ROLE OF CENTRAL EICOSANOIDS IN THE DEVELOPMENT OF ANGIOTENSIN II-SALT HYPERTENSION IN THE RAT

By

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ABSTRACT

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Human essential hypertension (HTN) causes end organ damage, cardiovascular disease and premature death. While increased sympathetic nerve activity (SNA) is a principal risk factor for the development of HTN, the central mechanisms that drive high sympathetic outflow remain unclear. Increased SNA and blood pressure (BP) can be triggered by high salt intake (2% NaCl) and the hormone angiotensin II (AngII). Our lab has extensively studied a rat model of HTN caused by these two factors in combination (AngII-salt HTN), and have shown that SNA contributes to the HTN and blocking whole-body eicosanoid (prostaglandin, thromboxane, lipoxygenase) synthesis with cyclooxygenase (COX) inhibitors attenuated both the increased SNA and HTN development. My project was to determine how eicosanoid products contribute to AngII-salt HTN. In initial experiments, I showed that whole-body COX inhibition failed to reverse established AngII-salt HTN in rats (similar to human hypertensives). In contrast, COX inhibition only during the first several days of 14-day AngII-salt treatment successfully prevented subsequent HTN development and sympathetic support of BP (i.e. neurogenic pressor activity). I concluded that COX products exert important physiological effects only during the early phase. I next investigated the roles of the two isoforms of COX and found that COX-1 specific products drive the development of AngII-salt HTN. Because most eicosanoid products in peripheral tissues (blood vessel,

kidney) lower BP, I decided to test if central (i.e. brain) eicosanoids, acting early in the process of AngII-salt HTN development, cause a long-lived increase in SNA and BP. I chronically administered the COX inhibitor into the brain at a dose designed to block COX in the brain, and showed that this prevented increase in SNA and HTN development. I concluded that one or more COX-1 products in the brain contribute to HTN development. I also performed PCR and Western blot analysis of COX pathway-associated gene and protein expression in known cardio-regulatory regions of the brain. This revealed only modest changes for the most part, but I observed significant changes in the prostaglandin D2 (PGD2) pathway (downstream from COX) in the OVLT (cardio-regulatory brain region), choroid plexus (CP) and cerebrospinal fluid (CSF). Importantly, I showed increased lipocalin-prostaglandin D synthase (L-PGDS) expression in the CP and CSF, the main sites of L-PGDS synthesis and secretion, respectively. These findings were the first ever to implicate brain PGD2 in HTN. Thus, to investigate this finding in more detail, I measure brain levels of PGD2 with mass spectrometry and found high levels in CSF and rostral ventrolateral medulla (RVLM) of HTN rats. PGD2 in the brain binds mainly to the G-protein coupled receptor DP1R. Immunofluorescence staining revealed down-regulation of DP1R in the RVLM during the early phase of AngII-salt HTN; perhaps predictable in the presence of increased agonist concentration. Finally, blockade of L-PGDS prevented the increase in PGD2 levels in the RVLM during the early phase of AngII-salt treatment and attenuated subsequent HTN development. In conclusion, the results of my studies suggest a novel mechanism for neurogenic HTN development: PGD2 generated in the brain from L-PGDS acts on DP1R in the RVLM, which ultimately leads to increased SNA, neurogenic pressor activity and HTN.

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KEY TO ABBREVIATIONS

AA	Arachidonic acid
AngII	Angiotensin II
AP	Area postrema
AT1R	Angiotensin II receptor type 1
BP	Blood pressure
CG	Celiac ganglion
c-PGES	Cytosolic prostaglandin E synthase
cPLA2	Cytosolic phospholipase A2
CO	Cumulus oophorus cell
CP	Choroid plexus
CRTH2	Chemoattractant receptor homologous receptor molecule expressed on T helper 2 cells (or DP2)
CSF	Cerebrospinal fluid
CVO	Circumventricular organ
DBP	Diastolic blood pressure
DP1	Prostanoid D receptor-1
DP2	Prostanoid D receptor-2 or (CRTH2)
EP1-4	Receptor subtypes for prostaglandin E2
FP	Prostaglandin F receptor
H-PGDS	Hematopoietic prostaglandin D synthase
HR	Heart rate
HTN	Hypertension

ICV	intracerebroventricular
im	intramuscular
IML	Intermediolateral cell column
ip	intraperitoneal
L-PGDS	Lipocalin type prostaglandin D synthase
LOX	Lipoxygenase
LT	Leukotriene
LTR	Leukotriene receptor
MAP	Mean arterial pressure
mPGES-1	Microsomal prostaglandin E synthase-1
mPGES-2	Microsomal prostaglandin E synthase-2
NTS	Nucleus tractus solitaries
OVLT	Organum vasculosum lamina terminalis
PL	Phospholipid
PGD2	prostaglandin D2
PGE2	prostaglandin E2
PGF2α	prostaglandin F2α
PGFS	Prostaglandin F synthase
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PGI2	prostaglandin I2 or prostacyclin
PGIS	Prostacyclin (Prostaglandin I2) synthase
PGT	Prostaglandin transporter

PVN	Paraventricular nucleus
RAS	Renin angiotensin system
RVLM	Rostral ventrolateral medulla
SBP	Systolic blood pressure
sc	subcutaneous
SFO	Subfornical organ
SNA	Sympathetic nerve activity
SNS	Sympathetic nervous system
SSNA	Splanchnic sympathetic nerve activity
TP	Thromboxane receptor
Tx	Thromboxane
TS	Thromboxane synthase
VSMC	Vascular smooth muscle cell

CHAPTER 1

INTRODUCTION

1. Hypertension

High blood pressure, medically termed hypertension (HTN), is a principal risk factor for cardiovascular diseases. A recent report published in 2013 on the occasion of World Health Day by the World Health Organization (WHO) addresses HTN as “a global public health problem” and a “*silent killer”* (1). One in three Americans adults over 18 years of age is hypertensive (196). HTN is a modifiable risk factor that predisposes to the development of cardiovascular and renal diseases; it is the major factor responsible for myocardial infarction (69%), stroke (77%), and chronic heart failure (74%) (99). In the US and the world, healthcare costs due to the impact of HTN have surpassed billions when considering direct medical expenses and lost productivity (101). Patients who are clinically diagnosed with HTN are prescribed multiple drugs like diuretics, angiotensin receptor inhibitors, angiotensin converting enzyme inhibitors etc., to control their blood pressure (BP) and prevent multiple organ damage. Lifestyle modifications suggested to reduce blood pressure include a nutritionally balanced diet low in sodium and fat content along with regular exercise. In particular, it has been suggested that lowering sodium intake population-wide from 3,300 mg to 2,300 mg per day could reduce the number of cases of high BP and save billions of dollars annually on health care (178).

1.1. Classification of hypertension

A pressure of ≤120 mmHg exerted against the walls of blood vessels when the left ventricle of the heart is contracting and ≤80 mmHg when the heart is relaxing are typical systolic and diastolic blood pressures found in healthy young adults, respectively.

Therefore, adult humans with BP at or below 120/80 mmHg are considered "normotensive" (28).

Individuals can exhibit different stages of HTN that are defined by the cardiovascular risk encountered with different levels of elevated BP. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure categorized HTN into prehypertension (120-129/80-89 mmHg), stage 1 hypertension (140-159/90-99 mmHg) and stage-2 hypertension (≥160/≥100 mmHg) (28). The stage of prehypertension is considered a grey area during HTN development because the relative risk for cardiovascular disease development is low, but such patients have an increased chance of progressing to true hypertension. Clinical management of patients with prehypertension is controversial at the same time.

1.2. Causes of hypertension

Only 5 to 10 percent of patients have a clearly identifiable etiology for their HTN, such as pheochromocytoma, primary aldosteronism, Cushing's syndrome, coarctation of aorta, or adrenal cortical tumors. Patients with a known cause for their HTN are generally curable once the underlying cause is removed and are said to have "secondary hypertension" (87). The remaining 90 to 95 percent of patients have HTN with no single identifiable cause: this category is termed "essential" or "primary" hypertension. The term "essential" is a misnomer that was coined in the 1940s-1950s based on the mistaken belief that increased BP was essential to maintain adequate tissue perfusion through an abnormally constricted arterial system. Modifiable risk

factors that increase the probability of developing essential HTN include increased body weight, excessive salt intake or alcohol consumption, environmental stresses, and physical inactivity (3).

Non-modifiable risk factors include age, sex and genetics. Human hypertension is a polygenic trait with many gene variants contributing to a quantitative phenotype such as blood pressure. It is impossible to point at any one particular gene as the cause of HTN, instead it arises from the interaction of many genes with small effects (10). Therefore, identification of new genes requires novel statistical approaches based on many thousands of patients (142).

There are few outward symptoms of HTN, hence the designation “silent killer”. Severe HTN occasionally is often signaled by headache and/or vertigo. Stroke, left ventricular hypertrophy and vascular disease leading to myocardial ischemia and congestive heart failure are frequent complications that account for much of the morbidity and mortality associated with HTN (82, 198).

1.3. Treatment of hypertension

Numerous categories of hypertensive drugs are prescribed to patients including diuretics, beta-blockers, alpha-blockers, centrally acting sympatholytics, vasodilators, calcium channel blockers, ACE inhibitors, angiotensin receptor blockers and renin, or a combination of these drugs (28). Most patients ultimately need to take drugs from two or more of these classes to achieve adequate control of their HTN. Some individuals do

not reach therapeutic BP targets even when taking three or more drugs and are therefore described as having resistant hypertension. In recent years catheter-based renal sympathetic denervation has shown efficacy (50, 238) in lowering blood pressure in some patients with resistant hypertension, emphasizing the important role of the sympathetic nervous system in the pathophysiology of HTN.

2. Neural regulation of blood pressure

Hippocrates, “The Father of Medicine”, over 2500 years ago appreciated the link between the mind and physical health. In recent decades, many details have been discovered about how the brain regulates BP (145, 244). Changing neuronal activity in a number of distinct “cardio-regulatory” neuronal regions of the brain controls BP by affecting cardiac function, blood vessel tone, hormone secretion, body fluid volume regulation and other factors (88). The primary sites in the brain containing neurons that regulate cardiovascular function are the spinal cord, brainstem, and hypothalamus. In addition, a network of peripheral afferent and efferent autonomic neurons comprises a short-term and long-term feedback control system that allows the brain to tightly regulate BP (36). BP control is mostly invested in the sympathetic nervous system (SNS), one of two major arms of the efferent autonomic nervous system (the other being the parasympathetic nervous system). Anatomically, short preganglionic neurons of the SNS originate from the thoracolumbar region of the spinal cord, in particular from the cell bodies of intermediolateral (IML) cell column. They send their axons to the paravertebral and prevertebral ganglia and adrenal medulla. Long postganglionic fibers project from the ganglia to various effector organs, where they release the primary

sympathetic neurotransmitter norepinephrine (NE) along with numerous other co-transmitters.

Widespread physiological responses to increased SNS activity (SNA) were initially characterized as the “fight or flight response.” With regard to BP regulation, sympathetic innervation of effector organs like the peripheral blood vessels, sinoatrial node and myocardium allows precise *minute-to-minute* control of BP via changes in arterial vasoconstriction, heart rate, and stroke volume (86, 88). While *long-term control* of BP was generally thought to be determined by the kidney pressure natriuresis mechanism (78, 87), it is now well accepted that the SNS plays a key role not only in short term but also in long-term control of BP (36, 50, 86, 128). For example, one autonomic pathway that operates over the long-term to control BP is renal SNA (49), which regulates renal vascular resistance, sodium and water excretion, and renin release.

2.1. Central nervous system

As noted earlier, neural pathways originating in the brain maintain BP primarily by affecting SNA. Activity in these pathways is determined both by the intrinsic firing rates of neurons and by the concerted action of circulating hormones (e.g angiotensin II, leptin), chemicals (e.g. glucose), afferent neural inputs from the periphery (e.g. baroreceptors and chemoreceptors) and inputs from other brain regions (e.g. cerebral cortex) on pathway neurons.

Brain regions known to be important in BP regulation that are considered important for signaling include the amygdala in the cerebral cortex, the paraventricular nucleus (PVN), preoptic nuclei and dorsomedial nucleus in the hypothalamus, the A5 noradrenergic nuclei in the pons, the rostral ventrolateral medulla (RVLM) and nucleus of the solitary tract (NTS) in the brainstem, and circumventricular organs such as the hypothalamic subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT) and the brainstem area postrema (AP) (59, 96, 208). Circumventricular organs (CVO) are the first brain structures to come into contact with peripheral hormonal signals that regulate SNA, like angiotensin II (176). Neuronal connections link these CVOs to other cardioregulatory regions involved in control of SNA and BP (217).

2.2. Baroreceptor reflex

Although many factors influence SNA at rest and during physiological stresses, the arterial baroreceptor reflex exerts overriding control of SNA over short time intervals (seconds to minutes). The brain receives continuous inputs from arterial baroreceptor neurons, i.e. neurons with cell bodies in the nodose and petrosal ganglia and deformation-sensitive nerve endings situated in the carotid sinus and the aortic arch. Increased arterial pressure stretches the blood vessel wall and activates these nerve endings, which causes the baroreceptor neurons to discharge and inhibit sympathetic vasomotor activity. Peripheral afferent fibers arising from the baroreceptor neurons synapse on first order neurons of the NTS located in medulla (brainstem). These NTS neurons excite second-order neurons through glutamatergic synapses (140). The

glutamate sensitive NTS second-order neurons convey the baroreceptor signal to the caudal ventrolateral medulla (CVLM), which sends inhibitory signals to neurons within the RVLM (86, 186). RVLM neurons are tonically active and provide most of the “basal” excitatory drive to sympathetic preganglionic neurons in the spinal cord. The reduced activity in RVLM neurons caused by baroreceptor activation causes a fall in sympathetic nerve discharge to the blood vessels and heart and a decline in blood pressure (and heart rate) (186, 197).

Aside from its role in the baroreceptor reflex, the RVLM serves as a “final common pathway” for most neural regulation of BP by causing sympathetically mediated changes in heart rate, stroke volume and systemic vascular resistance. This role of the RVLM became evident when researchers observed dose dependent reductions in SNA and BP when the inhibitory neurotransmitter gamma amino butyric acid (GABA) was microinjected into the RVLM in experimental animals (98, 182).

Among other brain nuclei, the PVN is considered a particularly key integrative center for control of SNA and BP (125, 170). The PVN regulates SNA and BP by direct neuronal connections to: 1) the NTS and RVLM in the brainstem, and 2) spinal sympathetic pre-ganglionic neurons. The PVN receives input from many important integrative centers of the hypothalamus (e.g. the SFO, median preoptic nucleus, arcuate nucleus, suprachiasmatic nucleus), pons (lateral parabrachial nucleus), and brainstem (NTS and RVLM) (187). Although the PVN is known to play an important role in stress, metabolism, growth, reproduction, immune, gastrointestinal, and renal function, recent

studies point to an important function of the PVN in regulating SNA and BP in hypertension as well (4, 31, 125, 148).

3. Sympathetic nervous system activity (SNA) in hypertension

In varied experimental animal models such as spontaneously hypertensive rats (116), renovascular hypertension (21), angiotensin II hypertension (162), angiotensin II-salt hypertension (175, 268), Dahl salt sensitive rats (146) and mineralocorticoid hypertension (119), there is evidence for increased SNA. There also is compelling evidence for increased SNA in human hypertensive patients. For example, a substantial subset (around 50%) of essential hypertensive patients has elevated SNA and/or sympathetic support of BP (45, 48, 80, 81, 134, 205). This evidence comes from a variety of techniques for assessing SNA such as measurement of plasma norepinephrine or norepinephrine spillover, direct recording of single or multi-unit sympathetic nerve fiber firing rate, measurement of plasma catecholamine in hypertensive population the depressor response to ganglionic blockers, and changes in blood pressure after surgical sympathectomy (191, 205).

3.1. Physiological mechanisms of increased sympathetic effects on BP

Broadly speaking, sympathetic effects on blood pressure, which in my dissertation I call neurogenic pressor activity, can be caused by two general sets of physiological effects classified as “prejunctional” and “postjunctional”. Prejunctional mechanisms determine the concentrations of NE in the neuro-effector junctions of target tissues (blood vessels, heart, kidney), and include: 1) increased centrally-mediated sympathoneural or adrenal

medullary outflow (this is generally described as “increased SNA”), 2) increased ganglionic neurotransmission and 3) increased NE release or decreased uptake by sympathetic nerve terminals (often called “increased neurotransmission”) (86). The postjunctional mechanisms are 1) vascular remodeling, 2) augmented vascular, cardiac or renal adrenoreceptor-mediated tissue responses, 3) altered cardiac or vascular ion channels, 4) abnormal intracellular signaling, 4) increased release of intracellular calcium from stores, or 5) decreased actions of physiological antagonists such as nitric oxide (86). The *net effect* of all these factors determines the degree to which the sympathetic nervous system determines blood pressure, i.e. neurogenic pressor activity.

3.2. What causes increased SNA and neurogenic pressor activity in HTN?

A myriad of factors contribute to increased SNA or neurogenic pressor activity in human hypertension. Broadly these could be categorized into genetic factors and behavioral or environmental factors (48). The increase in SNA seen in many hypertensive patients is partly caused by genetic factors (56, 245). Another prime cause for increased SNA is behavioral stress (47, 183). Obesity (6), insulin resistance (150) and inactivity (154) also are believed to enhance SNA in hypertensive patients. Finally, sleep deprivation (184), hypoxia (277) and inflammation (144) could account for increased SNA in human hypertension. From a physiological control system perspective, increased SNA could be a consequence of baroreceptor, chemoreceptor or metaboreceptor impairment (36, 37). Other physiological factors possibly responsible for increased SNA in hypertension include: increased plasma or tissue osmolality (173), elevated levels of circulating

hormones like angiotensin II (92, 193), and both inadequate or excess intake of salt (18, 108) .

A major goal of my research project is to more fully elucidate specific brain signaling mechanisms that can cause increased SNA that result in increase BP. To this end, my experiments employed two factors that are well known to be important in the development of HTN, namely angiotensin II and high dietary salt intake. Thus, before proceeding further I will give brief overviews on the physiology of the renin-angiotensin-aldosterone system and salt, with specific emphasis on how they can affect SNA.

4. Renin angiotensin aldosterone system

The renin angiotensin aldosterone system (RAAS) producing the effector molecule angiotensin II (AngII) in response to physiological stimuli is a classic endocrine system (246). The main components of the classical renin angiotensin aldosterone system (Figure 1-1) include:

Renin: The juxtaglomerular (JG) cells that surround the renal afferent arterioles secrete renin. It is a protease enzyme synthesized first as an inactive precursor pro-renin.

Angiotensinogen: It is a large protein with over 450 amino acids and is synthesized in the liver. The renin angiotensin system cascade starts with the release of enzyme renin from the kidney. Renin then acts on the precursor angiotensinogen in the blood to form AngI.

Angiotensin I: AngI is a decapeptide formed by renin activity. It has limited physiological activity, but is hydrolyzed by circulating and locally expressed angiotensin converting enzyme to the physiologically active AngII.

