Central Mechanisms Mediating Ang II-Salt Hypertension

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Abstract

Statement of problem

Plasma angiotensin II (Ang II) increases blood pressure (BP) through the activation of brain angiotensinergic pathways and the aldosterone-mineralocorticoid receptors (MR)-epithelial Na^+ channel (ENaC)-endogenous ouabain (EO) pathway. The response of BP to circulating Ang II is enhanced by high salt intake, but the central mechanisms mediating this elevated response are not known.

Methods of investigation

Study 1) Male Wistar rats were divided into 4 groups and treated with regular salt diet (0.4% NaCl), high salt diet (2% NaCl), sc Ang II infusion (150 ng/kg/min), or sc Ang II infusion together with 2% salt diet for 14 days; plasma aldosterone and corticosterone levels, CYP11B2 mRNA in adrenal cortex and the mRNA levels of Ang II type 1 receptors (AT_1R), CYP11B1 (11-β hydroxylase), CYP11B2 (aldosterone synthase), MR, 11βHSD2, ENaC α, ENaC β and ENaC γ in the subfornical organ (SFO), paraventricular nucleus (PVN), supraoptic nucleus (SON) and rostral ventrolateral medulla (RVLM) were measured.

Study 2) MR blockers (eplerenone, spironolactone), ENaC blocker (benzamil), AT_1R blocker (losartan) or vehicles were centrally infused in rats treated with Ang II plus high salt, and BP and heart rate (HR) were recorded by telemetry; plasma aldosterone and corticosterone levels and CYP11B2 mRNA expression in adrenal cortex were measured.

Major findings

Ang II alone caused a small increase in BP. Ang II together with 2% salt diet markedly

increased the BP and plasma aldosterone level. Sc Ang II decreased 11βHSD2 and MR mRNA expression in the PVN, increased AT_1R and ENaC γ expression in the PVN, and increased AT_1R mRNA expression in the RVLM. Other genes tested in the four brain nuclei were not affected by sc Ang II or high salt diet. BP and plasma aldosterone increases in response to Ang II and salt, as well as CYP11B2 mRNA expression in adrenal cortex, were largely prevented by central infusion of eplerenone, spironolactone, benzamil or losartan.

Main conclusion

BP and plasma aldosterone responses to Ang II-salt are under the control of central mechanisms, and MR-AT_1R activation in the brain plays a critical role in Ang II-salt induced hypertension.

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ACE: Angiotensin-Converting Enzyme
aCSF: Artificial Cerebrospinal Fluid
ACTH: Adrenocoritotropic Hormone
Ang II: Angiotensin II
AS: Aldosterone Synthase
AT_1R: Ang II type 1 receptors
AT_2R: Ang II type 2 receptors
AVP: Arginine Vasopressin
β_2AR: beta2 Adrenergic Receptor
BP: Blood Pressure
BBB: Blood Brain Barrier
CaMK: Calcium/calmodulin-dependent Protein Kinases
cAMP: Cyclic Adenosine Monophosphate
CGx: Celiac Ganglionectomy
CNS: Central Nervous System
CRH: Corticotropin-Releasing Hormone
CVOs: Circumventricular Organs
CYP11B1: Cytochrome P450 11 B1, 11-β hydroxylase
CYP11B2: Cytochrome P450 11 B2, Aldosterone Synthase
CYP17: 17α-hydrolyase
CYP21*:* 21-hydroxylase
DAG: Diacylglycerol
ENaC: Epithelial Sodium Channel
eNOS: endothelial Nitric Oxide Synthase
EO: Endogenous Ouabain
GABA: Gamma-Amino Butyric Acid
ICV: Intracerebroventricular
IML: Intermediolateral Cell Column
IP3: Inositol 1,4,5-trisphosphate
KO: Knockout
LT: Lamina Terminalis
Iv: Intravenous
LV: Left Ventricle
MAP: Mean Arterial Pressure
MC2R: Melanocortin Receptor
MnPO: Median Preoptic Nucleus
mPVN: magnocellular subdivision of the PVN
MR: Mineralocorticoid Receptor
NADPH: Nicotinamide-Adenine Dinucleotide Phosphate
NE: Norepinephrine
NO: Nitric Oxide
NOX: NADPH Oxidase
NTS: Nucleus Tractus Solitarii
OLC: Ouabain-like Compounds

OVLT: Organum Vasculosum of the Lamina Terminalis
PB: Parabrachial Nucleus
PGK: Phosphoglycerate Kinase
PIP2: Phosphatidylinositol 4,5-bisphosphate
PKA: Protein Kinase A
PKC: Protein Kinase C
PKD: Protein Kinase D
PLCβ: Phospholipase C-β
pPVN: parvocellular subdivisions of the PVN
PRR: (pro)Renin Receptor
PVN: Paraventricular Nucleus
RAS: Renin-Angiotensin System
RIA: Radioimmunoassay
ROS: Reactive Oxygen Species
RSNA: Renal Sympathetic Nervous Activity
RV: Right Ventricle
RVLM: Rostral Ventrolateral Medulla
Sc: Subcutaneous
SFO: Subfornical Organ
SHR: Spontaneously Hypertensive Rats
siRNA: small interfering RNA
SNA: Sympathetic Nervous Activity
SON: Supraoptic Nucleus
StAR: Steroidogenic Acute Regulatory
TFA: Trifluoroacetic Acid
VP: Vasopressin
VSMCs: Vascular Smooth Muscle Cells
WKY: Wistar-Kyoto

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Contribution of collaborators

The mRNA measurements, the aldosterone and corticosterone assays, the body weight, LV/RV weight and water intake measurement, the BP and HR calculation and the data analysis were done by Jiao Lu. The animal surgeries (Ang II minipump and telemetry implantation, BP measurement and icv infusion surgeries) were done by Dr. Monir Ahmad. The plasma Ang II assay and the blockers preparation were done by Ms. Roselyn White. The mRNA measurements were done under the supervision of Dr. Hongwei Wang. The aldosterone and corticosterone assays were done under the supervision of Ms. Roselyn White. The whole study was under the supervision of Dr. Frans Leenen.

Authorization

Permission for using figure 3 in Hattangady, N.G., Olala, L.O., Bollag, W.B., Rainey, W.E. 2012. Acute and chronic regulation of aldosterone production. Molecular and cellular endocrinology 350, 151 -162 was obtained. This figure is shown in the current thesis as Figure I1.

Permission for using figure 3 in Gabor, A., Leenen, F.H. 2012. Central neuromodulatory pathways regulating sympathetic activity in hypertension. Journal of applied physiology 113, 1294-1303 was requested, but not required. This figure is adapted and shown in the current thesis as Figure I2.

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1. Introduction

Overview

It is well known that both Ang II and high salt intake have effects on the BP.

In kidneys and arteries, Ang II increases BP directly by causing vasoconstriction or indirectly by enhancing Na^+ and water reabsorption which increases cardiac output and thus BP either induced by Ang II itself or Ang II-stimulated aldosterone (Lavoie et al., 2003; Willians et al., 2001). However, the actions of circulating Ang II and aldosterone on kidneys and arteries may not be enough to induce chronic hypertension (Leenen, 2014). The role of the brain in Ang II-hypertension is of great importance. Ang II can activate neurons in the SFO/OVLT with projections to the PVN/RVLM and increase sympathetic nerve activity (SNA) and thereby BP (Leenen, 2014). Ang II also stimulates vasopressin synthesis in hypothalamic magnocellular neurons, which is released into circulation from the posterior pituitary gland (Han et al., 2015). In addition, neurons in the SFO, OVLT, SON, PVN are involved in regulating drinking behavior and sodium appetite through AT_1R stimulation by Ang II (McKinley et al., 1996; Walch et al., 2014; Zardettosmith et al., 1993). On high salt diet, Na^+/K^+-ATPase and ENaCs are involved in the regulation of the renal function to maintain Na+ and fluid balance in response to high salt intake (Liu et al., 2011; Wang et al., 2016a). A decrease in renal capability to excrete extra Na+ will result in blood volume increase and thus cause BP increase (Guyton, 1991). In addition, plasma sodium increase caused by high salt intake increases ENaC surface expression, stiffens vascular endothelial cells, and consequently induces vascular stiffness (Jeggle et al., 2016; Oberleithner et al., 2007). High salt diet also elevates CSF[Na+] in salt sensitive rats and activates aldosterone-EO pathway and angiotensinergic pathways in the CNS, leading to

sympathetic hyperactivity and BP increase (Huang et al., 2006; Huang et al., 2004; Leenen, 2010).

Ang II and high salt intake do not work separately to affect BP. In fact, the response of BP to circulating Ang II depends on the level of salt intake. When Ang II and high salt diet are given together, the increase of BP is much higher compared with Ang II alone (Osborn et al., 2010). The mechanisms contributing to hypertension induced by Ang II together with a high salt intake (Ang II-salt hypertension) remain unclear.

Similar to high salt induced hypertension, there are two main pathways in the brain involved in Ang II-induced hypertension, angiotensinergic pathway and aldosterone-MR-ENaC-EO neuromodulatory pathway (Gabor et al., 2012). Angiotensinergic pathway is activated by the binding of Ang II to AT_1Rs in the SFO (Zimmerman et al., 2004). Most of the chronic angiotensinergic activation depends on aldosterone-MR-ENaC-EO pathway (Huang et al., 2011), in which aldosterone, MR and ENaCs are key factors (Leenen, 2010; Wang et al., 2003b).

The SFO, PVN, SON, and RVLM are major cardiovascular regulatory brain nuclei. In angiotensinergic pathway, Ang II and AT_1Rs are present in the SFO, PVN, SON, and RVLM (Oldfield et al., 1989; Wang et al., 2003b). In the aldosterone-MR-ENaC-EO pathway, CYP11B2 is an enzyme required for aldosterone synthesis (Geerling et al., 2009; Gomez-Sanchez et al., 2010), and CYP11B2 mRNA is present in the SFO, PVN, and SON (Wang et al., 2010). 11βHSD2 mRNA is expressed in brain nuclei like the PVN and SON, which increases the binding of aldosterone to MR by converting corticosterone into an inactive metabolite (Haque et al., 2015; Zhang et al., 2006). Both MR and three ENaC subunits are distributed in the SFO, PVN, and SON (Amin et al., 2005).

Circulating Ang II acts on the SFO and the organum vasculosum of the lamina terminalis (OVLT) which lack the blood brain barrier (BBB). The neuronal activation of the SFO and the OVLT is relayed to the PVN directly or via the median preoptic nucleus (MnPO) (Wright et al., 1993). The PVN sends projections to the RVLM or directly to the intermediolateral cell column (IML), increasing SNA and BP (Pyner et al., 1999; Yang et al., 1998). Chronically, the SON receives projections from the SFO and OVLT, and increases aldosterone release in magnocellular neurons of the SON which may stimulate EO production and vasopressin release in the magnocellular neurons of the SON/mPVN (Gabor et al., 2012).

A few studies on Ang II-salt hypertension have illustrated the involvement of brain pathways. Acute inhibition of neurons in the PVN by microinjection of GABA-A receptor agonist muscimol significantly decreased SNA and BP, which indicates that Ang II-salt hypertension involves increased excitability of PVN neurons (Bardgett et al., 2013). Chronic intracerebroventricular (icv) administration of the sodium channel blocker benzamil prevented Ang II-salt hypertension in rats, suggesting that benzamil-sensitive sodium channels in the brain, possibly ENaC, are involved in the development of Ang II-salt hypertension (Osborn et al., 2014).

The following topics will be discussed in literature review

1. Renin-angiotensin system
2. Regulation of aldosterone biosynthesis
3. Angiotensinergic pathways in the brain
4. Aldosterone-ouabain pathway in Ang II hypertension

5. Central mechanisms mediating Ang II-salt hypertension

6. Possible peripheral mechanisms mediating Ang II-salt hypertension

1.1 Renin–Angiotensin system (RAS)

1.1.1 Systemic RAS

The RAS is a hormonal pathway involved in the regulation of BP and fluid balance. Renin is a circulating enzyme synthesized and released by the juxtaglomerular cells in the kidney, in response to stimuli such as sympathetic activity, and decreases in arterial BP and NaCl level in renal filtrate, sensed by macula densa in juxtaglomerular apparatus through Na-K-2Cl cotransport (Schlatter et al., 1989). A decrease in luminal NaCl concentration activates the signaling in macula densa cells and stimulates renin secretion by juxtaglomerular cells. The specific pathways are poorly defined (Damkjær et al., 2013). Upon release, it initiates a signaling pathway involving a series of enzymatic steps (Yee et al., 2010). Specifically, renin catalyzes the conversion of angiotensinogen secreted by the liver into angiotensin I (Ang I). Ang I is subsequently converted to Ang II through the cleavage by angiotensin-converting enzyme (ACE) from lung and kidney. Ang II causes vasoconstriction through activation of AT_1R in arterial smooth muscle cells (Lavoie et al., 2003). By stimulation of AT_1R in the kidney, Ang II enhances water and sodium retention through tubular re-absorption, leading to an increase of effective circulating volume. Ang II stimulates the release of aldosterone from adrenal cortex, which also promotes water and sodium re-absorption (Williams et al., 2001). In addition, through actions on the central nervous system (CNS), Ang II increases SNA and stimulates the secretion of anti-diuretic hormone arginine vasopressin (AVP) from the pituitary gland, resulting in increased water

reabsorption in the collecting duct.

The Ang II-AT_1R signaling involves several classical G-protein signal transduction mechanisms in vascular smooth muscle cells (VSMCs), the phospholipase C-β (PLCβ) induced increase of intracellular Ca^{2+} and protein kinase C (PKC), and the opening of Ca^{2+} channel and Ca^{2+} influx into cells. These are also linked to Ang II mediated aldosterone production, which will be discussed in detail. In addition, AT_1R coupled to G protein inhibits adenylated cyclase and decreases the production of cyclic AMP (cAMP) (Dinh et al., 2001). cAMP targets to protein kinase A (PKA), which has vasorelaxation effect (Bomzon et al., 2001). Thus, Ang II by inhibiting cAMP singaling pathway can cause vasoconstriction. In addition, the cAMP signaling also has effects on growth and proliferation of VSMCs (Bornfeldt et al., 1999).

There is another axis in the RAS which also contributes to the BP regulation, the ACE2-Ang-(1-7)-Mas receptor axis. ACE2 (ACE-related carboxypeptidase) catalyzes Ang II to Ang-(1-7), which binds to the Mas receptor existing in renal proximal tubular cells, neuronal cells, cardiac myocytes, and afferent arterioles (Moon, 2013). The ACE2-Ang-(1-7)-Mas receptor axis induces concentration-dependent vasorelaxation through several mechanisms, such as stimulating the synthesis of the vasorelaxant prostacyclin in VSMCs and inducing the phosphorylation of endothelial nitric oxide synthase (eNOS) in endothelial cells which leads to nitric oxide (NO) release (Pernomian et al., 2014).

In addition to the systemic RAS, many tissues, for example, kidney, heart, adrenal, brain, vasculature, have a local RAS.

1.1.2 Intrarenal RAS

All the components of RAS exist in kidney (Bruneval et al., 1986; Gomez et al., 1988). ACE is expressed in the proximal tubule, collecting duct, and the endothelial cells. AT_1R is widely expressed in the intrarenal vasculature, glomeruli, and tubules of the kidney (Moon, 2013). The formation of Ang II in kidney is independent of the systemic RAS (Carey et al., 2003). Compared with the plasma Ang II level, the Ang II concentration in interstitial fluid is much higher (Nishiyama et al., 2002). Unlike the systemic RAS with renin suppression in juxtaglomerular cells by negative feedback, upregulation of renin production in the distal nephron may cause continued formation of intrarenal Ang II, and therefore the hypertensive state can be amplified or maintained (Prieto-Carrasquero et al., 2005). Des aspartyl-Ang II (Ang III, Ang-(2–8)), a metabolite of Ang II, is more selective for Ang II type 2 receptors (AT_2R) over AT_1R. By AT_2R activation in the kidney, it induces a significant natriuretic response and possibly regulates BP through renal Na^+ excretion. Similarly to the ACE2-Ang-(1-7)-Mas receptor pathway, this newly revealed Ang III/AT_2R pathway also shows opposing actions to the intrarenal actions of ACE-Ang II- AT_1R axis (Carey, 2015; Moon, 2013).

1.1.3 Brain RAS

All components of the RAS have been found in different regions of the brain. Early studies have shown that renin mRNA levels are low in the brain and is mostly expressed in neurons, although it can also be detected in glial cells (Fuxe et al., 1980; Hermann et al., 1987; Hirose et al., 1978). In particular, renin is mainly produced in neurons of brain nuclei which have significant roles in cardiovascular regulation, including the SFO, PVN, parabrachial

nucleus (PB), and RVLM (Lavoie et al., 2004a; Lavoie et al., 2004b; und Halbach et al., 2006). Recently, prorenin, a precursor of renin, has been found in the brain with a 10-fold higher level than renin (Li et al., 2012). Both renin and prorenin bind to the (pro)renin receptor (PRR) (Nguyen et al., 2002). Angiotensinogen is widely expressed in astrocytes and is also in neurons of brain nuclei such as the SFO, PVN, SON, RVLM, and PB (Deschepper et al., 1986; McKinley et al., 2001; Thomas et al., 1992). Studies in rats have detected the ACE expression in the SFO, OVLT, PVN, SON, RVLM, etc., without identifying the cell type in these brain nuclei (Chai et al., 1987; Cheung et al., 2006; Dean et al., 2005).

With all the enzymes of RAS present in the brain, Ang II can be synthesized locally. Binding of renin or prorenin to the PRR results in elevated formation of Ang II (Danser et al., 2005; Nguyen et al., 2004). Overexpression of human PRR in Neuro-2A cells increased mRNA expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoforms, NADPH oxidase (NOX) activity and reactive oxygen species (ROS) content (Peng et al., 2013), while knockdown of PRR in the brain of mice caused a decrease of BP, AT_1R expression, and vasopressin levels (Li et al., 2012). In contrast, ACE2 plays an inhibitory role in Ang II-dependent hypertension in the CNS. Overexpression of ACE2 in the SFO decreased AT_1R expression and prevented the Ang II induced pressor response while knockout of ACE2 in mice resulted in a significant increase of NADPH oxidase and superoxide dismutase (SOD) activities in the brain (Feng et al., 2008; Xia et al., 2011).

1.2 Aldosterone biosynthesis and regulation

Aldosterone is a steroid hormone and is synthesized in the cells of the zona glomerulosa, which is the outer layer of the adrenal cortex (Hu et al., 2012). Aldosterone is synthesized from cholesterol, which is transferred from the outer mitochondrial membrane to the inner mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein to initiate the synthesis (Stocco, 2000). CYP11A1, the cholesterol side-chain cleavage complex located in the inner mitochondrial membrane, catalyzes cholesterol to form pregnenolone (Lieberman et al., 2001; Shih et al., 2011). Pregnenolone is subsequently oxidized to progesterone by 3β-hydroxysteroid dehydrogenase in the endoplasmic reticulum (Simard et al., 1993). 21-hydroxylase (CYP21) hydroxylates progesterone to 11-deoxycorticosterone in the endoplasmic reticulum (Miller et al., 2010). Aldosterone synthase (AS) CYP11B2 then catalyzes the last three steps of the aldosterone synthesis in the inner mitochondrial membrane, first 11β- hydroxylation to form corticosterone, then the other hydroxylation to generate 18-OH-corticosterone, and finally 18-methyloxidation to yield aldosterone (Curnow et al., 1991).

There are two rate-limiting steps in the aldosterone synthesis process, the translocation of cholesterol to the inner mitochondrial membrane by StAR protein and the catalysis by aldosterone synthase CYP11B2 (Lenzini et al., 2007). The former is related to the acute regulation of aldosterone synthesis while the latter controls chronic aldosterone production (Bassett et al., 2004; Cherradi et al., 1998).

Aldosterone biosynthesis and secretion is controlled by various regulators, among which Ang II, plasma potassium, and adrenocorticotrophic hormone (ACTH) are major regulators (Williams et al., 1972).

1.2.1 Effects of Ang II on aldosterone production

The acute actions of Ang II on aldosterone production in glomerulosa cells are through the activation of several G protein signaling transduction pathways upon its binding to the AT_1R, thus modulating StAR phosphorylation as well as its expression (Hattangady et al., 2012). Chronic effects of Ang II on aldosterone production is through its regulation of the enzymes involved in aldosterone synthesis, particularly CYP11B2 (Bassett et al., 2004).

In Sprague-Dawley rats, subcutaneous (sc) infusion of Ang II at 200 ng/kg/min for 55 hours increased the mRNA signal intensity of CYP11B2 in the outer adrenal cortex as measured by in situ hybridization and increased plasma aldosterone level by more than 2 fold (Peters et al., 1998). Similarly, sc infusion of Ang II at 200 ng/kg/min in Wistar Kyoto rats for 7 days significantly increased mRNA expression of CYP11B2 in adrenal gland as well as increased plasma aldosterone concentration by 1.5-fold (Ye et al., 2003). Sc infusion of Ang II at 80 ng/min in Sprague-Dawley rats for 28 days increased plasma aldosterone by 2.5-fold (Conte et al., 2011). A higher dose of sc Ang II at 500 ng/kg/min increased plasma aldosterone by 25-fold in Wistar rats after 14 days (Huang et al., 2010).

The phosphoinositide-specific phospholipase C (PLC) pathway activated by Ang II involves hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) and the generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), the two second messengers (Bollag et al., 1991; Kojima et al., 1984). IP3 initiates aldosterone secretion through the increase of cytosolic calcium concentration and the activation of calcium/calmodulin-dependent protein kinases (CaMK), which is essential for mediating aldosterone production (Ganguly et al., 1992). DAG stimulates PKC, which is involved in the regulation of adrenal steroidogenesis (Kapas et al., 1995). PKC inhibits the transcription

of 17α-hydrolyase (CYP17) via cFOS (Sirianni et al., 2010). Stimulated by Ang II, the DAG or PKC-activated protein kinase D (PKD) increases CYP11B2 expression and stimulates aldosterone secretion (Romero et al., 2006). In addition, Ang II also induces an increase in calcium influx by CaM kinase II (Yao et al., 2006). The calcium influx has been demonstrated to be essential for the sustained aldosterone secretion (Ganguly et al., 1994). The increased intracellular calcium and activated CaMK signaling induced by PLC pathway are critical in regulating transcription of CYP11B2 via several transcription factors, such as ATF1, CREB and NURR1 (Johannessen et al., 2007; Lu et al., 2004; Pezzi et al., 1997). Ang II binding to AT_1R also increases cholesterol uptake for steroidogenesis by elevating the expression of LDL and HDL receptors (Hattangady et al., 2012; Pilon et al., 2003) (Figure I1).

