that the inhibitory response was due to Ang (1-7).

2.4. Data Summary and Conclusion

The present study examined the direct effect of Ang (1-7) on the neuronal activity in isolated neurons and it's interaction with Ang II in neurons cultured from SHR and WKY rats. The major findings of the present study are:

1) Ang II increase neuronal firing rate in both WKY and SHR neurons.

2) The chronotropic effect of Ang II was significantly enhanced in SHR neurons as compared with WKY neurons.

3) The enhanced chronotropic effect of Ang II is mediated by a PI3-kinase dependent signaling pathway in SHR neurons

4) Blockage of PI3-kinase did not alter the chronotropic action of Ang II in WKY neurons.

5) Ang (1-7) alone did not alter the neuronal firing rate in either WKY or SHR neurons.

6) Ang (1-7) significantly abolished the enhanced chronotropic response to Ang II in SHR neurons.

7) Ang (1-7) did not alter the chrontropic action of Ang II in WKY rat neurons

Ang (1-7) counter regulate the over-activated neuronal response to Ang II in SHR neurons. Previously, it was suggested that Ang II acts at AT1R in SHR and WKY rat neurons to produce an increase in firing rate via signaling pathway that involves parallel activation of PKCα and CaMKII (Pan *et al*., 2001; Sumners *et al*., 2002) Figure 6. In addition, AT1R in SHR neurons were reported to be linked to a PI3 kinase-signaling pathway that is responsible for the enhanced chronotropic response to Ang II exclusively in SHR Figure 6. The present studies suggested that Ang (1-7) abolished the enhanced chronotropic effects of Ang II exclusively in SHR neurons. Based upon these observation and previous studies we hypothesize that Ang (1-7) may deactivate or inhibit the additional PI3-kinase signaling pathways in SHR neurons. Various pharmacological agents as well as dominant negative mutants for PI3-kinase were used and described in the following chapters to test this hypothesis and to further investigate the signaling pathways responsible for this action of Ang (1-7) in SHR neurons.

ACE2, an enzyme that cleaves Ang II to Ang (1-7) (Doobay *et al*., 2007; Sakima *et al*., 2005; Yamazato *et al*., 2007) is present in various brain cardiovascular regulatory regions. Yamazato *et al*. observed significant reduction of ACE2 expression at protein levels in SHRs as compared to WKY rats. In addition the overexpression of this enzyme in the RVLM did not alter the BP in WKY rats; however, it induced a long-term decrease in BP in SHR (Yamazato *et al*., 2007). Our results from this study revealed the possible cellular mechanism of antihypertensive effect of ACE2 in the brain, suggesting that this effect of ACE2 could be mediated by lowering Ang II levels and by increasing Ang (1-7) levels in brain cardiovascular regulatory areas.

Several studies (Sakima *et al*., 2005; Zimmermn *et al*., 2011; Hocht *et al*., 2006) have focused on the *in vivo* central effect of Ang (1-7) in the regulation of cardiovascular function. To clarify and further dissect the functional role of Ang (1-7), we observed the direct effect of Ang

42

(1-7) on neuronal activity on neurons cultured from the brainstem/hypothalamus of SHR and WKY rats. The present study demonstrates that Ang (1-7) did not alter neuronal firing rate in either SHR or WKY rat neurons (Figure 7 & 8). However, coadministration of Ang (1-7) with Ang II significantly attenuated the chronotropic response evoked by Ang II in SHR neurons (Figure 12), indicating that Ang (1-7) may counter-regulate Ang II actions in the central control of BP exclusively in SHR. The results are consistent with other *in vivo* observations indicating that the opposing effect of Ang (1-7) on responses to Ang II is enhanced in animal models of hypertension both in the central nervous system and in the peripheral vasculature (Hocht *et al*., 2006; Santos *et al*., 2000). Höcht *et al* (2006) have reported that intrahypothalamic injection of Ang II induced a significantly greater pressor response in SHR compared with WKY rats and that the coadministration of Ang (1-7) with Ang II reduced the pressor response to Ang II in SHR but not in WKY rats. Similarly, Benter *et al*. (1995) have demonstrated that Ang (1-7) attenuates Ang II-induced vasoconstriction in SHR but not in WKY rats. However, several other studies (Fontes *et al*., 1994; Silva-Barcellos *et al*., 2001) have demonstrated that microinjection of Ang (1-7) increases BP and the pressor response to Ang (1-7) is similar to that evoked by Ang II. This discrepancy may be due to differences in animal species, the doses of Ang (1-7) used in the experiments, or other experimental conditions. For example, a low dose of Ang (1-7) (50 ng) in the brainstem/hypothalamus did not alter BP in either SHR or WKY rats. In contrast, a high dose of Ang (1-7) (250 ng) significantly increases BP in SHR and this pressor effect of Ang (1-7) is blocked by irbesartan, indicating that the pressor effect of Ang (1–7) is mediated by an AT1-receptor-dependent mechanism (Hocht *et al*., 2006). One question raised in this study centers on the cellular mechanism(s) underlying the Ang (1–7)-induced counterregulatory effect

of Ang II action in SHR. Further studies were focused on unraveling the intracellular signaling

pathways responsible for this interaction.

CHAPTER III. THE SIGNAL TRANSDUCTION PATHWAYS: ROLE OF PI3-KINASE AND PTEN

3.1. Introduction

A large body of evidence suggests that the heptapeptide angiotensin Ang (1-7), is a potent endogenous effector hormone of the RAS (Campagnole-santos *et al*, 1992; Santos RAS *et al*, 2003). Accumulating evidences indicate that the peripheral actions of Ang (1-7) have beneficial effects in cardiovascular diseases (Loot *et al*., 2002; Ferrario *et al*., 2005; Santos *et al*., 2000; Santos RAS *et al*, 2003). Ang (1-7), has been found to counter-regulate the actions of Ang II in the peripheral cardiovascular system. Furthermore, attesting to the importance of this function is the impressive clinical therapeutic benefits achieved by ACE inhibitors and Ang II receptor antagonists, which could increase the plasma levels of Ang (1-7), further indicating the beneficial effects of these agents could be partly mediated through Ang (1-7). By acting through GPCR-Mas, Ang (1-7) promotes vasodilation, antiproliferation and antihypertrophy in peripheral system (Kucharewicz *et al*., 2002; Polizio *et al*., 2007; Sampio *et al*., 2007; Trask and Ferrario, 2007; Santos *et al*, 2004; Benter *et al*., 2006; Grobe *et al*., 2007). But very few studies are focused on the central role of this heptapeptide. To our knowledge, no data are available studying the direct effect of Ang (1-7) on neurons cultured from brainstem/hypothalamus of SHR and WKY rats.

Ang (1-7) is found to be involved in BP regulation and in brain it is mainly present in central nuclei related to BP regulation, such as brainstem areas and hypothalamus (Chappell *et al.,* 1989; Bunnemann *et al*, 1990, Becker *et al.,* 2007, Metzger *et al*, 1995). Also centrally, its functions in contrast to Ang II are hypotensive effect, increased sensitivity of baroreceptor mechanism (Campagnole-santos *et al*, 1992; Santos *et al*, 2003). Ang- (1–7) has been shown to act as an

important neuromodulator of cardiac baroreflex mechanisms, leading to an increased sensitivity of this system (Campagnole-santos *et al*, 1992). In addition, central Ang (1-7) prevents norepinephrine release and induces depressor responses in hypertensive rats, increases bradykinin levels, potentiates the hypotensive effects of bradykinin and increases vasopressin and nitric oxide (NO) release (Gironacci *et al*., 2004). These effects are found to be mediated by Mas-R which has been identified as Ang (1-7) binding site and function-mediator (Santos *et al.,* 2003, Grobe *et al.,* 2007, Mercure *et al.,* 2008). Despite the dramatic beneficial effects of Ang (1-7) in the peripheral system and the observation that Ang (1-7) is present in various cardiovascular regulatory brain areas, the role of this peptide in neural control of blood pressure and in the pathogenesis of hypertension is poorly defined.

Elevated sympathetic nerve activity (SNA) is present in most forms of hypertension and a causal relationship is suggested by the well-documented anti-hypertensive efficacy of sympatholytic drugs (for eg. α and β adrenergic receptor antagonists). In previous studies, it has been found that through AT1-R, Ang II-induced increase in neuronal firing which is significantly greater in the neuronal cells from the brainstem/hypothalamus co-culture of SHR compared with WKY control rats (Sun *et al.,* 2003, Sun *et al.,* 2002, Veerasingham *et al.,* 2003). This enhanced response of SHR neurons to Ang II was a result of preferential linking of the AT1R to the PI3-kinase signal transduction pathway (results from this study Figure 6, and Figure 16 & 24). These results are supported by *in vivo* observations that PI3-kinase inhibition within the RVLM decreases blood pressure of the SHR to the levels similar to that of WKY rats (Seyedabadi *et al.,* 2001). .Messenger RNA levels of specific class I PI3-kinase subunits were elevated within the RVLM and paraventricular nucleus (PVN) of the SHRs compared with the WKY rats (Yang *et al.,* 1999, Veerasingham *et al.,* 2005). These studies suggest that an elevated

PI3-kinase signaling pathway in presympathetic brain regions of the SHR is critical for hypertension which supports our hypothesis that PI3-kinase is responsible for the enhanced chronotropic response to Ang II in SHR neurons and that the central action of Ang (1-7) on BP regulation is mediated by antagonising PI3-kinase.

Several studies have indicated that the counter-regulatory effects of Ang (1-7) against Ang II actions in the animal models of hypertension (Ferrario *et al*., 1998; Chappell *et al*., 1998). Our studies demonstrated above also indicate that Ang (1-7) counter-regulates the Ang II induced enhanced neuronal activity in SHR neurons as compared to WKY neurons. However, the molecular mechanisms underlying Ang (1-7)-mediated counter-regulation of Ang II, are unknown. The aim of this study was to identify the underlying mechanism(s) of Ang (1-7) action in the brain cardiovascular regulatory area. Previously, we demonstrated that that the enhanced chronotropic effect of Ang II in SHR neurons is mediated by PI3-kinase pathway. In the previous studies described in chapter 1, Ang (1-7) alone did not alter the neuronal activity in both WKY and SHR neurons. However, it significantly abolished the Ang II-induced enhanced neuronal activity exclusively in SHR neurons but no significant effect was observed in WKY rat neurons. The data from us and other research groups lead us to hypothesis that the action of Ang (1-7) is mediated by antagonizing PI3-kinase in SHR neurons.

Recently, a PI3-kinase antagonizing molecule, phosphatase and tensin homologue deleted on chromosome ten (PTEN), has been identified. PTEN is a dual protein-lipid phosphatase which dephosphorylate the secondary messenger produced by PI3-kinase (Hlobilkova *et al*., 2003). The result of PI3-kinase activity is the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (or PtdIns(4,5)P_2) (PIP2), into the secondary messenger phosphatidylinositol (3,4,5)-trisphosphate (or PtdIns(3,4,5)P_3),(PIP3). This metabolite is an intracellular second messenger, which

47

mediates downstream signaling by recruiting and activating 3-phosphoinositide-dependent kinase 1 (PDK-1) followed by the activation of PKB/Akt. PTEN dephosphorylates PIP3 to its precursor, PIP2, thereby blocking the cascade of events generated PI3-kinase. In the heart, the interaction of kinase and PTEN has been shown involved in cardiac hypertrophy, heart failure, preconditioning. Considering that Ang II induced enhancement of the chronotropic effect is mediated by PI3-kinase activity in SHR neurons as compared to WKY neurons and that the enhanced chronotropic action of Ang II is counter-regulated by Ang (1-7) in SHR neurons, thus, we hypothesize that the counter-regulatory action of Ang (1-7) is mediated by antagonizing PI3-kinase through stimulation of PTEN activity in SHR neurons.

In this chapter, we studied the intracellular signaling pathway mediated by the inhibitory action of Ang (1-7) on the Ang II induced neuronal activity in SHR vs. WKY rat neurons. More specifically, the role of Mas-R and PTEN was studied in the central action of Ang (1-7) on neuronal activity in the neurons cultured from brainstem/hypothalamus of SHR and WKY rats using various biochemical and pharmacological tools.

3.2. Materials and Methods

3.2.1. Materials and animals:

Twelve-week-old SHR and WKY rats were obtained from Charles River Farms (Wilmington, MA). These rats were used as breeders to produce a constant supply of newborn SHR and WKY rat pups to prepare neuronal cultures. Rats were housed at $25 \pm 2°C$ on a 12:12-h light-dark cycle and provided with food and water ad libitum. All animal protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee.

Dulbecco's modified Eagle's medium (DMEM) was obtained from GIBCO (Grand Island, NY). A-779 was purchased from American peptide company. BPV was purchased from EMD Millipore. Trypsin was purchased from Worthington biochemical corporation. Phosphate detecting kit was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Ang (1-7), Ang II, losartan, plasma-derived horse serum (PDHS), poly-l-lysine, ARC, DNase I, and other chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO).

3.2.2. Preparation of neuronal cultures:

Neuronal co-cultures will be prepared from the hypothalamus and brain stem of newborn SHR and WKY rats exactly as described previously (Sumners *et al.,* 1990). Briefly, trypsin (375 U/ml) and DNase I (496 U/ml) dissociated cells will be resuspended in DMEM containing 10% PDHS and will be plated on 35-mm Nunc plastic tissue culture dishes precoated with poly-L-lysine at 3.0 x 10^6 cells/dish. After the cells will be grown for 3 days at 37°C in a humidified incubator with 95% O2 and 5% CO2, they will be exposed to 1 μM ARC for 2 days in fresh DMEM containing 10% PDHS. ARC will be then removed and the cells will be incubated with DMEM (plus 10% PDHS) for an additional 11–14 days before use. This protocol will allow the neurons to recover from the isolation procedure, develop extensive network of neuritis, and express neuron specific properties comparable to those seen in the *in vivo* situation. At the time of use, cultures will consist of 90% neurons and 10% astrocyte glia cells, as determined by immunofluorescent staining with antibodies against neurofilament proteins and glial fibrillary acidic protein (Sumners *et al*., 1990).

3.2.3. Electrophysiological recording:

Spontaneous action potentials (APs) will be recorded from WKY and SHR neurons at room temperature using the whole cell patch-clamp technique in current-clamp mode as described

previously. Recordings will be taken from a morphologically distinct population of multipolar neurons. The recordings will be made using an Axon Digidata 1200 B interface (Axon Instruments, Burlingame, CA) as described previously (Zhu *et al.,* 2001). Briefly, cells will be bathed in Tyrode's solution containing (in mM) 140 NaCl, 5.4 KCl, 2 MgSO$_4$, 2 CaCl$_2$, 0.3 NaH$_2$PO$_4$, 10 dextrose, and 10 HEPES, pH 7.4. Neurons will be superfused at a rate of 2–4 ml/min. The patch electrodes will have resistance of 2–4 MΩ when filled with an internal pipette solution containing (in mM) 140 KCl, 4 MgCl$_2$, 10 dextrose, 10 HEPES, 4 ATP, and 0.1 GTP, pH 7.2. The whole cell configuration will be obtained by applying negative pressure to the patch electrode. The resting membrane potential (RMP) will be defined as the potential within a 1-s time period during which there will be no spontaneous APs. The neuronal firing rate will be measured as the number of fully developed APs per second (Hz) essentially as described previously (Zhu *et al.,* 2001). In individual experiments, test agents will be added sequentially in the superfusate.

3.2.4. Immunocytochemistry:

Neuronal cultures from neonatal rats will be washed briefly with ice-cold Dulbecco's phosphate-buffered saline (PBS) and then fix for 15 min with PBS containing 0.1% Tween 20 (PBS-Tween) and 4% formaldehyde. Dishes will be then washed briefly with PBS-Tween. Bovine serum albumin (BSA) (5%) in PBS-Tween will be added to the dish for 30 min at 37°C to reduce nonspecific binding, followed by an additional wash with PBS-Tween. Primary antibodies (goat Mas1 (polyclonal), 1:50, anti--NeuN monoclonal antibody, 1:500), will be diluted in a 0.3-ml total volume of PBS-Tween, will be added to the dish and incubated overnight at 4°C. After thrice 10-min washes with PBS-Tween, the neurons will be incubated with secondary antibodies (Alexa Fluor 488 donkey anti-goat IgG, 1:1,000; Alexa Fluor 594

goat antimouse IgG, 1:1,000), washed thrice for 10 min each time with PBS-Tween, and mounted with antibleaching medium. The staining will be detected with a confocal fluorescence microscope. The fluorescent images will be collected and analyzed with computer software.

3.2.5. PTEN activity assay:

PTEN activity in extracts from neuronal cultures will be measured essentially using phosphate detection kit. Neuronal cell will be treated with (100nM) Ang (1-7), Ang (1-7)+(10μM) A-779, Ang (1-7)+(10μM) BPV, or PBS for 15 minutes. Cells will be washed with ice-cold PBS and scrape with phosphatase lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 50 mM NaF, 1% NP-40, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin), sonicate for ten seconds, and briefly centrifuge at 2000 x g for 5 minutes. Supernatants will be collected and centrifuged for an hour at 20,000 RPM at 4˚C. Carefully, the supernatant will be removed and the pellet will be dissolved in PTEN Buffer (25 mM Tris-Cl, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 10 mM DTT) which will be used for phosphatase activity assays. The PTEN solution will be added first, followed by addition of PIP3 substrate (200 uM) to start the reaction. The plate will be sealed and placed on shaker for 30 seconds. The reaction will be stopped by addition of Malachite Green solution (100 μL/well) to each well of controls, phosphate standards, and PTEN reactions. The plate will be further incubated on a plate shaker for 15 minutes at room temperature to develop color. The absorbance will be read at 620 nm. The standard curve will be drawn with Abs. 620 nm as Y axis and pmol free phosphate as X axis. Free pmol phosphate will be determined from each reaction or control by interpolation from the standard curve.

3.2.6. Western blot analysis:

Akt and p-Akt protein levels in neuronal cultures will be assessed by Western blot analysis as described previously. Briefly, neuronal cultures will be washed with ice-cold PBS and scraped into a lysis buffer containing 20 mM Tris HCl (pH 6.8), 150 mM NaCl, 10% glycerol, 1% NP-40, and 8 µl/ml inhibitor cocktail (125 mM PMSF, 2.5 mg/ml aprotinin, 2.5 mg/ml leupeptin, 2.5 mg/ml antipain, and 2.5 mg/ml chymostatin). The samples will be sonicated twice for 5 s each and will be centrifuged at 10,000 rpm for 10 min at 4°C. Supernatants will be transferred into new tubes and stored in a -80°C freezer. The protein concentration will be determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). An aliquot of 20 µg of protein from each sample will be separated on a 10% SDS-PAGE gel and will be transferred onto nitrocellulose membranes for 2 h at 100 V. After a 10-min wash in PBS-T, membranes will be blocked in PBS-T containing 10% milk for 1 h, followed by an overnight incubation in rabbit anti-Akt and rabbit Anti p-Akt antibody (dilution 1:500) at 4°C. After a 15-min wash in PBS-T, four 5-min washes in PBS-T will be carried out, and membranes will be then incubated for 2 h in an anti-rabbit peroxidase-conjugated antibody (dilution 1:15,000). Densitometry of pAkt normalized to Akt. Immunoreactivity will be detected by enhanced chemiluminescence autoradiography (ECL Western blotting detection kit, Amersham Pharmacia Biotechnology), and film will be analyzed with Quantity One Software (Bio-Rad).

3.2.7. Data analysis:

Results are expressed as means ± SE. Statistical significance was evaluated with the use of a one-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at $P < 0.05$.