Angiotensin converting enzyme (ACE): The enzyme ACE is mainly expressed in the lungs and to some extent in other tissues like vascular endothelial cells, kidneys, forebrain circumventricular organs and the adrenal (25). ACE catalyzes the conversion of angiotensin I into angiotensin II. ACE inhibitors are widely used as antihypertensive drugs.

Angiotensin II (AngII): It is an octapeptide produced by the renin angiotensin pathway. Physiological effects of AngII include: potent vasoconstriction; stimulation of certain brain regions controlling SNA, thirst responses and vasopressin release; angiogenesis; release of aldosterone from the adrenal cortex; and direct actions on renal tubular cells to cause sodium absorption (68, 246).

Angiotensin II receptors: Most established physiological effects of AngII are mediated through interaction with cell membrane receptors. The known AngII receptors are angiotensin type 1 receptor and angiotensin type 2 (AT1 and AT2) that belong to the superfamily of seven transmembrane spanning G-protein coupled receptors. AT1 mediates vasoconstriction, cardiac hypertrophy, fibrosis and inflammation. AngII-AT1 signaling triggers aldosterone release and sodium retention (250). Actions of AngII at AT1 receptors are in some instances partially opposed by actions at AT2 receptors. AT2 receptor stimulation activates phospholipase A2, and nitric oxide release and thereby produces anti-inflammatory, vasodilatory and anti-proliferative effects (65, 230). AT1 receptor blockers are effective in lowering vascular tone and blood pressure along

with reducing catecholamine, vasopressin and aldosterone release (230).

Aldosterone: The adrenal cortex is the site of synthesis of the steroid hormone aldosterone. Aldosterone acts primarily on the epithelium of the renal collecting tubules and distal tubules to cause an increase in the reabsorption of sodium and secretion of potassium resulting in sodium conservation and enhanced excretion of the potassium ion (261). Angiotensin II stimulates secretion of aldosterone from adrenal cortex and aldosterone in turn acts on renal tubules to increases blood volume and blood pressure.

The RAAS controls BP after being activated by one or more physiological cues. For example, a reduction in renal perfusion pressure (or salt deprivation) stimulates the release of renin from the JG cells of the kidney into circulating blood. Under most circumstances, the rate-limiting step in the formation of AngII is the rate of release of renin from the kidney. In addition to reduced perfusion pressure and salt depletion, renin secretion is stimulated by activation of beta-1 adrenergic receptors on JG cells by norepinephrine released from renal sympathetic nerves (9). In addition to the classical renin-angiotensin system (RAS), tissue specific RAS have been identified in brain, blood vessels, heart, adrenal gland, testes, ovaries, skin, adipose tissue and leukocytes (34). These tissue-specific RAS have roles like control of blood flow, cell growth and repair, to name a few. It remains unclear how important tissue-specific RAS are to normal physiological function and the degree to which they are actually independent of the classical RAS (100, 258, 274).

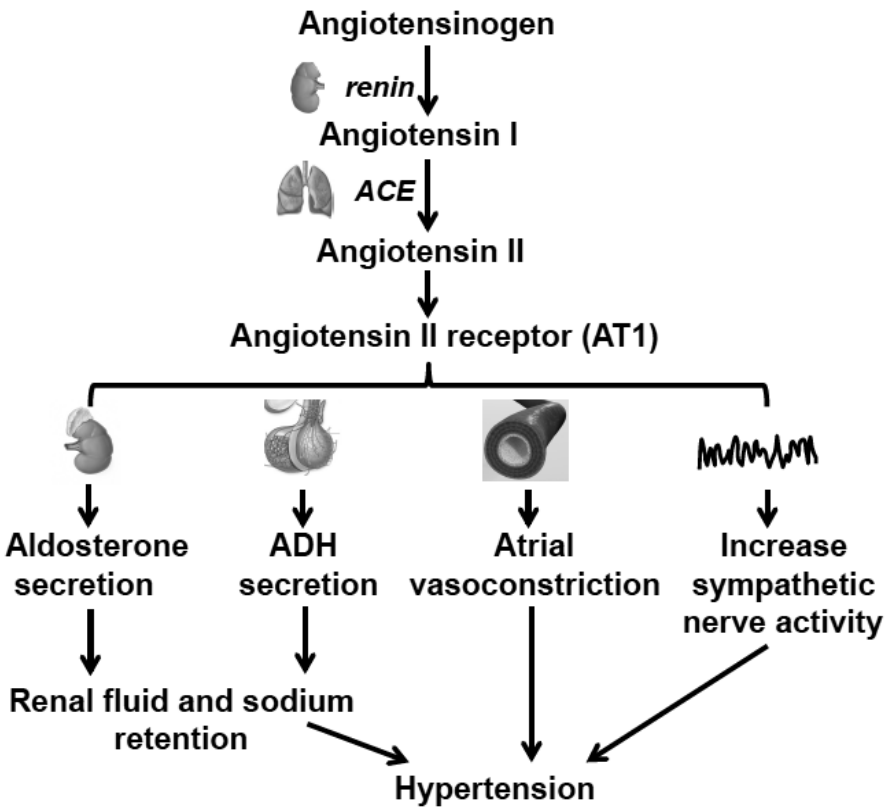


Figure 1-1: Renin-angiotensin-aldosterone system. Circulating renin from kidney cleaves liver angiotensinogen to form angiotensin I (AngI). In the lung, angiotensin-converting enzyme (ACE) acts on AngI to form angiotensin II (AngII). AngII acts mainly on the angiotensin type 1 receptor (AT1) to cause vasoconstriction, sympathetic activation, and the secretion of vasopressin and aldosterone.

5. Angiotensin II as a cause of increased SNA in hypertension

Abundant evidence supports the idea that AngII can increase SNA and/or sympathetic support of blood pressure by acting on both the brain and peripheral SNS (24, 85, 141, 268). With regard to brain mechanisms, pivotal work by Brody and colleagues revealed the critical role of brain CVOs in the AngII-induced HTN. Neurons in CVOs like the SFO and OVLT innervate neurons in the PVN that sends projections to the RVLM in the brain stem, and directly to sympathetic neurons in the spinal intermediolateral cell column (IML), and thereby modulate sympathetic tone (185). Although there is support for the idea that AngII could be a player in the elevated SNA observed in some human hypertensives, that idea remains controversial (79, 141). This is likely due to: 1) important differences in the experimental animal models used to study the question; 2) the disparate approaches that have been used to quantify SNA in experimental animals and human patients; and 3) the often disregarded impact of other factors (e.g. salt intake) on the physiological actions of AngII (see below).

6. Salt

Salt is regarded as an essential human commodity. If we take pages from the annals of history, we find many wars were fought to have control over salt. The Indian civil disobedience movement against the taxation of salt was a hallmark of the Indian Independence movement in 1930, where Gandhi led freedom fighters in what was called the “Salt March to Dandi” to make salt from the sea. With modernization, the use of salt has been under scrutiny because its widespread use as a food preservative has produced higher levels in the body, with possible adverse consequences. The human

body is 60% water, a handful of solutes, mainly sodium, potassium, calcium, magnesium, chloride, bicarbonate, phosphate, organic anions, and proteins. “Salt” is made up of sodium and chloride and is the major contributor to human extracellular and serum osmolality. In these fluids the concentration of sodium is tightly regulated in the range of 135-145mnol/L and physiological disturbances occur if the concentration becomes higher or lower. In large population groups, average dietary salt intake has been shown to be an important risk factor for hypertension and related cardiovascular diseases. In contemporary western society, sodium intake is around 100-200 mmol/day (240). Groups of individuals with intake of sodium below 50 to 100mmol/day have a lower incidence of cardiovascular diseases (42). Groups with higher daily intake than average are at increased risk for hypertension and modest reduction in salt intake reduces blood pressure (97, 240).

6.1. Salt sensitivity of blood pressure

Despite the results from population studies cited above, individuals exhibit different BP responses to changes in salt intake. Some individuals respond to an increase in salt intake with a prompt rise in BP of over 10 mmHg and are called “salt-sensitive,” whereas others show no change in BP and are called “salt-resistant”. Some studies claim that as many as 50% of hypertensive subjects are salt-sensitive (55, 163, 252), although others report a lower fraction (61). Factors affecting salt sensitivity are race, birth weight, age, renal function and diabetes (77, 122, 251). The physiological mechanisms underlying salt sensitive hypertension are not fully known. Impaired renal sodium excretion has been hypothesized as one explanation (89-91), but some studies

find no difference in sodium handling between salt-sensitive and salt-resistant hypertensive humans and instead show critical differences in regulation of vascular tone between the two groups (20, 41). High salt intake decreases renin secretion (218), and the resulting decrease in circulating AngII is a major factor in facilitating renal salt excretion (90). Interestingly, however, this decrease in renin-secretion is blunted in some salt-sensitive hypertensive patients (16, 256, 266) and these same subjects exhibit higher SNA than salt-resistant hypertensives (266). What could account for the increased SNA in these individuals? The answer may be that under certain conditions (especially inappropriately high RAS activity) the brain is capable of “sensing” high dietary salt intake and responding in a way that leads to elevated SNA and BP (6, 18, 136, 216, 217, 226).

6.2 Brain salt sensors and sympathetic nerve activity

An organism tightly maintains the homeostasis of water and solute content in the body. In experimental animals, raising the sodium concentration in the cerebrospinal fluid by infusion of hypertonic saline into the lateral ventricles of the brain causes a rapid increase in BP and SNA (217). This effect is mediated through “salt sensors” or sodium sensors in the brain (216). The central sodium sensing mechanism was proposed to be critical in salt sensitive hypertension because infusion of the sodium channel blockers amiloride and benzamil into the cerebral ventricles successfully attenuated HTN development in salt-sensitive animal models (2, 169). The most sensitive osmoreceptors are localized in CVOs like the SFO, OVLT and AP. As noted earlier, these structures lack a blood brain barrier and therefore are exposed to alterations in

blood osmolality and hormone levels (e.g. AngII) (227). In response to increase salt and other signals that influence the SNS (like AngII), projections from the OVLT and SFO stimulate the median preoptic nucleus and thereby activate excitatory interneurons projecting to the PVN to cause sympathoexcitation as described earlier (18, 216).

In summary, both circulating AngII and high salt intake salt can affect brain circuits that increase SNA and blood pressure via converging neural pathways that originate in the forebrain CVOs and ultimately include both the PVN and RVLM (176). This combined action may be operative in at least a subset of human patients with salt-sensitive essential hypertension. *A primary goal of the research in this dissertation was to employ an experimental animal model of AngII-dependent, salt-sensitive hypertension to develop a more detailed understanding of the brain signaling mechanisms responsible for the increased SNA and BP initiated by salt and AngII.*

7. Angiotensin II-salt model of hypertension

Our lab and others have previously characterized in detail an experimental model of HTN (see (176) for more details) produced by chronically infusing exogenous AngII into male Sprague-Dawley rats ingesting a diet containing 2% NaCl by weight (normal rat chow contains 0.4% NaCl). The HTN is very likely caused by multiple mechanisms (including actions of AngII on the vasculature and kidney), but King et al. (127) demonstrated that generalized sympathetic outflow (measured using both plasma NE concentrations and whole-body NE spillover) was greater in rats infused with AngII and fed a 2%NaCl diet (AngII-salt) than in rats receiving either AngII or salt treatment alone.

As noted above, high salt diet typically reduces RAS activity but the study by King et al. supports the idea that one possible mechanism of salt-sensitive HTN is increased SNA caused by inappropriately high AngII levels during high salt intake. Sympathetic effects on BP in the AngII-salt model also was assessed by measuring neurogenic pressor activity from the acute fall in BP after eliminating all postganglionic sympathetic activity with a ganglion blocking drug; the results strongly support a role for SNA in the development of AngII-salt HTN (126). Surprisingly, however, measurement of regional NE spillover and chronic, direct microneurographic recordings of SNA revealed no changes in either lumbar or renal SNA in rats with AngII-salt HTN (175, 268) and neither renal nor lumbar sympathectomy affected AngII-salt HTN development (175). On the other hand, splanchnic denervation significantly attenuated AngII-salt HT (128) suggesting that increased splanchnic SNA was the major player in the neurogenic component of HTN in the model. Furthermore, it appears that both increased splanchnic vascular resistance (133) and decreased vascular capacitance (126) mediate the effects of splanchnic sympathetic activity on AngII-salt HTN. Finally, central sodium sensors have an important part in AngII-salt HTN, since blockade of brain sodium channels with benzamil attenuates HTN development in the model (unpublished data).

An advantage of using the AngII-salt model to study sympathetic mechanisms in the development of hypertension is that there is a clear pre-hypertensive phase, followed by a short “developmental” phase, and finally an “established” phase, all occurring over a period of less than two weeks. Furthermore, neurogenic pressor activity during each of these phases can be assessed in relatively undisturbed, conscious animals using

telemetric measurement of BP and a ganglion-blocking drug as described earlier. Importantly, based on such analyses, *the mechanisms causing AngII-salt HTN appear to differ during the developmental and established phases of the model.* During the first 3-5 days of AngII infusion (the early developmental phase), non-neurogenic mechanisms seem to have the largest role in increasing BP, whereas during the late developmental and established phases of hypertension, neurogenic mechanisms become predominant (133). Thus, studying the AngII-salt model potentially allows for a more clear separation of cause and effect when seeking brain mechanisms that *initiate* changes in BP as opposed to those that occur in *response* to HTN development. A hypothetical scheme for sympathetically mediated increases in BP in AngII-salt HTN is shown in Figure 1-2.

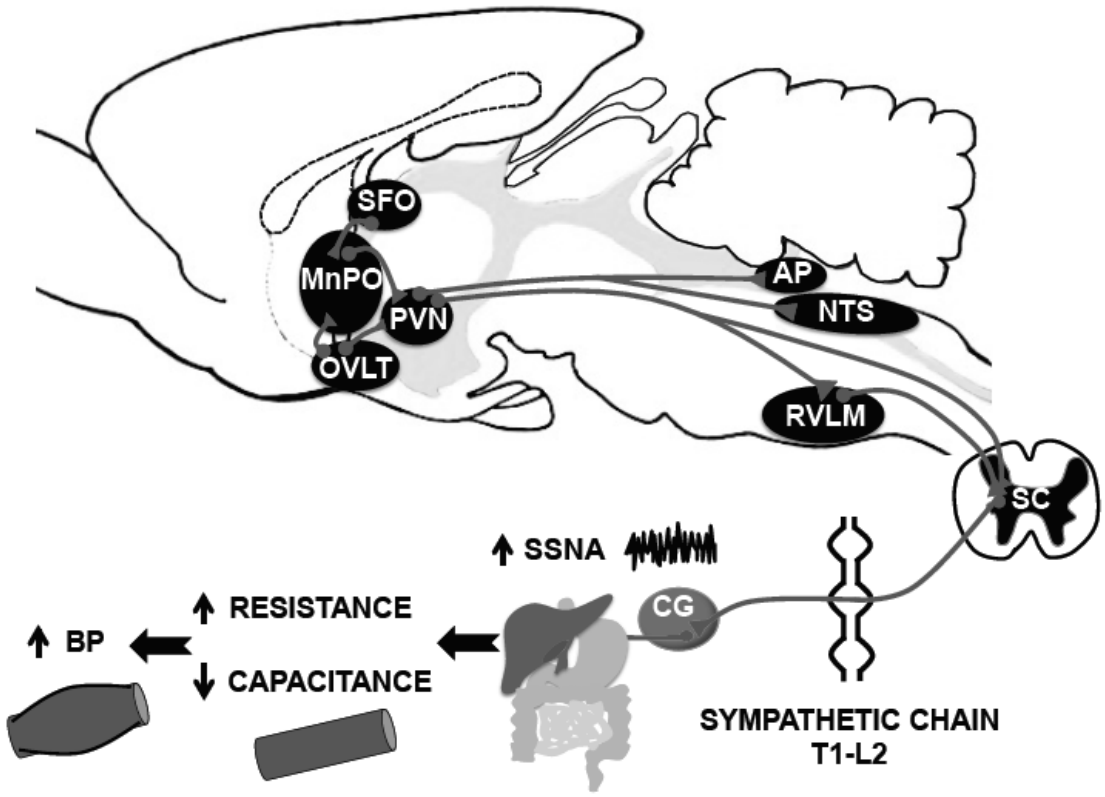


Figure 1-2: Brain sites potentially involved in changes in sympathetic nerve activity and blood pressure in AngII-salt hypertension (AngII-salt HTN). Changes in osmolarity due to high salt diet, and/or peripheral AngII levels, are sensed by the SFO and OVLT that then signal mainly through the MnPO, PVN and RVLM to cause increased splanchnic sympathetic nerve activity. The resultant increase in total peripheral resistance and decrease in vascular capacitance cause HTN. Not shown in the figure is the potential for modulation of SNA by baroreceptors activated by increased BP. SFO-subfornical organ, OVLT-organum vasculosum lamina terminalis, MnPO-median preoptic nucleus, PVN-paraventricular nucleus, RVLM-rostral ventrolateral medulla.

8. Eicosanoids

Eicosanoids are a class of lipid mediators that carry information from one cell to another (35). These cellular messengers have various physiological and pathophysiological roles in fever, inflammation, pain, sleep, gastrointestinal function, bone remodeling, allergic asthma, luteolysis and parturition (35). The term “eicosa” in Greek means 20 as eicosanoids are derived from polyunsaturated fatty acids containing 20 carbons (Figure 1-3). They play a vital role in host defense against adverse conditions, e.g. protection against bacterial pathogens. The eicosanoids are part of a family of biologically active lipids derived from the action of cyclooxygenases (COX) or prostaglandin synthases upon the twenty-carbon essential fatty acids or eicosanoids. Prostanoids can be further subdivided into three main groups, the prostaglandins, prostacyclins and thromboxanes. Prostaglandins (PGs) are the major eicosanoids studied in detail beginning with work by Von Euler in 1936, who first reported prostaglandins as vasodilators and muscle stimulating agents derived from the prostate glands of humans and experimental animals (243). PGs have been used clinically in patients with congenital ductus arteriosus to maintain duct patency until surgical correction (44). PGs regulate gastric secretion, increase uterine contraction and cause labor induction (64). Receptors for PGs are a class of 7-trans-membrane domain, G-protein coupled proteins with strong intra-class structural similarities (29).