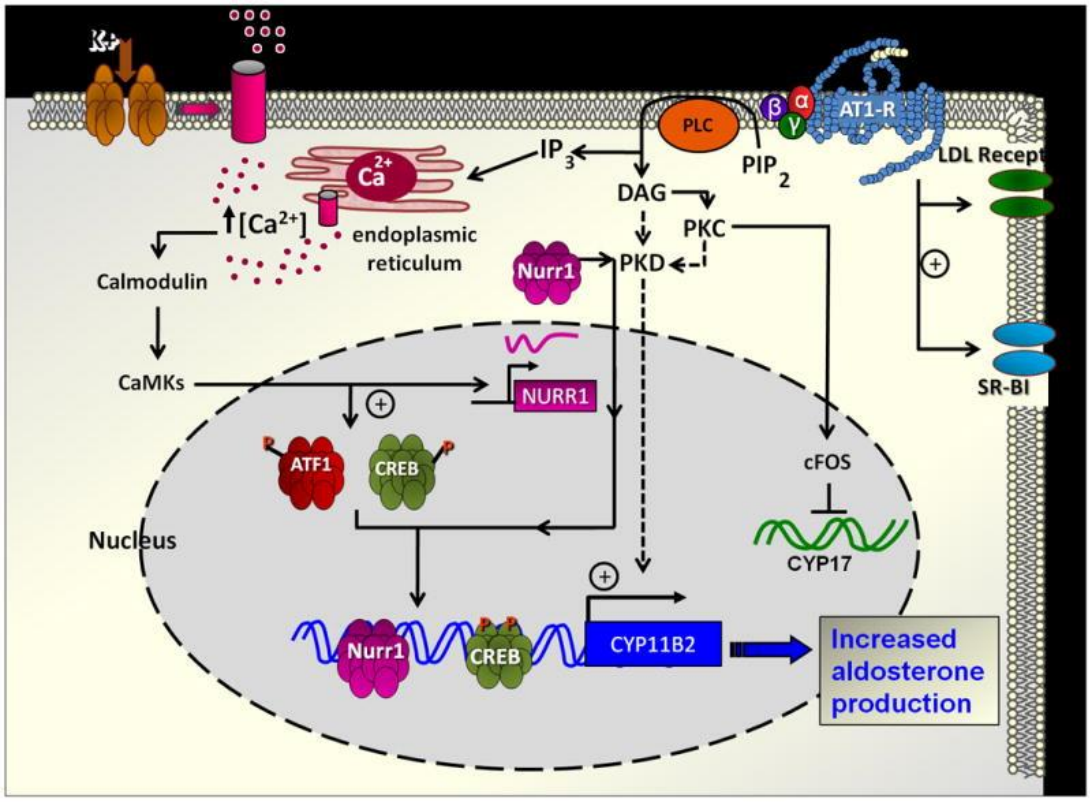

Figure I1. Schematic diagram of Ang II or potassium induced signaling in regulating chronic aldosterone synthesis in adrenal glomerulosa cells (Hattangady et al., 2012).

1.2.2 The effect of the extracellular potassium concentration on aldosterone release

Potassium conductance, carried by potassium leak channels such as TASK and TREK, largely determines the negative resting membrane potential of glomerulosa cells, with a much higher potassium level intracellularly than extracellularly (Spät et al., 2004). Small increases in extracellular potassium levels reduce the driving force for potassium and cause depolarization of the cell. The membrane depolarization of glomerulosa cells subsequently activates voltage-dependent calcium channels, stimulating calcium influx (Barrett et al., 1991; Cohen et al., 1988). The enhanced calcium influx induced by potassium increases

cytoplasmic calcium levels and activates various calcium-sensitive signaling pathways including CaMK signaling (Bollag et al., 1992). The calcium influx plays an important role in aldosterone production induced by elevated extracellular potassium concentration as well as Ang II (Lotshaw, 2001). This potassium-induced increase in calcium influx causes upregulation of CYP11B2 expression, which can be abolished by calcium channel blocker nifidipine (Denner et al., 1996; Yagci et al., 1996).

Potassium can increase CYP11B2 expression chronically. High potassium intake for 7 days in Long Evans rats significantly increased both the mRNA and protein levels of the enzymes that are involved in aldosterone synthesis in adrenal zona glomerulosa. Plasma aldosterone level was significantly increased after 1 day and remained elevated throughout the period of high potassium diet treatment, and after 7 days, aldosterone concentration was increased by about 3 fold (Tremblay et al., 1993; Tremblay et al., 1991). Similarly, high potassium intake for 5 days in Wistar-Kyoto rats increased CYP11B2 mRNA level by 3 fold in zona glomerulosa and plasma aldosterone level by 5 fold (Peters et al., 2007).

1.2.3 ACTH-mediated aldosterone secretion

ACTH binds to the melanocortin receptor (MC2R), activating the heterotrimeric G protein, Gs. The Gs protein subsequently stimulates adenylate cyclase, converting ATP to cAMP. cAMP then stimulates PKA, which is critical in ACTH-mediated aldosterone secretion from glomerulosa cells (Bollag, 2014). Intravenous (iv) administration of ACTH stimulated both plasma aldosterone and corticosterone concentration around 1 h after injection in rats (Ait-Chaoui et al., 1995; Hilfenhaus, 1977).

While ACTH is able to increase aldosterone secretion acutely, the chronic regulation of

ACTH on aldosterone does not work the same way. ACTH administration in rats caused an increase of mRNA level of CYP11B2 in adrenal capsular tissues at the initial 3 hour but CYP11B2 mRNA level was decreased at 24 hour compared with values in control rats (Holland et al., 1993). Consistent with this study, chronic ACTH administration in humans increased the plasma aldosterone level during the first two days, but it was gradually decreased to control or below control levels during the following days. Plasma renin activity was also only transiently increased by ACTH (Fuchs-Hammoser et al., 1980). In transgenic mice with ablation of pituitary pre-proopiomelanocortin cells that produce ACTH, the mRNA level of CYP11B1 was decreased but the CYP11B2 mRNA level remained constant (Allen et al., 1995). ACTH treatment in rats for 7 days decreased over 90% of aldosterone release into the adrenal vein in vivo as well as aldosterone production by adrenal capsules in vitro (Abayasekara et al., 1993).

1.2.4 Effects of ouabain on aldosterone production

Icv, iv, or sc administration of ouabain in rats for 2 weeks increased BP by 20 to 30 mm Hg, and chronic ouabain infusion also significantly elevates plasma aldosterone levels (Huang et al., 1994; Manunta et al., 1994). Icv infusion of Ang II in rats at 2.5 ng/min for 2 weeks significantly increased the plasma EO and aldosterone levels, and both were prevented by central infusion of MR blocker or aldosterone synthase inhibitor. It is possible that EO is released from pituitary and increases aldosterone release from adrenal cortex (Hamlyn et al., 2014; Huang et al., 2013).

1.3 Central mechanisms mediating Ang II hypertension

As shown in figure 2, the acute responses in the brain to circulating Ang II start with enhanced neuronal activity in the circumventricular organs (CVOs) including the SFO and the OVLT, located outside the BBB. The signal is relayed to the PVN via MnPO and subsequently to the RVLM from the PVN, inducing endogenous Ang II secretion in the PVN and neuronal activation in the RVLM (Wright et al., 1993). The chronic responses involve the SON, which receives projections from the SFO and OVLT, and increases the aldosterone release in magnocellular neurons, and in turn stimulates EO production in the magnocellular neurons in the SON/mPVN through the aldosterone-MR-ENaC-EO pathway (Gabor et al., 2012) (Figure I2).

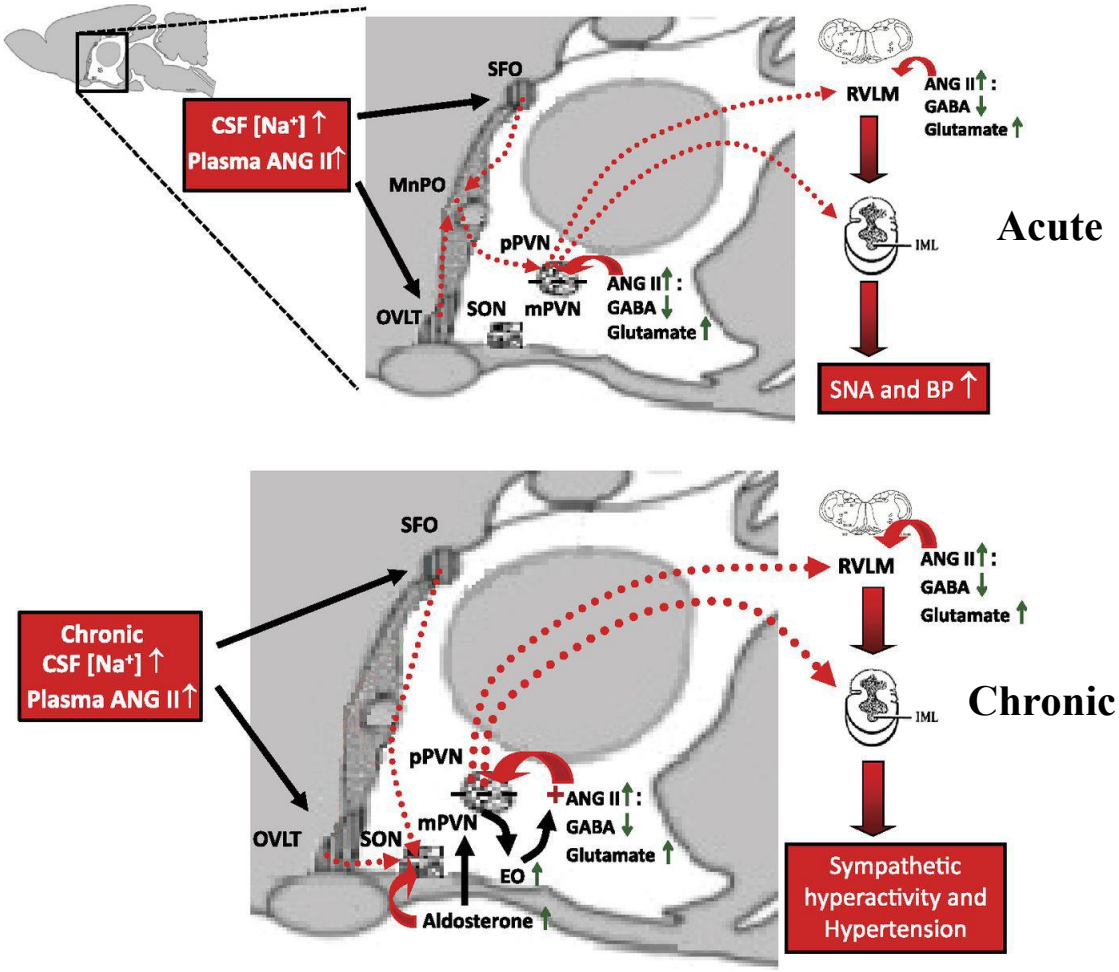

Figure I2. Acute and chronic responses in the brain to plasma Ang II (Adapted from Gabor et al., 2012).

1.3.1 Neuro-anatomy

1.3.1.1 SFO

The SFO is located in the dorsal third ventricle and belongs to the CVOs, which lack the normal BBB (Pfaff, 2002). Ang II is unable to cross the BBB, but activation of the CVOs can trigger the local production of Ang II in regions which are protected by the BBB (Braga et al., 2011). SFO is composed of an inner core and a peripheral region, projecting to different brain areas (McKinley et al., 1998). The SFO has projections to the magnocellular neurons in the SON and the PVN (Miselis, 1982), and excitatory projections to the

parvocellular region of the PVN (Coble et al., 2015; Cruz et al., 2008; Kantzides et al., 2003) (Figure I3). Robust staining of Ang II was found in both neurons cell bodies and fibers of the SFO in rats (Lind et al., 1984; Lind et al., 1985). Renin and angiotensinogen are coexpressed in the SFO (Lavoie et al., 2004a), and high concentration of ACE was also detected in the SFO (Saavedra et al., 1982), suggesting that Ang II can be locally synthesized as well as taken from circulation. Studies in transgenic mouse also support the local production of Ang II in the SFO (Sakai et al., 2007). AT_1Rs are expressed in the neurons in both the core and periphery regions of the SFO (Grob et al., 2004). Ang II at moderate plasma levels only activates neurons in the core region of the SFO while higher Ang II concentration stimulates additional neurons in the peripheral outer zone of the SFO with efferent projections to the SON and PVN, possibly mediating water drinking and vasopressin release stimulated by Ang II (McKinley et al., 1998).

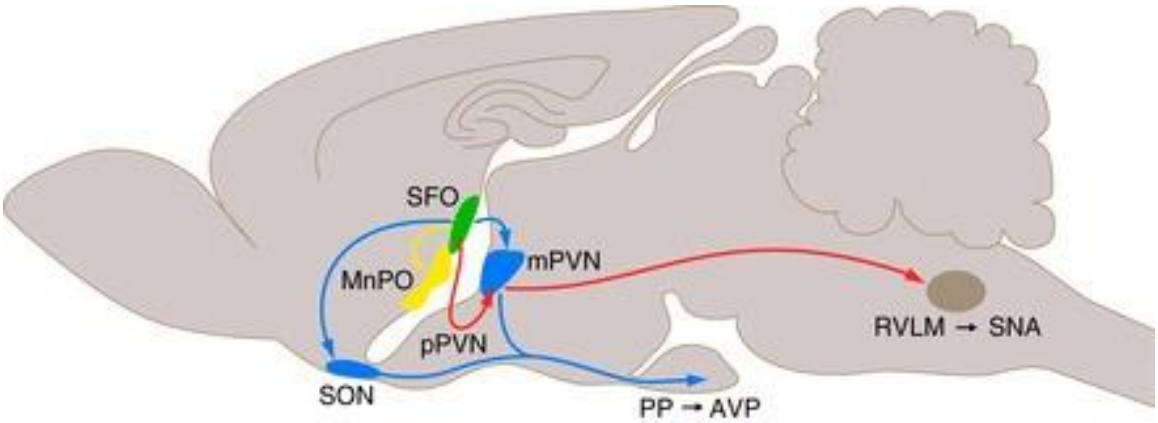

Figure I3. Neural circuitry among the SFO, PVN, SON and RVLM (Coble et al., 2014). The SFO projects to the parvocellular PVN (pPVN), which in turn either projects to the RVLM or directly to the brain stem to affect the SNA (red lines). The SFO also sends projections to the magnocellular neurons in the PVN and SON, which project to the posterior pituitary (PP) and regulate the release of vasopressin (AVP) (blue lines).

1.3.1.2 PVN

The PVN lies adjacent to the third ventricle and consists of magnocellular neurons and parvocellular neurons. Magnocellular neurons project to the posterior pituitary via the lateral and basal hypothalamus and are involved in vasopressin and oxytocin release (Hoffman et al., 1991) (Figure I3). Corticotropin-releasing hormone (CRH) has been found in the magnocellular neurons in PVN and SON, which stimulates the release of ACTH from pituitary (Dohanics et al., 1986; Ogasa et al., 1992).. Parvocellular neurons can be subdivided into dorsal, ventral, medial and the periventricular subgroups based on their projections, location, and firing pattern (Badoer, 2010; Swanson et al., 1983). Some parvocellular neurons have projections to the spinal cord and they synapse directly onto the sympathetic preganglionic motorneurons in the spinal cord (Shafton et al., 1998). Parvocellular neurons in the PVN also project to the RVLM, and neurons in the RVLM then project to the sympathetic preganglionic motorneurons, thereby influencing sympathetic tone (Dampney, 1994; Shafton et al., 1998). Ang II has been identified in parvocellular and magnocellular neurons of the PVN (Healy et al., 1984). High expression of AT_1R mRNA was detected in the parvocellular part of the PVN (Lenkei et al., 1997), and less in the magnocellular PVN (Aguilera et al., 1995). However, in both the mPVN and pPVN, AT_1R protein and binding densities are prominent (Chen et al., 2014; Huang et al., 2011). Almost all AVP producing neurons in the PVN and SON co-express AT_1R (Hatae et al., 2001). Release of endogenous Ang II has been found in the PVN upon stimulation (Harding et al., 1992; Wright et al., 1993).

1.3.1.3 SON

The SON is located ventrolaterally in the anterior hypothalamus adjacent to the optic chiasm. The magnocellular neurons of the SON project to the posterior pituitary and release either oxytocin or vasopressin into the blood stream (Armstrong, 1995) (Figure I3). Neurons in the perinuclear zone of the SON may act as interneurons and regulate neurohypophysial hormone release responding to physiological changes with regard to BP (Armstrong et al., 1997). The SON receives most projections from anterior peri-hypothalamic regions as well as the nucleus tractus solitarii (NTS) and the ventrolateral medulla in the brainstem (Cunningham et al., 1991; Hatton, 1990). There also exists an GABAergic projection to the SON from the arcuate nucleus in rats (Ludwig et al., 2000). Ang II is present in neurons of the SON (Healy et al., 1984). AT_1R mRNA and protein are present in the SON, and the AT_1R binding densities in the SON are lower compared with the mPVN and pPVN (Chen et al., 2014; Huang et al., 2011). Moreover, high AT_1R immunoreactivity was observed in cell bodies in the magnocellular SON (Phillips et al., 1993; Phillips et al., 1998). Double immunofluorescence revealed that all AVP-immunoreactive neurons co-express AT_1R and 60~70% AT_1R-immunoreactive neurons co-express AVP in the SON (Hatae et al., 2001).

1.3.1.4 RVLM

The RVLM is located in the brainstem region with projections to the preganglionic neurons of the sympathetic nervous system (Guyenet, 2006). The RVLM receives projections from the SFO and the PVN. Ang II exists in both cells and fibers in the RVLM whereas the cell types were not clarified (Krukoff et al., 1992; Lind et al., 1985). By the research on dual-

reporter transgenic mice, renin and angiotensinogen were found in adjacent glial cells and neurons in the RVLM (Lavoie et al., 2004a). In the RVLM, AT_1Rs are mostly present in the neurons projecting to sympathetic preganglionic neurons (Li et al., 1996). Microinjection of AT_1R antagonist losartan abolished the pressor and sympathoexcitatory responses induced by exogenous Ang II stimulation in rabbits (Hirooka et al., 1997). Endogenous Ang II in the RVLM is important for cardiovascular regulation since microinjection of Ang II antagonist in the RVLM decreased BP in both spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats, by -27±3 mmHg and -17±1 mmHg, respectively (Muratani et al., 1993). The pressor responses induced by microinjection of Ang II into the RVLM are dose dependent, and can be prevented by ganglionic blockade with hexamethonium, indicating the sympatho-excitatory role of Ang II in the RVLM in controlling BP (Ito et al., 2002; Muratani et al., 1991).

1.3.2 Angiotensinergic pathways

In brain nuclei involved in cardiovascular regulation, e.g. the SFO, PVN, SON and RVLM, Ang II binds to AT_1Rs and stimulates a G-protein signaling pathway. Activation of this signaling requires PKC, which leads to an increase in ROS production induced by NADPH oxidase, and thus elevates intracellular Ca^{2+} concentration and membrane potential (Wang et al., 2006). In addition, an increase in intracellular ROS and superoxide production inhibits the activity of voltage-gated potassium channels through two possible pathways, the first of which depends on inhibition of potassium channel function after NADPH oxidase activation by Ang II, the second of which depends on ROS inhibition of p38 mitogen-activated protein kinase (p38 MAPK), contributing to an increase in AT_1R

expression and a decrease in potassium channel proteins expression and activity (Zucker et al., 2005). The inhibition of potassium channel leads to an increase of action potential propagation and neuronal activity (Wigmore et al., 2000).

Microinfusion of Ang II or glutamic acid into the SFO provokes release of endogenous Ang II in the PVN (Wright et al., 1993). Ang II through AT_1R activation suppresses the inhibitory GABAergic input or increases the excitatory glutamatergic input from presynaptic terminals to postsynaptic neurons (Latchford et al., 2004; Li et al., 2003). The study by Li et al. reported that AT_1Rs could be located at presynaptic terminals that target spinally projecting neurons in the PVN, and Ang II increased discharge of spinally projecting neurons in the PVN through presynaptic inhibition of GABA release (Li et al., 2003). Microinjection of Ang II into the SFO increased renal sympathetic nervous activity (RSNA), and this effect was reduced by pre-injection of the glutamate receptor blocker into the PVN, suggesting that neuronal activation of SFO by Ang II is relayed to the PVN through angiotensinergic signaling, and activation of the glutamate receptors in the PVN presympathetic neurons causes sympathoexcitation and thus BP increase (Llewellyn et al., 2012). In addition, sc Ang II at 500 ng/kg/min increased AT_1R in the SON (Chen et al., 2014).

The PVN-RVLM pathway excites reticulo-spinal vasomotor neurons in the RVLM whose activity is critical for maintenance of ongoing SNA and resting BP (Pyner et al., 1999; Yang et al., 1998). In vitro, application of Ang II on PVN neurons that have innervations in the RVLM excited these PVN neurons, whereas AT_1R blocker almost prevented the responses to Ang II, indicating that Ang II activates PVN neurons projecting to the RVLM by activation of AT_1R (Cato et al., 2005). In vivo, microinjection of GABA receptor

antagonist into the PVN caused the increase of RSNA and BP, whereas pre-injection of AT_1R blocker into the RVLM reduced the increase of RSNA and BP by 44% and 38%, respectively, suggesting that neuronal activation of the PVN activates AT_1R in RVLM neurons (Tagawa et al., 1999). Microinjection of AT_1R blocker into the RVLM also prevented the BP increase induced by microinjection of Ang II into the PVN (Ku et al., 1999). Ang II infusion in the RVLM increased BP, HR as well as release of glutamate in the RVLM, and all these effects were attenuated by microinjection of AT_1R blocker into the RVLM (Zhu et al., 1998). In summary, AT_1R activation by increased Ang II release in the PVN or less GABA receptor activation by decreased GABA release in the PVN activates PVN neurons that innervates the RVLM, and via angiotensinergic signaling the neuronal activation in the PVN is relayed to the RVLM and activates AT_1R in the RVLM, causing increased release of glutamate, sympathoexcitation, and thus BP increase (Gabor et al, 2012).

1.3.3 Aldosterone-ouabain pathway in Ang II hypertension

1.3.3.1 Aldosterone-MR-ENaC-EO pathway

Key factors involved in Aldosterone-MR-ENaC-EO pathway

Aldosterone in the brain can originate from circulation or be synthesized locally (Gomez-Sanchez et al., 2005). The mRNA for the late-stage enzymes involved in aldosterone and corticosterone synthesis, CYP11B2 and CYP11B1, respectively, are expressed in the hypothalamus (Gomez-Sanchez et al., 2010). The mRNA for both CYP11B1 and CYP11B2 are present in the SFO, PVN, and SON (Wang et al., 2010). The mRNA of other steroidogenic enzymes such as CYP11A, CYP17, 3β-HSD, and CYP21, were also detected

in the brain (Yu et al., 2002). The mRNA abundance of CYP11B2 in the SFO, PVN and SON is low, with the range of CYP11B2/PGK1 level from 10^{-4} to 10^{-3}. In situ hybridization test in the SON has shown that CYP11B2 is mainly present in the magnocellular neurons (Wang et al., 2016b).

11β-HSD-2 is the key to identify which MR-expressing cells are aldosterone-sensitive (Geerling et al., 2009). MR and 11β-HSD-2 are present in brain nuclei like the SON and PVN (Amin et al., 2005; Han et al., 2005; Moisan et al., 1990; Sánchez et al., 2000; Wang et al., 2010). Furthermore, recent study has revealed that MR and 11β-HSD2 are co-localized in magnocellular neurosecretory cells in the PVN and SON (Haque et al., 2015). After icv infusion of aldosterone at 300 ng/h in conscious Wistar rats for 2 h, icv infusion of aCSF with 0.16 M Na+ significantly increased BP, RSNA and HR, compared with rats after icv infusion of vehicle for aldosterone (Wang et al., 2003a). Icv infusion of aldosterone dissolved in aCSF containing Na^+ in Dahl salt sensitive rats for 2 weeks increased BP, and the level of BP increase is proportional to Na^+ concentration in aCSF (Huang et al., 2005b). In addition to BP, icv infusion of aldosterone in aCSF with Na^+ in both Dahl salt sensitive rats and Wistar rats significantly increased ouabain-like compounds (OLC) in hypothalamus and RSNA (Huang et al., 2005b; Wang et al., 2003a). Icv infusion of a MR blocker prevented BP increase induced by chronic icv infusion of aldosterone (Gomez-Sanchez, 1986). BP, RSNA, and HR responses to acute icv infusion of Na+ after aldosterone were blocked by icv infusion of EO-binding Fab fragments (Digibind) or the ENaC blocker, benzamil. Icv infusion of benzamil also blocked the increases of hypothalamic EO content and BP caused by chronic icv infusion of aldosterone and Na+ (Wang et al., 2003a). The increases in BP and HR after chronic icv infusion of aldosterone

were also prevented by icv infusion of Digibind (to bind ouabain) (Huang et al., 2011). The aldosterone-MR-ENaC-EO pathway has been proposed: specifically, aldosterone binds to MR and increases ENaC activity, thus enhancing Na+ entry and increasing EO release (Leenen, 2010). EO, via inhibiting Na+/K+-ATPase and therefore lowering the membrane potential and increasing Ca2+ entry, enhances the excitability of presympathetic neurons to induce BP increase (Gabor et al., 2012).