3.3. Results and Data Analysis

3.3.1. Mas receptors expression in the cultured neurons:

The G protein-coupled receptor (GPCR) Mas (Mas-R) were recently described as functional receptor for Ang (1-7) (Santos *et al.,* 2003). Previous studies have described that most of the known peripheral actions of Ang (1-7) are mediated through Mas-R and these receptors are found to be highly expressed in the cardiovascular regulatory areas of the brain such as nucleus of solitary tract, RVLM, inferior olive, parvo and magnocellular portions of paraventricular hypothalamic nucleus (Becker *et al.,* 2007, Santos *et al.,* 2000).
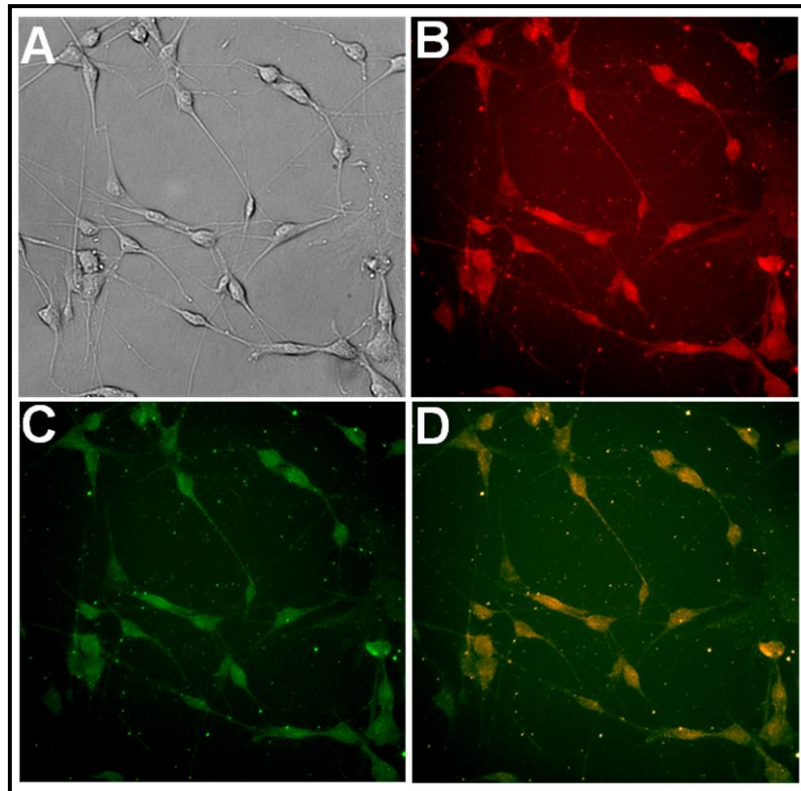

Figure 13: Mas-Receptor localization on cultured Neurons. Microscopic images were taken from the same fields of WKY rat neuronal cultures in optical phase (A) and fluorescent phase (B-D) after immunostaining with anti NeuN antibody (B) and with anti-Mas1 antibody (C). Overlap of B and C (D), indicating that Mas-R are neuronally located.

In the present study, we examined whether Mas-R present in the neurons cultured from hypothalamus and brainstems using immune-cytochemistry approach. Immunostaining was performed to evaluate the presence of Mas-R using anti-Mas1 antibody. To verify the cellular type in which Mas-R are present, we have co-stained neuronal cultures with a monoclonal antibody anti NeuN, a neuronal marker. The results are shown in Figure 13, indicating that the Mas-R are abundantly expressed in neurons cultured from brainstem/hypothalamus.
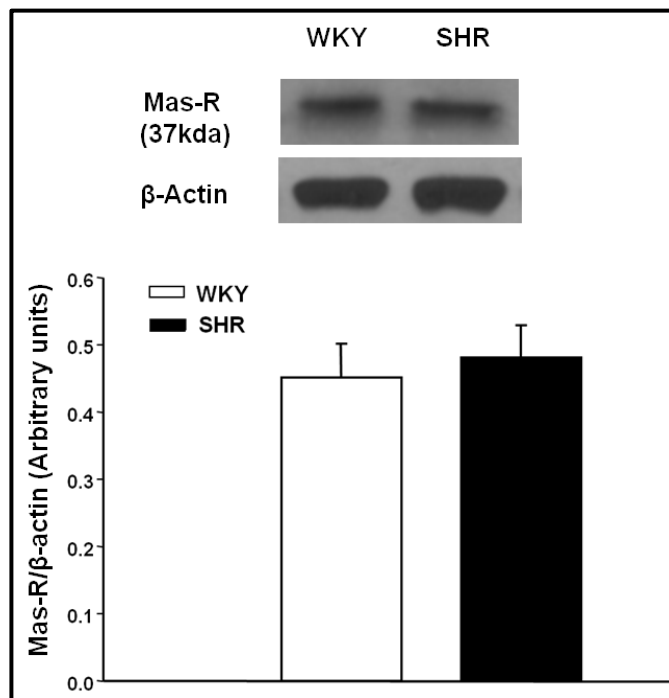
Figure 14: Mas-R expression in cultured Neurons: Mas-R in SH & WKY rat neurons were assessed by Western blot analysis as described in methods. **A:** Representative blots showing Mas-R levels **(Upper panel)**; and β-actin levels **(Lower panel)** in SH & WKY rat neurons. Densitometry of Mas-R was normalized to β-actin. **B:** bar graphs summarizing the expression level of Mas-Receptor in SH and WKY rat neurons. Data are means ± SE derived from three independent experiments. *P<0.05 indicates a significant difference from the corresponding nonstimulated (control).

The localization of Mas-R was observed on the cell bodies as well as in dendrites of neurons in both WKY and SHR neurons. No difference in the staining was observed between SHR and WKY rat neurons. Moreover, the Mas-R protein expression was also measured in neuronal cultures from SHR and WKY rats using Western blot (Figure 14) to further confirm the immunostaining results and to check the antibody specificity against Mas-R. It was observed that Mas-R was expressed in both SHR and WKY rat neuronal cultures. The results also indicated that there was no significant difference in Mas-R expression in SHR and WKY rat neuronal cultures (Figure 14). In summary, these data suggest that Mas-Rs are abundantly expressed in neurons cultured from brainstem/hypothalamus of both WKY and SHR rats specifically on the membrane of neuronal cell body and dendrites and that there was no significant difference in the Mas-R protein levels between neurons from SHR and WKY rats (Figure 13 & 14).

3.3.2. Effect of blockade of Mas receptor on the counter regulatory action of Ang (1-7) against Ang II:

In the current study, our objective was to determine whether the counter regulatory effect of Ang (1-7) against Ang II in SHR neurons is mediated through Mas-R. The effect of A-779, a specific Mas-R antagonist on the counter regulatory effect of Ang (1-7) against Ang II was examined from both SHR and WKY neurons. Neuronal firing was recorded using the whole cell configuration of patch clamp technique in current clamp mode on WKY and SHR neurons treated under the following sequential conditions: basal neuronal firing, superfusion with Ang II (100 nM), washout Ang II, superfusion with A-779 (10 μM), (a concentration shown previously to be effective; Gomes *et al*., 2010, Guo *et al*., 2010, Gwathmey *et al*., 2010) and Ang II+Ang (1-7) (100 nM)+ A-779.
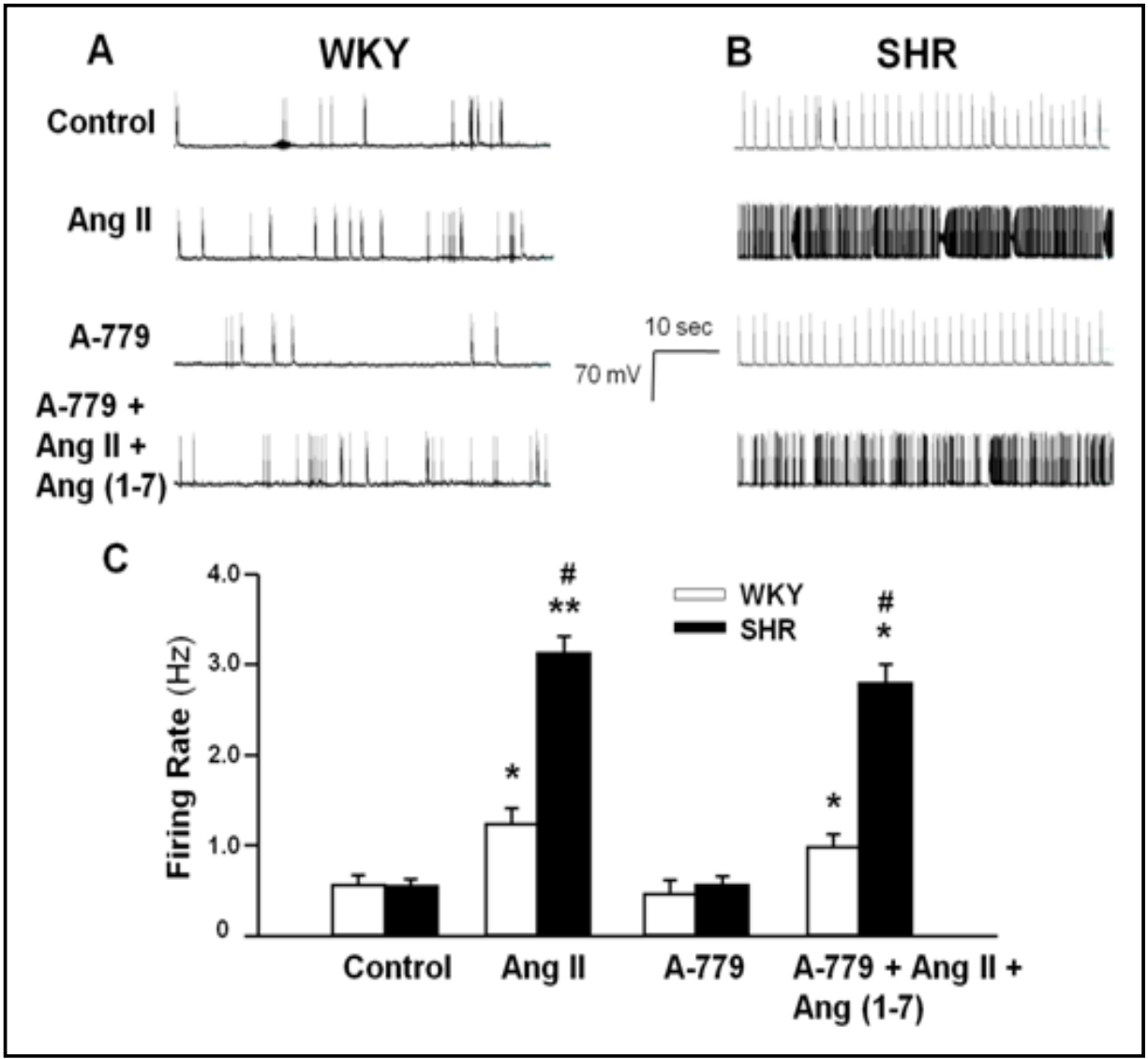
Figure 15: Effect of blockade of Mas-Receptor on the counter-regulatory action of ANG-(1–7) against ANG II. *A*: representative tracings showing the APs recorded from a single WKY rat neuron under the following sequential treatment conditions: basal firing rate (Con), superfusion with 100 nM ANG II, washout of ANG II, and superfusion with the Mas receptor antagonist A-779 (10 μM); this was followed by superfusion with A-779 + ANG II + ANG-(1–7) (100 nM). *B*: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in *A*. *C*: bar graphs summarizing the effect of ANG-(1–7) on the chronotropic action of ANG II in SHR and WKY rat neurons in each treatment situation described in *A*. Data are means ± SE from 7–9 neurons in each group. *$P < 0.05$, compared with respective control. **$P < 0.01$, compared with respective control. #$P < 0.01$, compared between SHR and WKY rat neurons under the same condition.

Figure 16: Effect of PI3-kinase inhibition on the chronotropic action of Ang II. Bar graphs summarizing the effect of LY (10 μM), a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, on the chronotropic action of ANG II in SHR and WKY neurons. Firing rate was recorded from neurons under the following treatment conditions: cont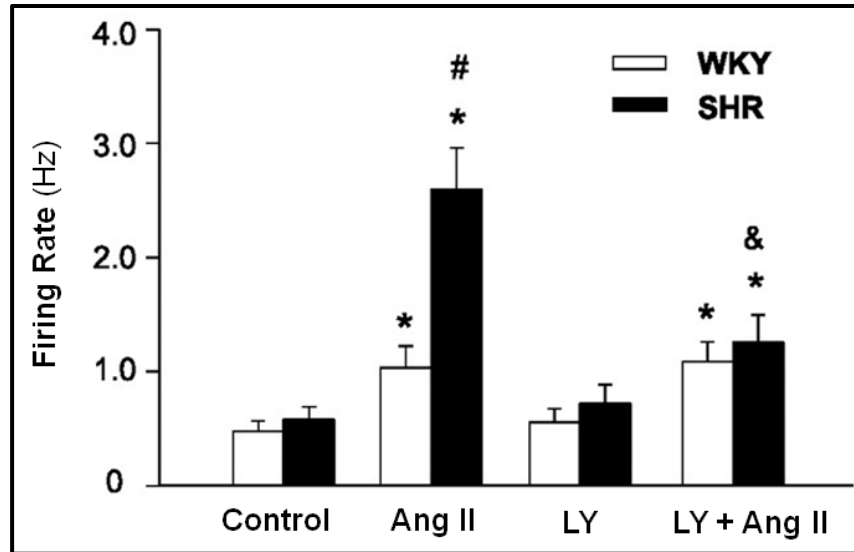rol, ANG II, washout of ANG II, and superfusion with LY, followed by superfusion with LY + ANG II. Data are means ± SE from 6–9 neurons. *$P < 0.05$, compared with respective control recordings. [#]$P < 0.01$, compared recording between SHR and WKY rat neurons under the same conditions. [&]$P < 0.05$, compared recording between ANG II and ANG II + LY in the same strain rat neuron.

The results are presented in Figure 15, demonstrating that Ang II increased neuronal firing rate in neurons cultured from both SHR and WKY rats and that this chronotropic action of Ang II was significantly enhanced in SHR neurons (from 0.55±0.08 to 3.12±0.19 Hz) as compared with WKY neurons (from 0.564±0.1to 1.238 ±0.16 Hz). Treatment with A-779, a Mas-receptor antagonist alone did not alter the basal firing rate in neurons from both SHR and WKY rats Figure 15. While, when coadministered with Ang (1-7), it blocked the counter regulatory effect of Ang (1-7) against Ang II action in SHR neurons (0.56±0.10 to 2.8±0.21 Hz) as compared to that of WKY neurons (0.47±0.15 to 0.984±0.13). The data showed that the inhibitory action of Ang (1-7) on the Ang II induced neuronal activity was abolished in presence of Mas-R

antagonist, A-779 Figure 15. These studies demonstrated that the inhibitory effect of Ang (1-7) on the chronotropic action of Ang II is mediated by a Mas receptor dependent mechanism in SHR. In summary, all the data indicated that A-779, Mas-receptor antagonist alone did not alter the basal firing rate in neurons from both SHR and WKY rats. However, when coadministered with Ang (1-7), it blocked the counter regulatory effect of Ang (1-7) against Ang II action in SHR neurons.

3.3.3. Effect of PI3-kinase inhibition on Ang II mediated effect on neuronal activity:

Our previous studies have established that Ang II stimulates PI3-kinase in both WKY and SHR neurons (Yang *et al*., 1996; Yang and Raizada, 1999). Thus the role of PI3-kinase in the Ang-II-induced increase in firing rate in SHR and WKY rat neurons was examined using the PI3-kinase inhibitors, LY294002 (10 µM). Similar to Ang (1-7), the PI3-kinase inhibitor, LY-294002 (10 µM, a concentration established in previous studies; Refs. Yang *et al*., 1999; Sun *et al*., 2003) alone did not alter the neuronal activity in both SHR (from 0.578±0.12 to 0.722±0.163 Hz) and WKY rat neurons (from 0.48±0.09 to 0.554±0.12 Hz) but when administered with Ang II, it significantly abolished the enhanced chronotropic action of Ang II in SHR neurons (before (0.578±0.12 to 2.6±0.36 Hz) and after (0.72±0.16 to 1.3±0.23) coadministration of LY-294002 respectively). It did not alter the chronotropic action of Ang II in WKY rat neurons (before (from 0.48±0.09 to 1.03±0.19 Hz) and after (from 0.55±0.12 to 1.08±0.17 Hz) coadministration of LY-294002 respectively) (Figure 16). These studies demonstrate that the Ang II increase neuronal activity in both SHR and WKY rat neurons and that chronotropic action of Ang II is enhanced in SHR neurons compared with WKY rat neurons. The enhanced chronotropic action of Ang II is mediated by PI3-kinase in SHR neurons. This inhibitory action of the PI3-kinase inhibitor LY-

294002 on Ang II mimicking the effect of Ang (1-7), indicating that they may share the same

intracellular signaling pathway by inhibiting PI3-kinase activity in SHR neurons.

Figure 17: Effect of Ang (1-7) on PI3-kinase activity expressed as ratio of p-Akt/Akt protein levels in SH and WKY rat neurons: Akt and p-Akt protein levels in SH & WKY rat neurons were assessed by Western blot analysis as described in methods under the following treatment conditions: PBS (control), ANG (1-7) (100 nM), Ang (1-7) plus Ang II (100 nM) and Ang II for 15 minutes at 37°C and 5% CO_2 in the humidified incubator. **A:** Representative blots showing p-Akt levels **(Upper panel)**; and Akt (total) levels **(Lower panel)** in SH & WKY rat neurons. **B:** bar graphs summarizing th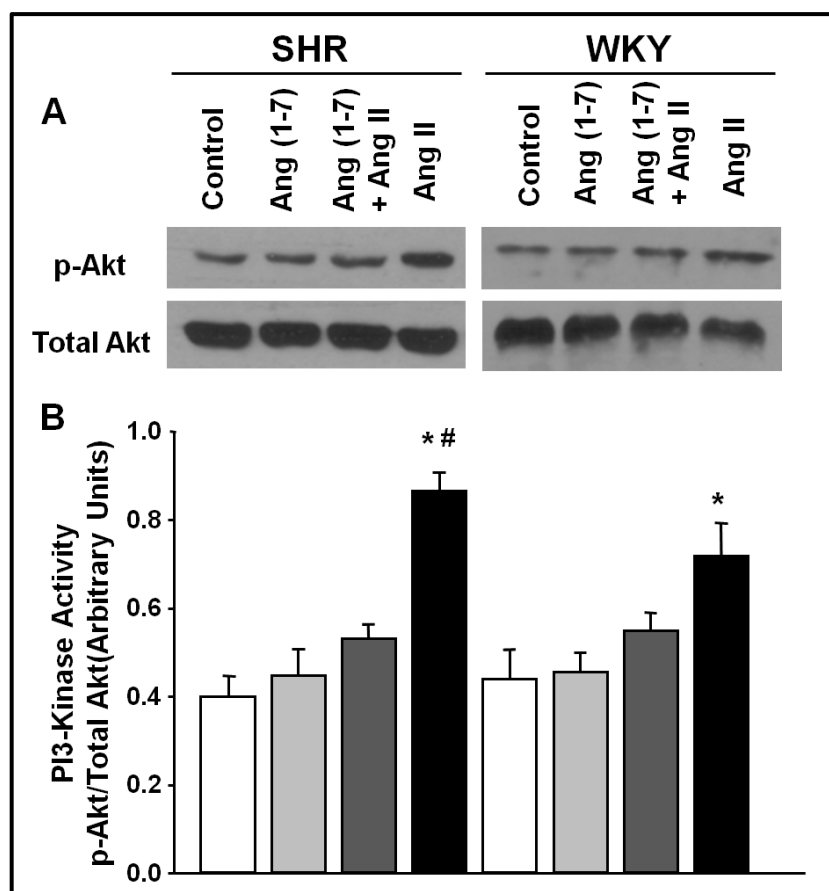e effect of Ang (1-7) on PI3-Kinase activity expressed as ratio of p-Akt/Akt protein levels in SH & WKY rat neurons. Data are means ± SE derived from three independent experiments. *$P<0.05$ indicates a significant difference from the corresponding nonstimulated (control); and #$P < 0.05$ compared recordings between SHR and WKY rat neurons under the same condition.