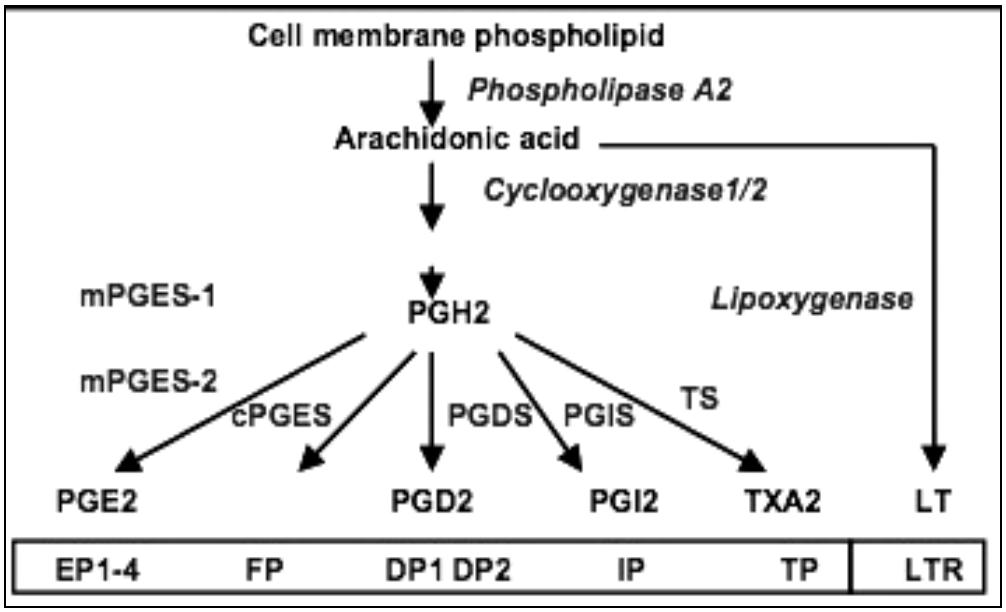


Figure 1-3: Eicosanoid biosynthesis and receptors: Eicosanoids are formed by a sequence of enzyme reactions after cell activation. First, cytosolic phospholipase A2 cleaves arachidonic acid (AA) from membrane phospholipids. AA is converted to prostaglandin H2 by the membrane bound cyclooxygenase (COX) enzyme and lipoxygenase converts AA to leukotrienes that act on the leukotriene (LT) receptor. PGH2 is further isomerized by prostaglandin synthases (PGIS, microsomal PGES1/2, cytosolic PGES, and PGDS) and thromboxane (TX) synthase to form PGI2, PGE2, PGF2α, PGD2 and TX, which act on their receptors IP, EP1-4, FP, DP1, DP2 and TP.

8.1. Synthesis, signaling and function of eicosanoids

Eicosanoids function as local hormones. They are not stored intracellularly, but instead are synthesized (as illustrated in Figure 4) “on demand”. Upon cell injury or other stimuli like binding of ligands such as bradykinin or angiotensin II, the rate-limiting enzyme phospholipase A2 translocates to the nuclear envelope, endoplasmic reticulum and Golgi apparatus to release arachidonic acid (AA) from membrane phospholipids (39). Among the PLA2 isoforms, Type IV cytosolic PLA2 (cPLA2) is the major player involved in eicosanoid synthesis because cells that do not contain this enzyme cannot synthesize eicosanoids. Oxygenation of AA by the rate-limiting enzyme COX (with two isoforms: COX-1 and COX-2) forms intermediate precursors viz., the prostaglandins PGG2 and PGH2 with a half-life of 3 minutes (35, 209). COX-1 and COX-2 are integral membrane proteins of the endoplasmic reticulum (ER) and nucleus. Subgroups of prostanoid synthases (acting on PGH2) and lipoxygenases (acting on AA directly) produce PGI2, PGE2, PGF2α, PGD2 and TxA2 and leukotrienes (LT). Products of this pathway have a very short half-life (20-30 seconds) (35).

The requirement for two distinct COX enzymes is not fully understood. COX-1, a 70kD protein is found ubiquitously in all tissues and acts as a constitutively active enzyme (209). The other isoform, COX-2 (72kD), produces prostaglandins in response to inflammatory stimuli. Separate genes encode the two forms of COX but the two forms of COX exhibit structural homology with almost identical catalytic sites. Other differences between COX-1 and COX-2 include varied subcellular localization, substrate specificity and the manner in which they are coupled to upstream and downstream

enzymes (241). In 1994, Picott, Loll and Gravito established the three-dimensional structure of COXs (138). The small differences in the catalytic domains of these enzymes have been exploited for development of isoform specific inhibitors.

The COX-1 isoform in the endoplasmic reticulum generates PG when there are high levels of AA substrate available to act on. The PG product is then released in an autocrine or paracrine manner to signal downstream through numerous cell-surface G-protein coupled receptors. The main role of the COX-1 products is to maintain homeostasis and hence the name “housekeeping protein.” COX-1 expression in kidney, stomach, vascular endothelium, and blood platelet supports the idea that it is expressed as a signaling mediator in tissues with specialized needs. In platelets the COX-1 product thromboxane is a potent vasoconstrictor and causes platelet aggregation (209).

COX-2 on the other hand is an inducible enzyme in most tissues. However the kidney and brain COX-2 is constitutively active. Stimuli like cytokines, growth factors and tumor promoters, produced as part of an inflammatory response, lead to eicosanoid synthesis from even low concentrations of the substrate AA in cell types involved in inflammatory responses like macrophages and monocytes. The prostanoids then help resolve the inflammation.

Listed below are some important eicosanoids (Figure 1-4) along with their important biological roles.

8.1.1. Prostaglandin E2: Prostaglandin E is synthesized from the precursor PGH2 by prostaglandin E synthases cytosolic-PGES (c-PGES) and microsomal-PGDS (m-PGES-1 and m-PGES-2). Cytosolic PGES is constitutively expressed in various tissues, however microsomal PGDS is a perinuclear protein. PGE2 has a very short half-life of 30 seconds. PGE2 acts locally by binding to its receptors EP1, EP2, EP3 or EP4. EP1 is a Gq coupled receptor that increases IP3 by calcium signaling. EP2 and EP4 are Gs coupled receptors that increase cAMP. The EP3 receptor is a Gi coupled receptor that decreases cAMP and increase calcium. PGE2 has proinflammatory effects: for example, LPS-induced PGE2 production causes harmful effects on neurons and enhances pain sensations. On the other hand, PGE2 blocks LPS-induced cytokine synthesis and neuroinflammation providing evidence for an anti-inflammatory effect (194).

8.1.2. Prostaglandin D2: PGD2 is the major prostaglandin synthesized in the brain. Its precursor PGH2 is acted upon by lipocalin type PGD2 (L-PGDS) or hematopoietic PGD2 (h-PGDS). L-PGDS is present in cells of a variety of tissues especially in the central nervous system. H-PGDS is predominant in the cytosol of immune and inflammatory cells. The CP, leptomeninges and oligodendrocytes synthesize L-PGDS in the CNS. It is unique in that it functions not only as a synthase to form PGD2 but also acts as a carrier for lipophilic molecules (235). L-PGDS is secreted into the cerebrospinal fluid and is also known as beta trace protein (172). Other sites of PGD2 synthesis are mast cells and leukocytes (dendritic cells and T helper 2 cells). PGD2 is metabolized into PGJ2 and 15d-PGJ2. PGJ2 is then converted into 15-deoxy-$\Delta^{12,\ 14}$-

PGJ2 and Δ^{12}-PGJ2. 15-deoxy-$\Delta^{12, 14}$-PGJ2 acts as a ligand for the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) and inhibits nuclear factor kappa light chain enhancer of kappa light chain enhancer of B cells (NF_KB) (195). A proinflammatory effect of PGD2 is mediated through the Gs-protein coupled DP1 receptor that increases cAMP; and the DP2 receptor, a Gi coupled receptor that decreases cAMP and increases intracellular calcium (194). PGD2 is involved in type I acute allergic responses, pain perception and regulating physiological sleep (95, 194, 233).

8.1.3. Prostaglandin I2: PGI2 is synthesized by prostacyclin synthase (PGIS) that is co-localized with COX-1 in the endoplasmic reticulum and constitutively expressed by endothelial cells (194). PGI2 is a potent vasodilator and inhibits platelet aggregation. PGI2 is metabolized by non-enzymatic hydrolysis to form the inactive product 6-keto-$PGF_{1\alpha}$. PGI2 acts through Gs, Gi or Gq coupled receptors to decrease cAMP and increase IP3 signaling. Relevant IP receptors are localized in kidney, liver, lung, platelets, aorta and heart (210).

8.1.4. Prostaglandin F2α*:* PGF synthase acts on PGH2 to form PGF2α. It is required for normal parturition (219) and plays an important role in ovulation and contraction of uterine smooth muscle cells. The PGF2α receptor FP is a Gq coupled receptor and its deletion reduces blood pressure and atherosclerosis (270). 15-keto-dihydro-PGF2α is

the main metabolic product of PGF2α and can be detected in the plasma and urine as an indicator of PGF2α produced in response to acute or chronic inflammation.

8.1.5. Thromboxane: Thromboxane A2 is synthesized by thromboxane synthase mainly in mast cells and macrophages. Later TxA2 is converted into inactive TxB2. The actions of TxA2 are through the Gq, G12/13 or small G protein coupled receptor TP that increases cAMP, intracellular calcium and IP3 signaling. TxA2 promotes platelet adhesion and smooth muscle contraction. TP receptor deletion has been linked to lower blood pressure response but also bleeding defects (225).

8.1.6. Non-steroidal anti-inflammatory drugs

Drugs that inhibit the synthesis of the eicosanoids are called non-steroidal anti-inflammatory drugs (NSAIDS). In 1899 the first NSAID, aspirin, was introduced; it revolutionized the drug industry by relieving patients of pain and inflammation (93). NSAIDs can be differentiated based on their ability to selectively inhibit COX-1 versus COX-2. The drugs that have much higher potency against COX-1 are so-called “COX-1 selective” inhibitors like naproxen and flurbiprofen. The selective COX-2 inhibitors include celecoxib and rofecoxib. Aspirin, a “non-selective COX inhibitor” actually produces more COX-1 than COX-2 inhibition (Cox-2/COX-1 IC50 ratio of 4.3, which is high and similar to COX-1 specific NSAIDS) (93, 248).

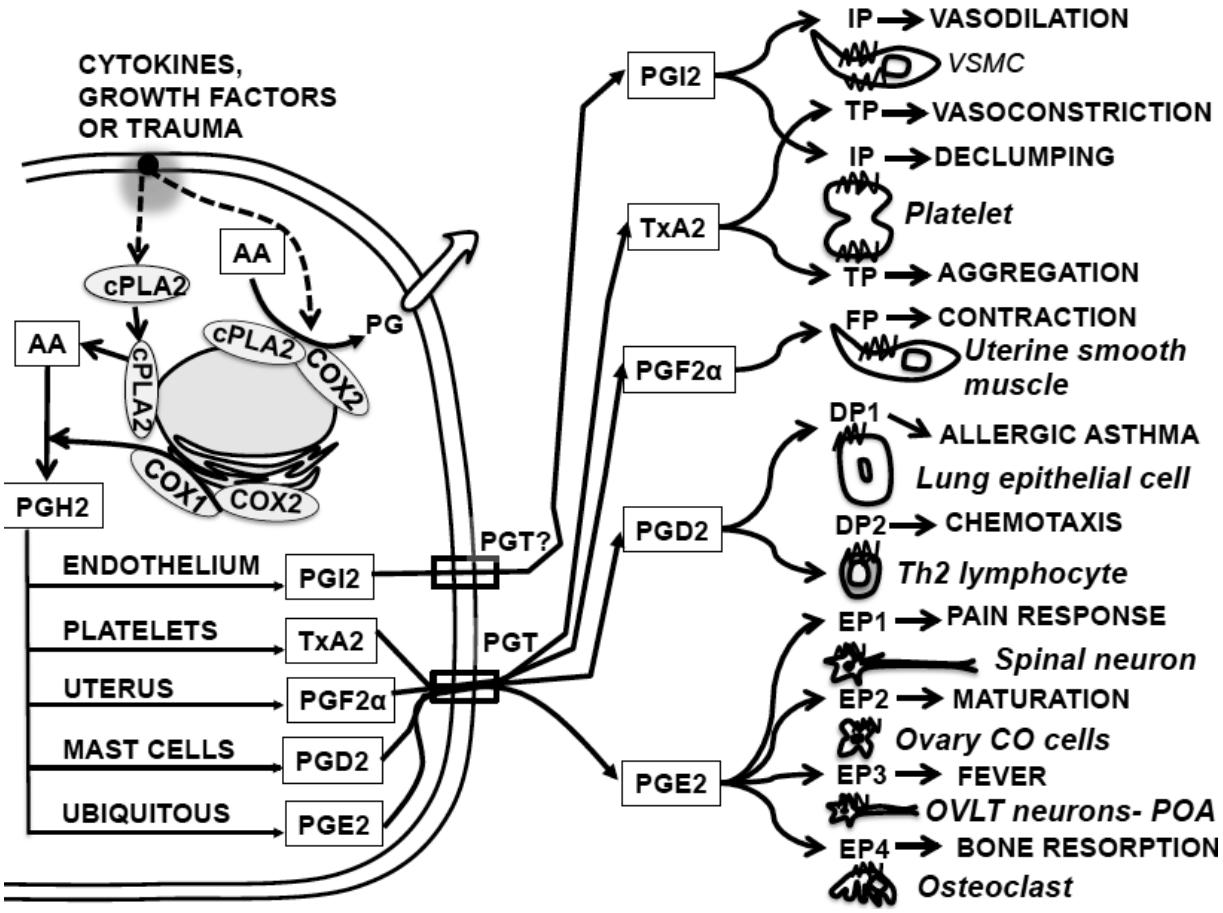


Figure 1-4: Prostaglandin synthesis and actions. Cytokines, growth factors or trauma triggers translocation of type IV cytosolic phospholipase ($cPLA_2$) and release of arachidonic acid from membrane lipids. Metabolism by COX-1 or COX-2 forms intermediate PGH_2 that is converted to PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TxA_2 (thromboxane). The synthesized prostaglandins then exert autocrine or paracrine actions by acting on prostaglandin transporter (PGT) or other carriers. The effector specific effect is caused by interaction with different receptors like EP_1, EP_2, EP_3, EP_4, DP_1, DP_2, FP, IP, TP. CO=cumulus oophorus cell. VSMC=vascular smooth muscle cell. [Adapted from C D Funk Science 2001; 294: 1871-1875].

8.2. Eicosanoids in the brain

Next to adipocytes, the brain contains the highest amount of lipids: around 36-60% (221). Arachidonic acid (AA) and docasohexanoic acid (DHA) are the two main polyunsaturated fatty acids present in the brain, located in the sn-2 position of the phosphoglycerides of neural cell membranes. Activation of phospholipase A2 liberates AA from those phosphoglycerides. In the brain cPLA2 is expressed in astrocytes (220), endothelial cells of cerebral blood vessels (213), glial cells, pia matter and choroid plexus (135). Cyclooxygenases (COX-1 and COX-2) metabolize free AA to form eicosanoids by synthetic pathways similar to those described earlier in this chapter. COX-1 in the brain is expressed constitutively in hippocampal CA3 and CA4 neurons, granular neurons of the neocortical layer, microglial cells in the white and grey matter in all brain regions (267), spinally projecting PVN neurons (264) and SFO (22). On the other hand, the inducible enzyme COX-2 is expressed in neurons in neo-cortices and allo-cortices, hippocampus, amygdala, dendritic spines involved in synaptic signaling, PVN neurons, locus coeruleus neurons and astrocytes surrounding the blood vessels (123). Both AA and DHA can be metabolized into leukotrienes and hydroxyl derivatives by lipoxygenase, whereas AA can form lipoxins by the action of 5-lipoxygenase (224). In the brain, 5-lipoxygenase is expressed in neurons and glial cell; however, the brain microvessel endothelium is devoid of 5-LOX (272).

COX-1 and COX-2 catalyze the formation of PGH2, the intermediate metabolite of AA. PGH2 is further transformed into PGE2, PGD2, PGI2 and TxA2 by various synthases in the brain. PGE2 is synthesized by three different PGE synthases viz., the constitutive

cytosolic prostaglandin E synthase (cPGES); membrane bound mPGES-2 and the inducible mPGES-1. In rat brain, neurons and astrocytes of the ipsilateral cortex express mPGES-2 and SFO neurons express cPGES (22, 201). Rat and human brain L-PGDS is expressed in arachnoid trabecular cells (158), oligodendrocytes, leptomeninges and choroid plexus (232) and it synthesizes PGD2, the most abundant eicosanoid in the brain. The isoform hematopoietic PGDS is widely distributed in the periphery, and to some extent in the brain in the microglial cells (160), T-helper2 cells, antigen presenting cells and the mast cells (117). The eicosanoid prostacyclin synthesizing enzyme prostacyclin (PGI2)-synthase is shown to be present both rats and humans in the cortical neurons, purkinje cells of the cerebellum but not in the glial cells (152). In addition to these, PGI2 synthase was also localized in blood vessels, microglial cells, oligodendrocytes and hippocampal neuron (207). The thromboxane synthesizing enzyme thromboxane synthase in the ovine brain is localized in the neuronal cell body and axons of NTS and RVLM along with ventricular ependymal cells, the cerebellar peduncle in the rostral pons and purkinje fibres (110).

Together, the products of COX pathway called the eicosanoids take part in critical neural functions. For example, PGE2 has been linked to physiological long-term potentiation, spatial learning and synaptic plasticity. Other critical roles of eicosanoids in the brain include resolution of inflammation, anti-inflammatory and neuroprotective functions (224). PGD2 is one of the several known humoral sleep inducing factors: it stimulates DP1 receptors localized in the leptomeninges of the basal forebrain causing release of adenosine that in turn acts as a paracrine sleep promoting signaling molecule (109). A dose dependent increase in sleep time occurs with increases in brain PGD2

levels. Studies with the PGD2 synthesis inhibitor selenium chloride, as well as transgenic and knockout mice, demonstrate the critical role of PGD2 as a sleep inducer (27, 94).

When there is injury to brain tissue, free radicals (reactive oxygen species) cause damage by oxidation of lipids. Lipid peroxidation can result in neurodegenerative diseases (224). Eicosanoids take part in the process of neuroinflammation as seen in diseases like Alzheimers, multiple sclerosis and epilepsy. The pathogenesis of neuroinflammation is characterized by neural injury that causes migration of microglia and adhesion of leukocytes to the site of injury. Microglia produces cytokines like tumour necrosis factor-α and interleukin-1β along with chemokines and vascular cell adhesion molecule at the site of injury. These cytokines and chemokines then increase cPLA2 activity (54). This cPLA2 activity is the source of increased release of pro-inflammatory eicosanoids. The importance of eicosanoids in neurodegenerative diseases is shown by the fact that long-term use of NSAIDs has been proven beneficial in delaying the onset and slowing the progression of the disease (74). COX-2 mediated synaptic signaling further elucidates its role in inflammation especially in neurons (265). Effectiveness of COX inhibitors like aspirin and ibuprofen in reducing fever, sleepiness and anorexia (the “sickness syndrome”) during systemic inflammation show that eicosanoids play a critical role during inflammation by signaling in the brain (202). In rat models PGE2 is suggested to act on the EP3 receptors localized the median preoptic nucleus neurons to cause fever (167).