Both mRNA and protein for all three subunits of ENaC (α, β, and γ) have been shown to be expressed in rat brain, including SON, OVTL and mPVN (Amin et al., 2005; Wang et al., 2010). Dense cell staining of α-ENaC was detected in all three CVOs (OVLT, SFO, and AP) (Miller et al., 2013). ENaC α is colocalized with vasopressin or oxytocin in magnocellular neurons in the SON and PVN, but ENaC β and ENaC γ are colocalized only with vasopressin in the magnocellular neurons. ENaCs contribute to the membrane potential in neurons expressing vasopressin or oxytocin (Teruyama et al., 2012). EO-containing neurons are distributed in the PVN, SON and scattered in the other hypothalamic areas like OVLT (Yamada et al., 1992a; Yamada et al., 1992b). EO immunoreactivity was detected in cultured cells originated from mouse hypothalamus, and application of aldosterone in culture media increased EO production whereas addition of MR blocker abolished the increase (Yoshika et al., 2011). This suggests that aldosterone by binding to MR stimulates EO release, likely from the magnocellular neurons in the PVN and SON.

Activation of aldosterone-MR-ENaC-EO pathway can enhance the activity of angiotensinergic pathways

The RSNA, BP and HR responses caused by icv infusion of ouabain were inhibited by icv infusion of the AT_1R blocker losartan, suggesting that AT_1R activation, likely by increased release of Ang II in brain nuclei, is involved in regulating the sympathoexcitation and BP increase by ouabain (Huang et al., 1996). AT_1R blocker in the PVN prevented the BP and HR responses to both icv and intra-PVN infusion of aldosterone in aCSF with high Na+ concentration as well as intra-PVN ouabain, suggesting that AT_1R activation also mediates the aldosterone stimulated pressor response (Gabor et al., 2009). Icv infusion of aldosterone in Wistar rats for 2 weeks significantly increased the mRNA and protein level of AT_1R, as well as increased ACE and NADPH oxidase subunits in the PVN, but decreased neuronal NO synthase in the PVN, and these effects were largely prevented by icv infusion of benzamil or Digibind, indicating that aldosterone increases ACE, AT_1R and oxidative stress in the PVN via ENaC and EO (Huang et al., 2011). These findings suggest that activation of MR-ENaC-EO pathway by aldosterone can enhance the activity of angiotensinergic pathways in the hypothalamus, thereby inducing sympathetic hyperactivity and hypertension.

1.3.3.2 Aldosterone-MR-ENaC-EO pathway in response to circulating Ang II on regular salt intake

Chronic sc infusion of Ang II in rabbits and Wistar rats on regular salt intake caused rapid and marked neuronal activation, which was detected by Fra-like immunoreactivity, in CVOs such as the SFO, the NTS, the PVN (mPVN, pPVN) and SON. The activation in the

SFO and NTS diminished over time, while the neuronal activation in the PVN and SON was sustained (Davern et al., 2007; Huang et al., 2010). Icv infusion of AS inhibitor or MR blocker significantly attenuated the neuronal activation induced by Ang II in the mPVN and pPVN without affecting the SFO and SON (Huang et al., 2010).

Chronic sc infusion of Ang II at 150 and 500 ng/kg/min increased BP by ∼8 and ∼20 mmHg the first few days, respectively, and then caused further increases with peaks of approximately +20 and +60 mmHg on days 10–14 (Huang et al., 2010). The icv infusion of an AS inhibitor or MR blocker fully prevented the increase in BP induced by chronic sc Ang II at 150 ng/kg/min and ∼70–80% of the increase by Ang II at 500 ng/kg/min. BP responses to sc Ang II at 500 ng/kg/min were also attenuated by Digibind by 50% (Huang et al., 2010).

Sc infusion of Ang II for 2 weeks also increased the mRNA expression of CYP11B2 in the lamina terminalis (LT), SFO, SON, and PVN and increased aldosterone level in hypothalamus in Wistar rats (Ahmadi et al., 2011; Huang et al., 2010; Xue et al., 2012). Icv infusion of an AS inhibitor prevented the increase in hypothalamic aldosterone but not the increase in plasma aldosterone induced by sc Ang II at 500 ng/kg/min (Huang et al., 2010).

The SFO is required for the BP response to chronic Ang II administration. SFO lesion significantly attenuated BP increase in response to 10 days of iv Ang II (10 ng/kg/min) in rats on normal salt diet compared with control group (3.7 ± 1.4 mmHg compared with 11.7 ± 3.0 mmHg BP increases at day 5 of Ang II infusion, and the increase continued till the end of Ang II infusion) (Hendel et al., 2005). Similarly, OVLT lesion also prevented Ang II-induced hypertension in rats on a regular salt diet (4 ± 1 mmHg BP increases compared

with 16 ± 4 mmHg) (Vieira et al., 2010). Knockdown of AT_1R or MR in the PVN in Wistar rats by small interfering RNA (siRNA) prevented the BP increase induced by sc Ang II infusion at 500 ng/kg/min, suggesting the pivotal role of the PVN as well as the activation of AT_1R and MR in the PVN for Ang II-induced hypertension (Chen et al., 2014). It has been proposed that circulating Ang II causes neuronal activation of the SFO, which results in neuronal activation of the PVN and SON via Angiotensinergic signaling, and chronically Ang II via aldosterone-MR-ENaC-EO pathway can cause continued activation of the PVN and induce hypertension (Huang et al., 2010).

1.4 Ang II-salt hypertension

1.4.1 The response of MAP to Ang II is dependent on level of salt intake

Sc Ang II infusion at 150 ng/kg/min had minimal effects on BP in rats on a low-salt diet but had larger effects in rats on a higher salt diet (Osborn et al., 2010) (Figure I4). Similarly, iv infusion of Ang II at 20 ng/kg/min did not affect BP in conscious mice with low sodium intake, but on high sodium intake increased BP by 16 mmHg (Cholewa et al., 2005). Ang II infusion at 5.0 ng/kg/min in dogs with high sodium intake (120 mM Na^+/day) resulted in higher levels of BP than Ang II with normal sodium intake (40 mM Na^+/day), 140% compared with 125% of control values (Cowley et al., 1976). Both CNS and peripheral mechanisms (i.e., the renal and arteries actions) may contribute to the larger increase of BP on high salt diet.

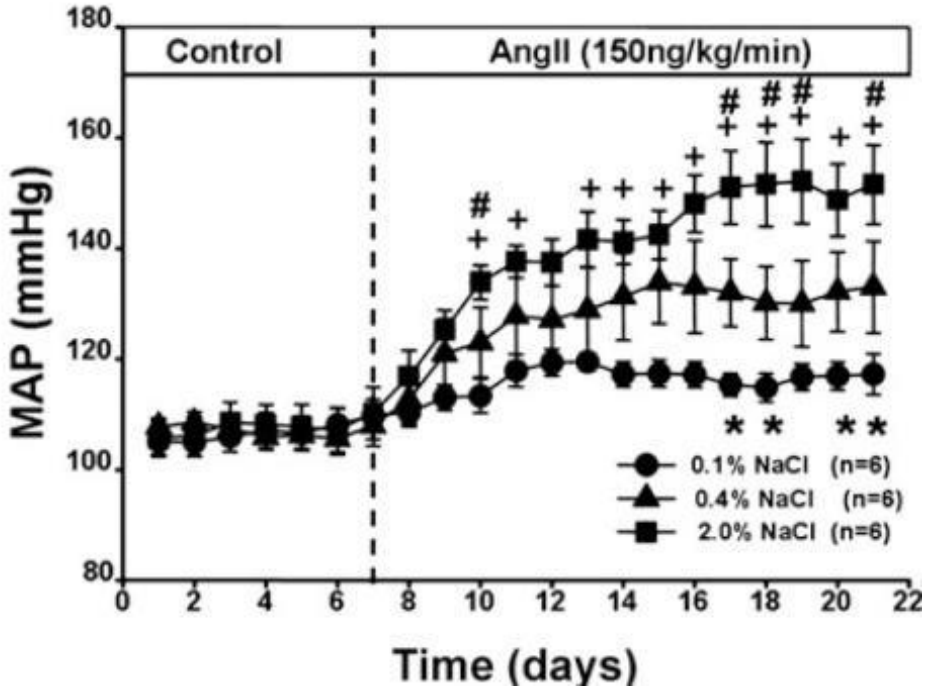

Figure I4. Salt sensitivity of Ang II–salt hypertension (Osborn et al., 2010)

1.4.2 Central mechanism of Ang II-salt hypertension

1.4.2.1 CNS mechanisms mediating responses to Ang II and salt

Possible CNS mechanisms mediating the interaction of Ang II and salt remain to be fully understood. It has been proposed that even a slight increase in plasma sodium from high dietary salt triggers excitation of the sympathetic nervous system via central sodium/osmoreceptor activation located in SFO/OVLT, ultimately increasing BP, and that Ang II may act directly on sodium-sensing neurons at these sites to amplify the sympathoexcitatory action of small increases in sodium levels (Brooks et al., 2005). It is possible that salt amplifies the effects of Ang II in the CNS, e.g., by enhancing responses to local aldosterone (Huang et al., 2005b; Wang et al., 2003a).

In rats with Ang II-salt treatment (sc Ang II at 150 ng/kg/min and 2% salt diet), the BP increase was modestly less in the SFO lesion rats compared with the sham control rats,

reaching statistical significance only on day 5 of Ang II treatment(~20 mmHg vs. 30 mmHg). These results indicate that the SFO may play only a modest role in the pathogenesis of Ang II-salt hypertension in rats, or other brains sites are also involved (Osborn et al., 2012).

A more recent study shows that OVLT lesion decreased the hypertensive response to sc Ang II at 150 ng/kg/min in rats on a 2% salt diet, 40±7mmHg increase in OVLTx rats compared to a 58±3mmHg increase in sham rats by day 10 of Ang II administration, based on which it was concluded that the OVLT is an important brain nuclei for the development of Ang II-salt hypertension in rats (Collister et al., 2013).

Hypertension induced by Ang II and a high salt diet involves increased excitability of PVN neurons with axonal projections to the RVLM (Chen et al., 2010). Acute inhibition of PVN by microinjection of GABA-A receptor agonist muscimol reduced SNA and BP in rats with Ang II–salt hypertension, indicating that ongoing PVN neuronal activity is necessary to maintain SNA and elevated BP (Bardgett et al., 2013). In rats with Ang II-salt hypertension, the discharge of RVLM vasomotor neurons under anesthesia was similar to rats received regular salt diet and infused with saline for 2 weeks, suggesting that elevated resting discharge of sympathoexcitatory neurons in the RVLM may not be required for the maintenance of Ang II-salt hypertension (Pedrino et al., 2013).

Icv administration of the ENaC blocker benzamil at 16 nmol/day completely prevented the BP response by the end of the 13 days' treatment in rats with Ang II-salt treatment, compared with a ~30 mmHg increase in the icv vehicle control group, indicating that benzamil-sensitive sodium channels in the brain are required for the development of Ang II-salt hypertension in rats (Osborn et al., 2014).

In summary, studies so far have shown that OVLT/SFO and PVN pathways contribute to AngII-salt hypertension. ENaC in these or other nuclei appears critical in Ang II-salt hypertension.

1.4.2.2 SNA and Ang II-salt hypertension

Studies attempting to relate Ang II and SNA have yielded disparate results. A possible explanation for these conflicting results is that there are other factors that affect the magnitude of the sympathoexcitatory and/or resulting pressor response to circulating Ang II, among which the level of salt intake seems to be a likely factor.

On a regular salt diet, iv Ang II at 50 ng/kg/min in rabbits for 7 days caused a sustained decrease of RSNA, and analysis of baroreflex response indicates that baroreflex action by sympatho-inhibition of RSNA was dominant over the Ang II induced central sympatho-excitatory effects at this dose and duration of treatment (Barrett et al., 2003). By comparison, sc Ang II at 20-30 ng/kg/min in rabbits for 3 months showed increased RSNA by 43% by the end of treatment and greater Fos-related immunoreactivity in the OVLT, PVN and SON compared with sham rabbits, suggesting that Ang II caused sympathetic activation by activating hypothalamic pathways (Moretti et al., 2012).

Intraperitoneal infusion of Ang II at 125 ng/min for 12 days did not increase plasma norepinephrine (NE) in rats with normal salt intake, but did so in rats with high salt intake (Sato et al., 1991). Moreover, chronic Ang II administration increased NE spillover only in rats on a high salt (King et al., 2008). In addition, the depressor response to ganglionic blockade was larger in Ang II infused rats on a high-salt diet compared with Ang II infused rats on a normal-salt diet or vehicle infused rats on high or normal-salt diet, and there was

no significant difference among the latter three groups (King et al., 2006). These results suggest that Ang II administration increases overall sympathetic activity in rats on a high salt diet rather than rats on a normal salt diet.

In rabbits with sc Ang II at 20 ng/kg/min and drinking saline for 21 days, BP increased and reached a plateau by the end of the 2nd week whereas RSNA started to increase then and was significantly different on day 21 compared with rabbits that drank tap water and did not receive sc Ang II (Guild et al., 2012). Sc Ang II at 76 ng/min for 2 weeks increased splanchnic SNA, as measured by splanchnic nerve electrodes on day 14, but the level of salt diet was not clear (Luft et al., 1989). Sympathetic innervation to the splanchnic circulation was selectively disrupted by celiac ganglionectomy (CGx), and this substantially attenuated Ang II-salt induced hypertension in rats whereas CGx had little effect on BP responses to Ang II with 0.4% salt diet for 14 days. Renal denervation had no significant effect on either Ang II-salt or Ang II alone induced pressor responses (King et al., 2007). In addition, sc Ang II together with 2% salt diet caused a sustained increase in total peripheral resistance without changing cardiac output, and CGx markedly attenuated the increase in aortic pressure, suggesting that Ang II-salt increases aortic pressure mainly by affecting splanchnic vascular resistance (Osborn et al., 2007). Similarly, mesenteric vascular resistance remained elevated only in rats with sc Ang II together with high salt diet but not regular or low salt diet, and its response to ganglionic blockade was enhanced selectively in rats on high salt diet. This study also supports the effect of Ang II-salt on splanchnic arterial resistance (Kuroki et al., 2012). Furthermore, during the development of Ang II-salt hypertension in rats, renal SNA decreased by 40% within the first week and subsequently returned to control level, but lumbar SNA remained unchanged

at control level, as SNA continuously recorded by a recording electrode on either the renal or lumbar nerve in conscious rats (Yoshimoto et al., 2010). These studies suggest that Ang II-salt hypertension is associated with increased splanchnic SNA, unchanged lumber SNA and decreased renal SNA (Osborn et al., 2010). It has been hypothesized that the increased SNA to the splanchnic vascular bed results in redistribution of blood volume, i.e., from the venous capacitance bed to the central and arterial compartment, leading to increased BP (Osborn et al., 2009; Osborn et al., 2011).

1.4.2.3 Vasopressin (VP)

VP is synthesized in the magnocellular neurons in the PVN and SON (Meeker et al., 1991). Chronic stimulation of V1 vasopressin receptors in normotensive rat causes sustained hypertension (Cowley et al., 1994; Szczepanska-Sadowska et al., 1994). Icv infusion of Ang II stimulates VP release (Andersson et al., 1972; Fyhrquist et al., 1979; Mouw et al., 1971). The increased VP release induced by icv or SON infusion of Ang II was inhibited by microinjection of AT_1R blocker losartan into the SON, suggesting that central action of Ang II in VP release involves AT_1R activation in the SON (Qadri et al., 1993). Consistently, in the PVN and SON, all VP immunoactive neurons co-express AT_1R, and the regulation of VP secretion in response to Ang II is mediated by AT_1R (Hatae et al., 2001). In transgenic mice with brain-specific RAS hyperactivity, the staining for VP in the SON was increased by 2-fold, and sc infusion of VP receptor antagonist normalized the hypertension, suggesting an important role for VP in hypertension mediated by activated brain RAS (Littlejohn et al., 2013).

1.4.3 Possible peripheral mechanisms of Ang II-salt hypertension

Renal and arteries actions

Circulating Ang II can also act directly on kidneys and arteries. Ang II via AT_1R causes the tubules of the kidneys to enhance sodium re-absorption and water retention by affecting the activities of key sodium transporters like ENaC, NCC, or NKCC2 (Bernstein et al., 2014). Kidney cross-transplantation among wild-type (+/+) and $AT_{1A}R$–deficient (–/–) mice created mice with renal $AT_{1A}R$ deficiency (D^-R^+) and those with systemic $AT_{1A}R$ deficiency (D^+R^-). In transplanted mice with renal $AT_{1A}R$ deficiency, BP was significantly lower compared with the D^+R^+ control group (99 ± 4 vs. 118 ± 5 mmHg). BP was similarly reduced in mice with systemic AT_{1A} R deficiency (99 ± 2 vs. 118 ± 5 mmHg). BP was further reduced in D^-R^- mice entirely lacking $AT_{1A}R$ (86 ± 3 mmHg). These results indicates that $AT_{1A}R$ in both the kidney and systemic tissues contribute to blood pressure regulation, and in a non-overlapping manner (Crowley et al., 2005). Furthermore, compared with wild-type mice with sc Ang II at the high rate of 1,000 ng/kg/min, the kidney $AT_{1A}R$ Knockout (KO) mice showed markedly attenuated BP (126 ± 5 vs. 166 ± 3 mmHg at week 3), which was similar to that of total $AT_{1A}R$ KOs in both kidney and systemic tissues, suggesting that the absence of renal $AT_{1A}R$ protects against hypertension induced by Ang II (Crowley et al., 2006). Ang II also stimulates arteriolar vasoconstriction. $AT_{1A}R$ deletion in vascular smooth muscle cells in mice (SMKO mice) significant reduced baseline BP by ∼7 mmHg as well as increased urinary Na^+ excretion. BP response to chronic sc Ang II at 400 ng/kg/min for 1 week or 1000 ng/kg/min for 4 weeks was significantly attenuated in SMKO mice (ΔMAP 8±2 mmHg vs. 25±5 mmHg, 16±4 mmHg vs. 30±3 mmHg, respectively), indicating the essential role of $AT_{1A}R$ in smooth muscle

cells in BP response to Ang II (Sparks et al., 2015).

1.5 Hypothesis

Systemic administration of Ang II to rats consuming a high salt diet increases the activity of Aldosterone-MR-ENaC-EO pathway and AT_1R in key brain sites such as the SFO, PVN, SON, and RVLM, leading to increased SNA and hypertension, significantly higher as compared with Ang II and regular salt diet.

1.6 Objectives

To evaluate BP response, plasma Ang II and aldosterone, and hypothalamus aldosterone after sc Ang II-salt.

To assess the mRNA expression of CYP11B1, CYP11B2, 11HSD2, MR, ENaCs and AT_1R in brain nuclei (SFO, PVN, SON, RVLM) and adrenal cortex after sc Ang II-salt.

To examine the effects of icv infusion of MR blocker, AT_1R blocker, or ENaC blocker on BP, plasma aldosterone, and mRNA levels of AT_1R and CYP11B2 in adrenal cortex after sc Ang II-salt.

2. Materials and Methods

2.1 Animals

Male Wistar rats (200–250 g, Charles River, Montreal, QC, Canada) were housed in a temperature (22 ºC) and humidity (30 %) controlled room on a 12:12-h light-dark cycle. Standard laboratory chow (120 μmol Na^+/g, 0.4% salt diet) and tap water were available ad libitum except where otherwise noted. The age of rats was around 8 weeks at arrival, and rats were allowed to acclimatize for 7 days before the start of Ang II-salt treatment.

All surgical and experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care, which conform to National Institutes of Health guidelines. Studies were approved by the University of Ottawa Animal Care Committee. All surgeries were performed under 2% isoflurane inhalation. Rats were pre-medicated with sc buprenorphine at 0.04 mg/kg before surgery.

2.2 Experimental protocols

2.2.1 Drugs and Dosages

Ang II was infused sc at 150 ng/kg/min via osmotic minipumps, Alzet model 2002. Ang II at this dose causes only a slight increase in plasma Ang II (Huang et al., 2010). Ang II was dissolved in 0.9% sodium chloride and was infused sc at 64.8 μg/day (based on the average body weight of 300g). With a pump flow rate of 12 μl/day, the concentration of Ang II in the pumps was 5.4 μg/μl.

aCSF was prepared following the same protocol as in Gabor et, 2011 (121 mM NaCl, 3.4 mM KCl, 1.2 mM MgCl2, 0.6 mM NaH_2PO4, 29 mM $NaHCO_3$, and 3.4 mM glucose (pH

7.4); osmolarity 296 mosmol/kg H2O) (Gabor et al., 2011).

Eplerenone was dissolved in aCSF with 4% acetonitrile and infused icv at 5 μg/day via osmotic minipumps, Alzet model 2004 (Huang et al., 2010; Huang et al., 2013). Based on the infusion rate of 6 μl/day, the concentration of eplerenone solution in the pumps was 0.83 μg/μl.

Spironolactone was dissolved in aCSF with 2% ethanol, and infused icv at 3 μg/day via osmotic minipumps, Alzet model 2004 (Huang et al., 2010). Based on the infusion rate of 6 μl/day, spironolactone concentration in the pumps was 0.5 μg/μl.

Benzamil was dissolved in aCSF with 15% propylene glycol, and infused icv at 28.8 μg/day via osmotic minipumps, Alzet model 2004 (Huang et al., 2005a). Based on the infusion rate of 6 μl/day, benzamil concentration in the pumps was 4.8 μg/μl.

Losartan in aCSF was infused at icv 300 μg/day via osmotic minipumps, Alzet model 2004 (Huang et al., 1999). Based on the infusion rate of 6 μl/day, losartan concentration in pumps was 50 μg/μl.

The concentration of acetonitrile, ethanol or propylene glycol listed above indicates their final concentration in the mixture with the blocker and aCSF (or in aCSF for vehicle group).

2.2.2 Experiment I) BP and aldosterone measurement in Wistar rats with Ang II-salt hypertension

Goal:

To study the effects of sc Ang II on BP and aldosterone in Wistar rats on high salt diet for 2 weeks.

Experimental Animals:

16 male Wistar rats were divided into 3 groups for the following treatments:

1.Control, 0.4% NaCl diet only. (n=4)

2. High salt (2% NaCl) diet only. (n=6)

3. Sc Ang II (150 ng/kg/day) + high salt (2% NaCl) diet. (n=6)

Osmotic minipumps were filled with Ang II (for infusion at 150 ng/kg/min) and implanted sc for 14 days infusion in group 3.

Initial and final body weights were measured.

Data from one rat in Ang II-salt group were significantly different from rats in the same group. Therefore, this rat was removed from group 3 (n=5 accordingly).

Method

One day before the end of the infusion period (the day before tissue collection), BP and HR were measured in conscious, undisturbed animals by a catheter in right femoral artery 4 hours after catheter was inserted (Detailed in 2.3).