3.3.4. Effect of Ang (1-7) on Ang II induced PI3-Kinase activity in SH and WKY neurons:

It was previously demonstrated that Ang II stimulated the PKB/Akt activation in both SH & WKY rat neurons but this stimulation was significantly higher in case of SHR as compared to WKY rat neurons. These observations indicated the exclusive involvement of PI3-kinase-PKB/Akt dependent signaling pathways in SHR neurons (*Yang & Raizada,* 1999). In the present study, we examined the effect of Ang (1-7) on Ang II stimulated PI3-kinase activity in SH & WKY rat neurons (Figure 17). Treatment of Ang II significantly increased the ratio of phosphorylated Akt/total Akt by 40% higher in case of SHR neurons as compared to WKY rat neurons (Figure 3). Alone, Ang (1-7) did not significantly alter the p-Akt/Akt expression in either SH or WKY rat neuronal cultures. When co-administered with Ang II (100 nM), Ang (1-7) (100 nM) blocked the stimulatory effect of Ang II on the PI3-kinase activity measured as ratio of phosphorylated Akt/total Akt in SHR neurons as compared to WKY rat neurons (Figure 17). These results suggest that the counter-regulatory effect of Ang (1-7) on Ang II stimulated PKB/Akt activity in SHR neurons is mediated by inhibition of PI3-kinase-PKB/Akt dependent signaling pathways.

3.3.5. Expression of PTEN in the cultured neurons:

Previously, it was demonstrated that PTEN is widely expressed in various mammalian tissues including the heart, brain, liver and lymphocytes (Crackower *et al.,* 2002, Kishimoto *et al.,* 2003, Stiles *et al.,* 2004, Seminario *et al.,* 2003). The function of PTEN is mediated by negative regulation of PI3-kinase pathway (Tomohiko *et al.,* 1998, Koul *et al.,* 2002, Kogan *et al.,* 1998). Here, we examined the presence of PTEN in cultured neurons from brainstem/ hypothalamus of SHR and WKY rats. Immunostaining technique was performed to evaluate the presence of PTEN protein using PTEN antibody. To verify the cellular type in which PTEN was present, we

co-stained neuronal cultures with monoclonal antibody anti Neu-N, a neuronal marker. The

results are presented in Figure 18, showing that PTEN is abundantly expressed in neurons

cultured from hypothalamus and brainstem. Moreover, the PTEN protein expression was also

measured in neuronal culture extracts from SHR and WKY rats using western blot to further

confirm the immunostaining results as well as to check the antibody specificity against Mas-R. It

was observed that PTEN was expressed in both SHR and WKY rat neuronal cultures. The results

presented in Figure 19 also indicated that there was no significant difference in PTEN expression

in SHR and WKY rat neuronal cultures. In summary, PTEN is abundantly expressed in neurons

cultured from hypothalamus and brainstems of both WKY and SHR rats specifically in the

cytosol of neuronal cells and dendrites and that there was no significant difference in PTEN

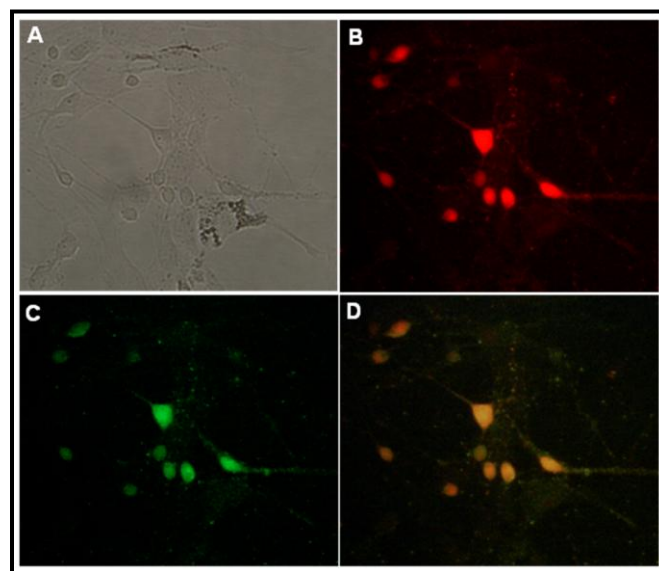levels between neurons from SHR Vs. WKY rats.

Figure 18: PTEN localization on cultured Neurons. Microscopic images were taken from the same fields of WKY rat neuronal culture in optical phase (A) and fluorescent phase after immunostained with anti Neu N antibody (B) and with anti-PTEN antibody (C). Overlap of B and C (D), indicating that PTEN is neuronally located.
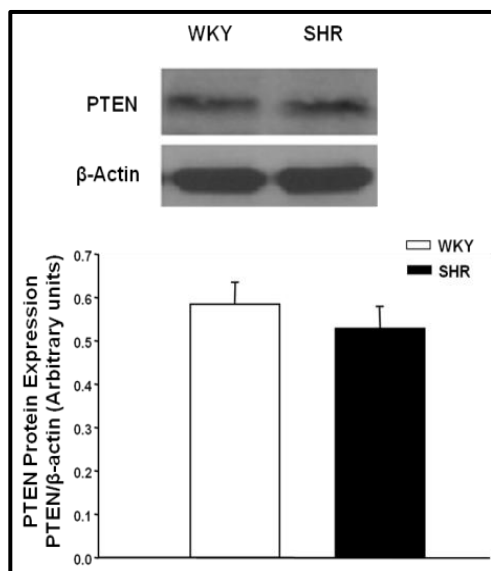
61

Figure 19: PTEN Expression in Cultured Neurons: PTEN protein levels in SH & WKY rat neurons were assessed by Western blot analysis as described in methods. **A:** Representative blots showing PTEN levels **(Upper panel)**; and β-actin levels **(Lower panel)** in SH & WKY rat neurons. Densitometry of PTEN was normalized to β-actin. **B:** bar graphs summarizing the expression level of PTEN in SH and WKY rat neurons. Data are means ± SE derived from three independent experiments. *P<0.05 indicates a significant difference from the corresponding nonstimulated (control).

3.3.6. Effect of PTEN inhibition on counter regulatory action of Ang (1-7) against Ang II:

Further experiments were performed to examine the possible mechanisms for the inhibitory effects of Ang (1-7) on the Ang II action in SHR neurons. It has been demonstrated that PTEN is the major phosphatase that inactivates PIP3 signaling pathways counter-regulating PI3- kinase action in several tissues including brain (Tomohiko *et al.,* 1998, Koul *et al.,* 2002, Kogan *et al.,* 1998). In addition, the enhanced chronotropic action of Ang II is mediated by PI3-kinase signaling pathway (Sun *et al.,* 2003, Sun *et al.,* 2002, Veerasingham *et al.,* 2003).

Thus, in the present study we examined the role of PTEN in the counter-regulatory effect of Ang (1-7) on Ang II action. In the current study, the effects of Bisperoxovanadium (BPV), a specific PTEN inhibitor on the counter regulatory effect of Ang (1-7) against Ang II were examined. Neuronal firing was recorded using the whole cell configuration of patch clamp technique in current clamp mode on WKY and SHR neurons treated under the following sequential conditions: basal neuronal firing, superfusion with Ang II (100 nM), washout Ang II, superfusion with BPV (10 μM a concentration shown previously to be effective; Ref. Ata *et al.,* 2011), followed by combination treatment with Ang II+ Ang (1-7) (100 nM)+BPV. The results

62

are shown in Figure 20. In compliance with previous studies, the data demonstrated that Ang II induced increases in neuronal firing rate in neurons cultured from both SHR (from 0.52 ± 0.07 to 2.97 ± 0.17 Hz) and WKY (from 0.505 ± 0.11 to 1.22 ± 0.19 Hz) rats and this chronotropic action of Ang II was significantly enhanced in SHR neurons. BPV, a PTEN inhibitor, alone did not alter the basal firing rate in neurons from both SHR (from 0.50 ± 0.11 to 0.52 ± 0.12 Hz) and WKY rats (from 0.52 ± 0.07 to 0.54 ± 0.03 Hz) Figure 20. However, when coadministered with Ang (1-7), it blocked the counter regulatory effect of Ang (1-7) against enhanced chronotropic effect of Ang II in SHR neurons (before (0.52 ± 0.07 to 2.97 ± 0.17 Hz) and after (0.54 ± 0.03 to 2.44 ± 0.28) addition of BPV) Figure 20. The results indicate that the inhibitory action of Ang (1-7) against Ang II action in SHR was abolished in presence of PTEN inhibitor, BPV suggesting that the inhibitory effects of Ang (1-7) against Ang II action in SHR neurons were mediated through PTEN activation. In summary, all the data demonstrate that PTEN inhibition did not alter the basal firing rate in neurons from both SHR and WKY rats. However, when coadministered with Ang (1-7), it blocked the counter-regulatory effect of Ang (1-7) against Ang II action in SHR neurons.

3.3.7. ANG (1–7) stimulates PTEN activity in SHR and WKY rat neurons:

Next, we examined the direct effect of Ang (1-7) on PTEN activity in SHR and WKY rat neurons. PTEN activity was examined using a PTEN detection kit, as described in the methods, in neuronal cultures treated with control (PBS), Ang II, Ang (1-7) (100 nM), Ang (1-7) plus A-779 (10 µM), or Ang (1-7) plus BPV (10 µM) for 10 min. The results are presented in Figure 21, indicating that Ang II did not significantly alter the PTEN activity in either SHR or WKY neurons. In contrast, Ang (1-7) treatment significantly increased the PTEN activity in SHR (from 74.1 ± 6.8 to 144.4 ± 14.9; $n = 4$; $P < 0.01$) and in WKY rat neurons (from 71.5 ± 6.5 to $133.4 \pm$

63

13.1; $n = 4$; $P < 0.01$) Figure 21. This stimulatory effect of Ang (1-7) on PTEN was completely abolished by the Mas receptor antagonist A-779, indicating this stimulatory effect of Ang (1-7) on PTEN is mediated by a Mas-dependent mechanism. Treatment of neurons with Ang II (100 nM) alone did not alter PTEN activity in neurons from either SHR or WKY rats Figure 21.

3.4. Data Summary and Discussion

In this study, we investigated the intracellular signaling pathways responsible for effect of Ang (1-7) on the chronotropic action of Ang II in SHR and WKY rat neurons. The results demonstrated that Ang (1-7) increases PTEN activity via stimulation of Mas receptors and abolishes the enhanced chronotropic effect of Ang II in the SHR neurons, indicating that Ang (1-7) counterregulates the chronotropic action of Ang II by a PTEN-dependent mechanism. This conclusion is supported by the following lines of evidence: *1*) Mas-Receptors were abundantly localized on cultured neurons from both SHR and WKY rats; *2*) the counterregulatory effect of Ang (1-7) on the Ang II induced chronotropic effect in SHR neurons was diminished by A-779, a selective Mas-R antagonist; 3) PI3-kinase inhibition also significantly abolished the enhanced chronotropic action of Ang II in SHR neurons; *4*) Pretreatment of Ang (1-7) significantly attenuated Ang II-induced increase in the phosphorylation of Akt in SHR vs. WKY neurons. 5) PTEN was also found to be abundantly expressed in cultured neurons; 6) the counterregulatory effect of Ang (1-7) on the Ang II action was also diminished by a PTEN inhibitor; 7) and treatment of neurons with Ang (1-7) significantly increased PTEN activity and this Ang (1-7) - induced stimulatory effect on PTEN was blocked by co-treatment with a Mas-receptor antagonist.
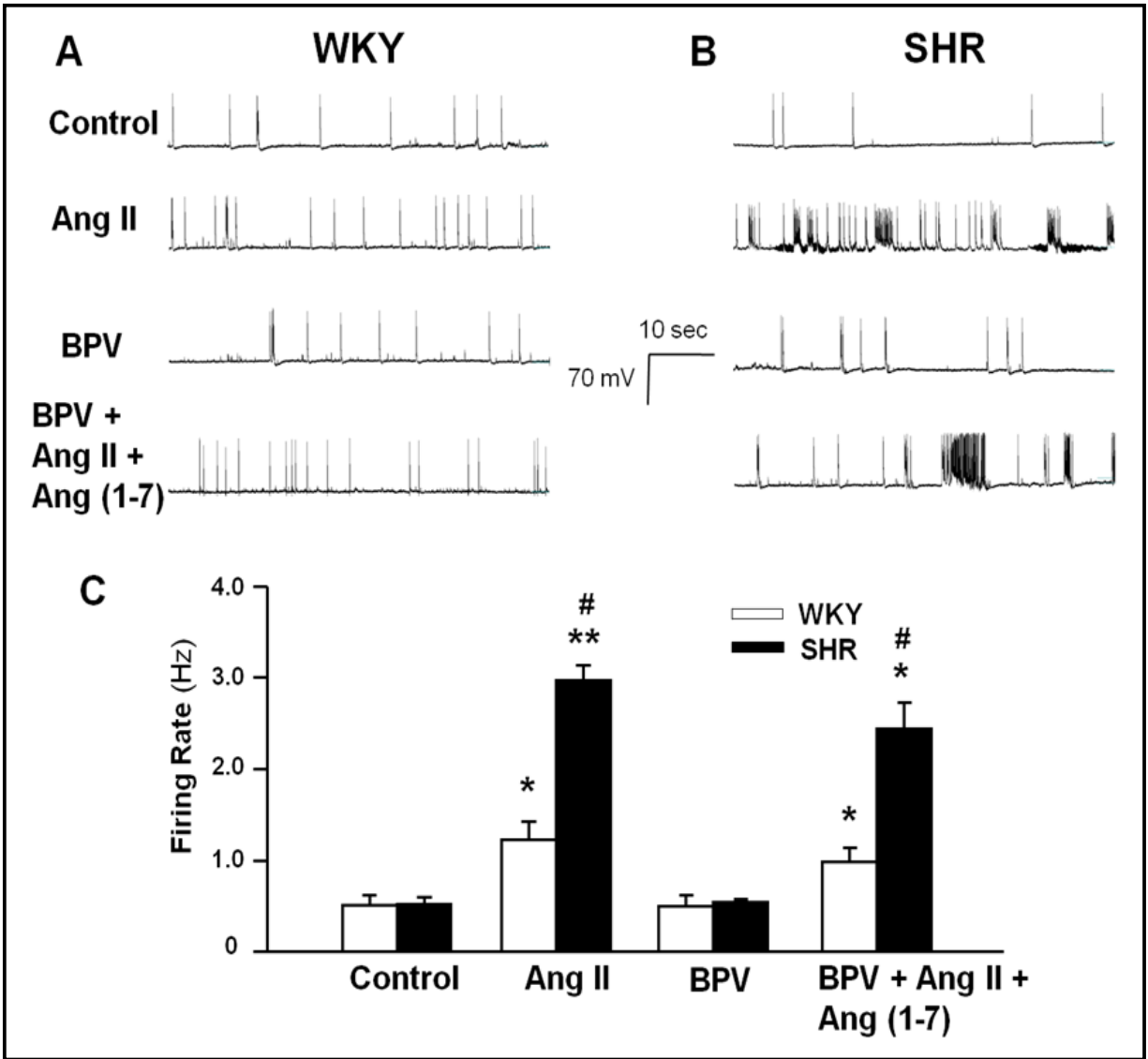
Figure 20: Effect of PTEN inhibition on the counterregulatory effect of Ang (1-7) against Ang II. *A*: representative tracings showing the APs recorded from a single WKY rat neuron under the following sequential treatment conditions: basal firing rate (Con), superfusion with 100 nM Ang II, washout of Ang II, and superfusion with the PTEN inhibitor bisperoxovanadium (BPV; 10 μM); this was followed by superfusion with BPV + Ang II + Ang (1–7) (100 nM). *B*: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in *A*. *C*: bar graphs summarizing the effect of BPV on the inhibitory action of ANG-(1–7) in SHR and WKY rat neurons in each treatment situation described in *A*. Data are means ± SE from 7–9 neurons. *$P < 0.05$, compared with respective control. **$P < 0.01$, compared with respective control. #$P < 0.01$, compared between SHR and WKY rat neurons under the same conditions.
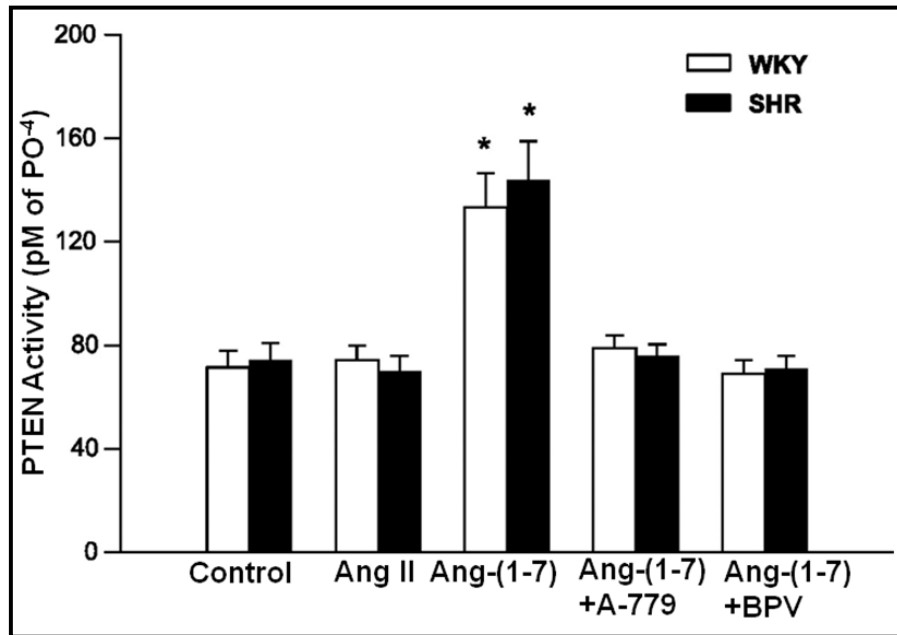
Figure 21: Effect of Ang II, Ang (1–7), A-779, and BPV on PTEN activity in neurons. PTEN activity was measured using immunoprecipitation, followed by biochemical phosphatase assay, as described in the methods. Neurons were incubated with Ang II (100 nM) or Ang (1–7) (100 nM) in the absence or presence of A-779 (10 μM) or BPV (10 μM) for 10 min at 37°C. Data are means ± SE derived from 4 experiments and ≥7 dishes in each experiment. *$P <$ 0.05, compared with respective control.

Ang (1-7), the main product of ACE2, has opposite properties to Ang-II in peripheral cardiovascular system (ref). Though the beneficial effects of Ang (1-7) on baroreceptor reflex, peripheral cardiovascular system were shown by various studies, however, the central action of Ang (1-7) on the cardiovascular regulation and the underlying mechanism of Ang (1-7) action are still not clear Previous studies have described that most of the known peripheral actions of Ang (1-7) are mediated through Mas-R and these receptors are found to be highly expressed in the cardiovascular regulatory areas of the brain such as nucleus of solitary tract, RVLM, inferior olive, parvo and magnocellular portions of paraventricular hypothalamic nucleus (Becker *et al.,*

2007, Santos *et al.,* 2000). Moreover, at the cellular level, Mas-R was predominantly present in neurons, as evidenced by colocalization of immunostaining for the neuronal marker, Neu-N, and the Mas receptor (Becker *et al.,* 2007). In the present study, we determined the localization and expression of Mas-R on the cultured neurons using double immunostaining technique and western blot (Figure 13 & 14). The results showed that Mas-Rs are abundantly expressed on the neurons cultured from brainstem/hypothalamus of SHR and WKY rats (Figure 13). Moreover, no significant difference in protein levels was observed between SHR and WKY rat neurons (Figure 14). The close proximity of Ang (1-7) and Mas-R in the brain cardiovascular regulatory regions indicate that the central effects of Ang (1-7) may be mediated through Mas-R, which is supported by our observation using A-779, a specific Mas-R antagonist in cultured neurons. The results demonstrated that alone A-779 had no effect on the basal neuronal activity in both SHR and WKY rat neurons, but when administered with Ang II and Ang (1-7), it significantly attenuate the inhibitory effect of Ang (1-7) on the enhanced chronotropic action of Ang II in SHR neurons, but no effect was observed in WKY rat neurons (Figure 15). These results indicated that the Ang (1-7) mediated inhibitory effects on Ang II induced chronotropic action were mediated through Mas-R in SHR neurons.