8.2.1. Role of eicosanoids in hypertension

The eicosanoids have both pro-hypertensive and antihypertensive effects (115). These lipid mediators are produced and act in multiple tissues like kidney, brain, blood vessels and immune system where they can influence blood pressure (168). Our lab's interest in the role of eicosanoids in hypertension originated in part from data on human HTN patients taking NSAIDs: most commonly an *increase* in BP was observed (11, 26). This suggests that the net effect of COX products on BP in patients with HTN is depressor. Interestingly, however, the effects of COX inhibitors on BP seem to vary depending on the type of HTN studied. For example, in normotensive and hypertensive patients with a stimulated renin angiotensin system, COX inhibitors lower blood pressure (113) . NSAID treatment also attenuates HTN development in human renovascular hypertension (111), and in renin-dependent animal models like two-kidney one-clip hypertension (214), AngII hypertension (168), and aortic coarctation induced hypertension (139). Therefore, it appears that the net effect of COX products in angiotensin-dependent HTN (unlike other forms) is to *increase* BP. Since a large fraction of human patients with HTN show a significant fall in BP when treated with renin-angiotensin system inhibitors (i.e. have angiotensin-dependent HTN), even when they have no evidence of an "activated" renin-angiotensin system, a better understanding of how COX products regulate BP in angiotensin-dependent HTN is needed. Some earlier work by others has addressed that topic, as described in the next section.

8.2.2. Effect of eicosanoids on AngII HTN development

Are eicosanoids one cause of AngII mediated HTN? COX products stimulate renin secretion, and COX inhibitors reduce plasma renin activity (62). This may explain why COX inhibitors blunt HTN development in some renin-dependent animal models like the aortic coarctation model (114), and two kidney one clip model (214), i.e. eicosanoid synthesis inhibition with NSAIDs likely lower BP by decreasing plasma renin activity. Aside from an action on renin secretion (and thus AngII formation) as a mechanism for eicosanoid effects, many studies have examined the role of eicosanoids in HTN caused by chronic administration of AngII itself (AngII HTN), thus bypassing any influence of renin. The most common approaches have been with the use of COX inhibitors (22, 57, 211) and COX knockout mice (22, 188, 260). The studies have yielded contradictory conclusions, possibly because they employed different treatment regimens, treatment durations and methods of measuring blood pressure. Furthermore, the relative importance of COX-1 versus COX-2 in AngII HTN is disputed (22, 188, 211).

Two main mechanisms have been proposed to explain pro-hypertensive effects of various eicosanoids in AngII HTN. Early work on this topic is well summarized by Nasjletti (119). He suggested that systemic and renal vasoconstriction induced by TxA2 and PGH2 played a critical part in AngII- and AngII-salt HTN. Wilcox and colleagues (70, 124, 129) also found that TxA2 induced renal vasoconstriction played an important role in AngII HTN. Interestingly, COX blockade during AngII HTN in dogs impaired hypertension development, but actually augmented renal sodium retention (76), so COX products don’t seem to exert a pro-hypertensive effect in AngII HTN by impairing renal

sodium excretion. Finally, a limited number of investigations have suggested a link between eicosanoids and increased SNA and neurogenic pressor activity in AngII HTN. The first support for this idea came from Luft and coworkers who reported an increase in directly recorded splanchnic SNA that was associated with increased eicosanoid synthesis in a chronic AngII HTN model in rats (143). Later, Wilcox's group (71) showed increased neurogenic pressor activity in rats made hypertensive by chronic infusion of a TxA2/PGH2 receptor agonist. Neither of these studies provided clear evidence, however, on specific tissue targets where eicosanoids might act to increase sympathetic regulation of BP.

8.2.3. Eicosanoids may increase BP and/or SNA by actions in the brain

Studies with acute ICV administration of various eicosanoids into brain showed that PGE2 and PGD2 cause pressor responses that require sympathetic nervous system activation. Similarly, stimulation of TxA2/PGH2 receptors in the brain increases BP (70). These finding provide the basis for the idea that brain eicosanoids may regulate BP by affecting SNA. Interestingly, the pressor response to ICV PGE2 was abolished by lesion of the median eminence (an important link between SFO, OVLT and PVN) (104) suggesting that "salt sensors" may be involved in eicosanoid mediated increases in sympathetic nerve activity and BP. Lesions of both SFO and OVLT have been found to reduce AngII-salt HTN in rats (30, 177). Very recently, Cao et al reported that COX-1 inhibition blocks AngII HTN and reduces SNA in mice by reducing PGE2 formation and therefore activation of EP1 receptors in the SFO (22). PGE2 induced ROS production (247) in the SFO was proposed to mediate the increased SNA and BP in AngII HTN

(275). Collectively, these observations strongly suggest that *angiotensin signaling in the brain may include recruitment of sympathoexcitatory and pro-hypertensive mechanisms mediated by COX-derived eicosanoids.*

Brain eicosanoids also activate SNA in other conditions like heart failure and stress, and studies on these conditions may provide insights into the specific brain pathways involved. For example, in a rat model of heart failure, PGE2 was reported to act directly on PVN neurons to increase SNA (269). Stress-related corticotropin releasing factor (CRF) signaling in the brain also brings out an intriguing connection between brain eicosanoids and SNA. Katafuchi et al has reported that ICV CRF or PGE2 increase splenic sympathetic nerve activity. The PGE2 mediated increase in SNA was blocked completely when the rats were pretreated with a CRF antagonist indicating a sequential signaling of CRF followed by PGE2 in causing pressor effect in rats (120). Furthermore, levels of CRF in the cerebrospinal fluid have been strongly correlated with those of L-PGDS in obese humans (43). A role for COX in sympathoexcitatory CRF signaling in the brain was suggested by the finding of increased COX1 and COX2 expression in pre-sympathetic PVN neurons by Yamaguchi et al (264). These same neurons were found to activate sympathetic pre-ganglionic neurons specifically in the celiac and stellate ganglia (239). With the reports of successful attenuation of AngII-salt HTN by celiac ganglionectomy, it is possible that the COX expressing PVN neurons play pivotal role in increased splanchnic sympathetic nerve activity in AngII-salt HTN (128).

8.2.4. Angiotensin II, neuroplasticity and brain eicosanoids

The brain can exhibit long-term “adaptation” in response to even brief stimuli by “*experience-dependent plasticity* causing long-lasting, functional and structural changes in brain circuits” (262). Interestingly, it has long been known that even a brief increase in circulating AngII levels can induce a long-term sensitization to effects of AngII on the brain that are produced days or weeks later; for example, stimulation of salt appetite (199). Recently, Johnson and colleagues showed that transient short-term exposure to circulating AngII also could accentuate or “sensitize” later (at least one week) development of AngII-HTN at least in part by brain mechanisms (262). Others also have shown that transient increases in circulating AngII can produce salt-sensitive hypertension later in life without the need for further exposure to AngII (107). Gabor and Leenen (69) recently summarized evidence that sympathoexcitation in AngII HTN involves both very rapid and very slow changes in brain cardio-regulatory pathways. Therefore, it is of great interest that brain eicosanoids have been shown to participate in long-term adaptation of neuronal pathways as part of the overall “stress response” (67).

9. Central hypothesis

Increased sympathetic activity and hypertension development caused by chronic infusion of angiotensin II in rats on high dietary salt intake are mediated by eicosanoid signaling in specific cardio-regulatory brain regions (See Figure 1-5)

Specific Aim 1: Test the hypothesis that AngII-salt HTN is associated with increased transcription of eicosanoid related genes in established cardio-regulatory brain regions during the early stages of HTN development.

Specific Aim 2**:** Test the hypothesis that COX products in the brain during the early stages of HTN development contribute to increased SNA and BP in AngII-salt HTN.

Specific Aim 3: Test the hypothesis that increased brain L-PGDS expression during early stages of HTN development contributes to elevated SNA and HTN.

Specific Aim 4**:** Test the hypothesis that PGD2 signaling through the DP1R causes increased SNA and BP in AngII-salt HTN.

10. Significance

Increased SNA contributes to human hypertension. Understanding how specific eicosanoids act on the brain to increase SNA and BP would help fill a gap in our knowledge of how dysregulation of SNA occurs in hypertension. The proposed work could help identify new therapeutic strategies for treating HTN and preventing cardiovascular disease.

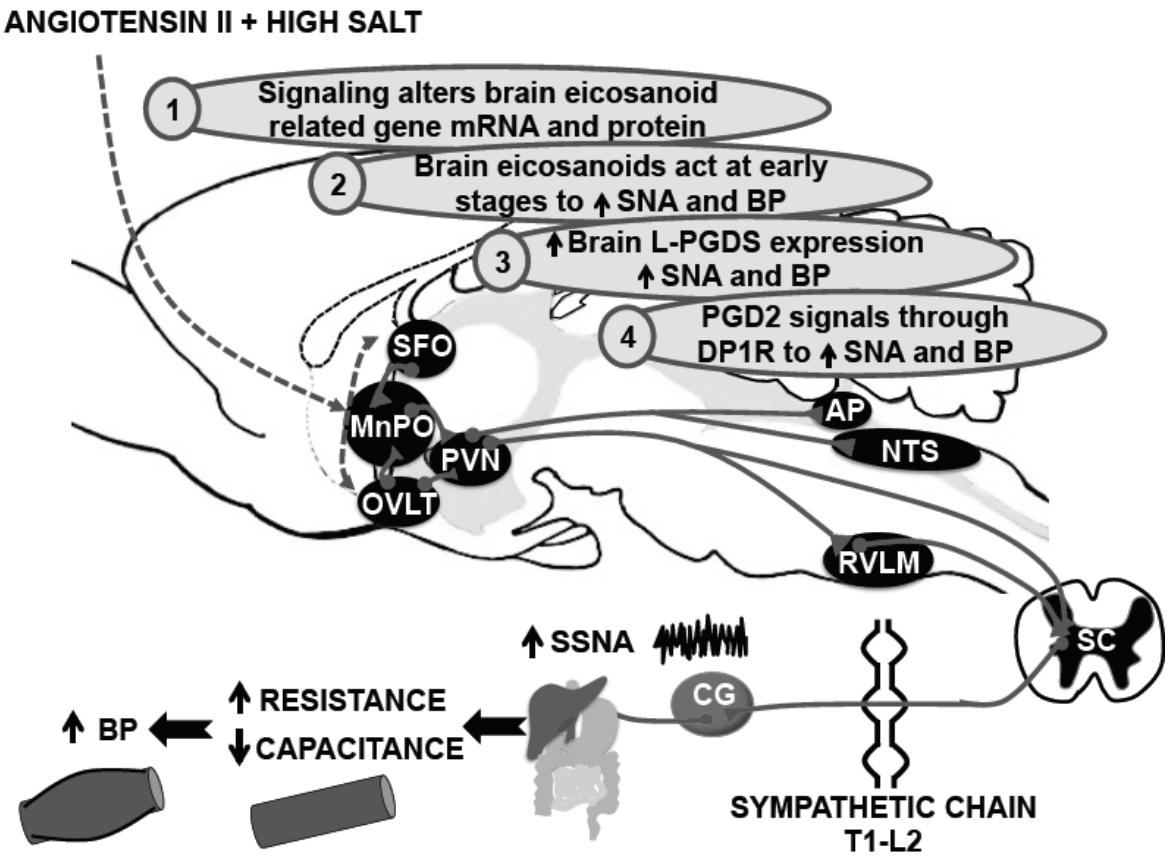


Figure 1-5: Hypothesis and specific aim: Chronic infusion of angiotensin II in rats on high dietary salt intake increase SNA and hypertension by altering eicosanoid signaling in brain. Abbreviations and acronyms are described in the text. CG = celiac ganglion

CHAPTER 2

MATERIALS AND METHODS

1. Animals

Male Sprague Dawley rats weighing 225-275g were obtained from Charles River laboratories (Wilmington, MA) and housed in groups of 3 prior to experimentation. The rats were maintained in light- and temperature-controlled animal rooms and were allowed free access to food and water. The daytime light cycle was from 6:30am to 6:30pm. After the surgical procedures, rats were housed individually in cages with cage tops containing high salt (2% NaCl) or normal chow diet (Research Diets, New Brunswick, NJ) that was provided throughout the experimental period along with ad-lib distilled water. All procedures were performed in compliance with the NIH's Laboratory Animal Care and Use guidelines and after approval from the Institutional Animal Care and Use Committee at Michigan State University.

2. General anesthesia and post-operative analgesia

Mini-osmotic pump and telemeter implantation surgeries were performed under isoflurane anesthesia. The rat was placed in an anesthetic chamber and breathed a mixture of 2 to 4% isoflurane in oxygen at a flow rate of 2.0 L/min for induction. The rats were then placed in a surgical area with nose cone and heated pad and anesthesia was maintained with a mixture of 0.5-2% isoflurane and oxygen at a flow rate of 2.0 L/min. Intracerebroventricular (ICV) cannula implantation surgery was performed using the injectable anesthetics ketamine (75mg/kg body weight) and xylazine (7.5mg/kg body weight), both administered intraperitoneally (ip) in combination.

The rats were given post-operative antibiotic and analgesics for a period of 24-48 hours depending on the surgery. Enrofloxacin (Baytril, Bayer Healthcare, Kansas), intramuscularly (im) at 5mg/kg body weight was used as the antibiotic. Carprofen (Rimadyl, Pfizer, NY), subcutaneously (sc) at a dose of 5mg/kg body weight was used as for analgesia.

3. Radiotelemetry implantation

The surgical site was prepared using chlorhexidine scrub. Under general anesthesia as described above, a 2mm lateral midline incision on the thigh and the femoral artery was exposed. The tip of a radio-telemeter (TA11PA-C40, Data Science International (DSI), St Paul, MN) catheter was introduced (approximately 4cm) through the left femoral artery into the abdominal aorta just cranial to aortic bifurcation. The body of the transmitter was placed in a subcutaneous pocket along the caudo-ventral abdomen. This device recorded blood pressure and physical activity for 10 seconds at every 10-minute interval. After surgical recovery, 24hr/day data values were transmitted via radio signals from the animals to DSI plate receiver positioned under the animals' cages and then to a computer data acquisition program (Dataquest ART 4.1, Data Sciences International, St. Paul, MN) for analysis. Variables recorded include; systolic, diastolic and mean (calculated) arterial pressure; heart rate (calculated); and activity level.

4. Mini-osmotic pump implantation

Mini-osmotic pumps (2ML2 and 2004 models, Alzet, Cupertino, CA) (Figure 2-1) were used for drug delivery in a chronic fashion with a constant rate of infusion.

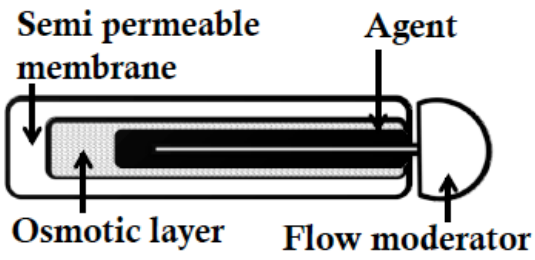


Figure 2-1: **Osmotic mini pump**

Osmotic pumps work on the principle of osmotic pressure difference between the inner chamber of the pump and the tissue where it is implanted. The pumps osmotic layer helps water to flux inside the pump through the semipermeable membrane. This flux causes the agent of interest (angiotensin II and other drugs) to be pumped out of the inner chamber at a constant and predetermined rate.

The 2ML2 mini-osmotic pumps had a flow rate of 0.083333 µl/min, and when filled with angiotensin II in powdered form dissolved in physiological saline (Sigma, St. Louis, MO) at appropriate concentrations produced a drug delivery rate of 150ng/kg body weight/minute. Mini-osmotic pumps filled with angiotensin II were incubated at 37°C for 4 hours or overnight before implantation to get a constant release of the drug from the day of implantation. The surgery was performed under general anesthesia (as described above). After making an incision in the dorsal region of the neck, a small subcutaneous pocket was made on the back of the rat to place the pump. The incision was closed with 5-0 silk, non-absorbable sutures.

5. Intracerebroventricular brain cannula implantation

After rats were anesthetized with ketamine and xylazine as described earlier, they were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). An incision was made in the skin on top of the skull. Cotton swabs were used to clean the surgical site to expose bregma on the skull surface. The coordinates relative to bregma, anterioposterior = -0.8mm, mediolateral = -1.5, and ventral = 4.2mm, were used to position the ICV cannula on the right side of the rat's skull. Stainless steel screws were attached to the skull and dental acrylic (Dentsply International inc., York, PA) was used to secure the cannula to the skull. Rats were housed in individual cages after surgery. At the end of the experiment, cannula placement was confirmed in each study. In case of using the brain for mRNA or western blot study, visual confirmation of the ICV site was done and in other cases either dye was injected or sections were stained to confirm the ICV cannula placement.

6. Femoral catheterization

A polyurethane catheter (RFA-01, Strategic Applications Inc., Chicago, IL) was used for arterial catheterization. Under general anesthesia with isoflurane, a midline incision on the thigh was made and the catheter tip was inserted into the abdominal aorta through the left femoral artery. The saline-filled catheter was connected to a Power Lab recorder (AD Instruments, Inc., Colorado Springs, CO) for blood pressure and heart rate measurements.

7. Animal euthanasia

Rats were euthanized by injecting Fatal Plus® (Pentabarbital, 390mg/kg, ip) followed by decapitation using guillotine. Rongeurs were used to quickly remove the brain out carefully by cutting the occipital bone and the skull attachments.

8. Microdissection of rat brain

Using a Microm HM525 cryostat maintained at -15^{0}C, 500 µm thick serial brain sections were obtained. The sections were then transferred to a cold stage maintained at the same temperature. The paraventricular nucleus (PVN), subfornical organ (SFO), organum vasculosum lamina terminalis (OVLT), rostral ventrolateral medulla (RVLM) and nucleus tractus solitarius (NTS) were microdissected using the Palkovits' microdissection technique (179) with a 500µm punch. A rat brain atlas (179) was used to identify and using a stainless steel needle of 1mm diameter, punch out the nuclei of interest. The isolated brain tissue was stored at -80^{0}C for future analyses.

9. Polymerase chain reaction (PCR) array and quantitative real time-PCR

RNA was extracted from brain punches using a GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich, St. Louis, MO USA). To evaluate the quality of extracted RNA, a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used and samples with low quality RNA (assessed by OD 260/280 ratio outside the range of 1.8 and 2.1) were excluded from further analysis. Reverse transcription for cDNA synthesis was performed with an RT^2 first strand kit (SABiosciences, Frederick, MD) using 100 ng of RNA from the samples.

9.1. PCR array: A custom Rat RT^2 profiler^{TM} PCR array (SABiosciences, Frederick, MD) with 21 eicosanoid related gene primers (Table 2-1) in a 96 well plate format was used. The PCR reaction mixture for the 96 well plates included 102 µl diluted cDNA synthesis reaction, 1048 µl RNA grade water, and 1150 µl RT^2 SYBR Green master mix. PCR reactions were carried out on ABI 7500 Fast real time PCR system (Applied Biosystems, Carlsbad, CA) using an RT^2 SYBR Green master mix (SABiosciences, Frederick, MD). The PCR conditions included a holding stage (95^0C for 10 mins) and cycling stage (95^0C for 15 sec, 60^0C for 1 minute and 72^0C for 35 seconds) followed by a melt curve to confirm the specificity of the amplified products. To control for DNA contamination, no template control was included. The Ct values were normalized to beta actin. The house keeping genes in the array were Lactate dehydrogenase (ldha), Ribosomal protein13a (Rpl13a) and β-actin. The fold change was calculated by $2^{\Delta\Delta CT}$ method.