After hemodynamic measurements, rats were left in a quiet room overnight, and kept undisturbed. The next morning, they were quietly taken one by one (in their cage) into necropsy room. The conscious rats were rapidly decapitated and trunk blood was collected for plasma aldosterone and corticosterone into pre-chilled tubes containing heparin. The tubes with blood samples were kept on ice, protected from light and then centrifuged at 3,000 rpm for 30 min at 4°C. Plasma aliquots were frozen at -80 till analysis.

The heart was rapidly removed, rinsed in ice cold saline, and the right ventricle was separated from the left ventricle and blotted dry. The right ventricle (RV) and left ventricle (LV) were weighed, then wrapped in foil, and snap frozen in liquid nitrogen. Adrenal

glands and kidneys were also snap frozen. Brains were quickly removed and frozen on dry ice. All tissues were stored at -80 ºC till analysis. For aldosterone analysis, hypothalamus and hippocampus were taken from brain; cortex and medulla of kidney were separated.

2.2.3 Experiment II) Effects of Ang II-salt on BP, plasma aldosterone and mRNA expression of genes in aldosterone-MR-ENaC-EO-AT$_1$R pathway

Goal:

To study the effect of sc AngII with or without high salt diet for 2 weeks on BP, plasma aldosterone and gene expression in brain nuclei (SFO, PVN, SON, RVLM), kidney cortex, kidney medulla, and adrenal cortex in Wistar rats.

Experimental Animals:

28 Wistar rats were divided into 4 groups for the following treatments:

1.Control, 0.4% NaCl diet only. (n=6)

2. High salt (2% NaCl) diet only. **(**n=6)

3. Sc Ang II only (150 ng/kg/day). **(**n=8)

4. Sc Ang II (150 ng/kg/day) + high salt (2% NaCl) diet. **(**n=8)

Osmotic minipumps were filled with Ang II (for infusion at 150 ng/kg/min) and implanted sc for 14 days infusion in group 3 and 4. Sham surgery was done for groups 1 and 2, without implantation of a pump.

Initial, after 5-6 days, and final body weight as well as water intake were measured.

Methods

Water intake was measured once per week (day 5-6, and day 12-13 after the start of treatment) during the 2 weeks' treatment period. For each measurement, the weight of the

water bottle was measured on 2 consecutive days and water intake was calculated by subtraction of bottle weight on second day from the preceding day's bottle weight.

One day before the end of the infusion period (the day before tissue collection), the right femoral artery was catheterized. After recovery for at least 4 hours, resting BP and HR was recorded for 20 min (Detailed in 2.3).

After hemodynamic measurements, rats were left in individual cages in a quiet room overnight, and kept undisturbed. The next morning, they were quietly taken one by one (in their cage) into necropsy room. The conscious rat was rapidly decapitated and trunk blood was collected for plasma aldosterone, corticosterone and Ang II into a pre-chilled 50ml tube containing heparin.

The tube was mixed thoroughly and 2 ml of heparinized blood was immediately transferred into a 2 ml microtube containing 200 µl ACE inhibitor solution for Ang II assay. Both the microtube and the 50 ml tube were kept in the ice, and then centrigued at 3000 rpm 4 °C as soon as possible. After centrifugal separation, minimal 1.0 ml plasma from the microtube for Ang II assay was transferred to a fresh prechilled 1.5 ml microtube. The plasma in the 50ml tube was separated into different microtubes for measurement of aldosterone and corticosterone (1 ml aliquot for measurement of aldosterone, around 50µL for corticosterone), and the rest of each plasma sample was kept in 5 ml tube. All plasmas samples were stored at -80 till analysis.

Brains were quickly removed and frozen in chilled methylbutane for later cryosectioning and punching of the SFO, PVN, SON, and RVLM for measurement of mRNA expression of AT_1R, CYP11B1, CYP11B2, MR, 11βHSD2, and ENaCs in these brain nuclei.

The heart was rapidly removed, rinsed in ice cold saline, and the right ventricle was

separated from the left ventricle and blotted dry. The RV and LV was weighed, and then snap frozen in methylbutane for measurement of aldosterone. Adrenal glands and kidneys were also weighed and snap frozen until needed. Cortex was isolated from adrenal glands for measurement of AT_1R, CYP11B1, and CYP11B2 expression. Kidney cortex and medulla was separated for measurement of MR, ENaCs and AT_1R mRNA expression.

Since the treatments for rats in experiment I and experiment II are the same, data from experiment I and experiment II were pooled together when the same parameters were measured.

2.2.4 Experiment III) Effects of central blockades on Ang II-salt induced BP and aldosterone responses

Goal:

To examine the effects of chronic icv infusion of MR blocker, ENaC blocker or AT_1R blocker on mRNA expression, plasma aldosterone, plasma corticosterone, plasma Ang II and BP in Wistar rats receiving a high salt diet plus sc Ang II infusion.

Experimental Animals:

A telemetry probe was implanted in the abdominal cavity and the catheter was inserted into the abdominal aorta. After 2 days, baseline HR and BP were recorded for 3 days (Detailed in 2.3).

Then an icv cannula and osmotic minipump (Alzet model 2004, flow rate 6µl/day) was implanted for the chronic icv infusion of a MR blocker, ENaC blocker, AT_1R blocker or their vehicles as controls. Both spironolactone and eplerenone were used as MR blockers. Spironolactone lacks specificity for MR, and also binds to progesterone and androgen

receptors (Lainscak et al., 2015). On the other hand, the binding affinity of eplerenone for MR is 20-fold lower compared with spironolactone (De Gasparo et al., 1987). Specifically, rats were divided into 7 groups for the following treatments:

1. Icv MR blocker eplerenone, n =5
2. Icv MR blocker spironolactone, n = 4
3. Icv ENaC blocker benzamil, n = 6
4. Icv AT_1R blocker losartan, n = 4
5. Icv vehicle for eplerenone, n = 4
6. Icv vehicle for spironolactone, n = 4
7. Icv aCSF as vehicle for benzamil and losartan, n =3

Two rats in the central blockade study experienced severe dehydration and loss of body weight, and were therefore euthanized. Data of these two rats were not included.

One Way ANOVA was performed for the BP of rats in 3 vehicle groups, and no significant differences were found. The rats in these groups were pooled together as the vehicle group.

Two days after the start of icv infusions, a second osmotic minipump (Alzet model 2002, flow rate 12 µl/day) filled with Ang II was implanted sc for 14 days infusion at 150 ng/kg/min. At the same time, all rats were started on a 2% high salt diet.

Body weight was recorded at the start and then again at the end of the 2 wk infusion period. Water intake was measured twice per week during the 2 weeks' treatment period. For each measurement, the weight of the water bottle was measured on 2 consecutive days and water intake was calculated by subtraction of bottle weight on second day from the preceding day's bottle weight.

BP recording was continued until the end of the 2 weeks' Ang II infusion. The last day, rats were placed in a quiet room overnight. The next morning before 11 am, they were taken one by one in their cage to the necropsy room and immediately killed by decapitation.

Trunk blood was collected into pre-chilled tubes containing heparin and mixed well. An aliquot of whole blood was transferred immediately to a chilled microtube containing ACE inhibitor for plasma Ang II assay. Blood was centrifuged at 3,000 rpm, 30 min, 4 °C, and the plasma for Ang II as well as aliquots of the heparinized plasma for aldosterone (0.6 ml), corticosterone (100 µl) were separated and frozen at -80 °C.

Brains were quickly removed and first inspected visually to confirm icv cannula placement. Whole brain was frozen in pre-chilled methyl butane, and then stored at −80 °C till punching of brain nuclei for gene expression studies. The LV was separated from the RV, weighed and snap frozen in liquid nitrogen. The adrenals and kidneys were weighed and frozen in pre-chilled methylbutane.

For all the three protocols, right after tissue collection or blood centrifugation for plasma, all samples were frozen at -80 °C until analysis. The values from brains, peripheral tissues and plasma samples reflect the state of rats on the day of tissue collection, which is the end of Ang II-salt treatment.

2.3 BP measurement

For exp. I and exp. II, one day before the end of the infusion period (the day before tissue collection), the right femoral artery was catheterized with a polyethylene PE 50 (I.D.=0.58 mm) fused to PE 10 (I.D.=0.28 mm) tubing. After recovery for at least 4 hours, the arterial line was connected to a pressure transducer interfaced with a PC using

Acqknowledge III software (ACQ 3.2, BIOPAC Systems, Inc., USA) for BP and HR measurement. After a 20-30 min rest, resting BP and HR was recorded for 20 min. From recorded curve, the most stable and representative segments were selected at 0, 10, 20 minutes. At each time point, 15 peaks of systolic and diastolic pressure were calculated. The average systolic and diastolic pressure were then extracted. For calculation of MAP, the following formula was used:

MAP= DP+1/3(SP-DP).

The HR (beats/minute) was also calculated in the same segment at each time point for 1 minute.

Averages of 0, 10, 20 minutes MAP and HR were calculated.

For exp. III, a telemetry probe (model TA11PA-C40, DSI) was implanted into the abdominal cavity of each rat and secured to the ventral abdominal wall. The catheter was inserted into the abdominal aorta. An analog adapter and data-acquisition system were used for calculating and storing the telemetry signal. The resting BP and HR were recorded for 3-s interval over a 1-min period each hour and the mean values per hour were calculated by the system. BP and HR records each day were then separated by daytime (7 AM–7 PM) and nighttime (7 PM–7 AM), and the average BP and HR during the day and the night were calculated. Two days after the implantation of telemetry probes, the records of BP and HR for three days were used as baseline before osmotic minipumps for Ang II were implanted.

2.4 Molecular Biology Assays

(mRNA abundance assays in brain nuclei and peripheral tissues)

2.4.1 Total RNA isolation

2.4.1.1 RNA isolation from kidney cortex, kidney medulla or adrenal cortex

Quick-Start Protocol of QIAzol Lysis Reagent from QIAGEN was followed for RNA extraction from peripheral tissues. Less than 100 mg of kidney cortex or kidney medulla was weighed and homogenized in 1ml QIAzol. Adrenal cortex was separated from adrenal medulla and was homogenized in 120 µl QIAzol l in microtube right after separation. After homogenization, additional 880 µl QIAzol was added to reach a total volume of 1ml. Kidney or adrenal homogenate was placed at room temperature for 10 min, and then 200 µl chloroform was added in each tube and was shook vigorously by vortexing for 15 s. After placed at room temperature for 2 min, samples were centrifuged at 12,000 g for 15 min at 4 °C. The upper aqueous phase was then transferred to a new tube and 500 µl isopropanol was added to each tube and was mixed thoroughly by vortexing. After 10 min incubation at room temperature, samples were then centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was discarded and the RNA pellet in each tube was washed by 1 ml 75% ethanol and centrifuged at 7,500 g for 5 min at 4 °C. After a second wash and centrifugation, the supernatant was removed completely and the RNA pellet was air-dried for 10 min. 50 µl or 100 µl RNase-free water was added for dissolving RNA from adrenal cortex or kidney, respectively. The RNA concentration as well as the ratios of 260/280 and 260/230 was measured by Nanodrop 2000 (Thermo Scientific). Samples were stored at -80 °C.

2.4.1.2 RNA isolation from brain nuclei

Serial 50-µm-thick slices were cryosectioned, and the brain micropunches of SFO, SON

and PVN and RVLM were taken with prechilled Drummond microdispensers. The tissue pellet was disrupted in 120 µl LBA buffer (1-Thioglycerol included) by using a pestle, which was driven by a pestle motor. Homogenized samples were frozen in -80 °C until RNA extraction.

Promega RNA Tissue miniprep kit was used and its protocol was followed for isolation and purification of RNA from brain nuclei. Additional 130 µl LBA + Thioglycerol buffer was added to each tube to reach a total volume of 250 µl. After 10 times pipetting to shear the DNA using a P1000 pipettor, the homogenates were centrifuged for 3 min at 14,000 g and transferred to a clear tube. 85 µl 100% isopropanol was then added to each tube and mixed by vortexing for 5 seconds. The lysate was subsequently transferred to a ReliaPrep™ minicolumn which was placed into a collection tube and centrifuged at 14,000 g for 1 min at 25 °C. The liquid in the collection tube was discarded and the minicolumn was placed backed into each collection tube. 500 µl of RNA Wash Solution (prepared in advance: 350 ml of 95% ethanol was added to the bottle which contains 206 ml of concentrated RNA wash solution) was then added to the ReliaPrep™ Minicolumn and was centrifuged at 14,000 g for 30 seconds.

The DNase I incubation mix was prepared by combining 24 µl of Yellow Core Buffer, 3 µl 0.09M $MnCl_2$ and 3 µl of DNase I enzyme. Total 30 µl DNase I incubation mix was then applied directly to the membrane inside the column. After incubation up to 1 h at room temperature, 200 µl of Column Wash Solution (ethanol included) was added to each ReliaPrep™ Minicolumn and was centrifuged at 14,000 g for 15 seconds. 500 µl of RNA Wash Solution (ethanol included) was then added and centrifuged at 14,000 g for 30 seconds. After that, the ReliaPrep™ minicolumn was placed into a new collection tube and

300 µl of RNA Wash Solution (ethanol included) was added and centrifuged at 17,000 g for 2 min. The ReliaPrep™ Minicolumn from the collection tube was transferred to an elution tube, and 15 µl Nuclease-Free water was added to the membrane and centrifuged at 14,000 g for 1 min. The elution tube containing the purified RNA was stored at -80 °C.

2.4.2 DNase I treatment of RNA isolated from peripheral tissues

Ambion's DNA-free TM kit was used for removal of genomic DNA from RNA preparations. 20 µg RNA isolated from kidney or adrenal tissues was used for each preparation. Nuclease-free water was added to each tube to make the total volume of 50 µl. 5 µl of 10 × DNase I buffer and 1 µl of the recombinant DNase I (rDNase, 2 Units/µL for stock concentration) were then added into each tube and mixed gently. Subsequently, samples were incubated at 37 °C for 30 min. 5 µl of resuspended DNase Inactivation Reagent was added and mixture was incubated for 2 min at room temperature with occasional mix by vortexing. After centrifugation at 10,000 g for 1.5 min at 4 °C, the supernatant from each tube was transferred to a new microtube. 5.5 µl of sodium acetate (NaAc, 3 M pH 5.2) and 110 µl of 100% ethanol were then added to each microtube and samples were stored at -20 °C overnight. The following day samples were centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was discarded and the RNA pellet in each tube was washed by 1 ml 70% ethanol and centrifuged at 10,000 g for 5 min at 4 °C. After a second wash and centrifugation, the supernatant was removed completely and the RNA pellet was air-dried for 10 min. 25 µl nuclease-free water was added for dissolving the RNA pellet. The RNA concentration, OD260/280, and OD260/230 were measured by Nanodrop 2000 (Thermo Scientific). Purified RNA samples were stored at –80 °C.

2.4.3 Reverse transcription

2.4.3.1 cDNA synthesis from purified RNA of peripheral tissues

First strand cDNA synthesis kit from Thermo Scientific was used for reverse transcription from purified RNA of peripheral tissues: kidney cortex, kidney medulla and adrenal cortex. 5 μg of total RNA was used as template RNA for each reaction system and 1μl of Oligo(dT)$_{18}$ primer (0.5 μg/μl for stock concentration) was added. Nuclease-free water was added to make the total volume of 12.5 μl. The mixture was heated at 65 °C for 5 min and quickly chilled on ice. 4 μL of 5 × Reaction Buffer, 0.5 μL (20 U) of Thermo Scientific™ RiboLock™ RNase Inhibitor (#EO0381), 2 μL (1 mM final concentration) of dNTP Mix(10 mM each, #R0191), and 1 μL (200 U) of RevertAid H Minus Reverse Transcriptase were added and the final volume of each reaction is 20 μL. The mixture was mixed gently, centrifuged briefly, and incubated for 60 min at 42 °C. The reaction was terminated by the mixture being heated at 70 °C for 10 min. The synthesized cDNA was diluted 10 times for quantification.

2.4.3.2 cDNA synthesis from purified RNA of brain nuclei

Thermo Scientific Verso cDNA Synthesis Kit was used for cDNA synthesis from purified RNA of brain nuclei. Maximum 4 μg of total RNA was used as template RNA for each reaction system and 1μl of Oligo(dT)$_{18}$ primer(0.5 μg/μl for stock concentration) was added. Nuclease-free water was added to make the total volume of 12 μl. The mixture was heated at 70°C for 5 min and quickly chilled on ice. 4 μl of 5 × cDNA synthesis buffer, 2 μl of dNTP Mix, 1μl of RT Enhancer, and 1 μl of Verso Enzyme Mix were added and the final volume of each reaction is 20 μL. The mixture was mixed gently, centrifuged briefly,

and incubated for 30 min at 42 °C. The reaction was terminated by the mixture being heated at 95 °C for 2 min. The synthesized cDNA was diluted 3 times for quantification.

2.4.4 Quantification of mRNA by real-time qPCR

2.4.4.1 Reaction system and program in real-time qPCR

Real-time qPCR was performed using the Roche LightCycler and fast-start DNA Master SYBR Green I dye (Roche Diagnostics, Laval, QC, Canada). In each reaction, 6 µl of nuclease-free water, 1µl of forward primer, 1µl of reverse primer, 10 µl of SYBR Green, and 2 µl of diluted cDNA (dilution rate as mentioned above) were added and the final volume was 20 µl. The stock concentration of each primer is 10 µM, after 1µl of primer was added in a 20 µl reaction system, the final concentration in the mixture is 0.5 µM. Quantitative normalization of mRNA expression was achieved using the expression of the constitutively expressed reference gene PGK1 as an internal control. Since the concentration of target gene is normalized to PGK1, the concentrations of cDNA were not measured specifically. Primers sequences (Table M1) were the same as what was reported in Wang et al, 2010 for ENaC three subunits, CYP11B1 and CYP11B2, in Amin et al, 2005 for MR and PGK1, in Wang et al, 2016 for 11βHSD2, and in Chen et al, 2014 for AT_1R.

PCR program: The pre-incubation for all genes is at 95 °C for 10 min, the amplification program is listed below with cycles vary from 30 to 45 (Table M2), the melting step is at 95 °C for 5 s followed by 70 °C for 1 min, and the cooling step is at 40 °C for 30 s.

Table M1. Primer Sequences for RT-qPCR

Gene	Sequence	Amplicon Length
PGK1	Forward: 5'- GCTGCAGAACTCAAATCTCT-3' Reverse: 5'- TGTGTGCAGTCCCAAAAGCA-3'	263 bp
AT_1R	Forward: 5' - GCACACTGGCAATGTAATGC-3' Reverse: 5'- GTTGAACAGAACAAGTGACC-3'	385 bp
MR	Forward: 5′- GCTCAACATTGTCCAGTACA - 3′ Reverse: 5′- GCACAGGTGGTCCTAAGATT- 3′	260bp
ENaCα	Forward: 5'- GTTCTGTGACTACCGAAAGCAGAG -3' Reverse: 5'- CGTAGCAGCATGAGAAGTGTGATG -3'	429 bp
ENaCβ	Forward: 5'-TGGATCACTGTCATCAAGCTAGTG-3' Reverse: 5'-TGGTACCAGCATCTTGACCCTATG-3'	440 bp
ENaCγ	Forward: 5'- CGTCAGTGGCACAAAGCCAA -3' Reverse: 5'- GAGAGCCTCCTCAAACCATG -3'	301 bp
11βHSD2	Forward: 5'-CGTCACTCAAGGGGACGTAT-3' Reverse: 5'-TACAACGGGGCTAAGGTCAG-3'	345 bp
CYP11B1	Forward: 5'- GTCTATAAACATTCAGTCCAA -3' Reverse: 5'- ATCTCGGATATGACACTCC -3'	324bp
CYP11B2	Forward: 5'- CCCTGGTAGCCTGAAGTTCATC -3' Reverse: 5'- TCTGAGAGCTGCCGAGTCTG -3'	203 bp

Table M2. Amplification program for RT-qPCR

	Denaturation	Annealing	Elongation
PGK1	95 °C, 10 s	62 °C, 15 s	72 °C, 13 s
AT_1R	95 °C, 10 s	62 °C, 15 s	72 °C, 20 s
MR	95 °C, 10 s	62 °C, 15 s	72 °C, 13 s
ENaCα	95 °C, 10 s	65 °C, 15 s	72 °C, 23 s
ENaCβ	95 °C, 10 s	65 °C, 15 s	72 °C, 23 s
ENaCγ	95 °C, 10 s	62 °C, 15 s	72 °C, 17 s
11βHSD2	95 °C, 10 s	60 °C, 15 s	72 °C, 17 s
CYP11B1	95 °C, 10 s	55 °C, 15 s	72 °C, 15 s
CYP11B2	95 °C, 10 s	62 °C, 15 s	72 °C, 14 s

2.4.4.2 Standard Curve in real-time qPCR

In real-time qPCR assay, the standard curve was generated by PCR amplification using a serial dilution of standard from 1000, 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 pg/µl. The recombinant plasmid containing the sequence of target gene was served as template for the qPCR. Various plasmid containing the specific target gene were constructed in Dr. Leenen's lab previously (Wang et al., 2010, Chen et al., 2014). The efficiency of each standard curve was above 1.8. A single point of 1 pg/µl from the standard was included in every qPCR assay.

The copy number was calculated based on the following equation from Li et al, 2006.

$$\text{DNA copy number} = \frac{6.02 \times 10^{23}\ (\text{copy/mol}) \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660\ (\text{g/mol/bp})}$$

DNA length was the length of the plasmid vector (3971 bp, pCR™II vector, Invitrogen) plus the length of the target gene (Table M1). Therefore, the detection of qPCR is at the range of copy number for different target genes from ~ 20 to 2×10^8copies/µl based upon the standard curve.

2.5 Radioimmunoassays (RIA)

2.5.1 RIA for aldosterone

2.5.1.1 Protocol of RIA for aldosterone

Plasma, brain and heart aldosterone were measured by RIA. The protocol from Huang et al (2013) for RIA of aldosterone was followed. The whole hypothalamus and hippocampus were dissected based on Glowinski and Iversen's method (Glowinski et al., 1966).

Brian and heart tissues were first homogenized in 10 volumes (1.5 ml) 100% methanol

using a pre-chilled polytron, and the tubes containing tissues were put in ice during homogenization. After centrifugation at 3,500 rpm for 30 min at 4 ºC using a Sorvall Legend RT, the supernatants were dried overnight in a vacuum concentrator. Each dried homogenate was re-dissolved in 3 ml 0.1% trifluoroacetic acid (TFA), and allowed to redissolve for a minimum 30 min with frequent vortexing. Each sample was then centrifuged for 30 min at 4 ºC to pellet insoluble material until the supernatant was clear, and the supernatant was applied to preconditioned Sep-Pak C18 cartridges.

Each cartridge was preconditioned by passing through 15 ml methanol followed by 10 ml ddH_2O. Tissue supernatant in TFA or 500 µl plasma was applied to pre-conditioned C18 cartridges and aldosterone was eluted with 4 ml 80% methanol after pre-washing with 10 ml ddH_2O followed by 4 ml 12% methanol.

The eluate was dried in a vacuum concentrator and then re-dissolved in 2.5 ml phosphate buffered saline (0.1 M PBS) containing 0.5% bovine serum albumin for the RIA (RIA buffer). Duplicate 0.5 ml aliquots were incubated with 100 µl each aldosterone antibody and ^{125}I aldosterone for 16-24 hours at 4 ºC. After separation with dextran-coated charcoal, the supernatants were counted using a Canberra-Packard AutoGamma counter. The aldosterone content was calculated by a standard curve, which was generated by the AutoGamma's curve fitting software using a serial dilution of aldosterone from 0, 1, 2.5, 5, 10, 25, 50, to 100 pg/0.5ml plus total count(without antibody).