The activation or inhibition of membrane ionic currents and their underlying channels by G-protein-coupled receptors can occur through direct coupling of a G-protein subunit to the channel (Brown *et al.*, 1993) or indirect modulation via protein kinases and phosphatases (Koepke *et al.*, 1990). In the present study, Ang (1-7) and LY-294002 inhibits the Ang II induced neuronal activity in SHR neurons (Figure 15 & 16). Based upon these studies, we hypothesized that Ang (1-7) and LY 294002 may share common signaling pathways i.e. by inhibiting or antagonizing PI3-kinase pathway which led us to hypothesize that phosphates may

be involved in the action of Ang (1-7) which antagonize the Ang II induced PI3-kinase signaling pathway. PTEN is major negative regulator of PI3-kinase signaling pathways in the physiological system. Previously, it was demonstrated that PTEN is widely expressed in various mammalian tissues including the heart, brain, liver and lymphocytes (Crackower *et al.,* 2002, Kishimoto *et al.,* 2003, Stiles *et al.,* 2004, Seminario *et al.,* 2003). The function of PTEN is mediated by negative regulation of PI3-kinase pathway (Tomohiko *et al.,* 1998, Koul *et al.,* 2002, Kogan *et al.,* 1998). Here, we examined the presence of PTEN in cultured neurons from brainstem/hypothalamus of SHR and WKY rats, indicating that PTEN is expressed in the cultured neurons.

One question raised in this study centers on the cellular mechanism(s) underlying the Ang (1-7) -induced counter regulatory effect of Ang II action in SHR. To unravel these intracellular signaling pathways, we hypothesized that Ang (1-7) stimulates PTEN, which downregulates the enhanced PI3-kinase by dephosphorylating PIP3, the direct product of PI3-kinase. At first, we examined the role of PTEN in the counter-regulatory effect of Ang (1-7) on Ang II action in SHR and WKY rat neurons using bisperoxovanadium (BPV), a specific PTEN inhibitor. Neuronal firing was recorded using the whole cell configuration of patch clamp technique in current clamp mode on SHR and WKY rat neurons. BPV alone did not alter the neuronal activity in both SHR and WKY rat neurons, but in presence of Ang II and Ang (1-7), it significantly abolished the negative effect of Ang (1-7) on enhanced effect of Ang II on neuronal activity in case of SHR neurons, but no significant effect was observed in case of WKY rat neurons (Figure 20). Secondly, PTEN activity in extracts from neuronal cultures was measured using a phosphate detection kit as described in the methods (Section 3.2.4). Immunoprecipitates were collected using antibody against PTEN from neuronal extracts under various treatment conditions.

Immnuoprecipitates were used to avoid the interference caused by other phosphatases and to measure the direct effect of Ang (1-7) on PTEN activity. The results indicated that Ang (1-7) significantly increased the PTEN activity in both WKY and SHR neuronal cultures (Figure 21). This stimulatory effect of Ang (1-7) on PTEN was completely abolished by the Mas receptor antagonist A-779 (Figure 21), indicating this stimulatory effect of Ang (1–7) on PTEN is mediated by a Mas-dependent mechanism. Treatment of neurons with Ang II (100 nM) alone did not alter PTEN activity in neurons from either SHR or WKY rats (Figure 21).

Incubation of neurons with Ang (1-7) significantly increases PTEN activity via stimulation of Mas receptor in both WKY and SHR neurons (Figure 21). In addition, we also observed that the counterregulatory effect of Ang (1-7) on the Ang II action is abolished by the PTEN inhibitor BPV in SHR neurons (Figure 20). Another concern in the current study is the role of PTEN in normotensive rat brain neurons because Ang (1-7) stimulates this enzyme in both WKY and SHR. Current observations demonstrated that inhibition of PI3-kinase in WKY rat neurons has no effect on neuronal activity or on the chronotropic action of Ang II. These results suggest that neuronal activity in WKY rat neurons is not linked to the PI3-kinase signaling pathway. Thus the stimulatory action of Ang (1-7) on PTEN activity in WKY rat neurons would not alter the chronotropic effect of Ang II. However, PTEN activation results in many cellular effects that are mediated by PDK1, Akt/PKB, and rac1/cdc42 (Blanco-Aparicio *et al*., 2007). In neurons, these signaling molecules are involved in the regulation of gene expression and dendrite outgrowth (Chen *et al*., 2010; Montenegro-venegas *et al*., 2010; Ojeda *et al*., 2011). Thus Ang (1-7) - induced PTEN activation in WKY rats may be linked to other cellular effects such as neurogenesis and apoptosis. Although there is no current evidence in the present study in favor or against this hypothesis, the possibility cannot be discounted. We believe, based on the

69

evidence, that in SHR neurons the Ang II-induced PI3-kinase activity leads to phosphorylation

of its downstream target, Akt/PKB (Yang *et al*., 1999). Previous studies (Crackower *et al*., 2002)

have concluded that PTEN is involved in dephosphorylation of p-Akt through inhibition of PIP3

production. Both Akt and PIP3 regulate the activity of ion channels or channel-associated

proteins, which are involved in neuronal action potential generation and neuronal activity

(Macrez *et al*., 2001; Northcott *et al*., 2002). Further *in vitro* and *in vivo* studies will be

necessary to validate the relevance of the proposed brain PTEN activity in BP control in SHR.

Nonetheless, these observations suggest that stimulation of PTEN alters Ang II action on SHR

brain neuronal activity and that the enzyme could be an important therapeutic target for the

control of neurogenic hypertension.

The potential mechanisms by which Ang (1-7) stimulates PTEN activity remain to be

clarified. PTEN protein has multiple domains that may harbor the basic residues essential for its

translocation to the phospholipid membrane and for its activation. The C terminal of PTEN

protein contains a cluster of serine and threonine phosphorylation sites that may regulate its

stability, activity, and recruitment to the membrane. Phosphorylation by protein kinases or

dephosphorylation by phosphatases on these domains could regulate PTEN activity (Vazquez *et

al*., 2006). More interestingly, the actions of Ang (1-7) have been shown to be mediated by Src

homology protein SHP-2 phosphatase, tyrosine phosphatase SHP-1, and mitogen-activated

protein kinase phosphatase (Gallagher *et al*., 2008; Gava *et al*., 2009; Sampio *et al*., 2007).

Whether those phosphatases are involved in PTEN activation in neurons is still unknown.

Another possible signaling mechanism underlying Ang (1-7) -induced PTEN activation could be

mediated by reactive oxygen species (ROS). The catalytic domain of PTEN can form a disulfide

bond between Cys124 and Cys 71 in the enzyme active site. Free oxygen radicals could induce

disulfide bonding within the active site of PTEN, rendering it inactive (Lee *et al.*, 2002). It has been reported that reduced ROS and increased nitric oxide are involved in the action of Ang (1-7) in different tissues (Zimmerman *et al.*, 2011). However, the role of ROS in the Ang (1-7) - induced PTEN activation in neurons is not currently known and is the focus of ongoing investigation.

In addition, several limitations of the present study should be pointed out. In this study, neurons were cultured from the brainstem/hypothalamus of SHR neonates. Neonatal SHRs are not yet hypertensive, and their BP is comparable to that of WKY rats at this stage of development. The SHR is a genetically hypertensive animal model, carrying hypertension-related genes that cause them to develop hypertension. Thus they are prehypertensive rats, showing some abnormalities that may or may not contribute to the development of hypertension later in life. Using these neuronal cultures from prehypertensive SHRs, we observed that Ang (1-7) has a counterregulatory effect on Ang II action via stimulation of PTEN activity. However, future studies will be necessary to confirm whether or not this phenomenon is related to the development of hypertension in the adult SHRs. Another limitation of the current study is that the neurons used were cultured from the hypothalamus, which contains a heterogeneous population of neurons. As such, we cannot be certain which of the neurons used in the current study connect to sympathetic nerves related to the regulation of cardiovascular function. Nevertheless, a key advantage of this cellular model is that it allows us to study the effects of Ang (1-7) on individual neurons in a manner that minimizes potential confounding factors related to inputs from other neuronal networks. Improved retrograde labeling techniques, which could be used to identify the presympathetic neurons but are technically difficult to perform in neonatal rats at present, may be useful in overcoming this limitation in future studies.

In conclusion, we demonstrated direct cross-talk between signaling pathways underlying the actions of Ang (1-7) and Ang II in neurons cultured from the brainstem/hypothalamus of SHR and WKY rats, indicating the central role of these peptides. Ang II stimulated neuronal activity, which is enhanced in SHR neurons, and coadministration of Ang (1-7) attenuates the Ang II-induced-chronotropic effect exclusively in SHR neurons. The *in vitro* observations suggest that Ang (1-7) stimulates PTEN activity via a Mas receptor, leading to a counterregulatory effect on Ang II action in SHR neurons.

CHAPTER IV. CHRONIC EFFECTS OF ANG (1-7) ON THE CHRONOTROPIC ACTION OF Ang II: ROLE OF PI3-KINASE AND PTEN

4.1. Introduction

The brain RAS plays a pivotal role in the central control of cardiovascular functions (Averill and Diz, 2000). Ang II is the major effector hormone of this pathway, which interacts with angiotensin type 1 receptor (AT1) subtype in the cardioregulatory brain areas, such as hypothalamic and brainstem nuclei to regulate its central actions such as blood pressure (BP), sympathetic outflow, fluid balance, baroreflexes, and secretion of neurohormones (Mckinley MJ *et al*., 2003, Zucker *et al*., 2006). Previous studies have demonstrated that Ang II increases neuronal firing rate via stimulating AT1R and this chronotropic action of Ang II is enhanced in the neuronal cells cultured from the brainstem/hypothalamus of SHR as compared to normotensive WKY rats (Sun *et al.,* 2003, Sun *et al.,* 2002, Veerasingham *et al.,* 2003). This enhanced response of SHR neurons to Ang II was a result of preferential linking of the AT1R to the PI3-kinase signal transduction pathway (Sun *et al.,* 2003). These results are also supported by *in vivo* observations that PI3-kinase inhibition within the RVLM decreases blood pressure of the SHR to the levels similar to that of WKY rats (Seyedabadi *et al.,* 2001). However, the role of chronic blockade of PI3-kinase in the Ang II-induced chronotropic response have never been investigated. Thus, one of the current study aims was to determine the effect of chronic PI3-kinase inhibition on the chronotropic effect of Ang II in SHR and WKY neurons.

There is also growing interest in the role of the small peptide Ang (1-7) in the peripheral and central cardiovascular regulation. Ang (1-7) and its Mas-R are found to be widely expressed in brain especially in the cardiovascular regulatory areas of the brain related to blood pressure regulation such as brainstem and hypothalamus (Chappell *et al*., 1989, Becker *et al*., 2007).

73

However, its actions in the central nervous system on cardiovascular regulation are not fully understood. Centrally, Ang (1-7) acts as an important neuromodulator, its functions in contrast to Ang II includes increased sensitivity of baroreceptor mechanism and prevents norepinephrine release in SHR (Campagnole-Santos *et al*., 1992, Santos *et al*., 2003). Exogenous administration of Ang (1-7) in the caudal ventrolateral medulla inhibited RVLM mediated pressor response and hence lowered arterial pressure (Silva *et al*., 1993). In the NTS Ang (1-7) facilitates arterial baroreflex bradycardia (Campagnole-Santos *et al*., 1989). In addition, intrahypothalamic injection of Ang II induced a significantly greater pressor response in SHR and this enhanced pressor response to Ang II was reduced by co-administration with Ang (1-7) (Hocht *et al*., 2006). Moreover, viral vector-mediated overexpression of ACE2, an enzyme responsible for converting Ang II to Ang (1-7), in the RVLM prevents the development of hypertension in SHRs (Yamazato *et al*., 2007). In addition, central-specifically overexpression of ACE2 significantly prevents Ang II-induced hypertension in mice (Feng *et al*., 2010). These studies suggest that Ang (1-7) acts as an important neuromodulator and may antagonize the hypertensive actions of Ang II in the brainstem/hypothalamus of SHR. In spite of significance progress in this area, understanding of the cellular and molecular mechanisms involved in these physiological actions of Ang (1-7) remains to be completely elucidated.

The data presented in previous chapters was focused on the acute effect of Ang (1-7), antagonizing the chronotropic effect of Ang II, was found to be mediated by the interaction between PI3-kinase and PTEN in SHR neurons (Modgil *et al*., 2012). However, the chronic effects of Ang (1-7) or the effect of chronic PI3-kinase inhibition on the chronotropic action of Ang II are still unknown. Thus, the aims of the current study were to detect the chronic action of Ang (1-7) and PI3-kinase to examine the hypothesis that Ang (1-7) increase PTEN gene

expression, which contribute to the chronic effect of Ang (1-7) antagonizing the chronotropic action of Ang II in SHR neuron versus WKY rat neurons.

4.2. Material and Methods

4.2.1. Animals:

Twelve-week-old male SHR and WKY rats were obtained from Charles River Farms (Wilmington, MA). These rats were used as breeders to produce a constant supply of newborn SHR and WKY rat pups to prepare neuronal cultures. Rats were housed at $25 \pm 2°C$ on a 12 h-12 h light-dark cycle and provided with food and water ad libitum. All animal protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee.

4.2.2. Preparation of neuronal cultures:

Neuronal co-cultures were prepared from the brain stem and a hypothalamic block of newborn SHR and WKY rats as described previously (Yao *et al*., 2008). Trypsin (375 U/ml) and DNase I (496 U/ml)-dissociated cells were resuspended in DMEM containing 10% PDHA and plated on poly-L-lysine-precoated 35-mm Nunc plastic tissue culture dishes. After the cells were grown for 3 days at 37°C in a humidified incubator with 95% air- 5% CO_2, they were exposed to 1 µM ARC for 2 days in fresh DMEM containing 10% PDHS. Then, ARC was removed and the cells were incubated with fresh DMEM (containing 10% PDHS) for further 9-12 days before use. At the time of use, cultures consist of 90% neurons and 10% astrocyte glia, as determined by immunofluorescent staining with antibodies against neurofilament proteins and glial fibrillary acidic protein in previous study (Summner *et al*., 1990).

4.2.3. Electrophysiological recordings:

Spontaneous action potentials (APs) were recorded from WKY and SHR neurons at room temperature using the whole cell patch-clamp technique in current-clamp mode as described in our previous publication (Yao *et al.*, 2008). Cells were bathed in Tyrodes's solution containing (in mmol/L) 140 NaCl, 5.4 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 0.3 NaH_2PO_4, 10 HEPES, and 10 dextrose; pH adjusted to 7.4 with NaOH. The patch electrodes (resistance from 3-4 MΩ) were filled with an internal pipette solution containing (in mmol/L) 140 KCl, 4 $MgCl_2$, 4 ATP, 0.1 guanidine-5'-triphosphate, 10 dextrose, and 10 HEPES; pH adjusted to 7.2 with KOH. Neuronal firing rate was measured as the number of fully developed APs (depolarization beyond 0 mV) per second (Hz).

4.2.4. Western blot analysis:

PTEN protein levels in neuronal cultures were assessed by western blot analysis using goat anti-PTEN antibody as primary and anti-goat peroxidase-conjugated antibody as secondary antibody. Briefly, neuronal cultures were washed with ice-cold PBS and scraped into a lysis buffer containing 20 mM Tris HCl (pH 6.8), 150 mM NaCl, 10% glycerol, 1% NP-40, and 8 µl/ml inhibitor cocktail (125 mM PMSF, 2.5 mg/ml aprotinin, 2.5 mg/ml leupeptin, 2.5 mg/ml antipain, and 2.5 mg/ml chymostatin). The samples were sonicated twice for 10 s each and centrifuged at 10,000 rpm for 10 min at 4°C. Supernatants were transferred into new tubes and was saved for protein assay and further stored in a -80°C freezer. The protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). An aliquot of 20 µg of protein from each sample was separated on a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes for 2 h at 100 V. After a 10-min wash in PBS-T, membrane was blocked in PBS-T containing 10% milk for 1 h, followed by an overnight incubation in primary

goat anti-PTEN antibody (dilution 1:500) at 4°C. After a 15-min wash in PBS-T, four 5-min washes in PBS-T was carried out, and membranes was then incubated for 2 h in an anti-goat peroxidase-conjugated antibody as secondary antibody (dilution 1:15,000). Densitometry of PTEN was normalized to β-actin. Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL Western blotting detection kit, Amersham Pharmacia Biotechnology), and film was analyzed with Quantity One Software (Bio-Rad).

4.2.5. Real-time RT-PCR:

We used real-time PCR to detect changes in the expression of PTEN in the neuronal cultures from WKY and SHR rats. The isolation of total RNA from the neuronal cultures was performed with RNeasy Mini Kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA purity and concentration was determined spectrophotometrically. For each RT-PCR reaction, 2 µg of total RNA was converted into cDNA with reverse transcriptase (Promega catalogue no. 3500). Genomic DNA was eliminated by DNase I. For real-time PCR, the reactions were conducted by placing 20 µl of material into 96-well plates with the SYBR Green PCR kit or TaqMan PCR Master Mix (Applied Biosystems). 5 µl of cDNA, 10µl of standard Master mix, PTEN (1µl) (Rn00356918_m1) and GAPDH probe (1µl) (Rn0000576699_m1) as an internal control, obtained from Applied Biosystem, CA, were used for each sample. RT-PCR was performed in an Applied Biosystems PRISM 7500 sequence detection system according to the protocol from the manufacturer. Ct value were obtained and relative change in gene expression was calculated using $2^{\wedge}\Delta\Delta Ct$ method. In each experiment, samples were analyzed in triplicate.
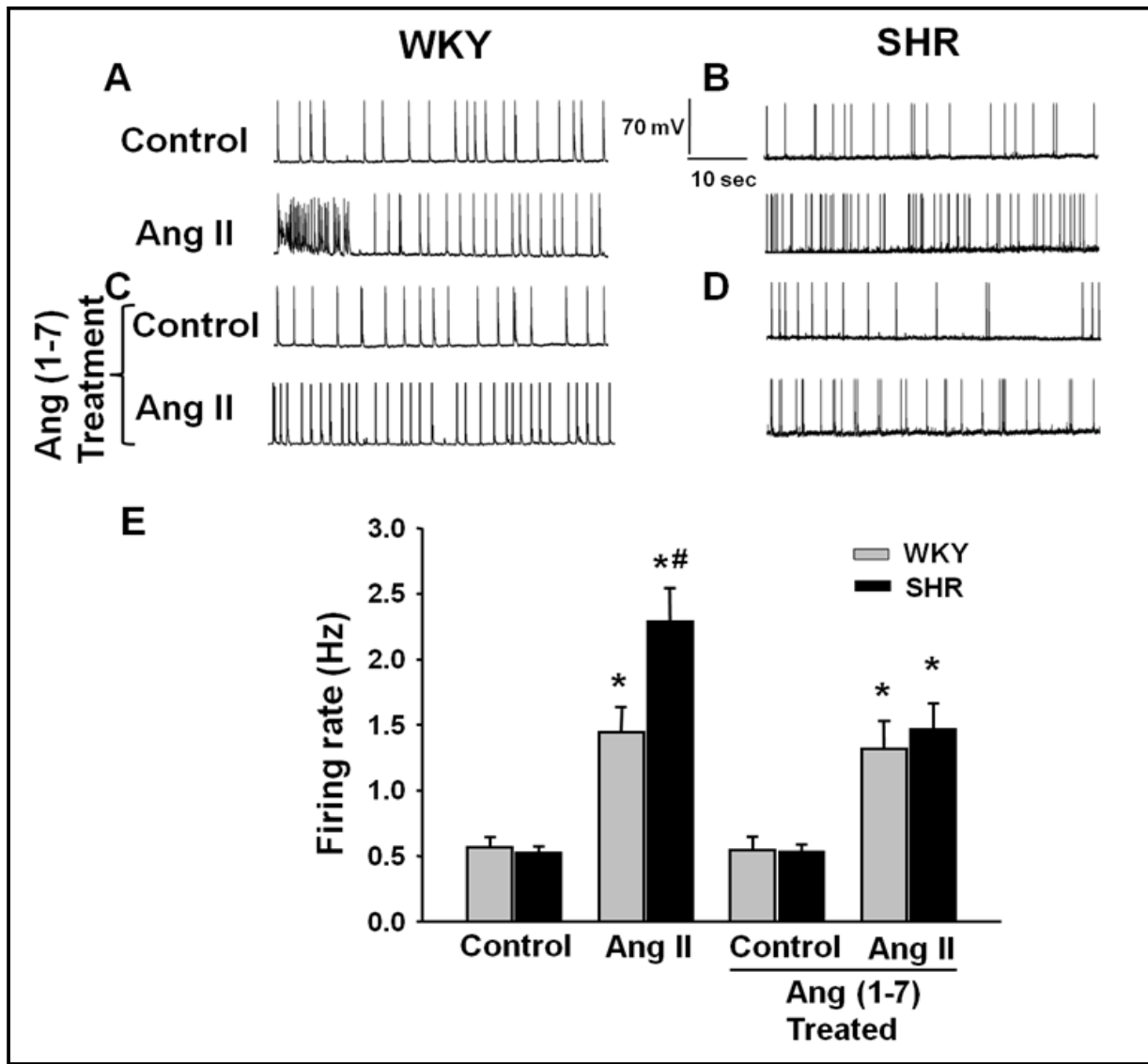
Figure 22: Effect of chronic Ang (1-7) on the chronotropic action of Ang II in rat neurons. Firing rate was recorded from neurons under each treatment condition. **A**: representative tracings showing the action potentials (APs) recorded from a single WKY rat neuron under the following sequential treatment conditions: superfusion with control (Con) solution (PBS); Ang II (100 nM). **B**: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in A. **C**: representative tracings showing the action potentials (APs) recorded from a single WKY rat neuron pretreated with Ang (1-7) (100nM; For 24 hrs) under the following sequential treatment conditions: superfusion with control (Con) solution (PBS); Ang II (100 nM). **D**: representative tracings showing the action potentials (APs) recorded from a single SH rat neuron pretreated with Ang (1-7) (100nM; For 24 hrs) under the same sequential treatment conditions as in C. **E**: bar graphs summarizing the effect of chronic pretreatment of Ang (1-7) on the chronotropic action of Ang II in SHR and WKY rat neurons. Data are means ± SE from 6 neurons for each group. *P < 0.01 compared with respective control recordings; and #P < 0.01 compared recordings between SHR and WKY rat neurons under the same condition.