Table 2-1: 96-well custom PCR array template for eicosanoid related genes

Number	Gene	Gene symbol	Gene RefSeq #
1	PG-endoperoxide synthase 1 (COX-1)	Ptgs1	NM_017043
2	PG-endoperoxide synthase 2 (COX-2)	Ptgs2	NM_017232
3	Arachidonate 5-lipoxygenase	Alox5	NM_012822
4	Alox5 activating protein	Alox5ap	NM_017260
5	Arachidonate 12-lipoxygenase	Alox12	NM_001105798
6	Arachidonate 15-lipoxygenase	Alox15	NM_031010
7	Thromboxane A synthase 1	Tbxas1	NM_012687

Table 2-1 (cont'd)

8	PGI2 (prostacyclin) synthase	Ptgis	NM_031557
9	PGE synthase 1(microsomal)	Ptges1	NM_021583
10	PGE synthase 2	Ptges2	NM_001107832
11	PGE synthase 3 (cytosolic)	Ptges3	NM_001130989
12	PGD2 synthase (brain)	Ptgds	NM_013015
13	PGD2 synthase 2 (hematopoietic)	Ptgds2	NM_031644
14	Thromboxane A2 receptor	Tbxa2r	NM_017054
15	PGI2 (prostacyclin) receptor	Ptgir	NM_001077644
16	PGE receptor 1 (subtype EP1)	Ptger1	NM_013100
17	PGE receptor 2 (subtype EP2)	Ptger2	NM_031088
18	PGE receptor 3 (subtype EP3)	Ptger3	NM_012704
19	PGE receptor 4 (subtype EP4)	Ptger4	NM_032076
20	PGD receptor	Ptgdr	NM_001135164
21	PGD receptor-like	Ptgdrl	NM_001030643
22	Beta actin	Actb	NM_031144
23	Lactate dehydrogenase	ldha	NM_012583
24	Ribosomal protein13a	Rpl13a	NM_017008

9.2. Quantitative RT-PCR: To amplify and simultaneously quantify the DNA of interest we performed real time polymerase chain reaction or quantitative PCR reactions. It was carried out on ABI 7500 Fast real time PCR system (Applied Biosystems, Carlsbad, CA) using SYBR Green master mix (Cat. No. 330500, SABiosciences, Frederick, MD). The

PCR reaction mixture included 1µl cDNA, 10.5µl RNA grade water, 1µl primer and 12.5µl SYBR Green master mix. The PCR conditions were the following: holding stage (95^0C for 10 min) and cycling stage (95^0C for 15 sec, 60^0C for 1 minute and 72^0C for 35 seconds) followed by melt curve to confirm the specificity of the amplified products.

10. Cerebrospinal fluid collection

The skin on the head was shaved and surgically prepared with 3 chlorhexadine scrubs and 3 alcohol scrubs. The surgical site was draped with sterile gauze and a small incision was made on the skin above the atlanto-occipital region. The head was placed in a stereotaxic apparatus and flexed downwards, so that it made a 45 ° angle to the rat's body. One end of a micro-hematocrit capillary tube was pulled with a pipette puller to get a pointed tip with 0.5mm diameter for insertion into the cisterna magna to collect the CSF. The other blunt end of the micro-hematocrit capillary tube was attached to a 7-10 cm length of Tygon® tubing. An 18G needle with a blunt tip was inserted into the other end of the Tygon® tubing and the needle was attached to a 1cc syringe. The plunger of the syringe was disconnected before cerebrospinal fluid (CSF) collection to allow its free flow. Once the spinotrapezius muscles on the rat's back region were separated from one other at the midline using cotton swabs, the landmark for CSF collection at the atlanto-occipital junction was seen. The pointed micro-hematocrit capillary tube was inserted into the atlanto-occipital junction and clear CSF (75 to 100 micro liters) flowing through the tube was collected in an Eppendorf® tube by either allowing it to flow freely or pulling the plunger if necessary.

11. Western blotting

The brains removed from rats anesthetized with pentobarbital sodium (50mg/kg ip) were extracted and then frozen on dry ice. Cryostat sections (500μm) from PVN, SFO, NTS, RVLM, CP and OVLT were made and punched using the Palkovits micro-dissection technique. Tissues were lysed using lysis buffer [0.5 mmol/l Tris·HCl (pH 6.8), 10% SDS, and 10% glycerol] with protease inhibitors (0.5 mmol/l PMSF, 10 μg/μl aprotinin, and 10 μg/μl leupeptin). Protein concentration was measured using the bicinchoninic acid protein assay (Sigma, St Louis, Mo), which was run on SDS-PAGE 10% gel or 12% gel and transferred to a nitrocellulose membrane and later blocked for 1 h with Odyssey® Blocking Buffer. Blots prepared were then incubated overnight (4°C) with primary antibody Anti-COX-1 (1:500; Millipore, Temecula, CA) or Anti-tubulin (1:2,000 dilution; Millipore; Temecula, CA) or Anti-PGDs (Cayman chemicals, Ann Arbor, MI) or anti-PLA2 (Novus biologicals, Littleton, CO). After rinsing the membrane 4 times for 5 minutes with tris buffered saline with tween-20 (TBS-T) and a final rinse in TBS, the blots were incubated using a secondary antibody for 1 h at room temperature. An Odyssey® imager was used to visualize the bands. For a positive control, one lane in the membrane had the purified form of the protein of interest.

12. Cyclooxygenase activity assay

The bifunctional enzyme COX exhibits both cyclooxygenase activity and peroxidase activity. The COX activity leads to conversion of the substrate arachidonic acid into PGG2 (hydroperoxy endoperoxide), and the peroxidase component converts PGG2 into PGH2, a precursor for the production of various other prostanoids through reduction

reactions. To determine the activity of the enzyme (both COX1 and COX2) in brain tissue, we used a cyclooxygenase fluorescent activity assay (Cayman Chemicals, Ann Arbor, MI). The assay utilizes the peroxidase component of the enzyme. The compound ADHP (10-acetyl-3, 7-dihydroxyphenoxazine), when added to tissue containing PGG2, forms a fluorescent compound resoruffin. This fluorescent compound is analyzed with an excitation wavelength between 530-540nm and an emission wavelength between 585-595nm. The test system included purified ovine COX-1 as a positive control.

Sample preparation: The rat brains were snap frozen on dry ice and stored at -80°C. 500µm brain sections were prepared using a cryostat at -15°C and brain punches from SFO, OVLT, PVN), RVLM, NTS and the third and fourth ventricle CP were homogenized in 50µl of lysis buffer (0.1M Tris HCl, pH 7.5, containing 100mM PMSF, leupeptin and aprotinin). The homogenized tissue was centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was then collected and frozen at -80°C before assay.

13. Blood collection and high performance liquid chromatography

For blood collection the rats were anesthetized using 0.5ml of Fatal Plus® (pentobarbital, 390mg/kg ip). 2-3ml of blood was collected using a 22gauge needle from the abdominal aorta. The blood was collected into 2ml Eppendorf® tubes and centrifuged at 14000 rpm for 10 minutes (Spectrafuge 16M Microcentrifuge). Plasma was collected from the top layer and placed in fresh Eppendorf® tubes without disturbing the pellet.

HPLC was performed using a Waters 510 HPLC pump (Millipore), Waters™717 Autosampler (Millipore) and Waters™ 996 photodiode Array Detector. Ketorolac

tromethamine (Cayman chemicals, Ann Arbor, MI) standards were prepared by dissolving 1mg in 1ml of saline. The working standard solutions (62.5pg/µl, 125pg/µl, 250pg/µl, 500pg/µl) were prepared by dilution of the primary stock solution with the saline. The plasma containing the compound was separated using Luna 5u C18 (5 µm particle size, 250 mm length x 4.6 mm I.D.) reversed-phase columns (Phenomenex, Torrance, CA). A 50:50 (v/v) mixture of acetonitrile containing 0.065% triethylamine and 1.65% glacial acetic acid (pH 4.3) was used as the mobile phase. The optimum flow rate of 1.0 mL/min was used for separation and the column was maintained at 20-22^{o}C. The detection wavelength was 314nm.

14. Immunofluorescent staining and confocal microscopy

Previously flash frozen slides containing 10µm thick sections of SFO, OVLT, PVN, RVLM and NTS were used for immunofluorescent staining by the MSU histopathology laboratory. Samples were cryoprotected and sections were placed in -80 ºC for storage until staining. Upon removal from -80ºC, slides were air dried overnight at room temperature and were placed in Tris buffered saline (pH 7.5) for 5 minutes for pH adjustment. Heat Induced Antigen Retrieval (ScyTek Laboratories, Utah) was performed at pH 6.0 in a rice steamer for 10 minutes at 100 degrees followed by an additional 20 minutes at room temperature. Slides were then blocked in 3% hydrogen peroxide in TBS for 10 minutes at room temperature. Autofluorescence was blocked using ammonium hydroxide and ethanol for 60 minutes at room temperature. Following these pretreatments, slides were loaded on a Dako Autostainer. Slides were blocked for non-specific protein with Normal Donkey Serum (Jackson ImmunoResearch, PA) for 30

minutes. An antibody cocktail of mouse anti-DP1 at a dilution of 1:100 (Thermo Scientific, MA) and rabbit anti-FOX/NeuN at a dilution of 1:1000 (Abcam, Cambridge, MA) in Normal Antibody Diluent (NAD) (Scytek – Logan, UT) was incubated for 60 minutes at room temperature. Secondary antibody cocktail of Donkey anti-Mouse Dylight 647 @ 1:500 and Donkey anti-Rabbit Dylight 405 @ 1:100 was made in 2% normal rat serum (Jackson ImmunoResearch, PA). Cover slips were placed on the slides after covering them with ProLong Antifade.

15. Confocal microscopy: An Olympus FluoView 1000 laser scanning confocal microscope was used to take 20X and 40X magnified images of the 10µm thick coronal brain sections. Differential interference contrast images and overlay images were taken to localize the receptors on the neurons and ependymal cells.

16. Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Mass spectrometry is an analytical technique that separates ionic species based on the electrical charge and atomic mass using magnetic and electrical fields. The main components of a mass spectrometer are the inlet for the sample, ion source, mass analyzer and an ion detector. After solid phase extraction the sample in the mass spectrometer is sprayed through a thin capillary in an electric field to create charged droplets. These droplets then pass through a heated inlet gas in the mass analyzer. Here they are separated according to their mass to charge (m/z) ratio. MS/MS is a two-stage system, where in the first stage an ion is preselected, and in the second stage, fragments induced by collision with an inert gas like argon or helium are analyzed.

Preparation of standards: All standards were obtained from Cayman Chemicals, Ann Arbor, MI.

a) PGE2 MS standard (Catalog No.10007211): 1mg/ml DMSO

b) PGD2 MS standard (Catalog No.10007202): 1mg/ml DMSO

c) PGJ2 MS standard (Catalog No.10007233): 500µg/100µl methyl acetate (5mg/ml)

d) Δ^{12}-PGJ2 lipid maps MS standard (Catalog No.10007234): 100µg/100µl methyl acetate (1mg/ml)

e) 15-deoxy-$\Delta^{12, 14}$-PGJ2 lipid maps MS standard (Catalog No.10007235): 100µg/100µl methylacetate (1mg/ml)

f) Arachidonic acid: Item # 90010 500mg/ml

16.1. Solid phase extraction of lipid mediators from brain punches

Solid phase extraction (SPE) was performed using OASIS max µElution Plate 30 µm (Waters Corporation, Milford, MA). The steps followed for SPE were first, sample preparation (total volume of 500µl). To the sample (brain punches from region of interest), 18Ω water (245µl), 0.01% butylated hydroxytoluene to prevent oxidation (1µl of 1% BHT (25mg BHT from Cayman chemicals, Ann Arbor, MI in 1ml methanol) and Internal standard (4µl of 500pg/µl of PGD2-d4) were added. Then 250µl of 4% phosphoric acid (Sigma Aldrich, St. Louis, MO) (4% H3PO4=2g in 50ml water) was added to disrupt the protein binding and help flow through the SPE cartridge. The sample was homogenized (Omi TH int. – for 5-10 seconds) and centrifuged at ~12000g for 10 min (Spectrafuge 16M-14000rpm). The supernatant was separated and the

volume was made to up to 750µl. The OASIS max µElution cartridge was conditioned with 100µl methanol and equilibrated with 100µl water.

For loading, 750 µl of sample was loaded to the cartridge and a flow rate of 1 drop/ sec was maintained. [Note: The flow through the liquid was collected the first time to perform extraction efficiency]. For the first wash, 200µl of 2% ammonium hydroxide (2ml NH4OH+100ml water) at pH 10-11 was used for removal the salt and charge the acidic analytes and lock them in the cartridge. [Note: The flow through the liquid was collected the first time to perform extraction efficiency]. For the second wash, 200µl of 1ml 100% methanol was used to remove the basic and the neutral interference. [Note: The flow through the liquid was collected the first time to perform extraction efficiency]. For elution, 50µl of a mixture of 100% methanol and 2% formic acid was used for eluting the lipid prostaglandins and AA from the cartridge. (0.227ml of 88% formic acid + 10ml methanol).

The eluted solution was placed in a screw cap vial (Part # 5182-0715, Agilent Technologies, Santa Clara, CA) with a vial insert (Part # 5183-2085) and blue screw cap (Part # 5182-0717). The vials were stored at -80°C before LC-MS/MS analysis using Waters Xevo TQ-S UPLC MS/MS (Waters Corporation, Milford, MA). The experiments were performed using the biochemistry RTSF facility at Michigan State University.

$$\text{Extraction efficiency} = \frac{\text{Samples with standard mixed before SPE}}{\text{Samples with standards spiked post SPE}} \times 100$$

The loading waste, washes were dried in vacuum and reconstituted in 50µl acetonitrile/water (1:1, V/V) for analysis. This determined the loss of analytes and was performed during the first experimental run.

16.2. Liquid-liquid extraction of lipid mediators from cerebrospinal fluid

Liquid-liquid extraction was used for processing cerebrospinal fluid (CSF). 100µl of CSF sample was added to 100 µl of 0.15M EDTA, 80µl of 45nM phosphoric acid and 20µl of methanol. The liquid-liquid extraction was performed as described previously (72) where samples were incubated with 600µl of ethyl acetate. After vortexing thoroughly, two layers were obtained. The top layer containing the analytes was separated and 600µl of ethyl acetate was added. After vortexing, the top layer was removed and subjected to a gentle stream of nitrogen at 45°C. The residues were reconstituted with 50µl of acetonitrile/ water/formic acid (20:80:0.0025, v/v, pH 4.0) and centrifuged for 2 minutes at 10,000g. It was transferred to a glass vial and stored at -80°C prior to injection into the Xevo TQ-S for analysis.

16.3. Chromatography

Mobile phase C consisted of 0.1% formic acid and mobile phase D consisted of 100% acetonitrile. Compounds were separated using Ascentis® Express C18 HPLC column (10cm x 2.1mm, 2.7µm) maintained at a temperature of 40°C. Initial conditions for separation were 1% mobile phase C and 99% mobile phase D at a flow rate of 0.3ml/min. The proportion of mobile phase C gradually increased to 99% for 14 minutes at a flow rate of 0.3ml/min. The total run time was 14 minutes.

16.4. Mass spectrometry

Mass spectrometry was performed using an Acquity UPLC system coupled to a Waters Xevo TQ-S mass spectrometer (Waters, Milford, MA) in an electrospray negative ionization mode. An extract volume of 10 µl was injected into the UPLC system and eluted with a gradient mixture of 0.1% formic acid and 100% acetonitrile. The mass spectrometer was optimized following tuning in multiple reaction monitoring (MRM) mode with a capillary voltage of 3.0 V, cone voltage of 35.0 V, source offset of 50.0 V, analyser LM resolution 1 of 2.93, HM resolution 1 of 14.60, ion energy 1 of 0.6, LM resolution 2 of 2.79, HM resolution 2 of 14.90, ion energy 2 of 0.9, desolvation temperature of 500°C, desolvation gas flow of 600L/Hr, cone gas flow of 150L/Hr and collision gas pressure of 7 bar and collision gas flow of 0.20mL/min.

The mass to charge ratio (m/z) of parent and daughter ions of individual compounds with their dwell time, cone voltage and collision voltage were as shown in table 1. Data was acquired and analyzed using Target Lynx™ Software. Calibration curves were constructed by plotting peak area ratio (standard to internal standard) versus the nominal concentration and were fit using least-square regression with 1/x weighting.

Table 2-2: Arachidonic acid and prostanoid mass to charge ratio, dwell time and voltage for mass spectrometry analysis

	Compound	Parent (m/z)	Daughter (m/z)	Dwell (s)	Cone (V)	Collision (V)
1	AA	303.2	259	0.125	45	16
2	Prostaglandin J2	333.1	271.1	0.1	33	16
3	Δ^{12}-PGJ2	333.1	189	0.1	21	16
4	15-deoxy-$\Delta^{12, 14}$-PGJ2	315.2	271.1	0.1	39	10
5	Prostaglandin E2	351.2	271	0.125	55	16
6	prostaglandin D2	351.2	203.1	0.125	55	22
7	Prostaglandin D2-d4	355.2	319	0.125	33	10

17. Statistical analyses

Within group differences were assessed by a one-way repeated measures ANOVA with post-hoc multiple comparisons using Dunnett's procedure (GraphPad Instat 3). Between group differences were assessed by a two-way mixed design ANOVA and post-hoc testing at each time point was performed using Bonferroni's procedure to correct for multiple comparisons (GraphPad Prism 4). For the PCR array between group differences were analyzed by one-sample t-tests with a hypothetical mean as 1. Student's t-test was used to analyze the western blot data. A p-value of < 0.05 was considered significant. All results are presented as means ± standard error of the mean (SE).

CHAPTER 3

CYCLOOXYGENASE-1 AND NOT CYCLOOXYGENASE-2 INHIBITION ATTENUATES ANGIOTENSIN II-SALT HYPERTENSION AND NEUROGENIC PRESSOR ACTIVITY IN THE RAT

1. Introduction

Angiotensin II is involved in the etiology of systemic hypertension through various mechanisms. These include direct contraction of blood vessels and subsequent remodeling of their structure (83), reductions in renal sodium excretion (174), release of aldosterone (253), and activation of the sympathetic nervous system (SNS) (32, 58). McGiff proposed the idea that the renin-angiotensin system influences eicosanoid formation and reported the release of prostaglandin-like substances during infusion of AngII (151). But are eicosanoids involved in AngII HTN? This question has been addressed in numerous previous studies through the use of COX inhibiting drugs (22, 57, 211) and COX knockout mice (22, 188, 260). However these efforts produced contradictory conclusions, possibly because the studies employed quite varied treatment regimens, treatment durations and methods of measuring blood pressure. Also, has noted earlier, eicosanoids cause both pro-hypertensive and antihypertensive effects by acting on the kidneys, blood vessels, endocrine organs and brain. In addition, the relative importance of COX-1 versus COX-2 in AngII HTN is disputed (22, 188, 211).