2.5.1.2 Sensitivity of aldosterone assay

500 µl plasma was applied to pre-conditioned C18 cartridges for aldosterone analysis. After aldosterone was eluted and dried, 2.5 ml RIA buffer was used to re-dissolve aldosterone.

For each sample, 0.5 ml of the aldosterone-RIA buffer solution was taken for RIA (containing 1/5 of the whole aldosterone in 500μl plasma, as calculated by 0.5 ml/2.5 ml). As a consequence, the amount of aldosterone for RIA (A pg) generated according to RIA standard curve should time 5 to stand for the amount of aldosterone in 500 μl plasma. Therefore, the plasma aldosterone concentration is A pg ×5/(500 μl), which equals to 10×A pg/ml. Serial dilution of aldosterone standard is from 0, 1, 2.5, 5, 10, 25, 50, to 100 pg/0.5ml, so the range of plasma aldosterone concentration that can be detected is from 0 to 1000 pg/ml, which covers the concentration of most of the plasma samples. When the amount of aldosterone for RIA from the sample is so high that it stays outside of the standard curve, 100 pg was used to indicate its amount and thus 1000 pg/ml to indicate the plasma aldosterone concentration.

In the serial dilution of aldosterone standard, the statistical difference between the 0 standard and the 1 pg standard (the first non-zero standard), which is 1.0 pg/tube, was used to calculate the detection limit (Huang et al, 2013). The sensitivity for plasma aldosterone was 1.0 pg ×5/(500 μl) =10 pg/ml, based on the protocol for plasma aldosterone mentioned above. The average aldosterone concentration in plasma is 17-fold as high as the detection limit. The average weight of hypothalamus in the current study was 74 mg, and the average amount in RIA tube was 74/5 mg=15 mg. Therefore, the sensitivity for aldosterone in hypothalamus was 1.0 pg/15 mg=0.067 pg/mg or 67 pg/g. The average aldosterone level in hypothalamus is 1.7-fold as high as the detection limit. The average weight of hippocampus in the current study was 160 mg, and the average amount in RIA tube was 160/5 mg=30 mg. Therefore, the sensitivity for aldosterone in hippocampus was 1.0 pg/30 mg=0.033 pg/mg or 33 pg/g. The average aldosterone level in hippocampus is 3.7-fold as

high as the detection limit.

2.5.2 RIA for corticosterone

For corticosterone measurement, the corticosterone ^{125}I RIA kit from MP Biomedicals was used. Each plasma sample was diluted 200 times and 100 µl of diluted plasma was used for assay. 100 µl of re-dissolved brain tissue in RIA buffer was used. ^{125}I labeled corticosterone, corticosterone antibody I were added to each sample and the mixture was incubated at room temperature for 2 hours. After incubation, corticosterone antibody II was added to each tube and centrifuged at 2,500 rpm for 15 min. The pellets were counted using a Canberra-Packard Auto Gamma counter. The corticosterone standards for plasma and tissue were from 0, 6.25, 12.5, 25, 50, 125, 250, to 500 pg/ml.

2.5.3 RIA for Ang II

The protocol for plasma Ang II measurement was the same as described in Ruzicka et al., 1995. 2 ml blood sample used for Ang II essay was centrifuged at 3,000 g for 5 min, and then plasma was applied to preconditioned SepPak C 18 cartridge. Angiotensin peptides were extracted by 2 ml eluent containing methanol, water and TFA (80:19.9:0.1), and subsequently evaporated till dryness. Ang II was separated from other angiotensin peptides by HPLC. The concentration of plasma Ang II was measured by RIA (Leenen et al., 2001). The reagents/equipment mentioned above are listed in table M3.

Table M3. Information of laboratory supplies

	Catalog #	Manufacturer
^{125}I-labeled aldosterone	07-108226	MP Biomedicals
Aldosterone	A9477	Sigma
Aldosterone antiserum	07-108216	MP Biomedicals
corticosterone ^{125}I RIA kit	07-120103	MP Biomedicals
DNA- free™ Kit, Ambion	AM1906	Life Technologies
LightCycler® 480 SYBR Green I Master	04707516001	Roche
QIAzol Lysis Reagent	79306	Qiagen
ReliaPrep™ RNA Tissue Miniprep System	Z6112	Promega
RevertAid H Minus First Strand cDNA Synthesis kit	K1632	Thermo Scientific
Sep Pak Plus C18 cartridges	WAT020515	Waters
Verso cDNA Synthesis Kit	AB-1453/A	Thermo Scientific

2.6 Statistical analysis

Values were expressed as mean± SE. The effects of the factor sc infusion of Ang II and the factor 2% salt diet as well as their interaction were analyzed by Two-Way ANOVA. One-way ANOVA with repeated measures was performed for BP and HR changes from baseline. For comparisons of other parameters, one-way ANOVA for multiple groups was performed followed by a Tukey's test. Student t-test was performed for comparison between two groups. Statistical significance was defined as $p<0.05$. Statistical analyses were performed using SPSS 19.

For Two Way ANOVA, comparisons are between Ang II vs. non-Ang II, high salt vs. regular salt, and the symbol Ang II*salt indicates the interaction between Ang II treatment and high salt intake. Previous studies have shown that high salt diet decreases plasma aldosterone (section 4.3). A t-test for salt alone and control was performed to assess the effect of salt by itself, as a supplementary test.

3. Results

3.1 The effects of sc Ang II alone or combined with high salt diet on MAP, HR, heart weight and water intake

By day 13, 2% high salt diet alone or sc Ang II alone at 150 ng/kg/min increased MAP by ~5 and 15 mmHg, respectively. In contrast, sc Ang II together with high salt diet caused a large (~50 mmHg) increase of MAP (Figure 1). Neither high salt diet nor sc Ang II had significant effect on HR (Table 1). Both high salt diet and sc Ang II increased LV weight, and LV weight was further increased by sc Ang II and high salt diet combined (Figure 2). Neither sc Ang II nor high salt diet affected RV weight (Table 1). High salt diet but not sc Ang II increased water intake at both first and second week after the start of treatment (Table 2). There were no differences in changes in body weight with different treatments (Table 3).

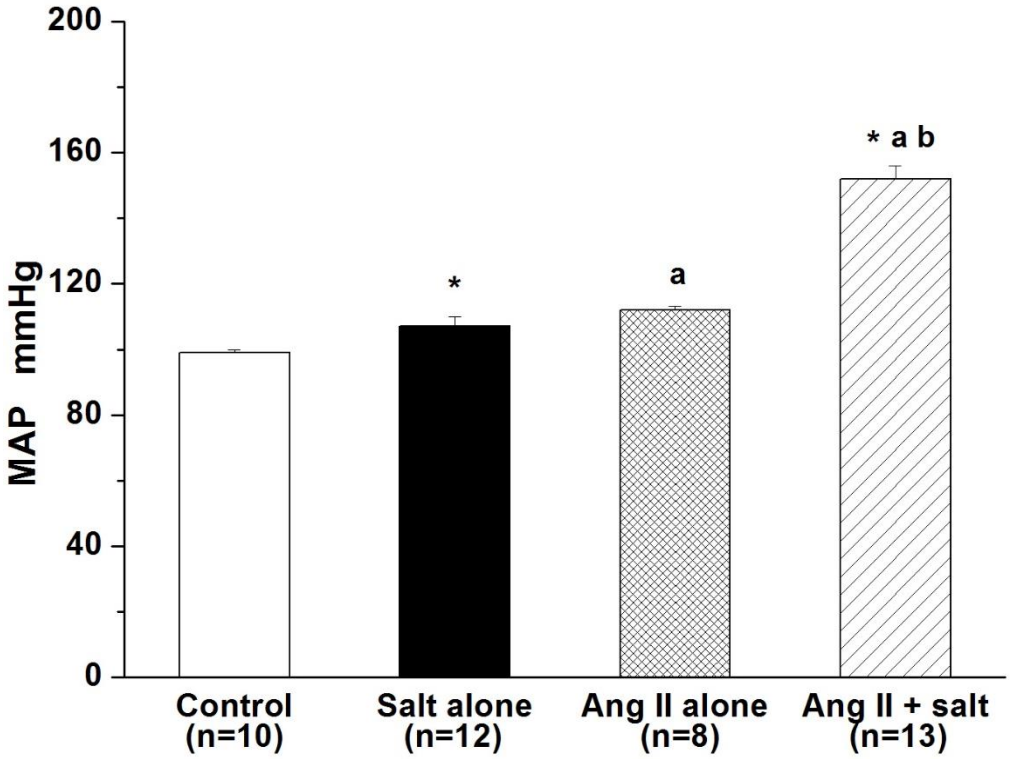

Figure 1. The effect of sc Ang II alone or combined with high salt diet on MAP.

Two Way ANOVA was performed to test the effect of the factor high salt diet or sc Ang II infusion on MAP and their interaction.

Factor high salt diet vs. regular salt diet, F=55.3, *P<0.001;

Factor Ang II vs. non-Ang II treatment, F=82.7, [a]P<0.001;

High salt*Ang II interaction: F=25.6, [b]P<0.001.

Table 1. Effects of sc Ang II alone or combined with high salt diet on HR and RV weight

	Control (n=10)	Salt alone (n=12)	AngII alone (n=8)	AngII + salt (n=13)
HR (bpm)	434±16	428±8	406±9	409±12
RV (mg/100 g BW)	47±3	45±2	49±2	52±2

Two Way ANOVA was performed. There were no significant differences. Data are mean values ± SE.

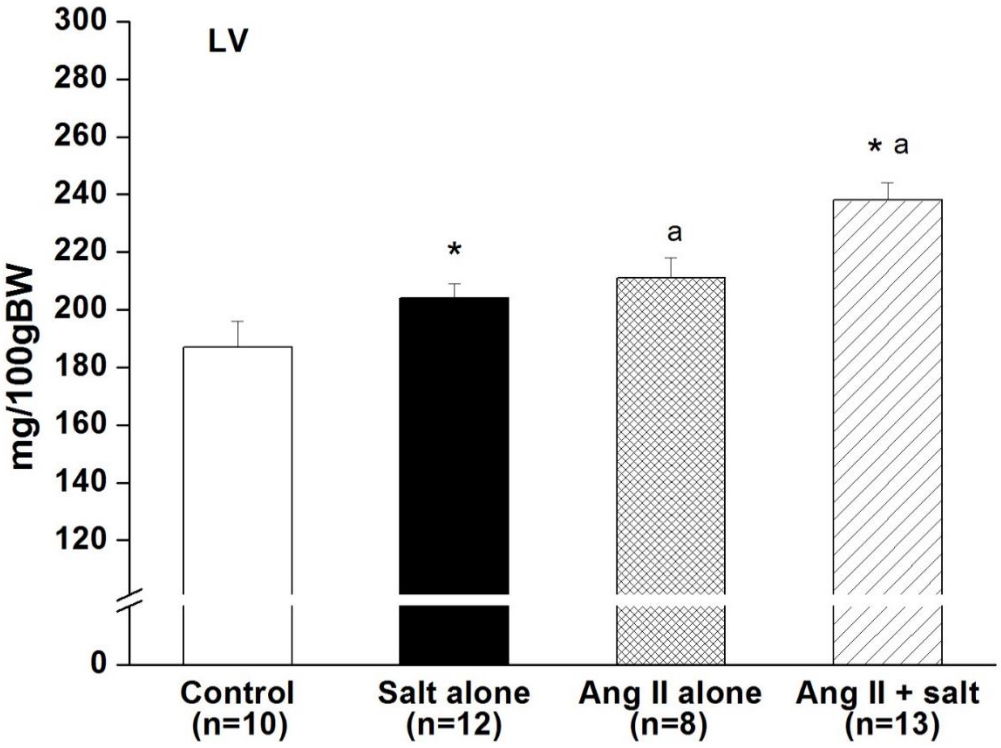

Figure 2. The effect of sc Ang II alone or combined with high salt diet on LV weight.

Two Way ANOVA was performed to test the effect of the factor high salt diet or sc Ang II infusion on LV weight and their interaction.

Factor high salt diet vs. regular salt diet, F=9.7, *P=0.004;

Factor Ang II vs. non-Ang II treatment, F=17.3, [a]P<0.001.

Table 2. The effect of sc Ang II alone or combined with high salt diet on water intake

	Control (n=6)	Salt alone (n=6)	AngII alone (n=8)	AngII + salt (n=8)
1st week (ml/100 g BW)	12±1	15±1*	12±1	19±2*
2nd week (ml/100 g BW)	11±1	14±1*	11±1	17±2*

Two Way ANOVA was performed. Data are mean values ± SE.

1st week: Factor high salt diet vs. regular salt diet, F=11.79, *P=0.002;

2nd week: Factor high salt diet vs. regular salt diet, F=15.9, *P=5.1E-4.

Table 3. Changes in body weight of rats with sc Ang II alone or combined with high salt diet

	Control (n=6)	Salt alone (n=6)	AngII alone (n=8)	AngII + salt (n=8)
BW-Initial (g)	304±13	289±11	293±8	293±13
BW-1 wk (g)	337±13	321±11	329±7	332±11
BW-final (g)	383±15	358±14	372±8	366±11
BW gain (g)	79±5	70±4	80±6	73±4

Two Way ANOVA was performed. Data are mean values ± SE. There were no significant differences.

3.2 Effects of sc Ang II alone or combined with high salt diet on plasma Ang II level and the levels of aldosterone and corticosterone in plasma and tissues

Sc Ang II did not affect plasma Ang II level. High salt diet alone tended to decrease plasma Ang II level (Table 4). Sc Ang II alone did not affect plasma aldosterone level while high salt diet alone tended to decrease it. However, sc Ang II combined with high salt diet markedly increased plasma aldosterone level (Figure 3). There was no significant difference in aldosterone level in LV or RV. Sc Ang II combined with high salt diet tended to increase aldosterone level in hypothalamus, and overall significantly in hippocampus (Table 5). There were no significant differences in plasma, hypothalamus, or hippocampus corticosterone level among the groups.

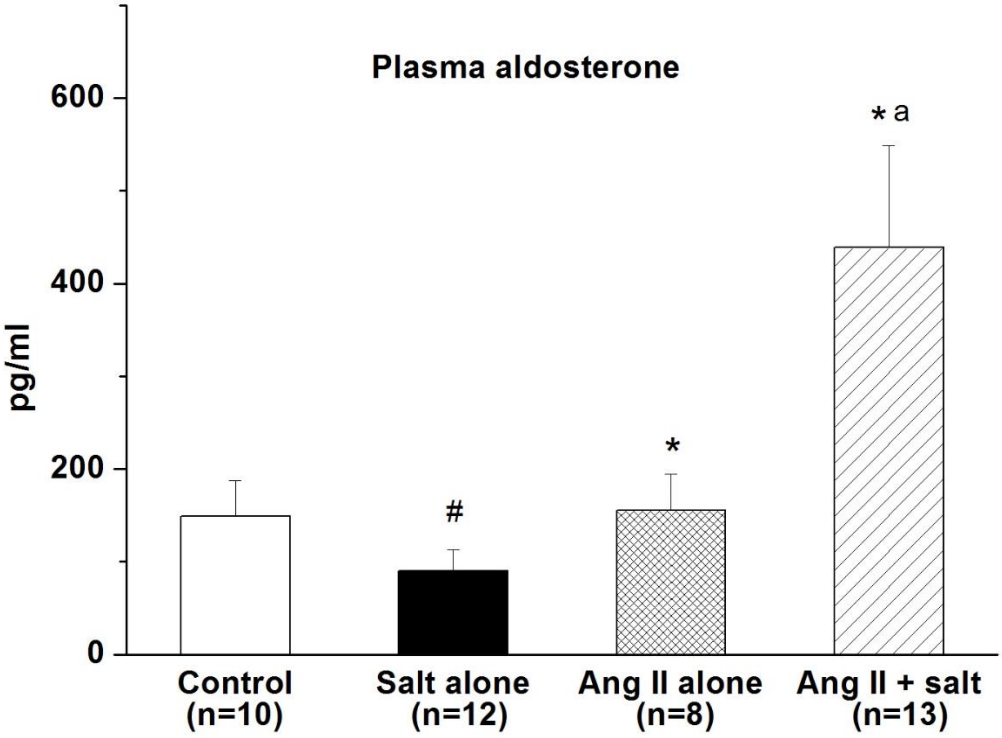

Figure 3. Effects of sc Ang II alone or combined with high salt diet on plasma aldosterone.

Two Way ANOVA was performed to test the effect of the factor high salt diet or sc Ang II on plasma aldosterone level.

Factor Ang II vs. non-Ang II treatment, F=5.90, *P=0.02;

High salt*Ang II interaction: F=5.57; [a]P=0.024.

T-test was performed to compare plasma aldosterone level between salt alone group and control group, [#]P=0.191 vs. control.

Table 4. Effects of sc Ang II alone or combined with high salt diet on corticosterone and Ang II levels in plasma and aldosterone level in heart

	Control (n=10)	Salt alone (n=12)	AngII alone (n=8)	AngII + salt (n=13)
LV aldo. (pg/g)	133±21	99±24	114±27	303±116
RV aldo. (pg/g)	146±58	163±49	---	540±220
Plasma corti. (ng/ml)	98±25	115±32	75±27	198±43
Plasma Ang II (pg/ml)	14.4±2.3	7.2±1.9[#]	12.2±3.0	13.6±1.9

By Two Way ANOVA, there were no significant differences. Data are mean values ± SE.

T-test was performed to compare plasma Ang II level between salt alone group and control group, [#]P=0.055 vs. control.

Table 5. Effects of sc Ang II alone or combined with high salt diet on aldosterone and corticosterone levels in the brain

	Control (n=4)	Salt alone (n=6)	AngII + salt (n=5)
Aldosterone			
Hypothalamus (pg/g)	84±24	79±15	153±37*
Hippocampus (pg/g)	71±15	58±23	212±69[a]
Corticosterone			
Hypothalamus (ng/g)	17±7	19±6	47±20
Hippocampus (ng/g)	19±8	18±8	40±9

One Way ANOVA was performed followed by Tukey's test.

Aldosterone in hypothalamus, Ang II+salt vs. Control, *P=0.232.

Aldosterone in hippocampus, between groups, F=3.904, P=0.049.

Ang II+salt vs. Salt alone, [a]P=0.056;

Ang II+salt vs. Control, P=0.119.

3.3 Effects of sc Ang II alone or combined with high salt diet on mRNA expression in peripheral tissues

In kidney cortex, neither high salt diet nor sc Ang II significantly affected the mRNA expression of AT_1R, MR and ENaC three subunits (Table 6).

Table 6. Effects of sc Ang II alone or combined with high salt diet on mRNA expression in the kidney cortex

	Control (n=6)	Salt alone (n=6)	Ang II alone (n=8)	Ang II + salt (n=8)
AT_1R/PGK1 ($\times10^{-1}$)	2.6±0.1	2.4±0.2	2.5±0.1	2.4±0.2
MR/PGK1 ($\times10^{-1}$)	2.7±0.5	2.8±0.5	2.1±0.3	2.2±0.4
ENaCα/PGK1 ($\times10^{-2}$)	7.1±0.9	5.9±0.5	7.5±0.9	6.6±0.7
ENaCβ/PGK1 ($\times10^{-2}$)	3.8±0.3	4.4±0.5	4.0±0.4	4.3±0.5
ENaCγ/PGK1 ($\times10^{-2}$)	6.0±0.7	5.7±0.4	5.3±0.5	5.3±0.5

Two Way ANOVA was performed. There were no significant differences. Data are mean values ± SE.

The mRNA expression of AT_1R and ENaC α subunit was decreased by high salt diet in kidney medulla (Figure 4, Figure 5). Sc Ang II infusion did not change the mRNA level of AT_1R, MR or ENaC three subunits in kidney medulla (Figure 4, Figure 5, Table 7).

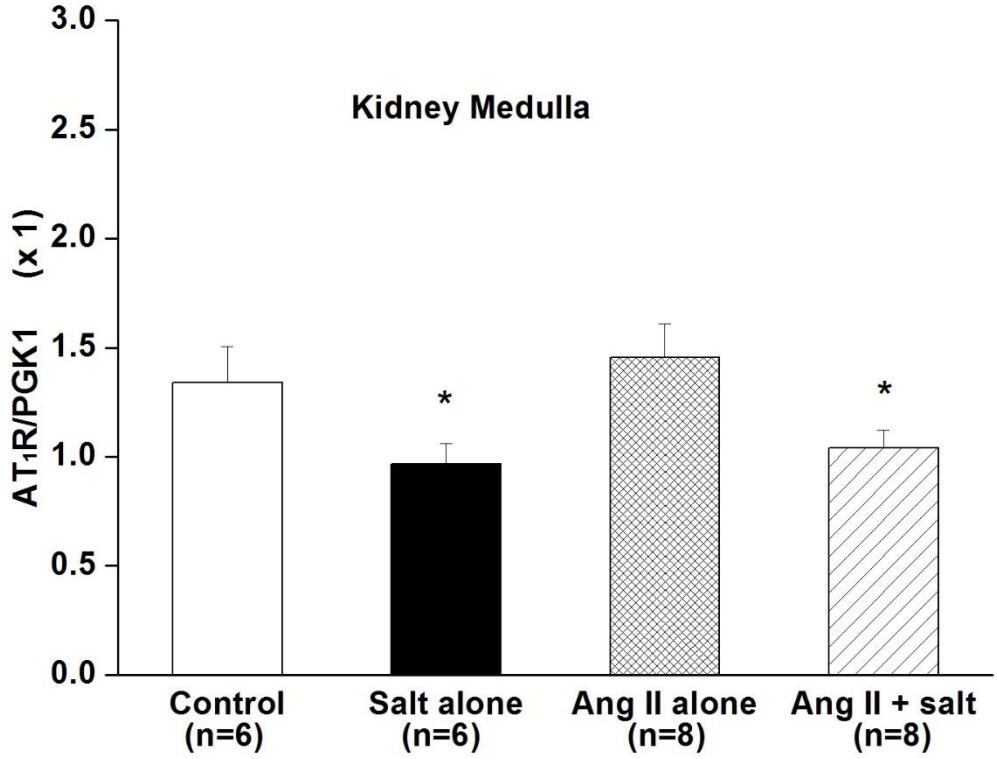

Figure 4. Effects of sc Ang II alone or combined with high salt diet on mRNA expression of AT_1R in the kidney medulla.

Two Way ANOVA was performed. Factor high salt diet vs. regular salt diet, F=9.93, *P=0.004.