4.2.6. *In vitro* lentiviral vector mediated gene transfer and confirmation of protein expression in neurons by fluorescence microscopy:

To examine the effect of chronic blockade of PI3-kinase on the chronotropic action of Ang II, lentiviral vector-mediated overexpression of dominant negative p85α subunit of PI3-kinase (DNp85α) was used. In the construct, DNp85α was driven by EF promoter (LV-EF α - DNp85α-IRES-eGFP). The gene-transfected cells were visualized by the expression of GFP under fluorescense microscope prior to electrophysiological recordings. The recordings were made only from neurons with positive transgenic expression of eGFP. In addition, we used LV-EF1α-eGFP as a control. Lentiviral particles (Lv-GFP or Lv- DNp85α) were prepared, titered and used for *in vitro* gene transfer as previously published by us (Zubcevic *et al*., 2009). The viral titer was $1\text{-}2x10^{10}$ transfection units (TU)/ml. Neurons from WKY and SH rats were treated with the virus particles containing either Lv-DNp85α or Lv-GFP (control) for 48 hours, followed by electrophysiological recording mentioned above.

4.2.7. Data analysis:

Results are expressed as means ± SE. Statistical significance wa evaluated with use of one way ANOVA followed by Newman-Keuls test. Differences considered significant at $P < 0.05$.

4.3. Results and Data Analysis

4.3.1. Chronic Ang (1-7) treatment attenuates the enhanced chronotropic response to Ang II in SHR neurons:

We first examined the effect of chronic treatment of Ang (1-7) on the chronotropic response to Ang II on neuronal firing rate in neurons cultured from the brainstem/hypothalamus of SH and WKY rats using the whole cell patch clamp configuration in current clamp mode. Neurons were pretreated with or without Ang (1-7) (100 nM; for 24 hrs) prior to recordings. In neurons,

without any treatment of Ang (1-7), Ang II superfusion significantly increased neuronal firing rate in both SHR (0.52±0.05 to 2.29±0.25) and WKY (0.57±0.07 to 1.44±0.19) neurons as shown in Figure 22 and the Ang II induced increase in neuronal firing was significantly enhanced in SHR neurons as compared to WKY rat neurons (Figure 22). The chronic treatment of Ang (1-7) (100 nM, 24 hrs) did not alter the Ang II-induced increase in neuronal firing rate in WKY rat neurons. However, it abolished the enhanced chronotropic action of Ang II in SHR neurons (0.53±0.05 to 1.46±0.19) (Figure 22). In addition, A-779 (10 µM), a Mas-R antagonist abolished the chronic inhibitory effect of Ang (1-7) on Ang II action in SHR neurons as compared to WKY neurons. Moreover, BPV (10 µM) the PTEN inhibitor, did not alter the neuronal activity in case of WKY neurons. In contrast, BPV blocked the inhibitory chronic effect of Ang (1-7) in case of SHR neurons.
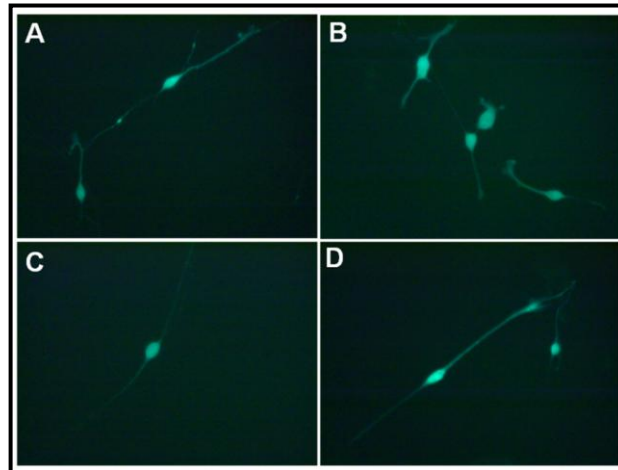

Figure 23: Confirmation of PI3-kinase knockdown: transgenic expression due to GFP. Identification of transgene expression (GFP) using fluorescence microscopy in the SH and WKY rat neurons following viral treatment (for >48 hrs). **A:** Lv-DNp85α treated WKY neurons **B:** Lv-DNp85α treated SHR neurons; **C:** Lv-GFP treated WKY neurons **D:** Lv-GFP treated SHR neurons

80

These studies demonstrate that the chronotropic action of Ang II is enhanced in SHR neurons as compared to WKY rat neurons and chronic treatment with Ang (1-7) attenuates the enhanced chronotropic action of Ang II exclusively in SHR neurons. In addition, this inhibitory effect of Ang (1-7) on the chronotropic effect of Ang II was abolished in presence of BPV, the PTEN inhibitor (0.78±0.11 to 2.32±0.3) (Figure 22), indicating that the chronic effect of Ang (1-7) was mediated through PTEN. In WKY rat neurons, neither chronic treatment with Ang (1-7) nor treatment with BPV alter the chronotropic action of Ang II.

4.3.2. Effect of chronic PI3-Kinase blockade on the chronotropic response to Ang II in SHR versus WKY rat neurons:

We have demonstrated previously that the enhanced chronotropic effect of Ang II in SHR neurons is mediated by PI3-kinase (Sun *et al.,* 2003, Sun *et al.,* 2009). In previous experiments, we examined the role of PI3-kinase in the chronotropic effect of Ang II using LY-294002 and wortmannin acutely. In the current studies, we examined the chronic effect of PI3-kinase blockade on the chronotropic action of Ang II, we chronically inhibited PI3-kinase activity by over-expression of dominant negative p85α subunit of PI3-kinase enzyme (DNp85α) using lentiviral vector (Lv-DNp85α). Lv-GFP was used as control. Neurons were treated with Lv-DNp85α or Lv-GFP for 48 hrs prior to recordings. The chronotropic effect of Ang II was present only in the neurons with positive transgenic expression of eGFP (Figure 23 $ 24). Lv-GFP treatment altered neither the basal neuronal firing nor the chronotropic response to Ang II as compared with the neurons without treatment in both SHR and WKY rats. The chronotropic response to Ang II was comparable between the neurons treated with Lv-GFP (0.61±0.13 to 1.38±0.24) and Lv-DNp85α (0.59±0.07 to 1.445±0.22) in WKY rat neurons (Figure 24). Whereas, compared to Lv-GFP control (0.49±0.08 to 2.24±0.33), treatment with Lv-DNp85α

(0.6±0.07 to 1.51±0.19) (Figure 24) significantly inhibited Ang II-induced enhancement in neuronal firing rate in SHR neurons. These results indicated chronic blockade of PI3-kinase also attenuate the enhanced chronotropic effect of Ang II in SHR neurons.

4.3.3. Effect of chronic treatment of Ang (1-7) on PTEN mRNA levels in SH and WKY rat neurons:

Further experiments focused on possible mechanisms for the counter-regulatory effect of Ang (1-7) on the chronotropic action of Ang II in SHR neurons. Our previous studies indicated the involvement of PTEN in the action of Ang (1-7) in neurons cultured from SHR rats (Modgil *et al*., 2012). The present study was designed to determine the chronic effect of Ang (1-7) on PTEN gene expression in SH and WKY rat neuronal cultures. Treatment of neuronal cultures with Ang (1-7) (100 nM, 24 hrs) significantly increased PTEN mRNA levels by 1.3 fold (n=3, p<0.05) in WKY rats neurons (Figure 25). In contrast, Ang (1-7) treatment with the same protocol induced a 2.1fold increases in PTEN mRNA levels (n=3, p<0.05). Ang (1-7)-induced increases in PTEN mRNA levels was significantly higher in SHR neurons (Figure 25), as compared to WKY neurons. This effect of Ang (1-7) on PTEN expression was completely blocked by co-treatment with A-779 (10 µM, 24hrs), a Mas-receptor antagonist (Figure 25). These results suggest that the Ang (1-7) stimulate PTEN gene expression in WKY/SHR neuronal cultures via a Mas receptor-dependent mechanism.

4.3.4. Effect of chronic treatment of Ang (1-7) on PTEN protein levels in SH and WKY rat neurons:

In this experiment, the chronic effect of Ang (1-7) on the PTEN protein levels in cultured neurons from SH & WKY rats. The results are presented in Figure XX, demonstrate that treatment of neurons with Ang (1-7) (100 nM, 24hrs) increases the PTEN protein levels by 39%

(n=3, p<0.05) in WKY rat neurons (Figure 26). In SHR neuronal cultures, Ang (1-7) induced a

45% (n=3, p<0.05) increase in the PTEN protein levels (Figure 26). This effect of Ang (1-7) on

PTEN expression was significantly blocked by A-779 (10 μM, 24 hrs), a Mas-receptor

antagonist (Figure 26). These results further suggest that the Ang (1-7) stimulated PTEN protein

expression in WKY/SHR neuronal cultures via a Mas receptor-dependent mechanism.

4.4. Data Summary and Discussion

It is well documented that Ang (1-7) antagonizes most of the actions of Ang II in the

peripheral system, but its action in brain has not been revealed. The present study examined the

chronic effect of Ang (1-7) on the chronotropic action of Ang II and the underlying intracellular

mechanism in SHR and WKY rat neurons. The results indicate that chronic treatment with Ang

(1-7) attenuate the enhanced chronotropic effect of Ang II via stimulation of PTEN expression,

which in turn antagonizes PI3-kinase activity, blocking Ang II-induced downstream signaling

transduction. This conclusion is supported by the following observations: (1) chronic treatment

with Ang (1-7) attenuates the Ang II-induced enhancement in chronotropic response in SH rat

neurons as compared to WKY neurons. (2) viral vector-mediated chronic blockade of PI3-kinase

also abolished the enhanced chronotropic effect of Ang II in SHR neurons as compared to WKY

rat neurons. (3) chronic treatment with Ang (1-7) significantly enhanced PTEN expression at

protein as well as mRNA levels in both SH and WKY rat neuronal cultures via a Mas-R

dependent mechanism. The data demonstrate that Ang (1-7) induces PTEN expression via Mas-

R, and depresses PI3-kinase signaling, which antagonizes the chronotropic action of Ang II in
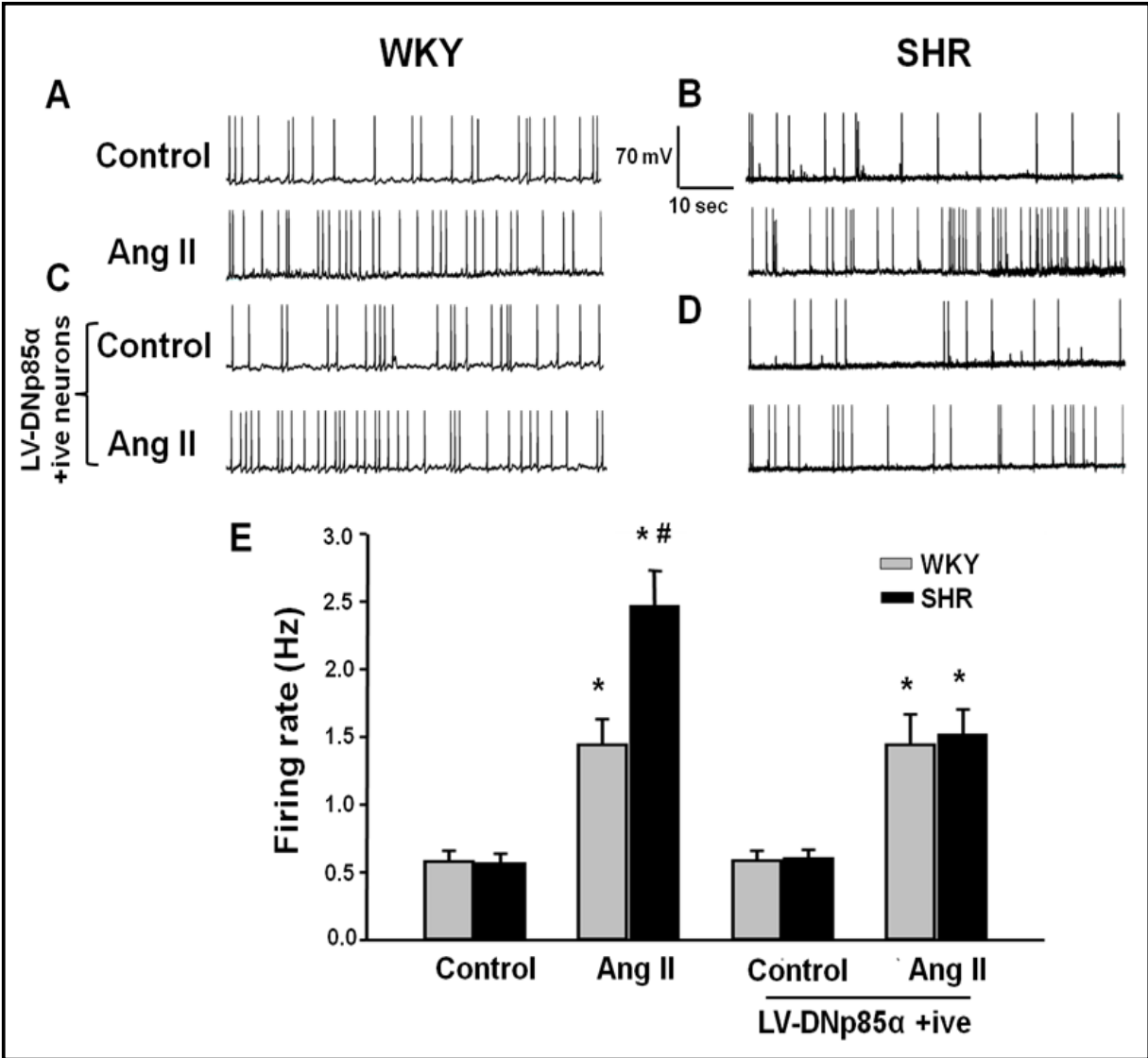
SHR neurons.

Figure 24: Effect of chronic PI3-Kinase blockade using lenti-virus vector, on the chronotropic action of Ang II. **A**: representative tracings showing the action potentials (APs) recorded from a single WKY rat neuron under the following sequential treatment conditions: superfusion with control (Con) solution (PBS); Ang II (100 nM). **B**: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in A. **C**: representative tracings showing the action potentials (APs) recorded from a single WKY rat neuron transfected with lentiviral vector expressing dominant negative form of PI3-kinase subunit p85α (Lv-DNp85α) under the following sequential treatment conditions: superfusion with control (Con) solution (PBS); Ang II (100 nM). **D**: representative tracings showing the action potentials (APs) recorded from a single SH rat neuron transfected with lentiviral vector expressing dominant negative form of PI3-kinase subunit p85α (Lv-DNp85α) under the same sequential treatment conditions as in C. **E**: bar graphs summarizing the effect of chronic blockade of PI3-Kinase on the chronotropic action of Ang II in SH and WKY rat neurons. Data are mean ± SE from 6-9 neurons. *P < 0.01 compared with respective control recordings; and #P < 0.01 compared recordings between SHR and WKY rat neurons under the same condition.
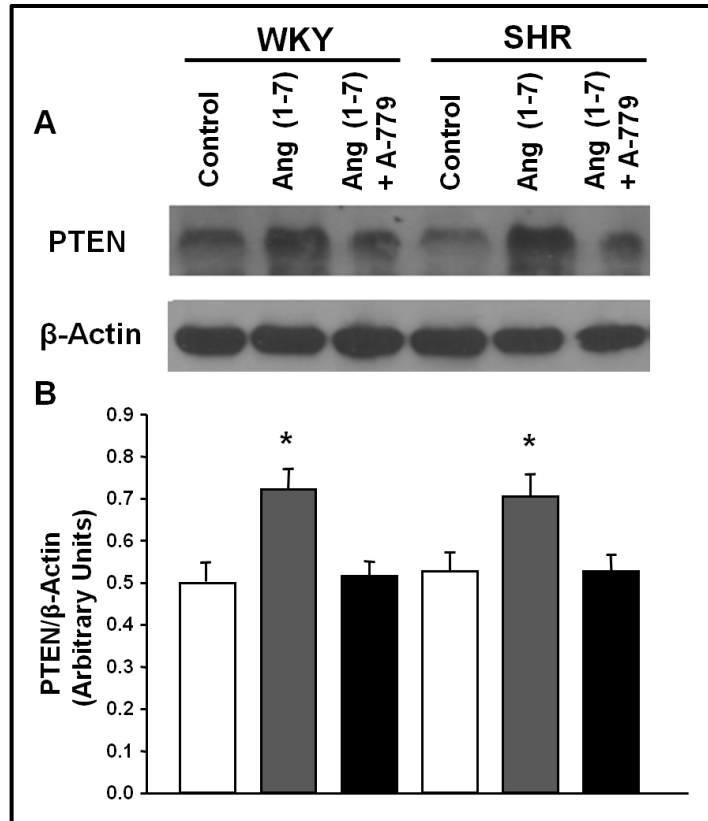
Figure 25: Effect of chronic treatment of Ang (1-7) on PTEN protein expression levels: PTEN protein levels in SH & WKY rat neurons were assessed by Western blot analysis as described in methods under the following treatment conditions: PBS (control), ANG (1-7) (100 nM), Ang (1-7) plus A-779 (10 μM). **A:** Representative blots showing PTEN levels **(Upper panel)**; and β-actin levels **(Lower panel)** in SH & WKY rat neurons. Densitometry of PTEN was normalized to β-actin. **B:** bar graphs summarizing the chronic effect of Ang (1-7) on PTEN expression in SH & WKY rat neurons. Data are means ± SE derived from three independent experiments. *$P<0.05$ indicates a significant difference from the corresponding nonstimulated (control).