As reviewed in Chapter 1, sympathoexcitation is an important cause of HTN during AngII infusion, particularly in the setting of high dietary salt intake (127, 203). Both older (115, 143) and recent (22) experiments indicate that the sympathetic nervous system is activated by eicosanoids in experimental AngII HTN and/or that eicosanoids are involved in mediating neurogenic hypertension. But the precise mechanisms by which eicosanoids cause sympathoexcitation remain to be fully elucidated. Therefore, in this study I used radiotelemetric methods and AngII-salt HTN protocol to determine the role

of eicosanoids in causing neurogenic HTN. In addition, pharmacological tools were used to determine the relative contribution of COX-1 and COX-2 to sympathetic control of BP in AngII-salt HTN.

2. Experimental Protocols

2.1. Selective COX-1 or COX-2 inhibition in chronic AngII-salt hypertensive rats:

Radiotelemetery- implanted rats, fed a high salt diet were used in this experiment. Following a 5-7 day of surgical recovery period and 3 days of baseline blood pressure recordings, DMSO (vehicle) or a selective COX-1 inhibitor SC560 (10mg/kg ip) or a selective COX-2 inhibitor nimesulide (10mg/kg ip) were injected once daily for the remainder of the study. The doses for COX-1 and COX-2 inhibitors were chosen based on previous reports (84, 242) of the use of 10mg/kg ip dose, successfully mimicking the effects of the widely used COX inhibitor aspirin as well as the successful reduction of eicosanoid levels in various tissues. Both SC560 and nimesulide are reported to cross the blood brain barrier (215) and therefore could act both peripherally and centrally. After 4 days of COX inhibition or DMSO injection, AngII or physiological saline infusion was initiated using a miniosmotic pump (2ML2, Alzet, Cupertino, CA). AngII was infused at the rate of 150ng/kg/min sc, for 14 days. MAP and HR were measured for the entire duration of the experiment. Animals were subjected to ganglionic blockade with hexamethonium (Sigma, St. Louis, MO; 30mg/kg ip) 10 days after starting AngII administration to assess neurogenic pressor activity (228). The fall in MAP was recorded 15 minutes after injecting hexamethonium, and the magnitude of that fall was used as an estimate of neurogenic pressor activity.

3. Results

3.1. Effect of selective COX-1 and COX-2 inhibition on chronic AngII-salt HTN:

COX-1 inhibition with SC560 (Figure 1): In vehicle (DMSO) treated rats (n=7) fed a high salt diet, MAP was 105±2 mmHg on pretreatment day 3 and gradually increased to 135±8 mmHg on day 14 of infusion. In the SC-560 treated group (n=10), during the pretreatment period or during treatment with SC560 alone MAP was not different from that of the vehicle treated group. During AngII administration, however, MAP rose to only 117±1 mmHg 14 days after AngII infusion in SC560 treated rats, and was significantly lower than that of the control group on days 6-14 of AngII infusion.

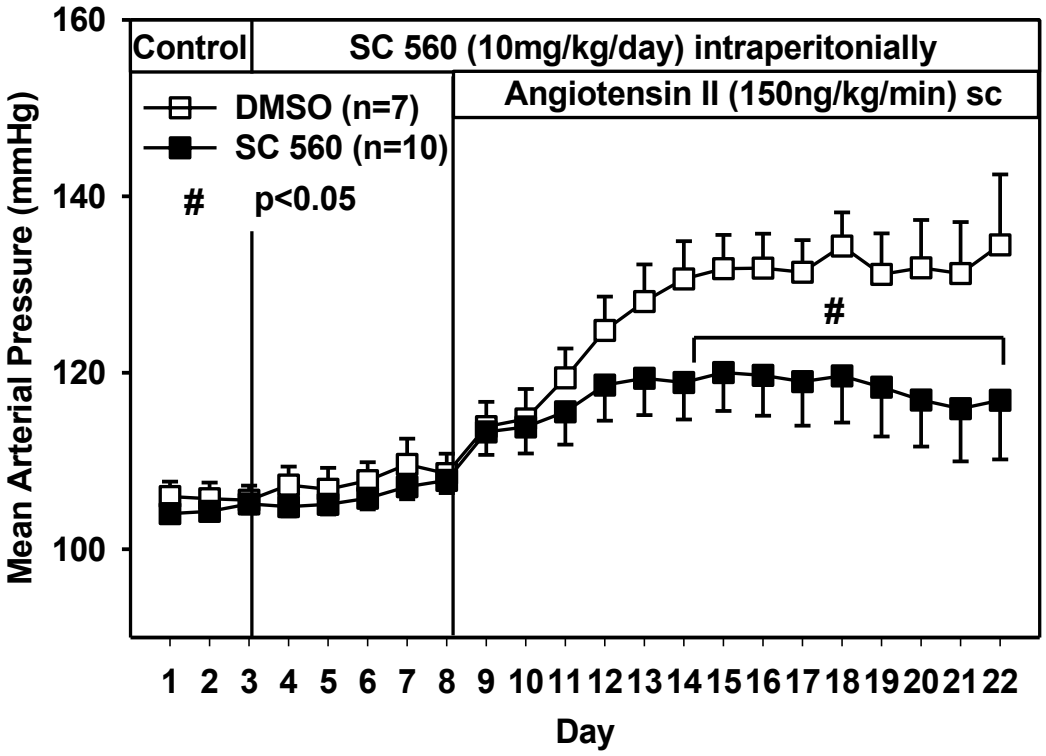


Figure 3-1: Effect of selective cyclooxygenase (COX)-1 inhibition on mean arterial pressure (MAP). Response to AngII in rats fed a high salt diet. Rats were treated daily with the selective COX-1 inhibitor SC560 or vehicle. # =p<0.05 SC560 vs. DMSO as vehicle.

COX-2 inhibition with nimesulide (Figure 2): Treatment with nimesulide alone did not affect MAP relative to the vehicle (DMSO) ip treated control group. Both groups had similar MAP during the control period. The vehicle treated group (n=5) on a high salt diet had a baseline MAP of 102 ± 2 mmHg on pretreatment day 3, which was similar the drug treatment group's (n=5) day 3 MAP of 101 ± 1 mmHg. From day 4 to 7, nimesulide treatment did not cause any change in MAP compared to the vehicle treated rats. After starting the AngII infusion, MAP in the vehicle treated group increased to 126 ± 6 mmHg at the end of infusion period. In the nimesulide treated group, MAP increased to 135 ± 12 mmHg. Therefore, AngII infusion caused the same magnitude of hypertension in vehicle and nimesulide treated rats. The data suggests that the COX-2 inhibitor causes a slight increase in blood pressure in AngII-salt HTN rats.

Neurogenic pressor activity: The maximum falls in MAP after ganglionic blockade on day 10 of AngII infusion in vehicle treated rats and in rats subjected to selective COX inhibition are shown in Figure 3. In experiments with SC560 (Figure 3A), ganglion blockade decreased MAP significantly more in control (39 ± 4 mmHg) versus drug-treated (27 ± 4 mmHg) rats. In experiments using nimesulide (Figure 3B), the fall in MAP with ganglion blockade was similar in control (58 ± 9 mmHg) and drug treated (62 ± 12 mmHg) animals.

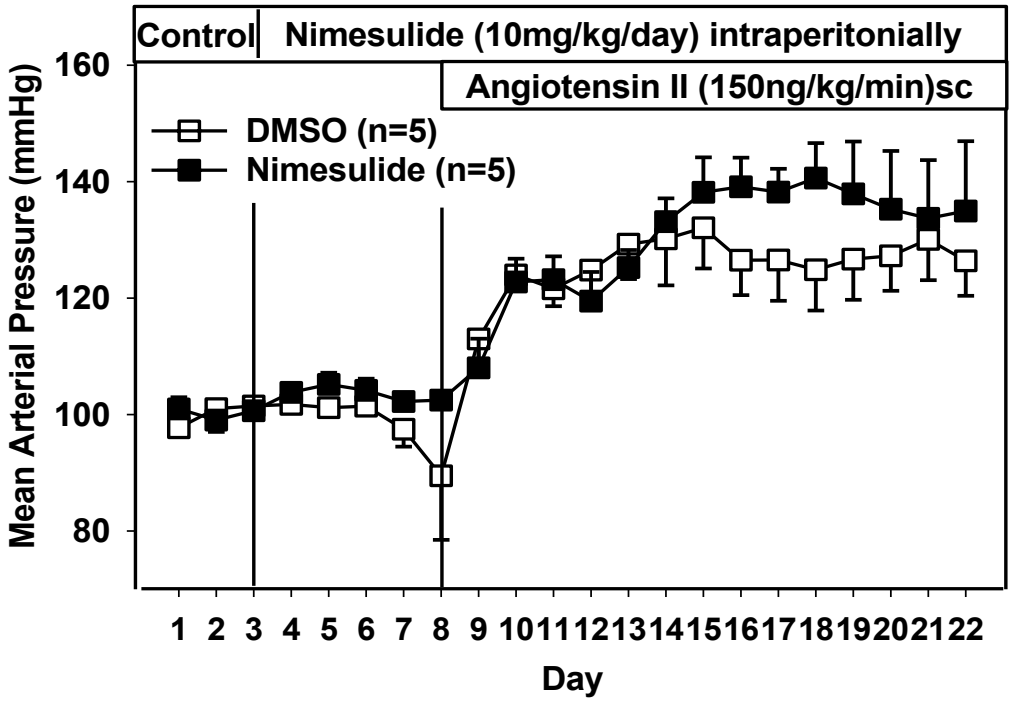


Figure 3-2: Effect of selective COX-2 inhibition on MAP. Rats were treated with the selective COX-2 inhibitor nimesulide or vehicle and were infused with angiotensin II subcutaneously.

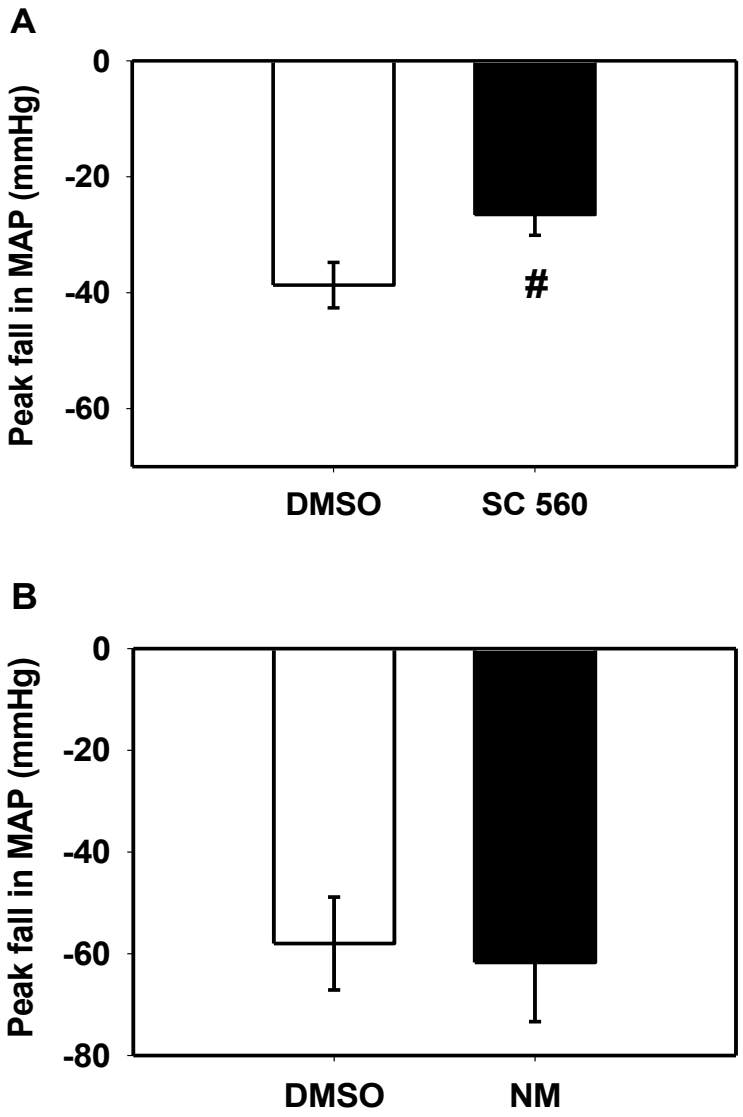


Figure 3-3: Depressor response to ganglionic blockade 10 days after AngII infusion in DMSO, SC560 and nimesulide (NM) treated rats. Peak fall in MAP in response to hexamethonium administration (30mg/kg ip) in SC560 (A) and NM (B) treated rats. # =p<0.05 SC560 vs. DMSO

4. Discussion

Previous investigators have used both pharmacological and gene knockout approaches to determine if eicosanoids play a critical role in AngII mediated hypertension, but their results have led to conflicting conclusions (22, 188, 260). Eicosanoid products can exert both pro- and anti-hypertensive effects: thus, their net effect on blood pressure will depend on where increased eicosanoids formation occurs and on which specific products are released. Earlier studies showed that dietary salt intake is one factor that influences the contribution of eicosanoids to AngII dependent hypertension (115, 157). More recent studies by King found that COX inhibition with ketoprofen, a non-selective COX inhibitor, did not prevent hypertension in rats on a normal salt diet (0.4%NaCl). However, in rats that were on a high salt diet (2% NaCl), COX-inhibition prevented the later phase of AngII-salt hypertension (beyond day 5 of AngII infusion), but not the early rise in MAP (days 1-4) of AngII infusion.

There are multiple potential explanations for why eicosanoids could make a higher contribution to AngII hypertension under conditions of high salt diet. However my lab and others have shown that high salt intake amplifies the role of the sympathetic nervous system in AngII HTN (176, 203, 260). Furthermore, Kings work showed that selective removal of the splanchnic sympathetic innervation significantly attenuated chronic AngII-salt HTN (128) during the later phase of HTN development, indicating that specifically splanchnic SNS activity is important for HTN development in this model. Since direct recordings in conscious rats with chronic AngII HTN showed an increase in splanchnic SNS activity that was associated with a significant rise in urinary excretion of cyclooxygenase products (143), we hypothesized that COX-derived prostanoids could

increase blood pressure in the AngII-salt model by stimulating SNS activity. This idea was further supported by the work that shows that ketoprofen treatment had a more prominent effect on AngII-salt hypertension development during the later days of AngII infusion (8).It has been previously reported that neurogenic mechanisms appear to play a greater role in this hypertension model after 4-5 days of AngII infusion (127, 128, 133). The increase in MAP on days 1-4 appears to be caused by either the direct pressor actions of AngII or perhaps renal sodium and water retention. The increase in neurogenic pressor activity only during the later phase of AngII-salt HTN may be due to an ability of COX products to “prime” subsequent changes in sympathetic regulation of BP. This idea will be addressed in more detail in Chapter 4 of my dissertation.

Earlier, King et al tested the role of COX products in the neurogenic actions of AngII in two ways. First the SNS activity was assessed using measurement of whole body norepinephrine spillover in ketoprofen treated rats. A statistically significant 71% increase in whole body NE spillover on days 7 and 14 of AngII infusion was seen in rats on a high salt, but not low salt, diet (127). In unpublished studies in rats treated with ketoprofen there was a much smaller (though statistically significant) increase in whole body NE spillover on day 7 of AngII infusion (36% vs. control) and a statistically non-significant increase (14% vs. control) on day 14 of AngII infusion in high salt fed rats. Therefore, the global SNS activation caused by AngII infusion in rats on high salt diet, as indicated by significant elevation in plasma NE and whole body NE spillover (127) was largely, but not completely, prevented by non-selective COX inhibition.

Furthermore, in King's studies measurement of neurogenic pressor activity (the acute contribution of the autonomic nervous system to arterial pressure) was used to test if eicosanoid-related SNS activity contributed to AngII-salt hypertension. Vehicle treated AngII-salt HTN rats exhibited a marked drop in MAP during ganglionic blockade; AngII-salt rats treated with ketoprofen showed a substantially smaller drop in pressure, although the difference was not statistically significant. This suggests that a eicosanoid-driven increase in SNS activity may be at least partially responsible for the hypertension in AngII-salt treated rats.

As a first step in my study I asked whether eicosanoids involved in AngII-salt HTN were derived from the enzymatic isoforms COX-1 or COX-2. It is known that AngII exerts physiologically significant pro-inflammatory effects that contribute to numerous cardiovascular diseases (38, 57). AngII appears to produce inflammatory responses predominantly in a localized manner in target tissues such as blood vessels (38) kidney (254) and brain (276). COX-2 is the isoform most strongly implicated in generating inflammatory eicosanoids, so it is logical to assume that COX-2 the key player in AngII-salt HTN. Some published studies support this conclusion. For example, pretreatment with COX-2 inhibitors like refecoxib and nimesulide attenuated AngII-HTN in Sprague Dawley rats (149, 190, 260). However, in contrast to this idea, other studies indicate that COX-1 is the source of eicosanoids involved in AngII HTN (7). In a recent report, Cao and colleagues showed that AngII HTN was attenuated in COX-$1^{-/-}$ mice but not COX-$2^{-/-}$ mice (22). As noted earlier, the contradictory reports from various laboratories could be attributed to the differences in animal models used, blood pressure

measurement methods like tail cuff plethysmography versus radiotelemetry, or the mode of drug administration.

My studies indicate that the key eicosanoids involved in AngII-salt hypertension are derived from the COX-1 and not the COX-2 isoform: hypertension development was markedly and significantly attenuated by treatment with the COX-1 inhibitor SC560, but not by treatment with the COX-2 inhibitor nimesulide. However, with selective COX-1 inhibition, we did not see as complete an attenuation of hypertension as we observed with the non-selective COX inhibitor ketoprofen. This may be due to 1) additional pharmacological actions of ketoprofen, or 2) involvement of COX-2 in the development of hypertension, although our results with the selective COX-2 inhibition do not support the latter idea. The doses of the inhibitors used to totally block COX enzyme could possibly fall on different stages of their dose response curves. This differential COX inhibition with a particular dose of NSAID could explain the increased attenuation of HTN with ketoprofen (2mg/kg, sc) compared to SC560 (10mg/kg, ip). It is further notable that COX-1 inhibition with SC560 significantly reduced neurogenic pressor activity in AngII-salt HTN rats. This result supports the hypothesis that eicosanoids increase arterial pressure in AngII-salt HTN in part by increasing SNS activity.

In conclusion, this study suggests that in rats fed a high salt diet, chronic AngII infusion stimulates the formation of eicosanoids from COX-1 that activate the SNS and increase arterial pressure. This data does not allow us to establish where these eicosanoids are produced, or where they act, to increase SNS activity or arterial pressure. However,

central injections of eicosanoids can increase SNS activity (171) and arterial pressure (112), and a recent study in mice strongly implicated *brain* COX-1 activity in AngII HTN (22). Thus, studies described in my next chapter focus on exploring the role of brain eicosanoids in AngII-salt hypertension.