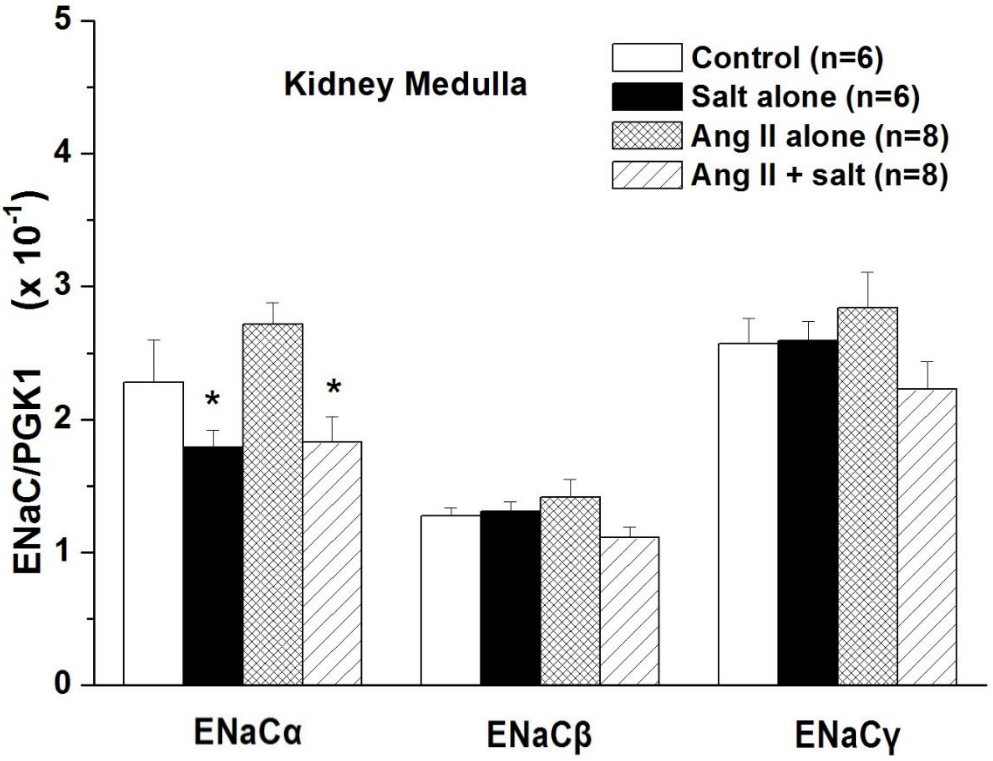

Figure 5. Effects of sc Ang II alone or combined with high salt diet on mRNA expression of ENaC three subunits in the kidney medulla.

Two Way ANOVA was performed.

ENaC α/PGK1, Factor high salt diet vs. regular salt diet, F=11.0, *P=0.003.

Table 7. Effects of sc Ang II alone or combined with high salt diet on mRNA expression of MR in the kidney medulla

	Control (n=6)	Salt alone (n=6)	Ang II alone (n=8)	Ang II + salt (n=8)
MR/PGK1 (×1)	1.4±0.1	1.5±0.1	1.5±0.1	1.3±0.1

Two Way ANOVA was performed. There was no significant difference. Data are mean values ± SE.

In adrenal cortex, high salt diet, or sc Ang II, or the two together had no effect on mRNA levels of AT_1R. Sc Ang II alone or combined with high salt diet similarly increased the mRNA expression of CYP11B2. In contrast, high salt diet alone significantly decreased it.

There was no interaction between sc Ang II and high salt for CYP11B2 mRNA abundance. High salt diet caused a slight increase in CYP11B1 mRNA expression in adrenal cortex (Table 8).

Table 8. Effects of sc Ang II alone or combined with high salt diet on mRNA expression in adrenal cortex

	Control (n=6)	Salt alone (n=6)	Ang II alone (n=8)	Ang II + salt (n=8)
AT_1R /PGK1 ($\times 10^{-2}$)	7.3±0.8	5.5±0.9	7.5±0.7	7.4±0.8
CYP11B1/PGK1 (×1)	4.5±0.3	6.1±0.4	5.2±0.3	5.1±0.4
CYP11B2/PGK1 ($\times 10^{-1}$)	3.4±0.5	0.8±0.3$^{\#}$	10.6±2.1*	7.8±3.5*

Two Way ANOVA was performed. CYP11B2/PGK1: Factor sc Ang II vs. non-Ang II treatment, F=7.361, *P=0.014.

T-test was performed to compare CYP11B2/PGK1 level between salt alone group and control group, $^{\#}$P=0.005 vs. control.

3.4 Effects of sc Ang II alone or combined with high salt diet on mRNA expression in brain nuclei

High salt diet, sc Ang II, or the two together had no effect on mRNA levels of AT_1R, CYP11B1, CYP11B2, 11βHSD2, MR or ENaC three subunits in the SFO (Table 9).

Table 9. Effects of sc Ang II alone or combined with high salt diet on mRNA expression in the SFO

	Control (n=5)	Salt alone (n=6)	Ang II alone (n=8)	Ang II + salt (n=8)
AT_1R/PGK1 ($\times10^{-2}$)	9.2±1.2	8.4±0.6	10.2±1.3	10.2±0.9
CYP11B1/PGK1 ($\times10^{-4}$)	3.2±0.4	3.1±0.7	3.7±1.2	4.4±1.5
CYP11B2/PGK1 ($\times10^{-4}$)	1.3±0.4	1.5±0.7	0.9±0.3	1.9±0.8
11βHSD2/PGK1 ($\times10^{-4}$)	6.9±0.3	7.8±0.5	8.0±0.6	7.5±0.4
MR/PGK1 ($\times10^{-1}$)	1.7±0.1	1.5±0.1	1.4±0.1	1.5±0.1
ENaCα/PGK1 ($\times10^{-3}$)	1.3±0.2	1.2±0.1	1.5±0.2	1.5±0.1
ENaCβ/PGK1 ($\times10^{-4}$)	7.6±0.9	7.2±1.2	8.8±1.1	7.7±1.7
ENaCγ/PGK1 ($\times10^{-4}$)	8.9±0.8	10.3±3.3	10.4±2.8	11.1±3.4

Two Way ANOVA was performed. There were no significant differences. Data are mean values ± SE.

In the PVN, sc Ang II alone or combined with high salt diet similarly increased mRNA levels of AT_1R (Figure 6) and ENaC γ (Figure 7). In contrast, sc Ang II alone or combined with high salt diet similarly decreased 11βHSD2 and MR mRNA expression in the PVN (Table 10). 2% salt diet alone had no effect on mRNA level of AT_1R, CYP11B1, CYP11B2, 11βHSD2, MR, or ENaCs in the PVN (Figure 6, Figure 7, Table 10).

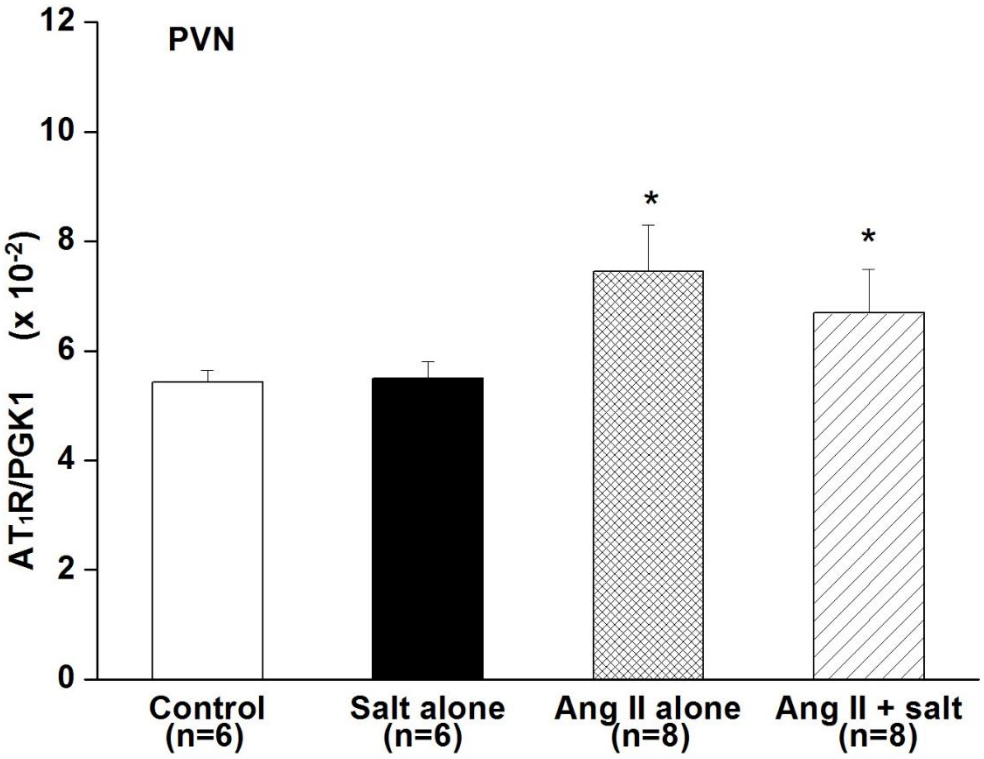

Figure 6. Effects of sc Ang II alone or combined with high salt diet on mRNA expression of AT_1R in the PVN.

Two Way ANOVA was performed. Factor sc Ang II treatment vs. non-Ang II treatment, F=5.67, *P=0.025.

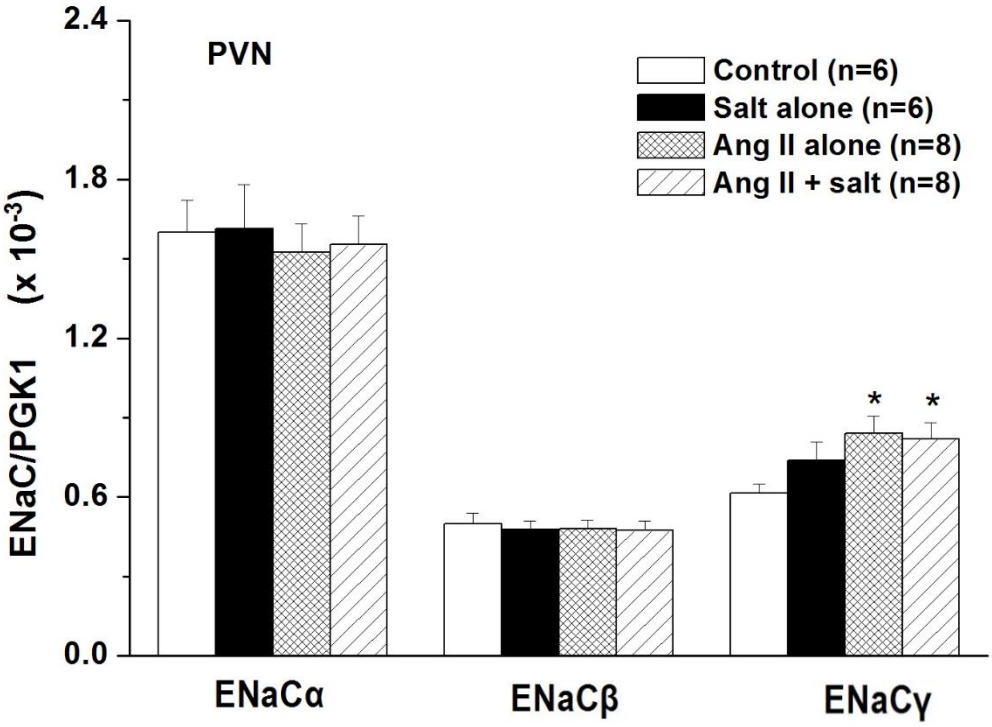

Figure 7. Effects of sc Ang II alone or combined with high salt diet on mRNA expression of ENaC three subunits in the PVN.

Two Way ANOVA was performed. ENaC γ/PGK1: Factor sc Ang II treatment vs. non-Ang II treatment, F=6.30, *P=0.019.

Table 10. Effects of sc Ang II alone or combined with high salt diet on mRNA expression in the PVN

	Control (n=6)	Salt alone (n=6)	Ang II alone (n=8)	Ang II + salt (n=8)
CYP11B1/PGK1 ($\times10^{-3}$)	1.2±0.1	1.4±0.2	1.3±0.1	1.3±0.1
CYP11B2/PGK1 ($\times10^{-4}$)	2.3±0.4	2.4±0.4	2.8±0.4	2.5±0.5
11βHSD2/PGK1 ($\times10^{-4}$)	10.8±0.7	10.5±0.7	9.4±0.5*	9.2±0.4*
MR/PGK1 ($\times10^{-2}$)	9.0±0.1	9.7±0.3	8.1±0.4*	7.7±0.5*

Two Way ANOVA was performed.

11βHSD2/PGK1: Factor sc Ang II treatment vs. non-Ang II treatment, F=5.60, *P=0.022.

MR/PGK1: Factor sc Ang II treatment vs. non-Ang II treatment, F=13.1, *P=0.0013.

Neither high salt diet nor sc Ang II had effects on mRNA levels of AT_1R, CYP11B1, CYP11B2, 11βHSD2, MR, or ENaCs in the SON (Table11).

Table 11. Effects of sc Ang II alone or combined with high salt diet on mRNA expression in the SON

	Control (n=6)	Salt alone (n=6)	Ang II alone (n=8)	Ang II + salt (n=8)
AT_1R/PGK1 ($\times 10^{-2}$)	1.7±0.2	1.8±0.1	1.7±0.2	1.6±0.1
CYP11B1/PGK1 ($\times 10^{-3}$)	4.0±1.4	4.6±1.5	5.6±1.4	6.1±0.8
CYP11B2/PGK1 ($\times 10^{-4}$)	1.7±0.6	0.8±0.2	1.2±0.2	1.3±0.5
11βHSD2/PGK1 ($\times 10^{-3}$)	1.1±0.1	1.1±0.1	1.2±0.1	1.0±0.0
MR/PGK1 ($\times 10^{-1}$)	1.7±0.1	1.8±0.1	1.7±0.1	1.6±0.1
ENaCα/PGK1 ($\times 10^{-3}$)	1.7±0.1	1.5±0.1	1.8±0.1	1.7±0.1
ENaCβ/PGK1 ($\times 10^{-4}$)	4.5±0.5	4.8±0.5	5.4±0.6	3.8±0.7
ENaCγ/PGK1 ($\times 10^{-4}$)	6.2±1.1	7.6±0.9	8.0±0.8	8.2±0.8

Two Way ANOVA was performed. There were no significant differences. Data are mean values ± SE.

Sc Ang II alone or combined with high salt diet similarly increased mRNA levels of AT_1R in the RVLM (Figure 8). Neither sc Ang II nor 2% salt diet affected mRNA levels of CYP11B1, CYP11B2, 11βHSD2, MR, or ENaCs in the in the RVLM (Table 12).

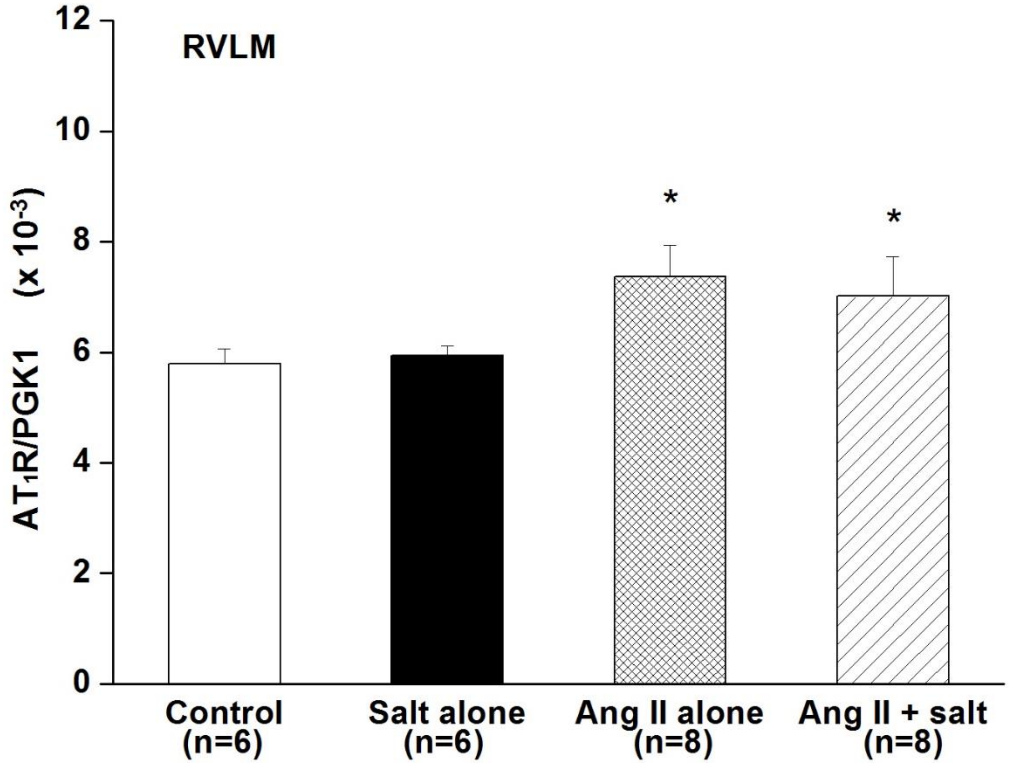

Figure 8. Effects of sc Ang II alone or combined with high salt diet on mRNA expression of AT_1R in the RVLM.

Two Way ANOVA was performed. Factor sc Ang II treatment vs. non-Ang II treatment, F=5.88, *P=0.023.

Table 12. Effects of sc Ang II alone or combined with high salt diet on mRNA expression in the RVLM

	Control (n=6)	Salt alone (n=6)	Ang II alone (n=8)	Ang II + salt (n=8)
CYP11B1/PGK1 ($\times10^{-3}$)	1.1±0.3	1.5±0.6	1.3±0.3	1.4±0.3
CYP11B2/PGK1 ($\times10^{-4}$)	2.3±0.7	3.0±1.0	4.8±1.0	3.9±1.0
11βHSD2/PGK1 ($\times10^{-4}$)	3.9±0.2	4.1±0.2	4.1±0.2	3.9±0.2
MR/PGK1 ($\times10^{-1}$)	1.7±0.1	1.8±0.1	1.5±0.1	1.6±0.1
ENaCα/PGK1 ($\times10^{-4}$)	8.9±0.3	9.1±0.3	9.6±0.5	9.7±0.7
ENaCβ/PGK1 ($\times10^{-4}$)	1.4±0.2	1.4±0.2	1.1±0.3	1.3±0.1
ENaCγ/PGK1 ($\times10^{-4}$)	2.9±0.2	3.2±0.4	4.0±0.7	3.2±0.6

Two Way ANOVA was performed. There were no significant differences. Data are mean values ± SE.

3.5 Effects of central blockades on MAP and HR in rats treated with sc Ang II and high salt diet

In Wistar rats with icv vehicle, sc Ang II at the infusion rate of 150 ng/kg/min combined with high salt diet caused a gradual increase in MAP by 50mmHg. In contrast, icv infusion of eplerenone, spironolactone, benzamil or losartan prevented ~80% of MAP increase caused by sc Ang II combined with high salt diet. There were no significant differences between the groups treated with different blockers (Table 13). Compared with baseline, there was 50mmHg increase of MAP in icv vehicle group by the end of Ang II-salt treatment. In contrast, there was only 10~15 mmHg increase of MAP in groups with icv blockers. The patterns of MAP changes at day and night are similar in all groups (Table 13, Figure 9, Figure 10).

Table 13. Baseline MAP and MAP of last 3 days or nights during Ang II-salt treatment with different central treatments

	Veh (n=11)	Eple (n=5)	Spir (n=4)	Ben (n=6)	Los (n=4)
Day					
Baseline (mmHg)	98±2	94±1	93±2	91±2	96±2
Last 3 days (mmHg)	149±8	107±5	102±3	98±3	102±5
	152±8	106±5	102±2	97±3	102±5
	148±7	104±3	104±0	102±3	104±5
Night					
Baseline (mmHg)	98±2	95±2	97±1	93±2	99±2
Last 3 nights (mmHg)	143±6	110±6	108±3	103±3	108±4
	150±7	110±5	108±1	101±3	109±4
	149±7	105±7	110±3	101±3	109±5

For each rat, average of MAP during the last 3 days or nights was calculated.

One Way ANOVA was performed followed by Tukey's test.

Average of MAP during last 3 days, Veh vs. each blocker, $P<0.001$;

Average of MAP during last 3 nights, Veh vs. each blocker, $P<0.001$;

No significant differences between blockers.

For each individual group, paired t-test was performed to compare the average of MAP during last 3 days or nights with their corresponding baseline.

Veh, average of MAP during last 3 days, $P<0.001$ vs. baseline;

Eple, average of MAP during last 3 days, $P=0.039$ vs. baseline;

Spir, average of MAP during last 3 days, $P=0.001$ vs. baseline;

Ben, average of MAP during last 3 days, $P<0.001$ vs. baseline;

Los, average of MAP during last 3 days, $P=0.291$ vs. baseline;

Veh, average of MAP during last 3 nights, $P<0.001$ vs. baseline;

Eple, average of MAP during last 3 nights, $P=0.049$ vs. baseline;

Spir, average of MAP during last 3 nights, $P=0.001$ vs. baseline;

Ben, average of MAP during last 3 nights, $P=0.001$ vs. baseline;

Los, average of MAP during last 3 nights, P=0.124 vs. baseline.

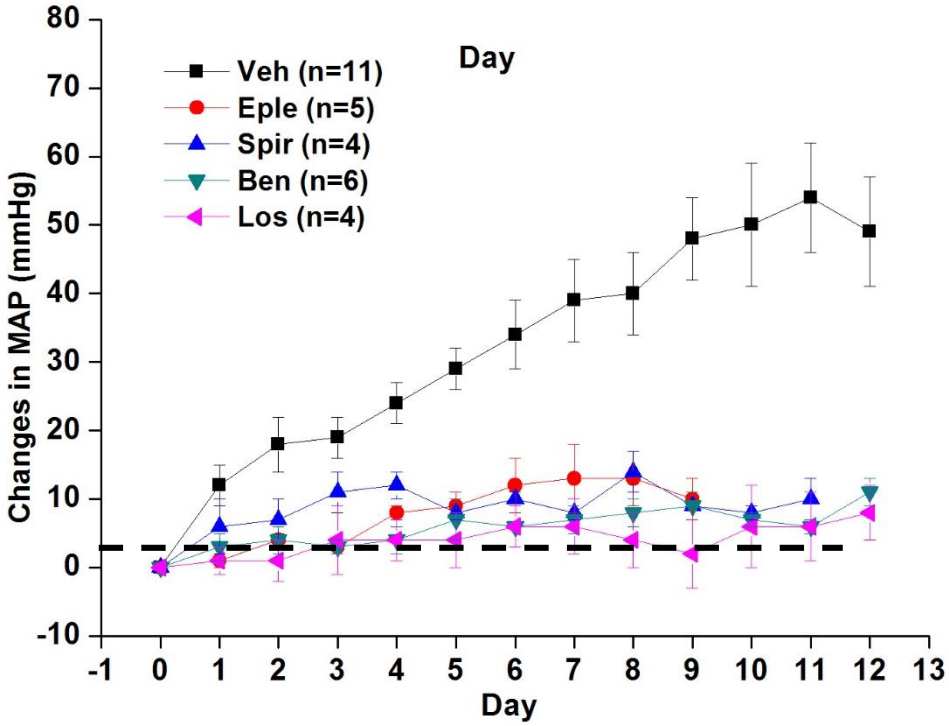

Figure 9. Effects of central blockades on MAP changes in the daytime by Ang II-salt treatment.

One Way Repeated Measures Analysis was performed.

Veh, F=15.9, P=7.10E-19 overall compared with baseline; P<0.05 starting from D1;

Eple, F=5.01, P=2.13E-4 overall compared with baseline; P<0.05 starting from D5;

Spir, F=3.93, P=2.69E-3 overall compared with baseline; P<0.05 starting from D1;

Ben, F=5.29, P=5.38E-6 overall compared with baseline; P<0.05 starting from D6;

Los, F=1.26, P = 0.277 overall compared with baseline.

One Way ANOVA on area under curve was performed.

Veh vs. Eple, P<0.001;

Veh vs. Spir, P<0.05;

Veh vs. Ben, P<0.001;

Veh vs. Los, P<0.001;

No significant differences between blockers.