We have demonstrated previously that the enhanced chronotropic effect of Ang II in SHR neurons is mediated by PI3-kinase (Sun *et al*., 2003, Sun *et al*., 2009). Acute administration of Ang (1-7) attenuated the enhanced chronotropic effect of Ang II in SHR via stimulation of

PTEN activity (Chapter 3). In the current study, we examined the chronic effect of Ang (1-7) on

the chronotropic response to Ang II in SHR and WKY rat neurons. Chronic treatment with Ang

(1-7) (Figure 22) and inhibition of PI3-kinase using lentiviral vector-mediated overexpression of

dominant negative p85α subunit of PI3-kinase (Figure 23 & 24) significantly abolished the

enhanced chronotropic action of Ang II in SHR neurons as compared with WKY rat neurons.

This inhibition reduces the enhanced firing response of SHR neurons in response to Ang II to the

level observed in neurons from WKY rat neuron exposed to Ang II (Figure 24). However, the

absence of any effects of LV- DNp85α on the neuronal activity in WKY neurons indicates that

the additional PI3-kinase pathway if exist, may be present only in SHR neurons.
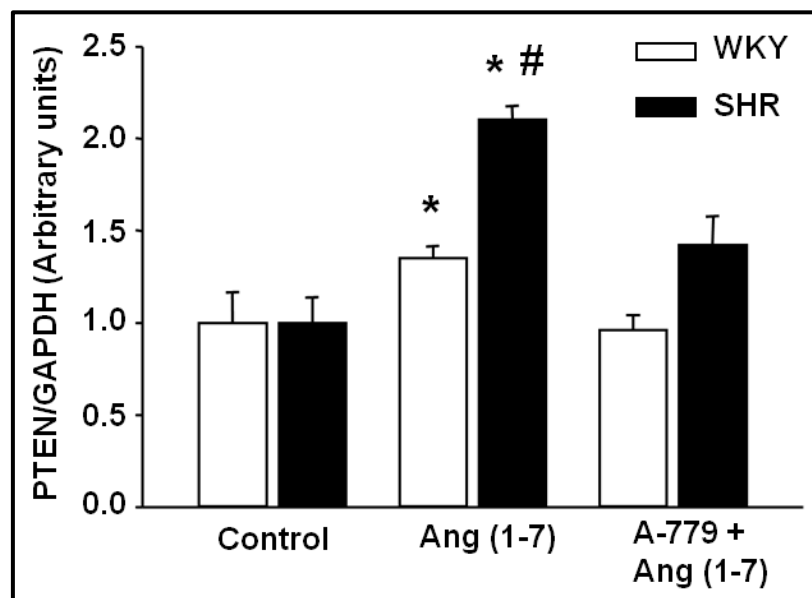
Figure 26: Effect of Ang (1-7) on the PTEN mRNA expression: PTEN mRNA levels in SH & WKY rat neuronal cultures under the following treatment conditions: PBS (control), ANG (1-7) (100 nM), Ang (1-7) plus A-779 (10 μM) for 24 hrs at 37°C. PTEN mRNA levels were detected with real-time RT-PCR as described in methods. Data are means± SE of the fold increase over control (n = 4 experiments). *P < 0.05 vs. control.

In physiological system, PI3-kinase has been shown to be negatively regulated or antagonized by PTEN. It is one of the major phosphatase that dephosphorylate PtdIns (3,4,5)P3 (PIP3), which is the major product of PI3-kinase and substrate for PTEN (Tomohiko *et al.,* 1998, Koul *et al.,* 2002, Kogan *et al.,* 1998). The results from the current study demonstrate that chronic treatment with Ang (1-7) antagonizing the chronotropic effect of Ang II by enhancing PTEN expression, leasing to enhancement of PI3-kinase and PTEN coupling in SHR neurons.

CHAPTER V. *IN VIVO* EFFECTS OF ANG (1-7) ON CARDIOVASCULAR FUNCTIONS AND ITS INTERACTION WITH ANG II IN THE RVLM AREA

5.1. Introduction

Increasing evidences indicate that a hyperactive brain RAS is critical in the development and maintenance of hypertension. The RVLM is a key integrative site within the medulla that participates in the tonic and baroreflex regulation of BP via sympathetic nerve activity. Pacemaker cells within this area provide the major excitatory input to sympathetic preganglionic neurons in the spinal cord that innervate sympathetic ganglia and adrenal medulla (Sun *et al.,* 1995*;* Dampney *et al.,* 2003). In addition, RVLM receives extensive inputs from other cardiovascular nuclei, including tonic excitatory input from the paraventricular nucleus (PVN) and NTS and inhibitory input from caudal ventrolateral medulla (Dampney *et al.,* 2002*;* Schreihofer *et al.,* 2002). The RVLM coordinate the propagation of angiotensin (Ang) II signals leading to hyperactivity of this hormone in hypertension (Veerasingham *et al.,* 2003). This conclusion is supported by the observations that the RVLM is considered as the final relay point before transmission of Ang II signals to periphery and provides supraspinal excitatory input to sympathetic preganglionic neurons in the regulation of blood pressure (Dampney *et al.,* 2002). Thus, the RVLM is considered as major relay point for the transmission of SNA. The importance of the RVLM is further highlighted by observations that AT1-R and Ang II sensitivity in this brain area of SHR and other rat models of hypertension are found to be increased (Muratani *et al.,* 1993; Ito *et al.,* 2002*;* Ito *et al.,* 2003). In addition both altered RVLM function and elevated SNA have been implicated in the pathogenesis of hypertension in several hypertensive animal models (Colombari *et al.,* 2001*;* Esler *et al.,* 2000*;* Mancia *et al.,*

2000; de Wardener *et al.,* 2001). Thus, regulation of the RAS activity in the RVLM is critical in a long-term regulation of neural control of blood pressure and other cardiovascular functions.

Ang (1-7) is another powerful CNS regulator of cardiovascular functions, leading to increased sensitivity of baroreflex mechanism, prevents norepinephrine release and induces depressor response in hypertensive rats. More interestingly, viral vector-mediated overexpression of ACE2, an enzyme responsible for converting Ang II to Ang (1-7), in the RVLM prevents the development of hypertension in SHRs (Yamazato *et al*., 2007). In addition, brain-specific overexpression of ACE2 significantly prevents Ang II-induced hypertension in mice (Feng *et al*., 2010). These effects of ACE2 could be mediated by lowering of Ang II levels or by increasing Ang (1-7) levels in this brain area. To test this hypothesis, we examined the effect of Ang (1-7) and Ang II microinjection into the RVLM brain area of SHR and WKY rats on the blood pressure and heart rate. In addition, the cellular and molecular mechanism(s) underlying the central action of Ang (1-7) was also examined in both SHR and WKY rats.

5.2. Materials and Methods

5.2.1. Animals:

Twelve-week-old male SHR and WKY rats were obtained from Charles River Farms (Wilmington, MA). Rats were housed at $25 \pm 2°C$ on a 12:12-h light-dark cycle and provided with food and water ad libitum. All animal protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee (Protocol A0741).

5.2.2. RVLM Microinjection and BP Recording:

Male WKY rats (9 to 10 weeks old) were anesthetized with a mixture of O_2 (1 L/min), and isoflurane (3%) was delivered through a nose cone. The rats were then placed in a stereotaxic

frame (David Kopf Instruments, Tujunga, Calif). Skin overlying the midline of the skull was incised, and a small hole was drilled bilaterally on the dorsal surface of the cranium according to the following coordinates: 1.9 mm lateral to the midline, 3.0 mm posterior to the lambdoid suture, 10 mm below the skull. Bilateral RVLM microinjections (50 nL) of Saline or Ang (1-7) or/and Ang II in absence or presence of various inhibitors were performed to examine the effect and underlying signaling pathways for exogenous Ang (1-7). The RVLM location of the microinjection site was verified by the pressor response to L-glutamate microinjection as described in our previous publication (Yamazato *et al.,* 2007). E-10 catheters fused to PE-50 catheters were prefilled with heparinized saline (100 IU/mL) and placed in the right femoral artery for acute recording of BP and HR. BP and HR were recorded using a pressure transducer, which was connected to a Bridge Amplifier (AD Instrument, Colorado Springs, Colo). Both BP and HR data were collected and analyzed with PowerLab software (AD Instruments). Blood Pressure (BP) and Heart Rate (HR) of Spontaneously Hypertensive Rats (SHR) and Wister Kyoto (WKY) rats were recorded using a pressure transducer and Power-Lab software before and after microinjection of Ang II and/or Ang (1-7) into the RVLM.

5.2.3. Data analysis:

Results are expressed as means ± SE. Statistical significance was evaluated with the use of a one-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at $P < 0.05$

5.3. Results and Data Analysis

5.3.1. Effect of Ang II microinjected into RVLM on BP and HR in SHR vs. WKY rats:

In the present study, our primary objective was to determine the cardiovascular regulatory effects of Ang II in the RVLM area of the brain in SHR and WKY rats. The rats were anesthetized with a mixture of O_2 (1 L/min), and isoflurane (3%) delivered through a nose cone. BP and HR were recorded before and after bilateral RVLM microinjections (50 nL) of saline or Ang II (100 pmol). BP and HR of SHR and WKY rats were recorded using a pressure transducer and Power-Lab software as described in the Methods (Section 5.2.2). The effects of Ang II microinjection into the RVLM were compared with the microinjection of saline control. As expected and demonstrated in Figure 27, RVLM microinjection of Ang II increase the MAP by 35 ± 3 mm of Hg and HR by 52 ± 4 bpm in SHR. Whereas, in WKY rats the MAP increases by 21 ± 4 mm of Hg and HR by 32 ± 7 bpm (Figure 27). The pressor effect of Ang II started immediate after the microinjection and peaked at 3 minutes after RVLM injection. The data demonstrated that the Ang II-induced increase in MAP and HR was significantly higher in case of SHR as compared to WKY rats. In summary, the results demonstrate that the microinjection of Ang II into the RVLM induced pressor response in both SHR and WKY rats and this pressor effect of Ang II was significantly enhanced in the SHR as compared to WKY rats.

5.3.2. Effect of Ang (1-7) microinjected into RVLM on BP and HR in WKY vs. SH rats:

Ang (1-7) was shown to be present as an endogenous constituent of all the major cardiovascular regulatory regions of the brain, in areas including the hypothalamus, medulla oblongata, and amygdala, as well as in adrenal glands and plasma of normal rats (Chappell *et al*., 1989). Thus, we examined the effect of Ang (1-7) microinjected into the RVLM of SHR and WKY rats on the MAP and HR (Figure 27). The rats were anesthetized with a mixture of O_2 (1

91

L/min), and isoflurane (3%) delivered through a nose cone. MAP and HR were recorded before

and after bilateral RVLM microinjections (50 nL) of saline or Ang (1-7) (300 pmol). MAP and

HR were recorded using a pressure transducer and Power-Lab software as described in the

Methods (Section 5.2.2). The effect of Ang (1-7) microinjection into the RVLM was compared

with microinjection of saline. In compliance with our *in vitro* studies (Figure 7 & 8), RVLM

microinjection of Ang (1-7) did not significantly alter BP and HR in case of SHR and WKY rats.
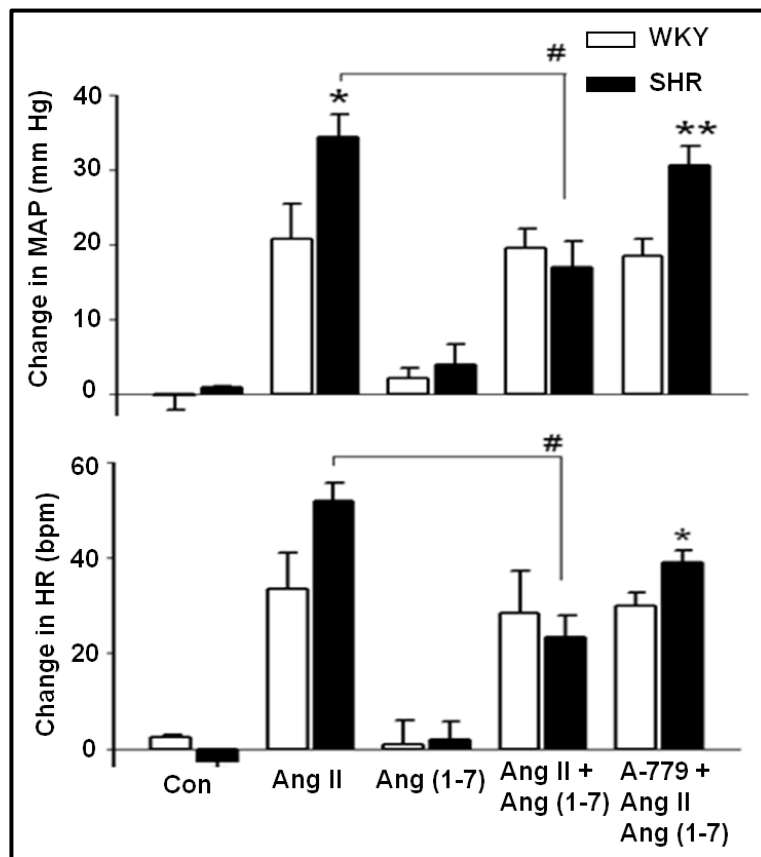

Figure 27: Effect of Ang II, Ang (1-7), Ang II+Ang (1-7), A-779 (central blockade of Mas-R) on MAP and HR after RVLM microinjection. Change in MAP (A) and HR (B) in SHR and WKY rats after RVLM microinjection of Ang II (100 pmol), Ang (1-7)(100 pmol), Ang II(100pmol)+Ang (1-7)(100pmol), and Ang II(100pmol)+Ang (1-7)(100pmol)+A779(1nmol). *P<0.05 vs. WKY rats. #P<0.01 vs. Ang II group.

5.3.3. Effect of Ang (1-7) microinjection on the pressor response to Ang II in the RVLM of SHR vs. WKY rats:

Our preliminary studies demonstrated that Ang (1-7) counter-regulates the chronotropic action of Ang II in the neurons cultured from hypothalamus and brainstem of SHR as compared to WKY rat. In the current study, we examined the effect of Ang (1-7) microinjection on the pressor response to Ang II in the RVLM brain area of SHR and WKY rats (Figure 27). The rats were anesthetized with a mixture of O_2 (1 L/min), and isoflurane (3%) delivered through a nose cone. BP and HR were recorded before and after bilateral RVLM microinjections (50 nL) of saline/Ang II or Ang II plus Ang (1-7). BP and HR were recorded using a pressure transducer and Power-Lab software as described in the Methods section (Section 5.2.2). The effect of Ang II microinjection (100 pmol) into the RVLM was compared in the absence or presence of Ang (1-7) (300 pmol). The results indicated that Ang II was found to have enhanced effect on MAP and HR in case of SHR as compared to WKY rats (Figure 27). This enhanced effect of Ang II on MAP and HR were abolished after co-administration of Ang (1-7) from 35±3 mm of Hg (before) to 18±3 mm (after) of Hg and 52±4 bpm (before) to 22±5 bpm (after) respectively in SHR (Figure 27). In contrast, co-administration of Ang (1-7) and Ang II did not alter Ang II effects on MAP and HR. Data are presented in Figure 27, demonstrate that Ang (1-7) antagonize the enhanced pressor effect of Ang II in neurons cultured from hypothalamus and brainstem of SHR as compared with WKY rats (Figure 27). However, RVLM microinjection of Ang (1-7) did not alter the Ang II response in WKY rats. In summary, the data suggest that alone Ang (1-7) in RVLM area did not alter the cardiovascular functions in either SHR or WKY rats. On the contrary, when Ang (1-7) was administered together with Ang II in the RVLM area of SHR brain, it counter-regulates the pressor response of Ang II in SHRs, in compliance with our *in*

vitro results (Figure 27). Whereas, in WKY rat brain no alteration in the effect of Ang II was observed, when administered together with Ang II.
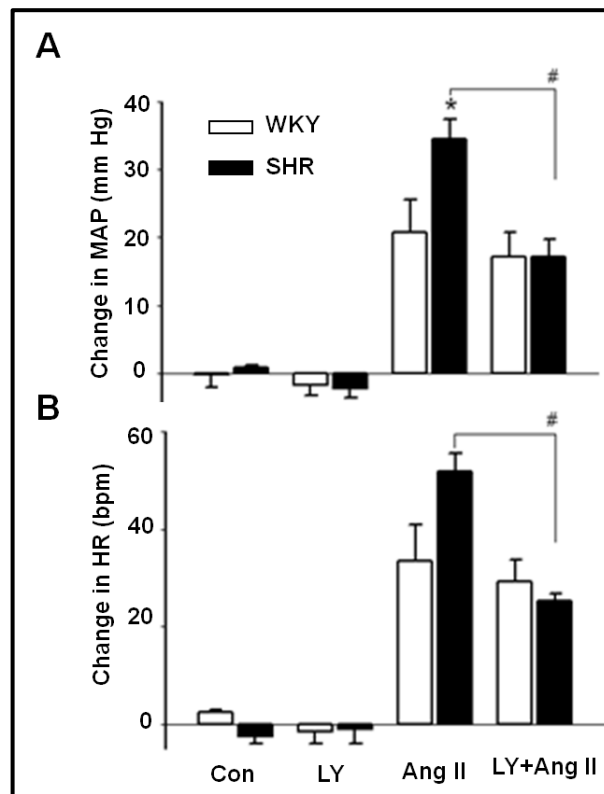
Figure 28: Effect of PI3-Kinase inhibition on Ang II induced increase in MAP and HR after RVLM microinjection. Change in MAP (A) and HR (B) in SHR and WKY rats after RVLM microinjection of AngII (100 pmol), LY(10nmol), LY(10nmol) + AngII (100 pmol) and PBS control (50nl). *P<0.05 vs. WKY rats. #P<0.01 vs. Ang II group.

5.3.4. Effect of PI3-Kinase inhibition on the pressor response to Ang II in WKY vs. SHR rats:

We previously demonstrated that enhanced response to Ang II in SHR neurons was a result of preferential linking of the AT1R to the PI3-kinase signal transduction pathway (Sun *et al.*, 2003; Sun *et al.*, 2009). It was also observed that the *in vivo* pressor response to Ang II was

significantly enhanced in SHR as compared to WKY rats. In this study our main goal was to evaluate the effect of LY-294002, a specific PI3-kinase inhibitor on Ang II action. The rats were anesthetized with a mixture of O_2 (1 L/min), and isoflurane (3%) delivered through a nose cone. BP and HR were recorded before and after bilateral RVLM microinjections (50 nL) of saline. Ang II (100 pmol), LY-294002 (10 nmol), or LY-294002+Ang II (100 pmol) were performed to examine the underlying signaling pathways. BP and HR were recorded using a pressure transducer and Power-Lab software as described in the Method section (Section 5.2.2). The pressor response to Ang II on these cardiovascular functions was compared with or without LY-294002. It was observed that Ang II has enhanced effect on MAP and HR in case of SHR and

the LY-294002, alone did not alter the MAP and HR in either SHR or WKY rats (Figure 28). On the contrary, when LY-294002 and Ang II were administered together, the enhanced effect of Ang II on MAP and HR was abolished from 35±3 mm of Hg to 19±2 mm of Hg and 52±4 bpm to 25±1 bpm, respectively in SHR but no significant effect was observed in case of WKY rats (Figure 28). The results depicted in (Figure 28) suggested that LY-294002 did not alter the Ang II- induced pressor response in WKY rat. However, it significantly attenuated the enhanced pressor response to Ang II in case SHR indicating that the enhanced effect of Ang II in SHR is mediated through PI3-kinase pathway.