CHAPTER 4

TRANSCRIPTIONAL AND TRANSLATIONAL REGULATION OF CENTRAL ENZYMES AND RECEPTORS IN THE EICOSANOID SYNTHESIS PATHWAY DURING THE DEVELOPMENT OF ANGIOTENSIN II-SALT HYPERTENSION IN THE RAT

1. Introduction

Samuelsson first described the eicosanoids in the brain in 1964 (200) where these "local hormones" are known to act as mediators of inflammation, fever, feeding, sleep and other functions. Relatively little evidence has been published supporting a role for brain eicosanoids in BP regulation, and I summarized that evidence in Section 8.2.3 of Chapter 1. As described earlier my dissertation, both other investigators and I found that treatment with COX inhibitors during AngII-salt HTN development does not affect BP during the early stage (1-4 days of AngII infusion) but significantly lowered BP during the later neurogenic phase of HTN (5-14 days after AngII infusion). Therefore, here I hypothesize that 1) *eicosanoids act early in HTN development to "prime" a later engagement of neurogenic pressor mechanisms,* and 2) *early changes in the expression of COX pathway enzymes and/or receptors in the brain could be the cause of this "priming" effect in AngII-salt HTN*. To address these hypotheses I did experiments designed to 1) identify the critical time period during with eicosanoids operate to affect AngII-salt HTN development, and 2) analyze eicosanoid pathway gene and protein expression during that critical time window in specific brain nuclei known to regulate sympathetic activity and BP.

2. Experimental protocols

2.1. Non-selective COX inhibition in AngII-salt treated rats with established HTN:

In order to test the effect of NSAID treatment on rats with established AngII-salt HTN the following experiment was performed. Rats were allowed a 5-day recovery period after telemeter surgery. After three days of control MAP and HR recordings the rats

were divided into two groups, both receiving AngII (Sigma) delivered at a constant rate of 150ng/kg/min using a mini-osmotic pump (2ML2, Alzet) implanted subcutaneously, starting on day 3 until day 17 (figure 1). On days 9, 10 and 11 of the AngII infusion period (after HTN was well-established), one group received once daily subcutaneous injections of either vehicle (saline, n=5) or the potent non-selective COX inhibitor ketoprofen (2mg/kg, n=7). The subcutaneous AngII pumps were removed on day 14 of AngII infusion to allow reversal of the hypertension. Then starting 4 days later rats from one group (n=5) received once daily subcutaneous injections of saline vehicle for three consecutive days while the other group received ketoprofen (2mg/kg, n=7).

Hexamethonium (30mg/kg, IP) was given during the control period (C2), drug treatment period (D3) and the angiotensin treatment period (A10) of the protocol, respectively, to determine the effect of ketoprofen treatment on neurogenic pressor activity in established AngII-salt hypertensive rats. The magnitude of the acute (10-30 minutes) fall in BP was used as an indicator of the degree of sympathetic regulation of blood pressure, as described in General Methods.

2.2. Non-selective COX inhibition prior to and during early stages of AngII-salt HTN: My next goal was to identify potentially critical time periods during which eicosanoids contribute to the development of AngII-salt HTN. To achieve this end, I investigated the effect of non-selective cyclooxygenase inhibition at two different time points during the development of AngII-salt HT. In the first set of experiments, after 3 control days of BP measurement either saline vehicle (n=3) or ketoprofen (2mg/kg, n=3)

was administered for 4 days prior to AngII infusion. Hexamethonium (30mg/kg, IP) was given once acutely 10 days after starting AngII to evaluate neurogenic pressor activity in the two groups of rats.

In the second set of experiments, after 3 control days of BP measurement ketoprofen (2mg/kg, sc.) or saline was administered once daily for 4 days (prior to starting AngII infusion) and then once daily for an additional week (during the first 7 days of AngII infusion). Neurogenic pressor activity was measured on day 10 of AngII infusion, as described above.

2.3. Brain eicosanoids in the early stage of AngII-salt HTN

2.3.1. Transcriptional and translational regulation of eicosanoid pathway related genes:

For performing PCR and western blot analysis of tissue punches collected from cardio-regulatory brain regions, a total of 22 radiotelemeter-implanted rats were used. After 3 days of control recording, AngII (150ng/kg/min, sc., n=12) or physiological saline (n=10) was delivered for 4 days using a mini-osmotic pump (Alzet, 2ML2). Then the rats were sacrificed, brains were collected, snap frozen in dry ice and kept at -80°C until further analysis as described in Chapter 2.

2.3.2. PCR array:

Expression level of 21 genes involved in eicosanoid signaling was measured in PVN, OVLT, SFO, CP, RVLM and NTS as described in Chapter 2.

2.3.3. Quantitative real time polymerase chain reaction (q RT-PCR):

To confirm L-PGDS expression in OVLT and to measure the cPLA2 gene expression in 4 day AngII treated and vehicle treated rat brains, punches were collected from PVN, SFO, OVLT, RVLM, NTS, third ventricle CP (3VCP), fourth ventricle CP (4VCP) and the middle cerebral artery (MCA) (n=5/group) and q RT-PCR was performed as described in Chapter 2.

2.3.4. Western blot analysis:

To analyze changes in eicosanoid pathway proteins during early development of HTN, the brain from 4 day AngII (n=7) or vehicle (n=5) treated rats were collected. Punches from PVN, SFO, OVLT, NTS, RVLM, 3VCP and 4VCP were analyzed as described in Chapter 2.

3. Results

3.1. Non-selective COX inhibition in established AngII-salt HTN rats:

The baseline levels of MAP in both groups were identical from control day 1 to control day 3. After starting AngII infusion MAP increased from 101±1 mmHg on control day 3 to 125±7 mmHg on day 8 of AngII infusion in the vehicle group, and from 102 mmHg on day 3 to 122±3 mmHg on day 8 in the group that would later receive treatment with ketoprofen (Figure 4-1A). Rats were then given either saline vehicle or ketoprofen (2mg/kg/day, ip) for 3 consecutive days. MAP was not affected by ketoprofen treatment in these animals with established hypertension, being 126±8 mmHg in the vehicle group and 128±3 mmHg in the ketoprofen group, respectively, on the last day of treatment

(Figure 4-1A). Ganglion blockade with hexamethonium (10 days after AngII infusion), on the second day of ketoprofen or vehicle treatment caused similar depressor responses in the vehicle treated (66±14 mmHg) and ketorprofen treated (55±8 mmHg) rats (Figure 4-1B).

After the 14-day AngII infusion period the osmotic pumps were removed and MAP fell over several days to 103±1 mmHg and 103±2 mmHg in the vehicle and ketoprofen groups, respectively. At this point we again administered ketoprofen or vehicle for 3 days and saw no change in MAP in either group (Figure 4-1A).

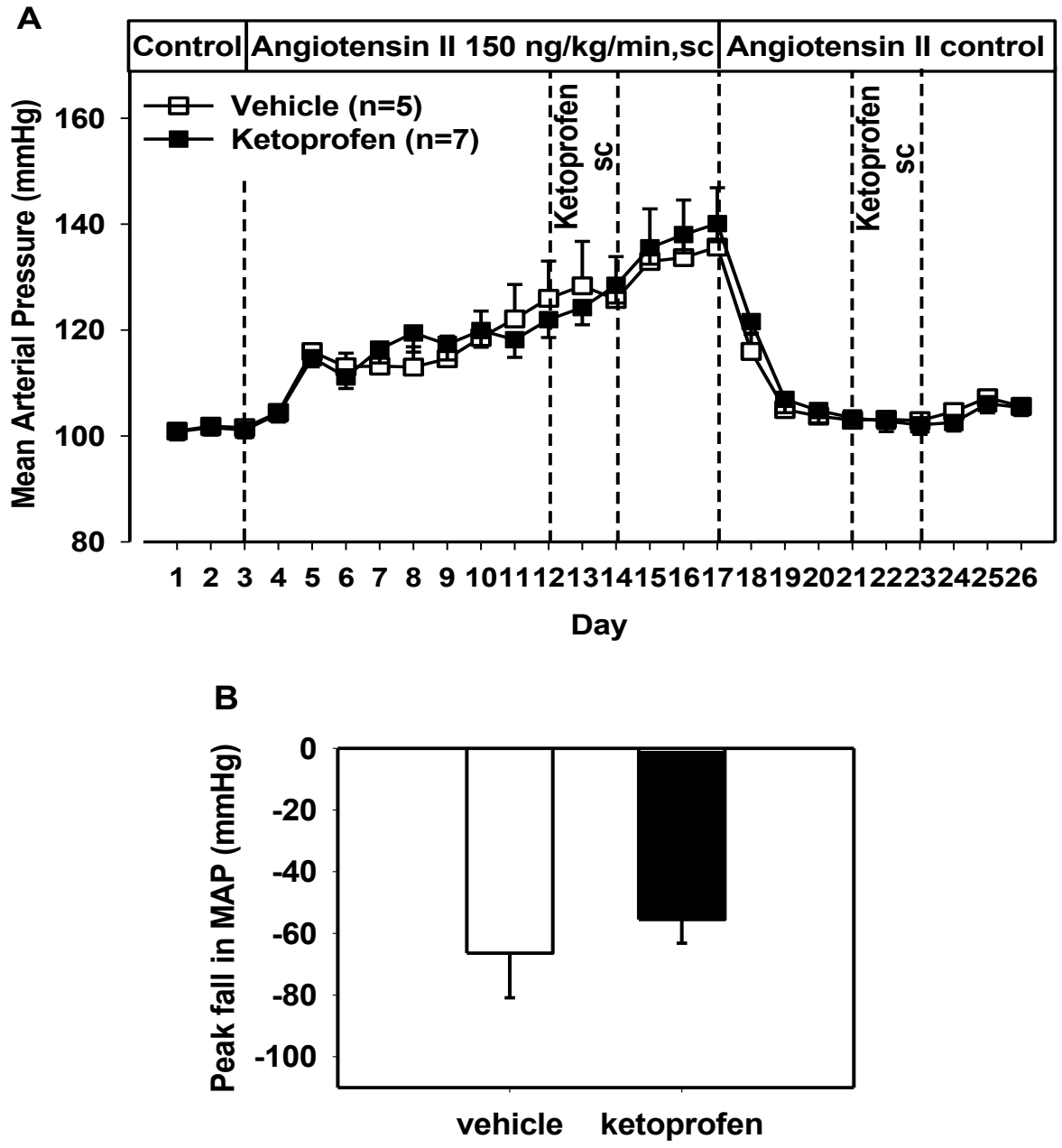


Figure 4-1: The effect of non-specific cyclooxygenase inhibition on MAP and neurogenic pressor activity in established AngII-salt HTN rats. A) MAP in rats on 2% NaCl diet given a three-day treatment of non-selective cyclooxygenase inhibitor or vehicle during and after infusion of AngII. B) Peak fall in MAP in response to ganglion blockade with hexamethonium during ketoprofen or vehicle treatment in rats with established HTN rats after 10 days of AngII infusion.

3.2. Non-selective COX inhibition prior to and during the early stages of AngII-salt HTN:

Figures 4-2 and 4-3 show data from rats treated with ketoprofen for different lengths of time prior to, or during the early days of, chronic AngII infusion. Figure 4-2A shows MAP in rats pretreated with vehicle (n=3) or ketoprofen (2mg/kg, sc, n=3) for 5 days prior to AngII infusion. After 14 days of AngII infusion MAP rose to 147±2mmHg in vehicle treated rats and to only 129±8mmHg in ketoprofen treated rats, but there was no statistically significant difference in MAP between the two groups. Acute depressor responses to hexamethonium on day 10 of AngII infusion were similar in vehicle (-57±5mmHg) and ketoprofen (-56±14mmHg) treated groups (Figure 4-2B).

Figure 4-3 shows results from studies in which ketoprofen administration was started 4 days prior to beginning AngII infusion and then extended for 7 more days (after the start of AngII infusion). Another group of animals received only saline vehicle treatment over the same time course. MAP was similar in the two groups prior to AngII infusion, e.g. 100±3 mmHg on D1 in vehicle treated rats and 103±1 mmHg on D1 in ketoprofen treated animals prior to starting AngII infusion (Figure 4-3A). In rats treated with vehicle only, MAP increased markedly during AngII infusion and was 141±10 mmHg by the end of the infusion period. In rats treated with ketoprofen, however, MAP rose initially up to A4 (125±3) during AngII infusion but was only 109±7 mmHg by the end of the infusion period (significantly lower than MAP in vehicle treated animals). The depressor response (Figure 4-3B) was significantly less in ketoprofen treated rats (-35±7 mmHg) than in vehicle treated rats (-92±12 mmHg).

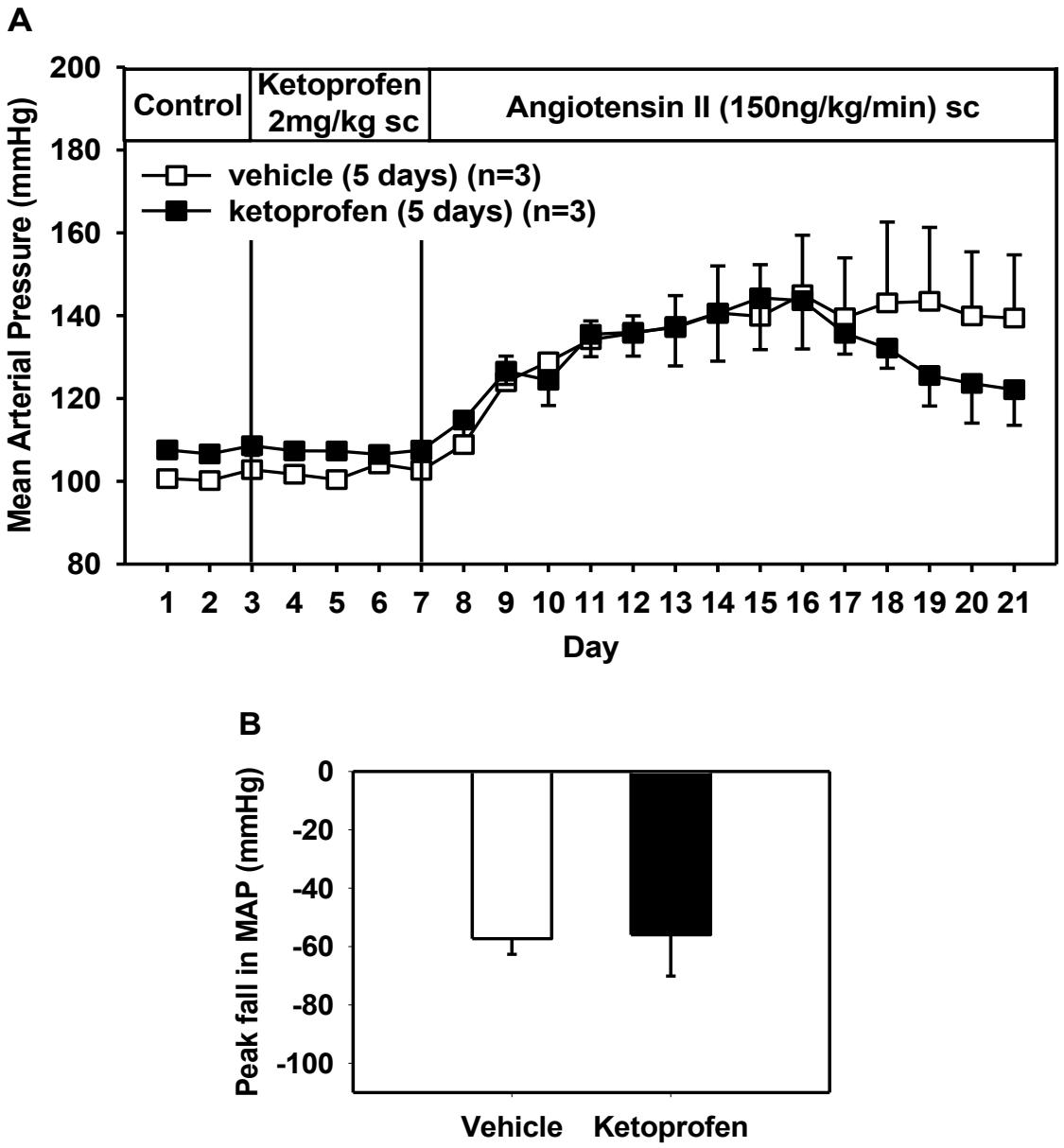


Figure 4-2: The effect of non-selective cyclooxygenase inhibition prior to AngII administration on AngII-salt HTN and neurogenic pressor activity. A) Mean arterial pressure in rats treated with cyclooxygenase inhibitor in one group and vehicle in another group for 5 days prior to making them hypertensive with AngII infusion. B) Peak fall in MAP in response to ganglion blockade after 10 days of AngII infusion.

= ($p<0.05$) vs. control rats.