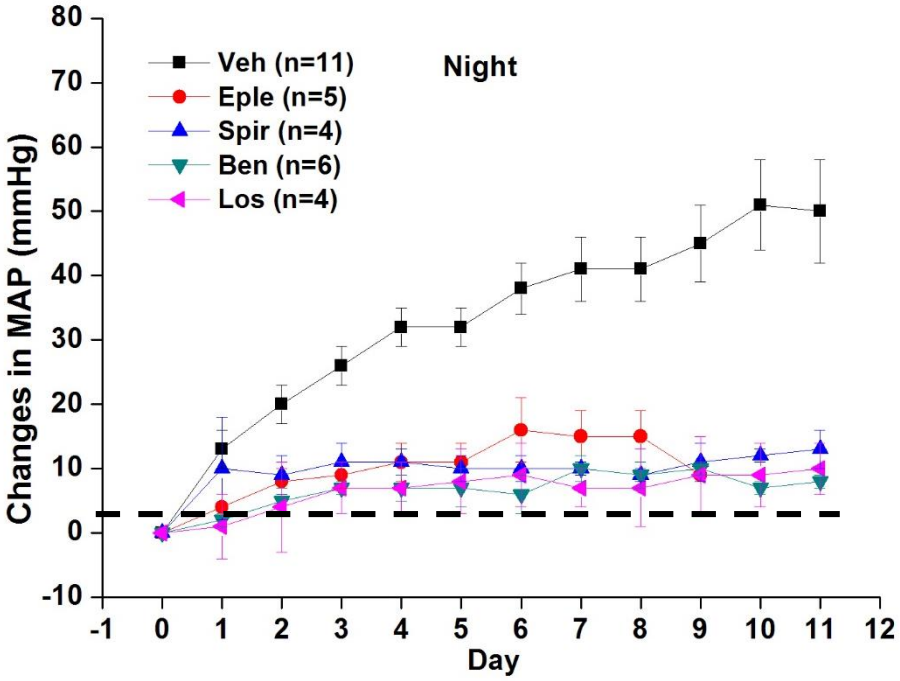

Figure 10. Effects of central blockades on MAP changes at night by Ang II-salt treatment.

One Way Repeated Measures Analysis was performed.

Veh, F=18.2, P=1.45E-19 overall compared with baseline; P<0.05 starting from D1;

Eple, F=4.19, P=9.21E-4 overall compared with baseline; P<0.05 starting from D2;

Spir, F=1.67, P=0.146 overall compared with baseline;

Ben, F=6.11, P=2.00E-6 overall compared with baseline; P<0.05 starting from D2;

Los, F=0.581, P = 0.843 overall compared with baseline.

One Way ANOVA on area under curve was performed.

Veh vs. each blocker group, P<0.001;

No significant differences between blockers.

In rats with icv vehicle, both day and nighttime HR decreased significantly compared with baseline. HR of rats with icv eplerenone, spironolactone and benzamil at day and nighttime or losartan at daytime decreased significantly overall compared with baseline (Figure 11, Figure 12). Nighttime HR of rats with icv losartan was not significantly different compared with baseline (Figure 12). Both day and nighttime HR changes of icv benzamil group were

significantly less compared with vehicle group. There were no significant differences between HR changes of vehicle group and of icv eplerenone, spironolactone, or losartan group.

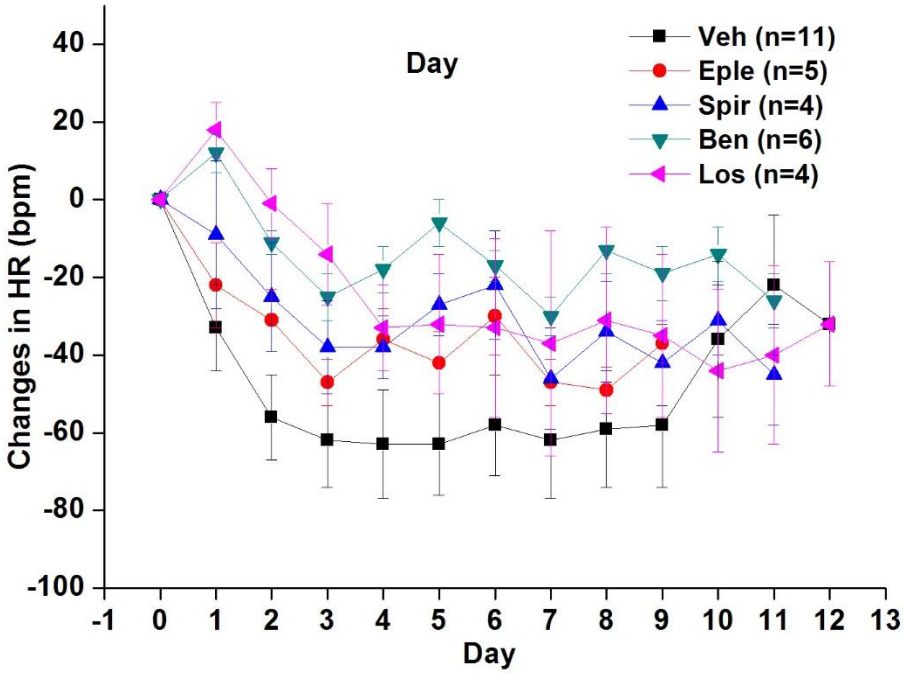

Figure 11. Effects of central blockades on HR changes in the daytime by Ang II-salt treatment.

One Way Repeated Measures Analysis was performed.

Veh, F=4.19, P=2.12E-5 overall compared with baseline;

Eple, F=6.78, P=1.25E-5 overall compared with baseline;

Spir, F=3.82, P=3.25E-3 overall compared with baseline;

Ben, F=6.26, P=5.21E-7 overall compared with baseline;

Los, F=3.85, P=5.36E-4 overall compared with baseline.

One Way ANOVA on area under curve was performed, between groups, F=3.002, P=0.033.

Veh vs. Ben, P=0.028.

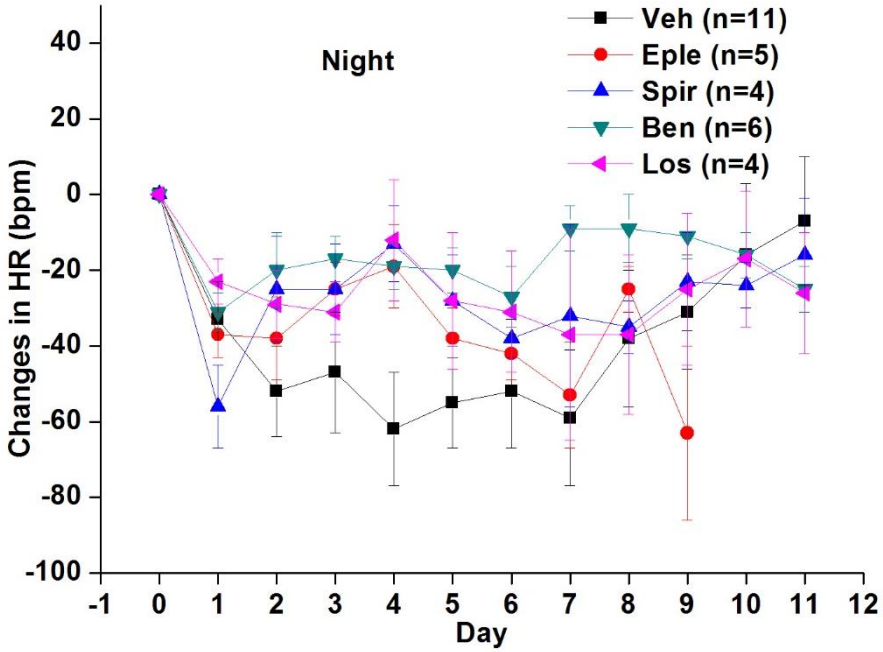

Figure 12. Effects of central blockades on HR changes at night by Ang II-salt treatment.

One Way Repeated Measures Analysis was performed.

Veh, F=4.25, P=1.78E-5 overall compared with baseline;

Eple, F=3.7, P=2.28E-3 overall compared with baseline;

Spir, F=2.77, P=1.93E-2 overall compared with baseline;

Ben, F=3.06, P=2.95E-3 overall compared with baseline;

Los, F=1.10, P=0.387 overall compared with baseline.

One Way ANOVA on area under curve was performed, between groups, F=2.699, P=0.055.

Veh vs. Ben, P=0.042.

3.6 Effects of central blockades on changes in body weight, LV and RV weight, and water intake in rats treated with sc Ang II and high salt diet

Body weight gain of rats with icv vehicle was lower compared with rats receiving central blockade (Table14). The LV weight of rats with icv vehicle was higher than the LV weight of rats with icv eplerenone or benzamil infusion. When all blockers were combined as one group, both the LV weight and RV weight of vehicle group were higher than blockers group (Figure 13, Table 15). There was no significant difference in water intake among rats with different treatments (Table 16).

Table 14. Effects of central blockades on body weight changes of rats with sc Ang II and high salt diet

	Veh (n=11)	Eple (n=5)	Spir (n=4)	Ben (n=6)	Los (n=4)
BW-Initial (g)	319±10	311±3	316±6	303±8	310±1
BW-1 wk (g)	346±12	343±4	344±7	331±8	352±2
BW-final (g)	366±10	376±3	380±8	368±10	377±3
BW gain (g)	47±3*	65±3	64±6	65±4	68±4

One Way ANOVA was performed followed by Tukey's test. BW gain: Veh vs. others, *P<0.05.

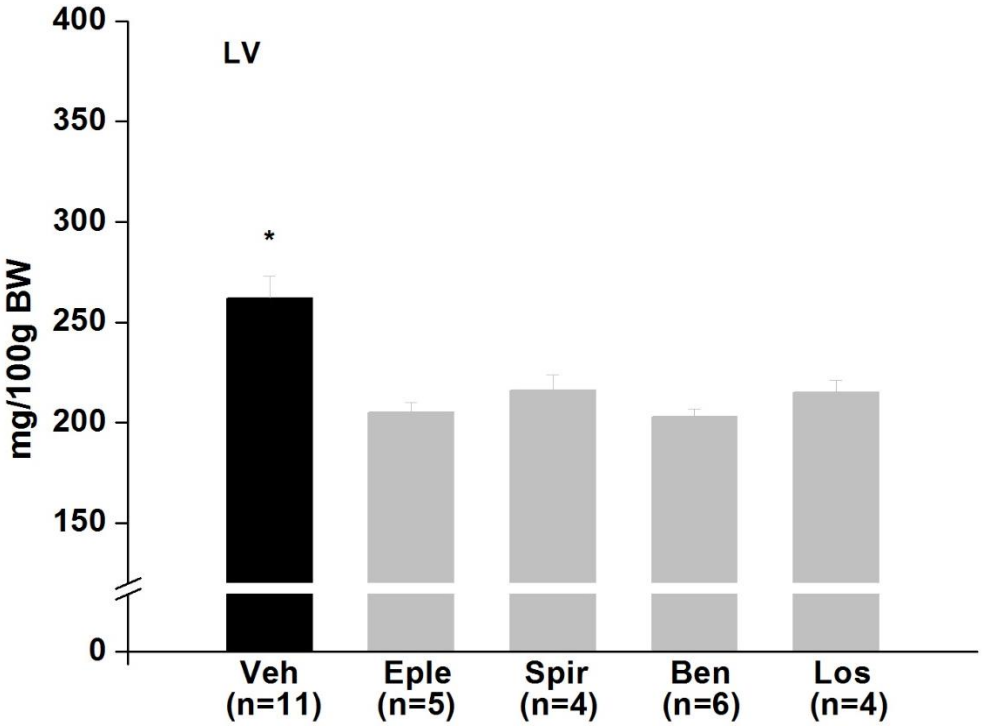

Figure 13. Effects of central blockades on LV weight of rats with sc Ang II and high salt diet

One Way ANOVA was performed followed by Tukey's test.

Eple vs. Veh, $P<0.05$; Ben vs. Veh, $P<0.05$.

Student t-test was performed to compare LV weight between Veh group and all blockers combined.

Veh vs. all blockers combined, *$P<0.001$.

Table 15. Effects of central blockades on RV weight of rats with sc Ang II and high salt diet

	Veh (n=11)	Eple (n=5)	Spir (n=4)	Ben (n=6)	Los (n=4)
RV (mg/100gBW)	57±3*	49±3	51±2	45±2	52±2

One Way ANOVA was performed. There was no significant difference among groups.

Student t-test was performed to compare RV weight between Veh group and all blockers combined.

Veh vs. all blockers combined, *$P<0.05$.

Table 16. Effects of central blockades on water intake of rats at first and second week of Ang II-salt treatment

	Veh (n=11)	Eple (n=5)	Spir (n=4)	Ben (n=6)	Los (n=4)
1st week (ml/100 g BW)	23±4	19±4	18±4	15±1	18±1
2nd week (ml/100 g BW)	21±2	18±2	17±3	18±2	15±2

One Way ANOVA was performed. There was no significant difference among groups. Data are mean values ± SE.

3.7 Effects of central blockades on plasma aldosterone, corticosterone, and Ang II levels in rats treated with sc Ang II and high salt diet

There were no significant differences in plasma Ang II level between rats with icv blockers and rats with icv vehicle (Table 17). Plasma aldosterone level was decreased by blockers compared with vehicle group (Figure 14). Central blockade by MR blockers, ENaC blocker or AT_1R blocker did not affect plasma corticosterone level, as compared with icv vehicle group (Table 17).

Table 17. Effects of central blockades on corticosterone and Ang II levels in plasma in rats with sc Ang II and high salt diet

	Veh (n=11)	Eple (n=5)	Spir (n=4)	Ben (n=6)	Los (n=4)
Plasma corti. (ng/ml)	108±35	83±42	58±17	113±57	53±17
Plasma Ang II (pg/ml)	24.0±15.6	12.1±2.6	26.1±5.0	23.9±1.7	14.2±5.0

One Way ANOVA was performed. There were no significant differences.

Student t-test was performed to compare plasma corticosterone and Ang II levels between Veh group and all blockers combined. There were no significant differences.

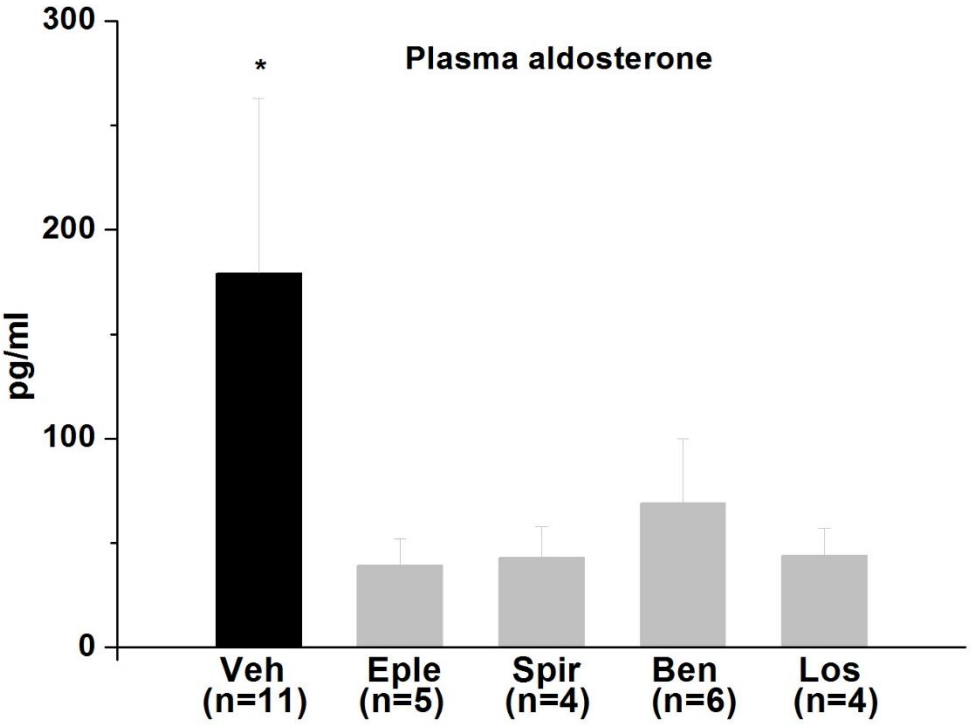

Figure 14. Effects of central blockades on plasma aldosterone in rats with sc Ang II and high salt diet

One Way ANOVA was performed. There were no significant differences.

Student t-test was performed to compare plasma aldosterone level between Veh group and all blockers combined. Veh vs. all blockers combined, *P=0.02.

3.8 Effects of central blockades on mRNA expression in adrenal cortex in rats treated with sc Ang II and high salt diet

Icv eplenerone, spironolactone, benzamil, or losartan each significantly decreased CYP11B2 mRNA level in adrenal cortex by more than 10 fold compared with icv vehicle (Figure 15). Icv blockers decreased mRNA expression of AT_1R in adrenal cortex. There were no significant differences in CYP11B1 mRNA level between rats with icv vehicle and icv blockers (Table 18).

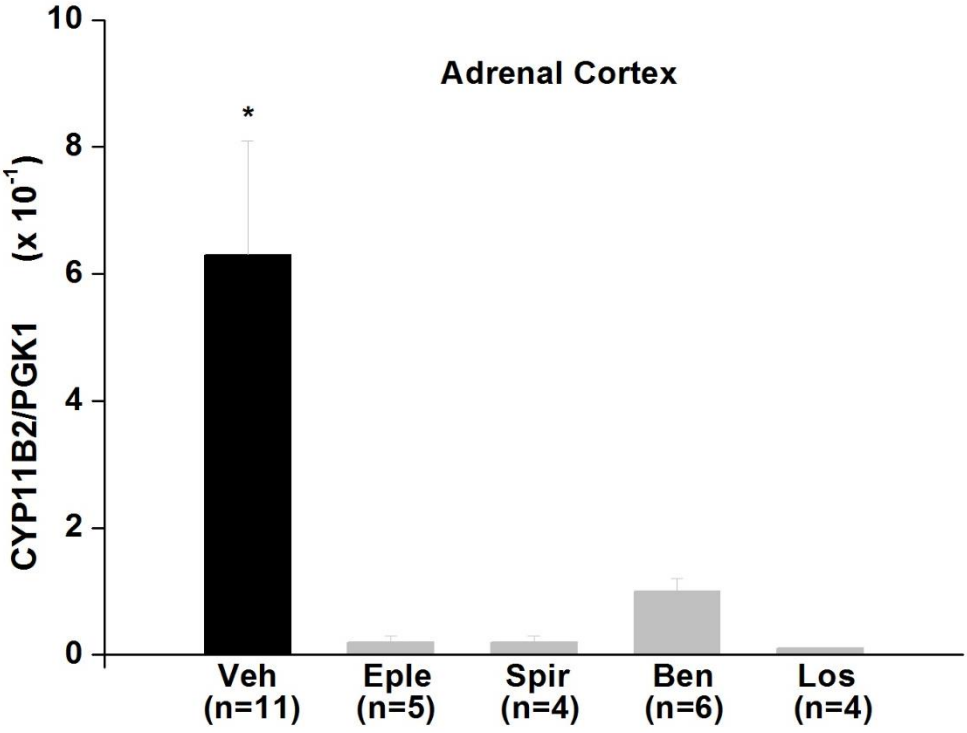

Figure 15. Effects of central blockades on mRNA expression of CYP11B2 in adrenal cortex in rats with sc Ang II and high salt diet

One Way ANOVA was performed followed by Tukey's test.

Eple vs. Veh, P<0.05; Spir vs. Veh, P<0.05; Ben vs. Veh, P<0.05; Los vs. Veh, P<0.05.

Student t-test was performed to compare CYP11B2/PGK1 level between Veh group and all blockers combined. Veh vs. all blockers combined, *P<0.001.

Table 18. Effects of central blockades on mRNA expression of AT_1R and CYP11B1 in adrenal cortex in rats with sc Ang II and high salt diet

	Veh (n=11)	Eple (n=5)	Spir (n=4)	Ben (n=6)	Los (n=4)
AT_1R /PGK1 ($\times 10^{-2}$)	6.0±0.5*	4.4±0.1	4.6±0.3	5.4±0.4	3.7±0.8
CYP11B1/PGK1 (×1)	6.0±0.4	6.3±0.5	6.1±0.4	5.6±0.8	5.5±0.7

One Way ANOVA was performed. There were no significant differences.

Student t-test was performed to compare AT_1R/PGK1 and CYP11B1/PGK1 levels between Veh group and all blockers combined. AT_1R/PGK1: Veh vs. all blockers combined, *P=0.008.

4. Discussion

4.1. Summary of main findings

The present study confirms that Ang II alone at a low infusion rate of 150 ng/kg/min causes only a small increase in BP (112±1 vs. 99±1 mmHg), and 2% salt diet markedly enhances the BP increase induced by this dose of Ang II to 152±4 mmHg. This study shows 3 major new findings. Firstly, BP increases in response to Ang II and salt are largely inhibited by central infusion of eplerenone, spironolactone, benzamil or losartan. Secondly, sc Ang II at 150 ng/kg/min decreases 11βHSD2 and MR mRNA expression in the PVN, but increases AT_1R and ENaC γ mRNA expression in the PVN and AT_1R mRNA in the RVLM. However, the changes of mRNA expression by sc Ang II alone or combined with high salt diet are similar. Other genes tested in the four brain nuclei are not affected by sc Ang II or high salt diet. Thirdly, sc Ang II alone or combined with high salt diet similarly increases CYP11B2 mRNA level in adrenal cortex, whereas high salt diet alone significantly decreases it. Ang II together with high salt diet significantly increases plasma aldosterone, while Ang II alone at this dose does not. As expected, high salt diet tends to decrease plasma Ang II and aldosterone levels.

4.2 BP response to Ang II and high salt diet

The BP response to Ang II and high salt is consistent with previous findings by Osborn et al. (2010). High salt diet significantly enhances the BP response caused by sc Ang II infusion. Icv infusion of AT_1R blocker, MR blocker or ENaC blocker prevents most of the increase in BP (~80%) induced by sc Ang II plus high salt diet. Osborn et al (2014) also reported that icv infusion of the ENaC blocker benzamil prevented the BP increase in

response to sc Ang II and high salt diet. These findings indicate that central AT_1R activation plays an important role in Ang II-salt hypertension, and also suggest that activation of AT_1R depends on aldosterone-MR-ENaC-EO pathway. Sc Ang II at 500 ng/kg/min markedly increases AT_1R and MR in the SFO, PVN and SON (Chen et al., 2014). Icv infusion of AS inhibitor or MR blocker significantly attenuates the increase in neuronal activity in the PVN and significantly attenuates the marked increase of BP by sc Ang II at 500 ng/kg/min. Icv infusion of an AS inhibitor fully prevents the modest BP increase by sc Ang II at 150 ng/kg/min (Huang et al., 2010). Similar mechanisms may apply in Ang II-salt induced hypertension, i.e., the AT_1R activation by Ang II-salt likely depends on activation of the MR-ENaC-EO pathway in the brain. The remaining ~10mmHg increase in BP may depend on other mechanisms, such as peripheral actions on arteries and/or kidneys elicited by circulating Ang II and high salt intake (see 4.5).

The BP response to sc Ang II alone at 150 ng/kg/min is smilar to what was reported by Huang et al., 2010 and Osborn et al., 2010. In other studies, e.g., King et al., 2008, Nunes et al., 2011 and Wu et al., 2000, larger increases in BP by sc Ang II at the same dose in rats were reported. There are some possible reasons to explain this discrepancy. (1)The amount of infused Ang II depends on the way of calculation. In the current study, 150 ng/kg/min was based on the initial body weight of 300g, but as the rats grow, the final body weight can reach 360~380g. (2) Difference in the age of rats can cause different response since the sensitivity of rats to Ang II is age dependant. Central responses to Ang II attenuated with age (Erdos et al., 2010). (3) Usually the level of potassium in diet is not reported. High potassium diet has vascular relaxation effect, probably by affecting the activities of K+ channel and the Na-K-ATPase in blood vessels (Dolson et al., 1995; Murphy et al., 1999).