5.3.5. Effect of central blockade of Mas-Receptors on the action of Ang (1-7) against Ang II in RVLM of WKY vs. SHR rats:

In spite of the evidences that the Mas-R are present in cardiovascular regulatory areas of the brain, not much is known about their functional relevance to Ang (1-7) action in brain. The characterization of Mas-R as Ang (1-7) receptors and high levels of these receptors in cardiovascular regulatory areas prompted us to evaluate whether the biological effects of Ang (1-

7) in these cardiovascular regulatory areas are mediated through Mas-R. The rats were anesthetized with a mixture of O_2 (1 L/min), and isoflurane (3%) delivered through a nose cone. BP and HR were recorded before and after bilateral RVLM microinjections (50 nL) of saline, Ang II (100 pmol), Ang (1-7) (300 pmol), or A-779 (1nmol)+Ang (1-7) (300 pmol)+Ang II. BP and HR were recorded using a pressure transducer and Power-Lab software as described in Methods section. The data presented in (Figure 27) demonstrated that Mas-R antagonist, A-779, blocked the inhibitory effect of Ang (1-7) on Ang II-induced pressor response on MAP (18±3 mm of Hg to 32±2 mm of Hg) and HR (22±5 bpm to 40±2 bpm) exclusively in SHR. In contrast, no significant effect was observed in case of WKY rats. In summary, Mas-R blockade by A-779 microinjection with Ang (1-7) and Ang II attenuated the inhibitory effects of Ang (1-7) on BP and HR against Ang II indicating that the inhibitory effect of Ang (1-7) is mediated through Mas-R in SHR.

5.3.6. Effect of central PTEN inhibition on the action of Ang (1-7) against Ang II action in RVLM of WKY vs. SHR rats:

Further experiments focused on the possible mechanisms for the central effect of Ang (1-7) on the Ang II action *in vivo*. It has been demonstrated that PTEN is one of the major phosphatase that inactivates PIP3 signaling pathways counter-regulating PI3-kinase action in several tissues. In addition, previously we demonstrated that the enhanced chronotropic effect of Ang II in SHR neurons is mediated by PI3-kinase (Sun *et al*., 2003; Sun *et al*., 2009). In the present study, our goal was to determine the effect of central PTEN inhibition on the Ang (1-7) action against Ang II in RVLM area of brain in SHR and WKY rats. The rats were anesthetized with a mixture of O_2 (1 L/min), and isoflurane (3%) delivered through a nose cone. BP and HR were recorded before and after bilateral RVLM microinjections (50 nL) of saline, Ang II (100 pmol), Ang (1-

96

7), Ang II+Ang (1-7), or BPV (10 nmol)+Ang (1-7) (300 pmol)+Ang II. BP and HR were recorded using a pressure transducer and Power-Lab software as described in Methods section (Section 5.2.2). Our *in vitro* preliminary data demonstrates that BPV, a selective PTEN inhibitor blocked the counter regulatory action of Ang (1-7) against Ang II in neurons cultured from SHR as compared to WKY rat neurons. *In vivo* data demonstrated that PTEN inhibitor, BPV, blocked the inhibitory effect of Ang (1-7) on Ang II-induced pressor response on MAP (12±4 mm of Hg to 27±3 mm of Hg) and HR (23±3 bpm to 44±5 bpm) exclusively in SHR as presented in (Figure 29). In contrast, no significant effect was observed in case of WKY rats. Similarly, central PTEN inhibition blocked the inhibitory effects of Ang (1-7) on Ang II-induced increases in BP and HR. In summary, the results indicate that the inhibitory effects of Ang (1-7) on Ang II-induced pressor response in SHR are mediated through PTEN activation in SHR.

5.4. Data Summary and Discussion

The present study examined the central role of Ang (1-7) and its interaction with Ang II in the RVLM of SHR and WKY rats. The results demonstrated that Ang (1-7) microinjection into the RVLM antagonize the pressor effect of Ang II by a Mas receptor- and PTEN-dependent signaling pathway in SHR rats. The conclusion is supported by the following observations: (1) Microinjection of Ang II into the RVLM increased BP in both SHR and WLY rats; (2) The pressor response to Ang II microinjected into the RVLM was enhanced in SHR, as compared to WKY rats; (3) microinjection of Ang (1-7) into the RVLM had no effect on the pressor response to Ang II in WKY rats; however attenuated the pressor response to Ang II in SHR; (4) The enhanced pressor response to Ang II microinjected into the RVLM was blocked by LY 294002, a PI3-kinase antagonist in SHR rats, mimicking the effect of Ang (1-7); (5) The Ang (1-7)-induced antagonizing effect against the pressor response to Ang II was blocked by BPV, an

97

PTEN inhibitor. (6) In addition, the inhibitory effects of Ang (1-7) against Ang II action in SHR are attenuated in presence of A-779, a Mas-R antagonist, indicating the involvement of Mas-R in the action of Ang (1-7). All of these results demonstrate that Ang (1-7) in the RVLM counter-regulate Ang II-induced enhanced pressor effect in SHR rats and this action of Ang (1-7) may be mediated by Mas-R and PTEN-dependent pathway. Thus, the coupling of PTEN and PI3-kinase exclusively in the cardiovascular regulatory area (RVLM) of SHR brain could be involved in the crosstalk between Ang II and Ang (1-7), hence, may play a major role in the development and pathogenesis of hypertension in SHR.

We have previously reported that the enhanced neuronal response to Ang II in SHR is mediated via PI3 kinase-dependent mechanisms (Sun *et al.,* 2003, Sun *et al.,* 2002, Veerasingham *et al.,* 2003). Thus, the central PI3-kinase signaling pathway is critical for the development of hypertension in SHR. Previous *in vivo* studies have also demonstrated that microinjection of Ang II into the RVLM, increase BP in both SHR and WKY rats and the pressor response to Ang II is enhanced in the SHR as compared with WKY rats (Li and Guyenet *et al.,* 1995). In contrast, PI3-kinase inhibition within the RVLM decrease BP of the SHR to the levels similar to that of their normotensive controls, WKY rats (Seyedabadi *et al.,* 2001) which is in compliance with our *in vivo* studies. The LY-294002, a specific PI3-kinase inhibitor abolished the enhanced pressor response to Ang II in case of SH rats, while no alteration in the effect of Ang II was observed in case of WKY rats (Figure 28). This data demonstrated the exclusive involvement of additional signaling pathway that is linked to PI3-kinase pathway in SHR.

Our preliminary data also revealed that Ang (1-7) antagonizes the enhanced chronotropic effect of Ang II in neurons cultured from hypothalamus and brainstem of SHR as compared with

WKY rats. In addition, overexpression of ACE2, an enzyme converting Ang II to Ang (1-7), in the RVLM, lowers BP of the SHR (Yamazato *et al.,* 2007). Though Ang (1-7) and its Mas-R are widely expressed in the brain cardiovascular regulatory regions, the action of Ang (1-7) in the central regulation of cardiovascular system and the underlying neuronal mechanisms are not fully understood.
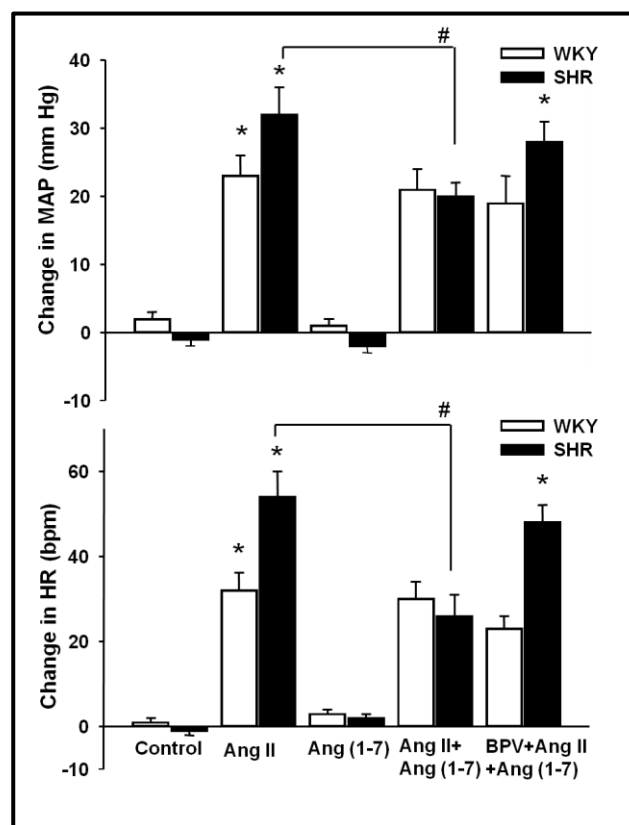

Figure 29: Effect of central PTEN inhibition on the action of Ang (1-7) against Ang II action in RVLM of WKY vs. SHR rats. Change in MAP (A) and HR (B) in SHR and WKY rats after RVLM microinjection of Ang II (100 pmol), Ang (1-7) (100pmol), Ang II (100pmol) + Ang (1-7) (300pmol), and AngI I (100pmol)+ Ang (1-7) (300pmol) +BPV (1nmol). *P<0.05 vs. WKY rats. #P<0.01 vs. Ang II group.

Most of the peripheral actions of Ang (1-7) are mediated through Mas-R and these receptors are found to be highly expressed in the cardiovascular regulatory areas of the brain such as nucleus of solitary tract, RVLM, inferior olive, parvo and magnocellular portions of paraventricular hypothalamic nucleus (Becker *et al.,* 2007, Santos *et al.,* 2000). To unravel the signaling pathways, our focus was to determine whether the central effect of Ang (1-7) in the RVLM was mediated through Mas-R or not. The data demonstrated that A-779, a specific Mas-R antagonist blocked the counter-regulatory effect of Ang (1-7) on Ang II induced enhanced pressor response exclusively in case of SH rats (Figure 27). These results strongly indicate that the central effects of Ang (1-7) in case of SH rats were mediated through Mas-R.

The next objective was to further explore the intracellular signaling pathways underlying the action of Ang (1-7), to validate PTEN mediated signaling pathway. PTEN is the major negative regulator of PI3-kinase signaling pathways. Thus, the role of PTEN was studied in the central action of Ang (1-7). BPV, a specific PTEN inhibitor, was found to abolish the central counter-regulatory effect of Ang (1-7) on Ang II induced enhanced increase in BP and HR in case of SH rats, while no effect was observed in case of WKY rats (Figure 29). The results demonstrate the involvement of PTEN in Ang (1-7) mediated effects against Ang II action in SHR.

In conclusion, our results demonstrated that the biologically active peptide Ang (1-7) counter-regulate the Ang II induced enhanced effect in case of SH rats and are involved in neurogenic BP regulation. Whereas, in case of WKY rats, Ang (1-7) alone as well as in combination with Ang II was not found to play any role in the central BP regulation. The counter-regulatory effect of Ang (1-7) on Ang II action in case of SH rats was mediated through Mas-R and may involve PTEN, which down regulates the Ang II induced enhanced PI3-kinase pathway and cause antihypertensive effect in case of SH rats.

CHAPTER VI. OVERALL DISCUSSION

The present study examined the actions of Ang (1–7) and Ang II as the major active peptides of the brain RAS in SHR and WKY rat neurons. Moreover, the major objective in the current study was to characterize the intracellular signaling mechanisms underlying PI3-kinasethe counter-regulatory effect of Ang (1-7) on Ang II action in neurons cultured from brainstem/hypothalamus of WKY vs. SH rats using various biochemical, pharmacological, molecular biology and electrophysiology techniques. The most significant observation of this study is the exclusive involvement of PTEN in Ang (1-7) induced inhibitory effect on the chronotropic actions of Ang II in the SHR neurons, as compared with WKY rat neurons. The results demonstrate that Ang (1-7) increases the activity and expression of PTEN via stimulation of Mas-R, which depresses the PI3-kinase activity, hence, abolishes the enhanced chronotropic effect and pressor response of Ang II exclusively in the SHR, implicating that Ang (1-7) counterregulates the chronotropic and pressor response of Ang II via a PTEN-dependent mechanism.

6.1. Ang II Mediated Central Blood Pressure Regulation

It is well known that the hypothalamus plays a major role in cardiovascular regulation and most forms of hypertension are associated with functional alterations in hypothalamus and RVLM, responsible for increase in sympathetic nervous system activity and decrease in sensitivity of baroreceptor reflex mechanism (Dampney *et al*., 2002). Significant increases in the discharge rate and differences in electrophysiological properties of hypothalamic neurons in SHR compared with WKY have been reported (Sun *et al*., 2002; Sun *et al*., 2003; Sun *et al*., 2009), indicating that this region may play a role in the genesis of the hypertension seen in the SHR. Another important cardiovascular regulatory brain area is the RVLM, which contains

presynaptic neurons of sympathetic nerves. Ang II microinjected into the RVLM induces

significant increases in MAP and HR in both SHR and WKY rats, and this pressor response

evoked by Ang II is also enhanced in the SHR (Silva-Barcellos *et al*., 2001). Accumulating

evidences point to Ang II and other derivatives of the central renin angiotensin system as

possible neurotransmitters or neuromodulators in specific pathways that connect major

cardiovascular and autonomic regulatory centers in the brain stem, hypothalamus, and forebrain.

6.2. Intracellular Mechanisms Responsible for Ang II Action on Neuronal Activity

Ang II is the major effector hormone of this pathway, which interacts with AT1-R subtype in

the cardioregulatory brain areas, such as hypothalamic and brainstem nuclei to regulate its

central actions such as blood pressure (BP), sympathetic outflow, fluid balance, baroreflexes, and

secretion of neurohormones (Mckinley MJ *et al*., 2003, Zucker *et al*., 2006). Summers *et al*

demonstrated the Ang II-AT1-R signaling using neuronal cultures from the newborn rat

brainstem/hypothalamus as a model system. Previous studies have demonstrated that Ang II

increases neuronal firing rate via stimulating AT1-R. This chronotropic effect elicited by Ang II

was found to be mediated by activation of both protein kinase C (PKC) and calcium/calmodulin-

dependent kinase II (Figure 6). In addition, this chronotropic action of Ang II is enhanced in the

neuronal cells cultured from the brainstem/hypothalamus of SHR as compared to normotensive

WKY rats (Figure 6) (Sun *et al.,* 2003, Sun *et al.,* 2002, Veerasingham *et al.,* 2003). Our *in vitro*

studies also showed that Ang II evoked an enhanced chronotropic response in neurons cultured

from SHR compared with WKY rats (Figure 9 & 10). This enhanced response of SHR neurons

to Ang II appears to be the result of the existence of an additional signal transduction pathway.

Many studies have linked AT_1R in SHR neurons to PI3-kinase signaling pathway in addition to

protein kinase C (PKC) and calcium/calmodulin-dependent kinase II pathways (Figure 6).

Blockade of PI3-kinase attenuates this action of Ang II exclusively in SHR neurons, suggesting that the increased PI3-kinase activity in the cardiovascular regulatory regions of SHR brain may be a unique signaling pathway that contributes to the hyperactivity of Ang II observed in these rats (Figure 16, 24 & 28). This speculation is supported by a previous study (Seyedabadi *et al*., 2001) showing that wortmannin, a PI3-kinase inhibitor, microinjected into the RVLM lowers BP in SHR but not in WKY rats. Thus, these studies suggest the functional role for PI3-kinase in the cardiovascular brain region of the SHRs. Similarly, in our preliminary studies, LY-294002, a specific PI3-kinase inhibitor, blocked the enhanced chronotropic effect of Ang II on neuronal activity of SHR neurons to the level observed in WKY rat neurons (Figure 16). This hypothesis was further substantiated by our *in vivo* studies which demonstrated that, blockade of PI3-kinase enzyme using LY-294002, in the RVLM, significantly abolished the enhanced chronotropic effects of Ang II on major cardiovascular functions i.e. BP and HR in SH rats, but no effect was observed in case of WKY rats (Figure 28). However, it is not known whether there are specific intracellular mechanisms that diminish or suppress the stimulatory actions of Ang II on neuronal firing in case of SHR neurons.

6.3. Central Blood Pressure Regulatory Effects of Ang (1-7) and ACE2

Ang (1-7) is another powerful CNS regulator of cardiovascular functions, leading to increased sensitivity of baroreflex mechanism, prevents norepinephrine release and induces depressor response in hypertensive rats. More interestingly, viral vector-mediated overexpression of ACE2, an enzyme responsible for converting Ang II to Ang (1–7), in the RVLM prevents the development of hypertension in SHRs (Yamazato *et al*., 2007). In addition, brain-specific overexpression of ACE2 significantly prevents Ang II-induced hypertension in mice (Feng *et al*., 2010). These effects of ACE2 could be mediated by lowering Ang II levels or

by increasing Ang (1–7) levels in this brain area. Several studies (Höcht *et al.*, 2006; Sakima et *al.*, 2005; Zimmerman *et al.*, 2011) have focused on the *in vivo* central effect of Ang (1–7) in the regulation of cardiovascular function. However, the cellular and molecular mechanism(s) underlying the central action of Ang (1–7) and its contribution to neurogenic hypertension is still not clearly established. Thus, to clarify and to further dissect the functional role and mechanisms of Ang (1–7), we observed the direct effect of Ang (1–7) on neuronal firing on neurons cultured from the brainstem/hypothalamus of WKY and SH rats.

We first examined the effect of Ang (1–7) and Ang II on neuronal firing rate in neurons cultured from the brainstem/hypothalamus of SHR and WKY rats using the whole cell patch-clamp configuration in current-clamp mode. This study demonstrates that Ang (1–7) does not alter neuronal firing rate in either SHR or WKY rat neurons (Figure 8). However, coadministration of Ang (1–7) with Ang II significantly attenuate the chronotropic response evoked by Ang II in SHR neurons (Figure 12), indicating that Ang (1-7) may counterregulate Ang II actions in the central control of BP exclusively in SHR. The results are consistent with other *in vivo* observations indicating that the opposing effect of Ang (1-7) on responses to Ang II is enhanced in animal models of hypertension both in the central nervous system and in the peripheral vasculature (Santos *et al.*, 2000, Höcht *et al.*, 2006). Höcht *et al.* (2006) have reported that intrahypothalamic injection of Ang II induced a significantly greater pressor response in SHR compared with WKY rats and whereas, the coadministration of Ang (1-7) with Ang II reduced the pressor response to Ang II in SHR but no effect was observed in WKY rats. Similarly, Benter *et al.* (1995) have demonstrated that Ang (1-7) attenuates Ang II-induced vasoconstriction in SHR but not in WKY rats. However, several other studies (Fontes *et al.*, 1994; Silva-Barcellos *et al.*, 2001) have demonstrated that microinjection of Ang (1-7) increases

BP and the pressor response to Ang (1-7) is similar to that evoked by Ang II. This discrepancy may be due to differences in animal species, the doses of Ang (1-7) used in the experiments, or other experimental conditions. For example, a low dose of Ang (1-7) (50 ng) in the hypothalamus did not alter BP in either SHR or WKY rats. In contrast, a high dose of Ang (1-7) (250 ng) significantly increases BP in SHR and this pressor effect of Ang (1-7) is blocked by irbesartan, indicating that the pressor effect of Ang (1-7) is mediated by an AT1-receptor-dependent mechanism (Höcht *et al*., 2006).