In addtion, there are other factors that may affect the BP changes in response to Ang II, such as gender of rats (Toering et al., 2015), strain of rats (Herin et al., 2003), model of minipumps (Kuroki et al., 2014).

4.3 Plasma Ang II and aldosterone responses to sc Ang II and high salt diet

Consistent with published results (Huang et al., 2010), sc Ang II alone at the dose of 150 ng/kg/min does not increase plasma Ang II or aldosterone, whereas high salt diet alone tends to decrease plasma Ang II and aldosterone. The effects of sc Ang II alone on plasma aldosterone are dose dependent. Sc Ang II at 200 ng/kg/min caused ~1.5-fold increase of plasma aldosterone, and sc Ang II at 500 ng/kg/min increased plasma Ang II by 4-fold and plasma aldosterone by more than 20-fold (Huang et al., 2010; Ye et al., 2003). A number of studies have shown that high salt diet suppresses plasma Ang II and aldosterone level in rats (Carillo et al., 2007; Crestani et al., 2014; Liang et al., 2007; Nagata et al., 2010; Thomson et al., 2006).

With regards to the effects of high salt diet alone or Ang II alone on CYP11B2 mRNA expression in adrenal gland, our results are as expected from previous studies. In our study, high salt diet (2%) alone decreased CYP11B2 mRNA level to ~1/4 and sc Ang II alone at 150 ng/kg/min increased it by ~3-fold. Consistently, Ye et al (2003) showed that high salt diet (3%) reduced adrenal CYP11B2 mRNA to 1/5 of control level, and sc Ang II infusion alone at 200 ng/kg/min significantly increased CYP11B2 mRNA level (Ye et al., 2003). Sc infusion of Ang II at 200 ng/kg/min increased CYP11B2 mRNA in adrenal cortex and increased plasma aldosterone by 2-fold in Sprague-Dawley rats (Peters et al., 1998).

Treatment of H295R cells (human adrenal cortex cell line) with Ang II at 10 nM for 6 hours caused an elevation of CYP11B2 mRNA level by ~7-fold, AS protein level by 2-fold, and AS activity and aldosterone by 3.5-fold (Rajamohan et al., 2012).

Our study shows that Ang II alone or Ang II-salt similarly increased CYP11B2 mRNA level in adrenal cortex but for Ang II-salt from a lower baseline versus high salt alone. Surprisingly, only sc Ang II at 150 ng/kg/min plus 2% salt diet significantly increased plasma aldosterone level. Previous studies related to the combined effect of Ang II and high salt on aldosterone level showed variable results. In rats with iv infusion of Ang II at 10 ng/min (~30 ng/kg/min) plus high salt infusion at 6 mmol/day, there was a significant BP increase of 55 mmHg by day 8 accompanied by a upward trend in plasma aldosterone, though the increase was not significant (Kanagy et al., 1990). Iv infusion of Ang II at 60 ng/min in Wistar rats on 0.66% salt diet for 12 days increased plasma aldosterone, but 8% salt diet reduced the increase of aldosterone by Ang II to control level (Ando et al., 1991). In this study, salt loading is at a very high level of 8%, which may exert a strong inhibitory effect and offset the action of Ang II on aldosterone.

The dissociation between adrenal CYP11B2 mRNA levels and plasma aldosterone levels in response to sc Ang II alone or combined with high salt may suggest that an increase in CYP11B2 mRNA level does not necessarily reflect an increase in enzyme activity. There are studies that show increased plasma aldosterone without an increase in CYP11B2 mRNA by certain stimuli, or vice versa. Sc Ang II infusion at 9 μg/h in lean Zucker rats increased plasma aldosterone by 2-fold, whereas adrenal CYP11B2 mRNA remained unaffected (Müller-Fielitz et al., 2012). Even for studies that show increases of CYP11B2 mRNA, protein, and aldosterone, the extent to which each was increased is not the same in

most cases. For example, in Rajamohan's study, there was a 6.8-fold elevation in CYP11B2 mRNA, 2-fold in AS, and 3.5-fold in AS activity and aldosterone by Ang II at 10 nM for 6 hours in H295R cells, as mentioned above (Rajamohan et al., 2012). This might be explained by changes in post-transcriptional regulation, e.g., the degradation of mRNA mediated by other intracellular regulatory molecules (Berk et al., 2000; Raff et al., 2002). For example, miR-10b is a negative post-transcriptional regulator of CYP11B2 gene and inhibits aldosterone production in H295R cells (Nusrin et al., 2014). Thus, quantifying also AS protein and enzyme activity may provide further insights.

Icv infusion of AT_1R blocker, MR blocker, or ENaC blocker significantly decreased mRNA level of CYP11B2 in adrenal cortex by more than 10-fold and significantly decreased plasma aldosterone level. These results suggest that circulating Ang II and high salt diet, through activation of the CNS MR-ENaC-AT_1R pathway, increases CYP11B2 expression and aldosterone synthesis in the adrenal cortex. Sc Ang II infusion at 200 ng/kg/min increased plasma aldosterone and BP in rats, both were significantly inhibited by bilateral infusion of AT_1R blocker losartan in the PVN, which suggests the central control of aldosterone synthesis by AT_1R activation in the PVN (Qi et al., 2013). Icv Ang II at 2.5 or 12.5 ng/min significantly increased plasma aldosterone, and icv AS inhibitor FAD286 or MR blocker eplerenone inhibited 50% of the increase induced by icv Ang II, indicating the control of adrenal aldosterone synthesis by CNS with MR- AT_1R activation (Huang et al., 2013). In rats on a high salt intake at 7.5 mmol/day by iv infusion, icv Ang II at 100 ng/min for 5 days significantly elevated plasma aldosterone by ~6.5-fold compared to icv saline control group (Bruner et al., 1985).

There are several possible CNS mechanisms that may regulate CYP11B2 expression in adrenal cortex and plasma aldosterone in response to sc Ang II and high salt intake. ACTH is not likely since ACTH mainly causes an acute increase of aldosterone secretion (Fuchs-Hammoser et al., 1980; Holland et al., 1993), and in our results CYP11B1 mRNA and plasma corticosterone were not increased by high salt diet or sc Ang II. Consistently, a previous study showed that there were no significant effects on plasma corticosterone and CYP11B1 mRNA level in adrenal by 3% salt diet or by sc Ang II at 200 ng/kg/min (Ye et al., 2003).

Secondly, an increase in SNA by Ang II and high salt diet may play a role. Many studies have reported elevated sympathetic activity by sc Ang II together with high salt (King et al., 2006; King et al., 2008; King et al., 2007). In rat glomerulosa cells, the beta adrenergic receptor (β_2AR) agonist isoproterenol markedly increased aldosterone production, while the β_2AR antagonist ICI-118 551 reduced isoproterenol-stimulated aldosterone secretion to baseline level. ICI-118 551 also significantly attenuated aldosterone production stimulated by Ang II (Pojoga et al., 2006). In isolated perfused pig adrenals, both splanchnic nerve activation and epinephrine perfusion induced a significant aldosterone release. In cultured bovine adrenocortical cells, the βAR antagonist propranolol completely blocked the steroidogenesis stimulated by epinephrine incubation (Ehrhart-Bornstem et al., 1995). In cultured bovine adrenal subcapsular cells, isoproterenol, epinephrine or norepinephrine elicited a 4- to 6-fold aldosterone secretion. Isoproterenol stimulated aldosterone secretion was significantly inhibited by the βAR antagonist alprenolol (Léan et al., 1984). In vivo, elevated plasma aldosterone level in untreated hypertensive stroke-prone rats was significantly decreased by β-blocker propranolol (Barone et al., 1996). In

humans, plasma aldosterone level was decreased by treatment with β-blocker nebivolol (Giles et al., 2015).

Thirdly, increased plasma EO level by Ang II or together with high salt may contribute to the enhanced aldosterone level. Chronic central infusion of Ang II markedly increases plasma EO and aldosterone, and central blockade by MR blocker prevents the increase of plasma EO and aldosterone by chronic icv Ang II (Huang et al, 2013; Hamlyn et al, 2014). Sc ouabain at 10 and 30 μg/kg/day induced a 2.9 and 7-fold increase in plasma aldosterone, respectively (Manunta et al., 1994). These findings suggest that Ang II and high salt may act centrally to induce EO release from pituitary and thereby may stimulate aldosterone production from adrenals.

The mean value of plasma aldosterone level in Ang II-salt group (Figure 3) is not consistent with the value in vehicle group in central blockade study (Figure 14). Firstly, it might be explained by different conditions for aldosterone assay. These two assays were done at different time separately, therefore the results can be different. Secondly, it could be because of the different responses of rats. In the first study, the BP of most of the rats in Ang II-salt group is similar (around 150 or 160 mmHg), while in the central blockade study, the BP of rats in vehicle group varies largely and ranges from 120 to 160 mmHg generally. There is a close but non-linear correlation between plasma aldosterone and BP (data not shown). When BP is below 150 mmHg, plasma aldosterone tended to change at a narrow range. In contrast, when BP goes from 150 to 170 mmHg or even higher, plasma aldosterone can be 10-fold higher.

In adrenal cortex, high salt diet, or sc Ang II, or the two together had no significant effect on mRNA levels of AT_1R, but central blockade in rats treated with Ang II and salt

significantly decreased mRNA expression of AT_1R in adrenal cortex, which is difficult to interpret. Ang II may increase adrenal AT_1R expression at high doses. In Wistar rats treated with 1% salt diet plus drinking saline for 1 week, the binding of AT_1R by Ang II in adrenal zona glomerulosa was modestly less compared with rats with 1% salt diet alone, whereas the binding in inner zones was significantly increased by salt loading (McNeill et al., 2000). Sc Ang II at 80 ng/min in Sprague-Dawley rats increased $AT_{1A}R$ mRNA level in adrenal (Harrison-Bernard et al., 1999). Further studies on the role of adrenal AT_1R signaling for aldosterone synthesis induced by Ang II together with high salt will be helpful for understanding the mechanisms of the increase in plasma aldosterone by Ang II-salt.

4.4 mRNA expression of genes in aldosterone-MR-ENaC-EO-AT_1R pathway in brain nuclei

The combined effects of Ang II and high salt on AT_1R expression in brain nuclei have not been studied before. In the current study, neither high salt diet nor sc Ang II had effects on mRNA levels of AT_1R in the SFO or SON. In the PVN and RVLM, sc Ang II alone or combined with high salt diet similarly increased mRNA levels of AT_1R. The increased AT_1R mRNA abundance in the PVN by sc Ang II suggests the activation of angiotensinergic pathways in the brain. Sc Ang II at 500 ng/kg/min significantly increased AT_1R expression in SFO, PVN, and SON (Chen et al., 2014). A number of studies have shown that increased AT_1R in the PVN contributes to sympathetic hyperactivity in rats (Chen et al., 2011; Huang et al., 2014; Qi et al., 2013). Sc Ang II infusion at 200 ng/kg/min increased AT_1R in the PVN and BP in rats, both were significantly inhibited by bilateral infusion of AT_1R blocker losartan in the PVN, suggesting that AT_1R activation in the PVN

contributes to sc Ang II induced hypertension (Qi et al., 2013). Similarly, intra-PVN infusion of AT_1R blocker candesartan fully reversed the BP increase caused by sc Ang II at 500 ng/kg/min in rats (Gabor et al., 2013). Previous studies have revealed the important role of AT_1R in the RVLM in contributing to hypertension. In two-kidney, one-clip (2K-1C) hypertensive Wistar rats, AT_1R protein level in the RVLM was significantly higher compared with sham rats, and bilateral injection of losartan into the RVLM reduced MAP and RSNA in 2K-1C rats, indicating that AT_1R activation in the RVLM is critical for sympathetic hyperactivity and hypertension in this model (de Oliveira-Sales et al., 2010).

In the present study, neither high salt diet nor sc Ang II affects corticosterone level in hypothalamus and hippocampus. In the SFO, PVN, SON or RVLM, CYP11B1 mRNA level is not affected by high salt diet or sc Ang II. Consistently, sc Ang II at 200 ng/kg/min or different salt diet had no effect on CYP11B1 mRNA level in brain tissues, as measured in hippocampus, cerebellum, hypothalamus and brain stem (Ye et al., 2003). These results suggest an insignificant effect on corticosterone in the CNS by sc Ang II, high salt or the two together. In contrast, icv Ang II at 2.5 or 12.5 ng/min increased corticosterone in the hypothalamus as well as plasma corticosterone, and icv FAD286 inhibited the increase in plasma corticosterone by ~50% without significantly affecting hypothalamic corticosterone (Huang et al., 2013). 8% salt diet in Dahl R rats significantly decreased corticosterone in hypothalamus whereas high salt tended to increase that in Dahl S rats (Huang et al., 2009). Icv Na^+-rich aCSF in Wistar rats for 2 weeks significantly increased corticosterone in the hypothalamus but did not affect CYP11B1 mRNA level in the SFO, PVN or SON, and icv FAD286 had no effect on hypothalamic corticosterone (Huang et al., 2008; Wang et al., 2010).

Sc Ang II together with high salt diet tended to increase aldosterone level in hypothalamus and hippocampus. In the SFO, PVN, SON or RVLM, CYP11B2 mRNA level is not significantly affected by high salt diet or sc Ang II. Sc Ang II alone at 200 ng/kg/min or 3% salt diet alone also had no effect on CYP11B2 mRNA level in hippocampus, cerebellum, hypothalamus or brain stem (Ye et al., 2003). Similar as for corticosterone, the actions of central Ang II or Na^+ on brain aldosterone are different from actions of circulating Ang II or Na^+. Icv Ang II at 2.5 or 12.5 ng/min increased aldosterone in hypothalamus as well as in plasma, and icv FAD286 attenuated the Ang II induced aldosterone increase in hypothalamus and plasma by ~60% and ~50%, respectively (Huang et al., 2013). 8% salt diet for 4 weeks increased hypothalamic aldosterone by ~35% in Dahl S rats and icv FAD286 prevented the increase. In contrast, high salt diet in Dahl R rats decreased aldosterone by ~65% in hypothalamus as well as in hippocampus (Huang et al., 2009). Icv Na^+-rich aCSF in Wistar rats for 2 weeks significantly increased aldosterone in hypothalamus but did not affect CYP11B2 mRNA level in the SFO, PVN or SON, and icv FAD286 prevented aldosterone increase (Huang et al., 2008; Wang et al., 2010). Together with the results on corticosterone, these findings suggest that a minor increase in plasma Ang II by sc infusion of Ang II at 150 ng/kg/min with high salt diet may not increase brain aldosterone or corticosterone.

In the SFO, SON or RVLM, neither high salt diet nor sc Ang II had effects on mRNA levels of 11βHSD2 or MR. However, mRNA levels of 11βHSD2 and MR in the PVN are decreased by sc Ang II alone or combined with high salt, which is opposite to the reported increase of MR mRNA and protein by sc Ang II at high dose of 500 ng/kg/min (Chen et al., 2014). The differences in dose of Ang II and model may explain this difference. In

Chen's study, Ang II was infused at 500 ng/kg/min and rats were on normal salt diet (Chen et al., 2014). The abundance of corticosterone in the brain is higher than aldosterone, and it has equal affinity to MR (Gomez-Sanchez et al, 2005; de Kloet et al, 2000). 11βHSD2 converts corticosterone to an inactive metabolite to increase the binding of aldosterone to MR (Geerling et al, 2006). Inhibition of 11βHSD2 in the PVN increased BP and renal SNA in Sprague-Dawley rats (Zhang et al., 2006), which give us the hint that in our study the decreased mRNA level of 11βHSD2 may result in activation of MR through increased binding of corticosterone to MR.

MR activation in turn can increase ENaC expression and activity (Masilamani et al., 1999). In the PVN, sc Ang II alone or combined with high salt diet similarly increased mRNA level of ENaC γ. In the SFO, SON, and RVLM, neither sc Ang II nor high salt affect mRNA expression of ENaC subunits. The increased ENaC γ mRNA in the PVN may indicate increased epithelial sodium channel activity on cell membrane. ENaC activation may increase EO production, which activates the α_2 Na^+ pump signaling and regulates the protein expression of ACE and AT_1R in the PVN induced by aldosterone (Huang et al., 2011). In addition, ENaCs have been implicated in controlling VP secretion. MR and all three subunits of ENaC are colocalized with VP in VP synthesizing magnocellular neurons in the SON and PVN. Benzamil induced membrane hyperpolarization in most of VP neurons in spontaneously firing and quiet cells, which suggests the involvement of ENaCs in regulating VP secretion by affecting the firing patterns of magnocellular neurons (Teruyama et al., 2012). However, the effect of sc Ang II or high salt on ENaC expression and activity in brain nuclei remains poorly understood.

mRNA expression in brain nuclei was similarly affected by Ang II alone or by Ang II-salt.

However, the mRNA abundance of some of the genes is low in brain nuclei and may not reflect the protein expression. Assessment of protein level, enzyme activity and neuronal activity may provide further insights in understanding the role of the CNS in Ang II and high salt induced hypertension.

4.5 Peripheral responses to Ang II and high salt diet

In kidney cortex, the mRNA levels of AT_1R, MR or ENaCs were not affected by high salt diet or sc Ang II. In kidney medulla, the mRNA expression of AT_1R and ENaC α subunit was similarly decreased by high salt diet alone or with Ang II, which suggests less sodium reabsorption.

Decreased mRNA expression of AT_1R in kidney by 3% high salt in Wistar rats was also reported (Jo et al., 1996). In agreement with our results, Stokes reported that 8% salt diet in Wistar rats decreased the mRNA level of ENaC α but not ENaC β or ENaC γ in inner medulla of kidney to 1/4 of the level in rats receiving 0.13% salt diet, whereas dietary salt had no significant effect on all 3 subunits of ENaC in cortex and outer medulla regions (Stokes et al., 1998). Similarly, 8% salt diet in Dahl salt-resistant rats significantly decreased the mRNA level of ENaC α in kidney compared with rats receiving 0.3% salt without affecting ENaC β or ENaC γ (Aoi et al., 2007). 8% salt diet in Dahl salt-resistant rats did not affect mRNA expression of ENaC subunits in inner medullary collecting duct, but decreased apical staining of ENaC β (Amin et al., 2011).

In the current study, the mRNA expression of AT_1R, MR or ENaC three subunits in the kidney were not affected by sc Ang II. The low dose of sc Ang II at 150 ng/kg/min in our study did not significantly increase plasma Ang II, which may explain the unaffected renal

mRNA expression. When higher doses of Ang II were used, increased mRNA or protein expression of ENaC α in kidney was reported in mice infused with Ang II at a dose of 2,500 ng/kg/min and in Sprague-Dawley rats at 400 ng/kg/min or 80 ng/min (Beutler et al., 2003; González et al., 2009; Wakui et al., 2010). Studies have shown that Ang II directly activates ENaCs in the cortical collecting duct via AT_1R (Peti-Peterdi et al., 2002; Sun et al., 2012).

It is well documented that Ang II can cause renal sodium and water reabsorption. Sc Ang II at 80 ng/min (~300 ng/kg/min) in Sprague-Dawley rats significantly elevated plasma Ang II and intrarenal Ang II, which can be from internalization of circulating Ang II or local Ang II production, each accounting for approximately half of the total renal Ang II level. Studies in transgenic mice highlight the role of renal AT_1R in BP regulation. Compared with wild-type mice in response to sc Ang II at 1000 ng/kg/min, BP was similarly attenuated in renal AT_1aR deficiency mice and in both renal and systemic AT_1aR deficiency mice, indicating the critical role of kidney in hypertension induced by Ang II (Crowley et al., 2006). Li and Gurley further demonstrate that AT1aRs specifically in renal proximal tubule regulate BP and promote hypertension by studies in transgenic mice with AT1R overexpression or deletion in renal proximal tubule in response to sc Ang II at 800 ng/kg/min and 1000 ng/kg/min, respectively (Gurley et al., 2011; Li et al., 2011). However, all these studies used higher doses of Ang II than were used in the present study, and were performed on regular salt. When Ang II is administrated at the low dose of 200 ng/kg/min, aldosterone level and the reabsorption of sodium and water are not significantly changed in Sprague-Dawley rats (Lachance et al., 1988). Therefore, sc Ang II at a low dose in our study may not act on kidney but whether it interacts with high salt to synergistically affect

renal mechanisms is not clear.

Ang II causes vasoconstriction via AT_1R activation in vascular smooth muscle cells (VSMCs), stimulating G-protein dependent pathways and consequently elevating intracellular calcium concentration. Vavrinec reported that Ang II at 30 ng/kg/min for 10 minutes caused significant vasoconstriction of afferent and efferent glomerular arterioles, and AT_1R expression was positively correlated with glomerular arterioles responses (Vavrinec et al., 2012). Recently, Sparks reported that in mice with $AT_{1A}R$ deletion in VSMCs, the BP in responses to sc Ang II at 1000 ng/kg/min was significantly attenuated in KO mice, associated with increased urinary Na^+ excretion. Compared with control mice that had 65% and 89% reduction of renal blood flow induced by sc Ang II at 0.1 μg/kg and 0.3 μg/kg, respectively, there was only ~7% decrease in responses to both doses in KO mice. This study highlights the significant role of $AT_{1A}R$ in VSMCs in regulating renal vascular and BP responses to Ang II (Sparks et al., 2015). High salt increases AT_1R mRNA and density in VSMCS and enhances the Ang II stimulated responses in VSMCs as pre-incubation of VSMCs with high concentration of NaCl for 24h significantly increased the angiotensin II induced intracellular calcium response (Nickenig et al., 1998).

To conclude, Ang II and high salt may act on the kidney in our study. However, this effect is not the primary response induced by Ang II since blockers in the brain inhibit most of the increase in BP. Nevertheless, peripheral actions may amplify the central effect while on its own the peripheral effect is minor.

4.6 Sensitivity of qPCR assay

The Ct values for the 0.0001 pg/μl standards are at the range of 31~33. The qPCR is

specific and sensitive enough to detect low copy numbers down to 20 copies/μl of the target gene. For most of the genes studied, the Ct values fall into the range of Ct values of standards. An analysis of melting curve was also performed to confirm the specificity of the PCR amplification. The mRNA abundance of CYP11B2 and ENaC β in brain nuclei is low with the Ct values at 29~33 and at 30~32, respectively, but are still mostly in detectable range. Both samples with the melting curve that is not a single clear sharp peak and samples with high Ct values were excluded in data analysis.

4.7 Conclusion

AT_1R mRNA expression is increased in both PVN and RVLM, while central blockade by AT_1R blocker markedly prevents the increases in BP induced by Ang II-salt, indicating that AT_1R activation in the brain plays a critical role in Ang II-salt induced hypertension. Similar to Ang II alone induced hypertension, this AT_1R activation by Ang II-salt may depend on activation of the MR-ENaC-ouabain pathway in the brain. The gene expression profile in the brain of rats with Ang II salt hypertension is similar to that of hypertension induced by Ang II alone. However, other components (e.g. ROS) or neuronal activities were not assessed, and may have changed in a different pattern, leading to higher SNA and plasma aldosterone.

4.8 Limitations

mRNA expression of relevant genes in AT_1R-aldosterone-MR-ENaC-EO pathway in major cardiovascular regulatory brain nuclei (SFO, PVN, SON, RVLM) was evaluated. However, the actual enzyme levels and activities are not necessarily reflected by mRNA level. In addition, the effects of central blockade on BP responses as well as plasma aldosterone

levels were assessed by icv infusion of blockers. In order to get more information on the specific pathways involved, microinjection of blockers in target brain nuclei can be further studied.

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