6.4. Intracellular Mechanisms of Ang (1-7) Action: Role of Mas-R

The G protein-coupled receptors (GPCR) Mas (Mas-R) were recently described as functional receptor for Ang (1-7) (Santos *et al.,* 2003). Previous studies have described that most of the known peripheral actions of Ang (1-7) are mediated through Mas-R and these receptors are found to be highly expressed in the cardiovascular regulatory areas of the brain such as nucleus of solitary tract, RVLM, inferior olive, parvo and magnocellular portions of paraventricular hypothalamic nucleus (Becker *et al.,* 2007, Santos *et al.,* 2000). To unravel the signaling pathways, the focus of our study was to determine whether the central effect of Ang (1-7) through neurons was mediated through Mas-R or not. Firstly, we confirmed the presence of Mas-R in the neurons cultured from brainstem/hypothalamus of SHR and WKY rat neurons. Mas-Rs were found to be abundantly expressed and no significant difference was observed in their expression in neurons cultured from brainstem/hypothalamus of both SHR and WKY rats. Moreover, Mas-R blockade attenuated the inhibitory effect of Ang (1-7) on Ang II induced chronotropic effect in case of SHR neurons indicating that this effect of Ang (1-7) is mediated through Mas-R (Figure 15). To check the functional relevance of our *in vitro* studies, our *in vivo*

data (Figure 27) also demonstrated that Mas-R blockade attenuated the inhibitory effect of Ang (1-7) on Ang II induced enhanced effect on BP and HR in SHR.

6.5. Intracellular Mechanisms of Ang II And Ang (1-7) Actions: Role of PI3-Kinase

Many studies have revealed the role of PI3-kinase in the enhanced chronotropic effect of Ang II in SHR neurons. Akt is an established downstream signaling kinase in PI3-kinase mediated signaling pathways in the physiological system. It was previously demonstrated that Ang II stimulated the PKB/Akt activation in both SH & WKY rat neurons but this stimulation was significantly higher in case of SHR as compared to WKY rat neurons (Yang & Raizada *et al*., 1999). These observations indicated the involvement of PI3-kinase-PKB/Akt dependent signaling pathways in SHR neurons (*Yang & Raizada,* 1999). In the present study, we examined the effect of Ang (1-7) on Ang II stimulated PI3-kinase activity in SH & WKY rat neurons. Treatment of Ang II significantly increased the ratio of phosphorylated Akt/total Akt in case of SHR neurons as compared to WKY rat neurons (Figure 17). When co-administered with Ang II, Ang (1-7) (100 nM) blocked the stimulatory effect of Ang II on the PI3-kinase activity measured as ratio of phosphorylated Akt/total Akt in SHR neurons as compared to WKY rat neurons (Figure 17). The results demonstrated that the counter-regulatory effect of Ang (1-7) on Ang II stimulated chronotropic effect on neuronal firing in SHR neurons is mediated via inhibition of PI3-kinase dependent signaling pathways.

6.6. Intracellular Mechanisms of Ang (1-7) Action: Role of PTEN

Our observation showed that Ang (1-7) or LY-294002 blocked the enhanced effect of Ang II on the neuronal activity specifically in SHR neurons to approximately similar level, which suggest that these may share a common signaling pathway i.e. by inhibiting PI3-kinase signaling. To further unravel the intracellular signaling pathway responsible for central actions of Ang (1-

7), we examined the role of phosphatase, which can down-regulate the enhanced PI3-kinase signaling in case of SHR neurons.

Our next studies were focused on the cellular mechanism(s) responsible for Ang-(1–7)-induced counterregulatory effect on Ang II action in SHR neurons. Based upon our hypothesis, some phosphatase may be involved in the counter-regulatory effect of Ang (1-7). PTEN is the major negative regulator of PI3-kinase signaling pathways. It is widely expressed in various mammalian tissues including the heart, brain, liver and lymphocytes (Crackower *et al.,* 2002, Kishimoto *et al.,* 2003, Stiles *et al.,* 2004, Seminario *et al.,* 2003). The function of PTEN is mediated by negative regulation of PI3-kinase pathway (Tomohiko *et al.,* 1998, Koul *et al.,* 2002, Kogan *et al.,* 1998). Here, we examined the presence of PTEN in cultured neurons from brainstem and hypothalamus of SHR and WKY rats. It was observed that PTEN was abundantly expressed in neurons cultured from brainstem/hypothalamus of both of SHR and WKY rats (Figure 18). In addition, no significant difference was observed in the PTEN expression among SH and WKY rat neurons (Figure 19).

Next, we moved to functional *in vitro* studies, the role of PTEN in Ang (1-7) induced counter-regulatory effect on Ang II stimulated enhanced chronotropic effect in SHR neurons was studied. To identify the intracellular signaling pathways, we hypothesized that Ang (1-7) might stimulate PTEN, which downregulates the enhanced PI3-kinase by dephosphorylating PIP3, the direct product of PI3-kinase. The present study provided the direct evidence for this hypothesis. Incubation of neurons with Ang (1-7) significantly increases PTEN activity via stimulation of Mas-R in both WKY and SHR neurons (Figure 21).

To further test our hypothesis, we examined the role of PTEN in the counter-regulatory effect of Ang (1-7) on Ang II action. In the current study, the effects of BPV, a specific PTEN inhibitor

on the counter regulatory effect of Ang (1-7) against Ang II was examined (Figure 20). It was

observed that the counterregulatory effect of Ang (1-7) on the Ang II induced enhanced

chronotropic effect was abolished by the BPV, exclusively in SHR neurons (Figure 20). Our *in

vivo* studies further authenticated our hypothesis. It was found that PTEN inhibition in the

RVLM region of the brain, abolished the inhibitory effect of Ang (1-7) on Ang II induced

enhanced effect on BP and HR in case of SHR as compared to WKY rats (Figure 29). Gathering

these results strongly suggest that PTEN may be involved in the inhibitory action of Ang (1-7)

against Ang II effect in case of SHR neurons.

Another concern in the current study is the role of PTEN in normotensive rat brain neurons

because Ang (1-7) stimulates this enzyme in both WKY and SHR. Current observations

demonstrated that inhibition of PI3-kinase in WKY rat neurons has no effect on neuronal activity

or on the chronotropic action of Ang II. These results suggest that neuronal activity in WKY rat

neurons is not linked to the PI3-kinase signaling pathway. Thus the stimulatory action of Ang (1-

7) on PTEN activity in WKY rat neurons would not alter the chronotropic effect of Ang II.

However, PTEN activation results in many cellular effects that are mediated by PDK1, Akt/PKB,

and rac1/cdc42 (Blanco-Aparicio *et al*., 2007). In neurons, these signaling molecules are

involved in the regulation of gene expression and dendrite outgrowth (Chen *et al*., 2010;

Montenegro-Venegas *et al*., 2010; Ojeda *et al*., 2011). Thus Ang (1-7) -induced PTEN activation

in WKY rats may be linked to other cellular effects such as neurogenesis and apoptosis.

Although there is no current evidence in the present study in favor or against this hypothesis, the

possibility cannot be discounted. We believe, based on the evidence, that in SHR neurons the

Ang II-induced PI3-kinase activity leads to phosphorylation of its downstream target, Akt/PKB

(Yang *et al*., 1999). Previous studies (Crackower *et al*., 2002) have concluded that PTEN is

involved in dephosphorylation of p-Akt through inhibition of PIP3 production. Both Akt and PIP3 regulate the activity of ion channels or channel-associated proteins, which are involved in neuronal action potential generation and neuronal activity (Macrez *et al*., 2001; Northcott *et al*., 2002). Further *in vitro* and *in vivo* studies will be necessary to validate the relevance of the proposed brain PTEN activity in BP control in SHR. Nonetheless, these observations suggest that stimulation of PTEN alters Ang II action on SHR brain neuronal activity and that the enzyme could be an important therapeutic target for the control of neurogenic hypertension.

6.7. Chronic Effects of Ang (1-7): Role of PI3-Kinase/PTEN

Our next focus was to study the chronic effects of Ang (1-7) neuronal activity and PTEN expression in SHR and WKY rat neurons cultured from brainstem/hypothalamus of SH vs. WKY rats. In the previous studies, we focused our attention on the acute effect of Ang (1-7) and postulated that the enhanced action of Ang II on the neuronal activity in SHR neurons is attenuated by Ang (1-7) via Mas-receptors (Modgil *et al*., 2012). Chronic treatment with Ang (1-7) or inhibition of PI3-kinase using lenti-viral vector expressing dominant negative construct of the p85α subunit of PI3-kinase (LV- DNp85α) significantly abolished Ang II mediated increase in neuronal firing rate in SH vs. WKY rat neurons (Figure 24). This inhibition reduces the enhanced firing response of SHR neurons in response to Ang II to the level observed in neurons from WKY rats exposed to Ang II. However, the absence of any effects of LV- DNp85α on the neuronal activity in WKY neurons indicates that the suggested additional PI3-K pathway if exist, may be present only in SHR neurons (Figure 24). Moreover, our previous studies indicated the involvement of phosphatase, PTEN in the action of Ang (1-7) in neurons cultured from SHR rats (Modgil *et al*., 2012). It was demonstrated that incubation of WKY/SHR neurons with Ang (1-7) increased PTEN activity (Figure 21) (Modgil *et al*., 2012). Here, we studied the chronic effect of

Ang (1-7) on the expression of PTEN in SH and WKY rat neuronal cultures. The data demonstrated that chronic treatment of Ang (1-7) significantly increase PTEN expression at both mRNA as well as protein levels in SH/WKY rat neurons (Figure 25 & 26).

The potential mechanisms by which Ang (1-7) stimulates PTEN activity remain to be clarified. PTEN protein has multiple domains that may harbor the basic residues essential for its translocation to the phospholipid membrane and for its activation. The C terminal of PTEN protein contains a cluster of serine and threonine phosphorylation sites that may regulate its stability, activity, and recruitment to the membrane. Phosphorylation by protein kinases or dephosphorylation by phosphatases on these domains could regulate PTEN activity (2006). More interestingly, the actions of Ang (1-7) have been shown to be mediated by Src homology protein SHP-2 phosphatase, tyrosine phosphatase SHP-1, and mitogen-activated protein kinase phosphatase (Gallagher *et al*., 2008; Gava *et al*., 2009; Sampaio *et al*., 2007). Whether those phosphatases are involved in PTEN activation in neurons is still unknown. Another possible signaling mechanism underlying Ang (1-7) -induced PTEN activation could be mediated by reactive oxygen species (ROS). The catalytic domain of PTEN can form a disulfide bond between Cys124 and Cys 71 in the enzyme active site. Free oxygen radicals could induce disulfide bonding within the active site of PTEN, rendering it inactive (Lee *et al*., 2002). It has been reported that reduced ROS and increased nitric oxide are involved in the action of Ang (1-7) in different tissues (Zimmerman, 2011). However, the role of ROS in the Ang (1-7) -induced PTEN activation in neurons is not currently known and is the focus of ongoing investigation. Moreover, these studies were primarily based on the use of pharmacological agents to inhibit the phosphatases, and we must use some caution in interpretation of this data. Further evidence with the use of PTEN dominant negative mutants will be needed to confirm these ideas.

In conclusion, we demonstrated direct cross-talk between signaling pathways underlying the actions of Ang (1-7) and Ang II using *in vitro* as well as *in vivo* model of SHR and WKY rats, indicating the central role of these peptides. Ang II stimulated neuronal activity or pressor response, which is enhanced in SHR neurons, and coadministration of Ang (1-7) attenuates the Ang II-induced-chronotropic effect exclusively in SHR brain. The results demonstrated that Ang (1-7) increase PTEN activity and expression via stimulation of Mas-R and down-regulate the PI3-kinase signaling pathway, hence, abolishes the enhanced chronotropic effect and pressor response of Ang II exclusively in the SHR neurons and brain respectively, implicating that Ang (1-7) counterregulates the chronotropic and pressor response of Ang II via PTEN-dependent mechanism.

CHAPTER VII. CONCLUSION

7.1. Overall Conclusion

In the present study, we determined the central action of Ang (1-7) and its interaction with Ang II in the central regulation of blood pressure and further elucidated the signaling mechanisms underlying these actions of Ang (1-7) using *in vitro* and *in vivo* hypertensive models. The overall conclusions from the *in vivo* and *in vitro* studies are summarized as following:
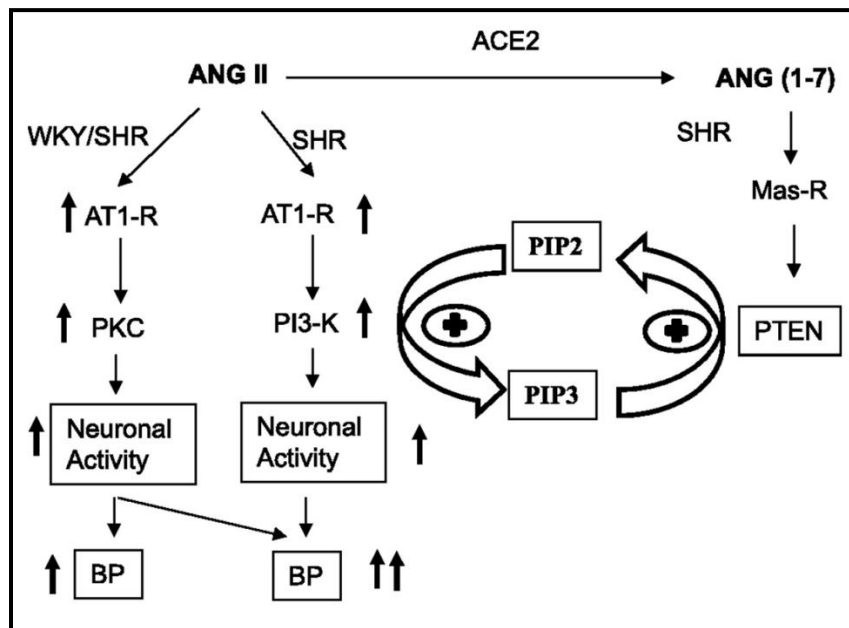

Figure 30: Diagram summarizing proposed signaling pathways for the counterregulatory effect of Ang (1–7) on Ang II action in SHR and WKY rat neurons. PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, soluble phosphatidylinositol-3,4,5-trisphosphate; Mas-R, Mas receptor. ACE2, angiotensin-converting enzyme 2; BP, blood pressure; AT1-R, Ang II type 1 receptor. (Modgil *et al.*, 2012)

7.1.1. *In Vitro* study on neuronal cultures from hypothalamus/brainstem of neonatal SHR and WKY rats:

(1) Ang II increases neuronal firing rate in both SHR and WKY neurons. The chronotropic effect of Ang II is significantly enhanced in SHR neurons as compared to WKY rat neurons.

(2) The enhanced chronotropic effect of Ang II is mediated by PI3-kinase pathway in SHR neurons. The PI3-kinase enzyme is involved exclusively in Ang II-induced effect in SHR neurons.

(3) Ang (1-7) alone has no effect on the basal neuronal firing rate. However, Ang (1-7) attenuates Ang II-induced enhanced chronotropic effect effect in SHR neurons whereas; it has no effect in WKY rat neurons.

(4) The counter-regulatory effect of Ang (1-7) on Ang II action is mediated by stimulation of PTEN activity, which antagonizes PI3-kinase signaling pathway located downstream of Ang II signaling.

(5) Chronic treatment of neurons with Ang (1-7) significantly increase PTEN expression and reduces the chronotropic effect of Ang II in SHR neurons. In addition, chronic inhibition of PI3-kinase using lentiviral vector-mediated over expression of dominant negative P85α subunit of PI3-kinase enzyme significantly attenuated the chronotropic action of Ang II exclusively in SHR neurons.

(6) The chronic antagonizing effect of Ang (1-7) on the chronotropic action of Ang II is mediated by stimulation of PTEN expression in SHR neurons.

7.1.2. *In Vivo* study on SHR and WKY rats:

(1) Microinjection of Ang II into the RVLM increases MAP and HR in both SHR and WKY rats. The pressor response to Ang II is enhanced in the RVLM of SHR as compared to WKY rats.

(2) RVLM injection of Ang (1-7) has no effect on MAP in both SHR and WKY rats. However, administration of Ang (1-7) into the RVLM significantly attenuates the pressor response to Ang II in SHR rats.

(3) The counter-regulatory effect of Ang (1-7) is blocked by PTEN inhibitor and Mas-R antagonist administered in the RVLM in SHR rats, as compared to WKY rats.

7.2. Future Research Directions

These studies were primarily based on the use of pharmacological agents to inhibit the phosphatases, and we must use some caution in interpretation of this data. Further evidence with the use of PTEN dominant negative mutants will be needed to confirm these ideas. Moreover, the potential mechanisms by which Ang -(1–7) stimulates PTEN activity remain to be clarified. PTEN protein has multiple domains that may harbor the basic residues essential for its translocation to the phospholipid membrane and for its activation. The C terminal of PTEN protein contains a cluster of serine and threonine phosphorylation sites that may regulate its stability, activity, and recruitment to the membrane. Phosphorylation by protein kinases or dephosphorylation by phosphatases on these domains could regulate PTEN activity (2006). More interestingly, the actions of Ang -(1–7) have been shown to be mediated by Src homology protein SHP-2 phosphatase, tyrosine phosphatase SHP-1, and mitogen-activated protein kinase phosphatase (Gallagher *et al*., 2008; Gava *et al*., 2009; Sampaio *et al*., 2007). Whether those

phosphatases are involved in PTEN activation in neurons is still unknown. Another possible

signaling mechanism underlying Ang -(1–7)-induced PTEN activation could be mediated by

reactive oxygen species (ROS). The catalytic domain of PTEN can form a disulfide bond

between Cys124 and Cys 71 in the enzyme active site. Free oxygen radicals could induce

disulfide bonding within the active site of PTEN, rendering it inactive (Lee *et al*., 2002). It has

been reported that reduced ROS and increased nitric oxide are involved in the action of Ang -(1–

7) in different tissues (Zimmerman, 2011). However, the role of ROS in the Ang -(1–7)-induced

PTEN activation in neurons is not currently known and is the focus of ongoing investigation.

7.3. Clinical Significance

With the alarming statistics for the global prevalence for hypertension, novel targets need to

be identified which can replace or improve the existing major therapeutic strategies such as ACE

inhibitors or AT1 receptor blockers. The latest discoveries provide a new axis to the brain RAS,

comprising ACE2, Ang (1-7) and Mas-R counter-regulating the classical ACE / Ang II / AT1

axis through generation of Ang (1-7) (Santos *et al.,* 2000, 2003, Ferrario *et al.,* 2005, Phillips and

de Oliveria *et al.,* 2008). Centrally, Ang (1-7) acts as an important neuromodulator, its functions

in contrast to Ang II includes increased sensitivity of baroreceptor mechanism and prevents

norepinephrine release in SHR (Campagnole-Santos *et al.,* 1992, Santos *et al.,* 2003). In

addition, large body of evidences has proven several beneficial effects of this peptide in the

peripheral cardiovascular system, which is often opposite to the effects elicited by Ang II (Santos

et al., 2000, 2003, Ferrario *et al.,* 2001, 2005, Grobe *et al.,* 2007, Mercure *et al.,* 2008). All of

the previous information suggested that Ang (1-7) may play an important role in the

pathogenesis of hypertension and cardiovascular diseases.

Our studies demonstrate that there is coupling of the SHR angiotensin type 1 receptors (AT1-R) to the PI3-kinase signaling pathway. PI3-kinase and PKB/Akt pathways are exclusively involved in the action of Ang II in SHR. The current study demonstrated the central effect of Ang (1-7) on the blood pressure regulation and on the action of Ang II both *in vitro* and *in vivo*. PTEN, negative regulator of PI3-kinase, is involved in the action of Ang (1-7). This study is highly relevant from the pathophysiological perspective and may turn out to have important pharmacological implications in the control of centrally regulated hypertension. For example, members of PI3-kinase and PTEN signaling cascades could serve as highly specific targets for pharmacological and/or gene therapy, because these pathways are only active in SHR. So, we believe that efforts of our group in this study raised the possibility to find new therapeutic targets based on the recently identified antihypertensive arm in RAS, ACE2 / Ang-(1–7) / Mas axis. Our studies indicate the importance of Ang (1-7) agonists or ACE2 activators, (which increases Ang (1-7) production and Ang II degradation), which in future may become therapeutic targets. We believe that targeting this recently identified arm in RAS would provide better cure for hypertension as compared to traditional therapies.

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