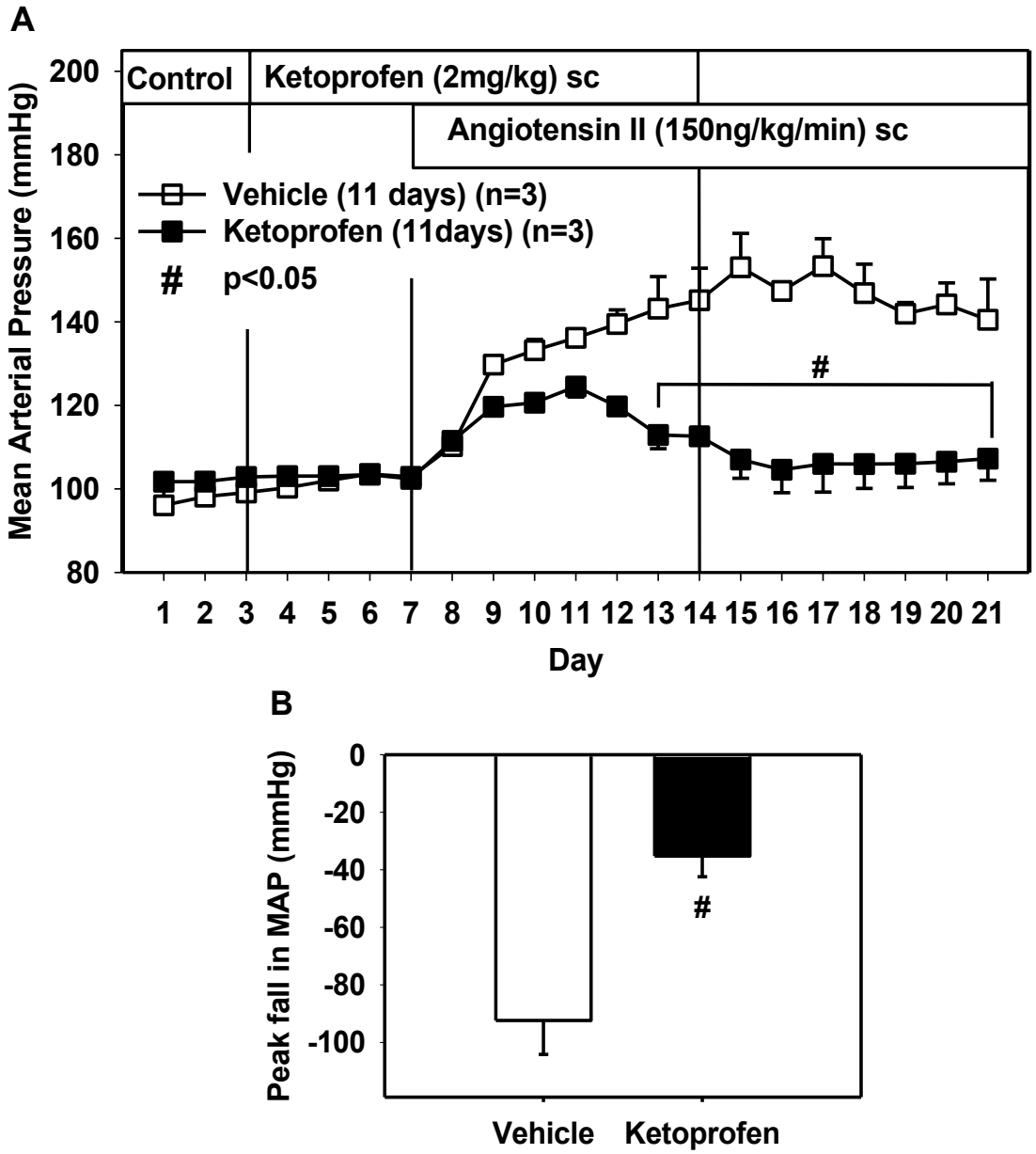


Figure 4-3: The effect of non-selective cyclooxygenase inhibition prior to and during the early phase of AngII administration on AngII-salt HTN and neurogenic pressor activity. A) MAP in rats after non-selective cyclooxygenase inhibition or vehicle treatment for 5 days prior to and first 7 days of AngII infusion. B) Peak fall in MAP in response to ganglion blockade with hexamethonium on day 10 (A10).

= ($p<0.05$) vs. control rats.

3.3. Gene array analysis of eicosanoid related gene expression in the brain of rats in the early stage of AngII-salt HTN:

PCR array analysis was used to measure transcriptional regulation of genes in brain samples obtained from rats on day 4 of AngII-salt administration. MAP on day 4 was 105±2 mmHg in control rats and 125±5 mmHg in AngII-salt treated rats. The PCR data are shown in Figure 4-4. Between group differences were analyzed by one-sample t-tests with a hypothetical mean as 1. AngII treated rats showed a significant up-regulation of Arachidonate 5-lipoxygenase (Alox5) (2.0 fold), PGD synthase 2-hematopoitic (Ptgds2) (1.6 fold) and down-regulation of PG-endoperoxide synthase 1 (Ptgs1) (0.9 fold) and prostacyclin synthase (0.6 fold), in the RVLM (n=4). In the OVLT (n=2), lipocalin type prostaglandin D synthase or PGD2 synthase (brain) (ptgds) (4.3 fold) expression was increased, whereas COX1, PGH synthase (0.2 fold) and PGD receptor (0.6 fold) expression were significantly down regulated. Eicosanoid gene expression was unaffected in the PVN, (n=5), SFO (n=5), NTS (n=5) and CP lining the third (n=5) and fourth ventricle (n=5). The p value of mRNA fold change for PGD2 synthase (brain) in CP of third ventricle was 0.0648.

3.4. Quantitative RT-PCR:

Q RT-PCR was performed in order to confirm the result of gene expression changes found in OVLT with PCR array. In rats (n=5) treated with AngII for 4 days, there was a significant 3-fold increase in L-PGD-synthase gene expression in the OVLT compared to vehicle treated rats.

Q RT-PCR was also performed for another rate limiting enzyme in eicosanoid synthesis, i.e. cPLA2, since it was inadvertently not included in the customized eicosanoid pathway PCR array we used. The enzyme cPLA2 expression was significantly higher in OVLT (2.2-fold, n=5), PVN (2.4-fold, n=5), NTS (9-fold, n=5) and MCA (22-fold, n=5) of AngII treated rats compared to vehicle treated rats. There were no changes in cPLA2 expression in RVLM or SFO.

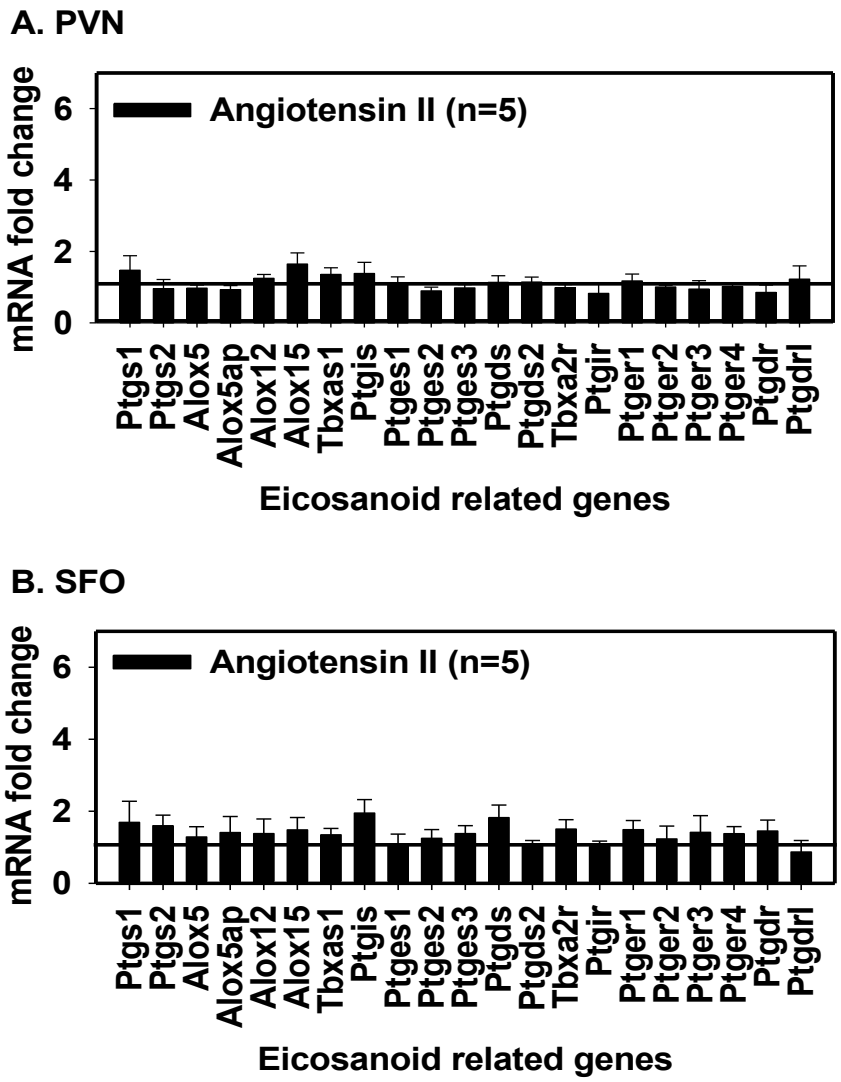
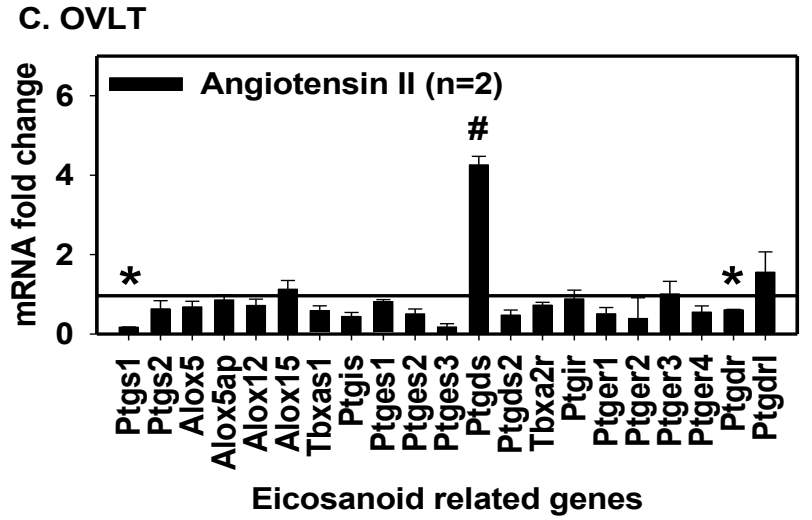
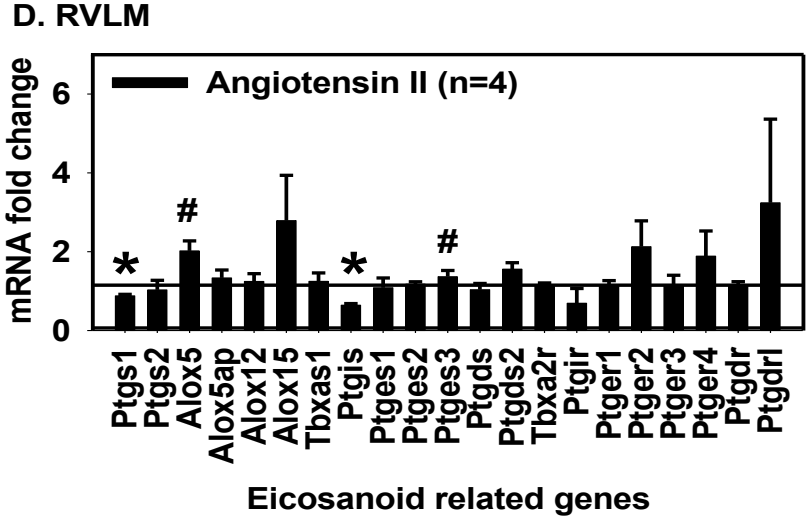


Figure 4-4: Eicosanoid related gene expression in rats treated with AngII or vehicle for 4 days. The mRNA fold change of eicosanoid related genes in samples collected on day 4 from A) PVN, B) SFO, C) OVLT, D) RVLM, E) NTS, F) MCA, G) CP-third ventricle (3VCP) and H) CP-fourth ventricle (4VCP). # Significant difference ($p<0.05$) upregulation of eicosanoid genes in AngII treated rats vs. vehicle treated rats. ∗ Significant downregulation of eicosanoid genes after AngII infusion compared to vehicle treated rats. Between group difference was analyzed by one-sample t-test with a hypothetical mean of 1.

Figure 4-4 (Cont'd)





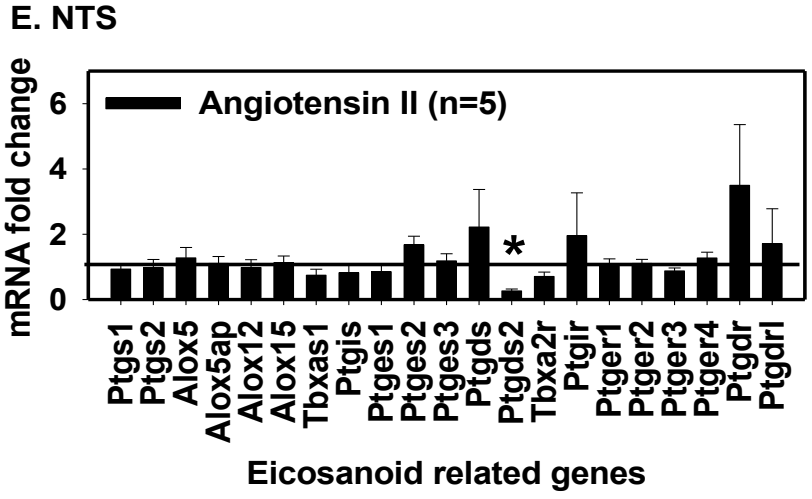
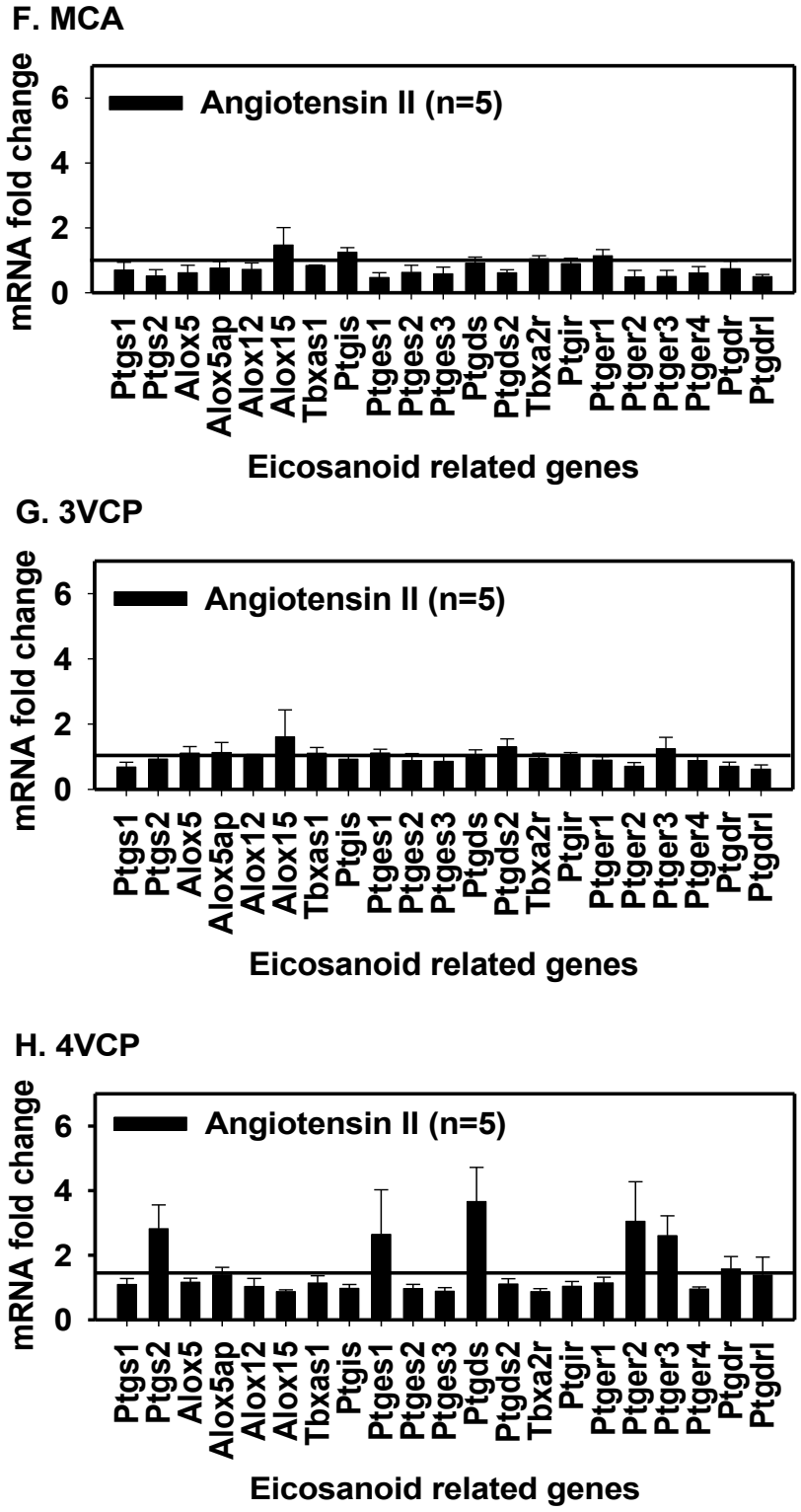


Figure 4-4 (Cont'd)



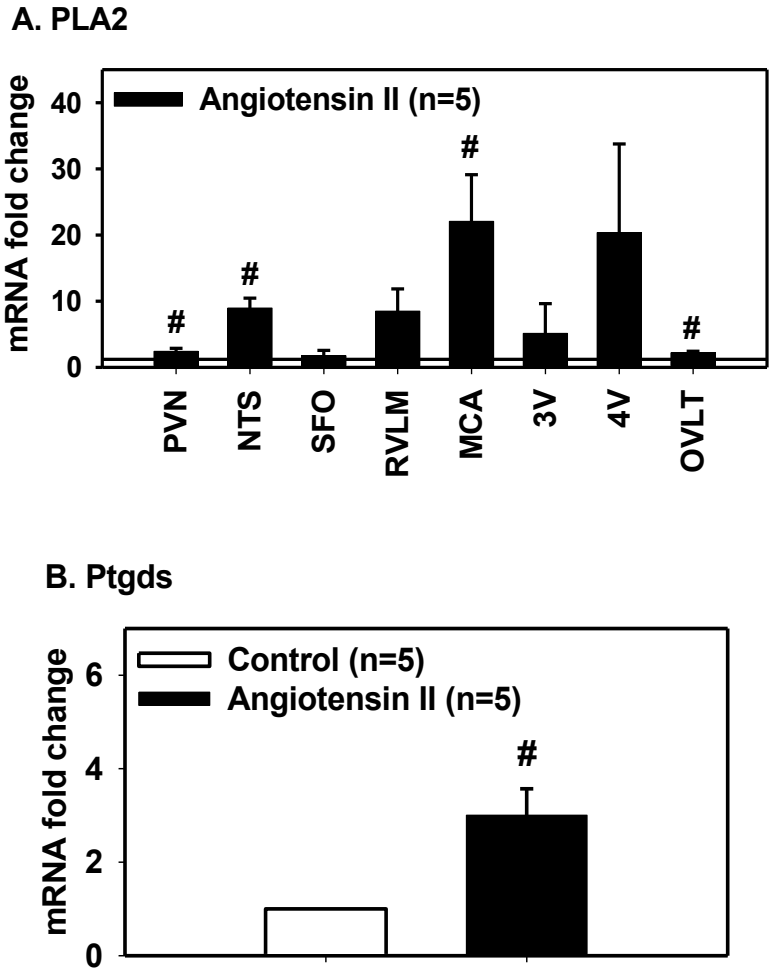


Figure 4-5: Phospholipase A2 and lipocalin prostaglandin D synthase gene expression using q-RT PCR in 4-day AngII or vehicle treated rats. (A) PLA2 gene expression in PVN, NTS, SFO, RVLM, MCA, third ventricle CP (3V), fourth ventricle CP (4V) and OVLT. (B) Lipocalin prostaglandin D synthase gene expression in 4 day AngII treated rats in OVLT. # = $p<0.05$, significant overexpression after 4 days of AngII infusion compared to vehicle treatment in rats. Between group difference was analyzed by one-sample t-test with a hypothetical mean of 1.

3.5. Western blot analysis:

Western blot analysis showed a significant overexpression of cPLA2 protein in the MCA and L-PGDS protein in the 3VCP (Figure 4-6). There were no changes in protein levels of COX-1 in AngII treated rats in MCA, SFO, OVLT, PVN or RVLM (Table 4-1).

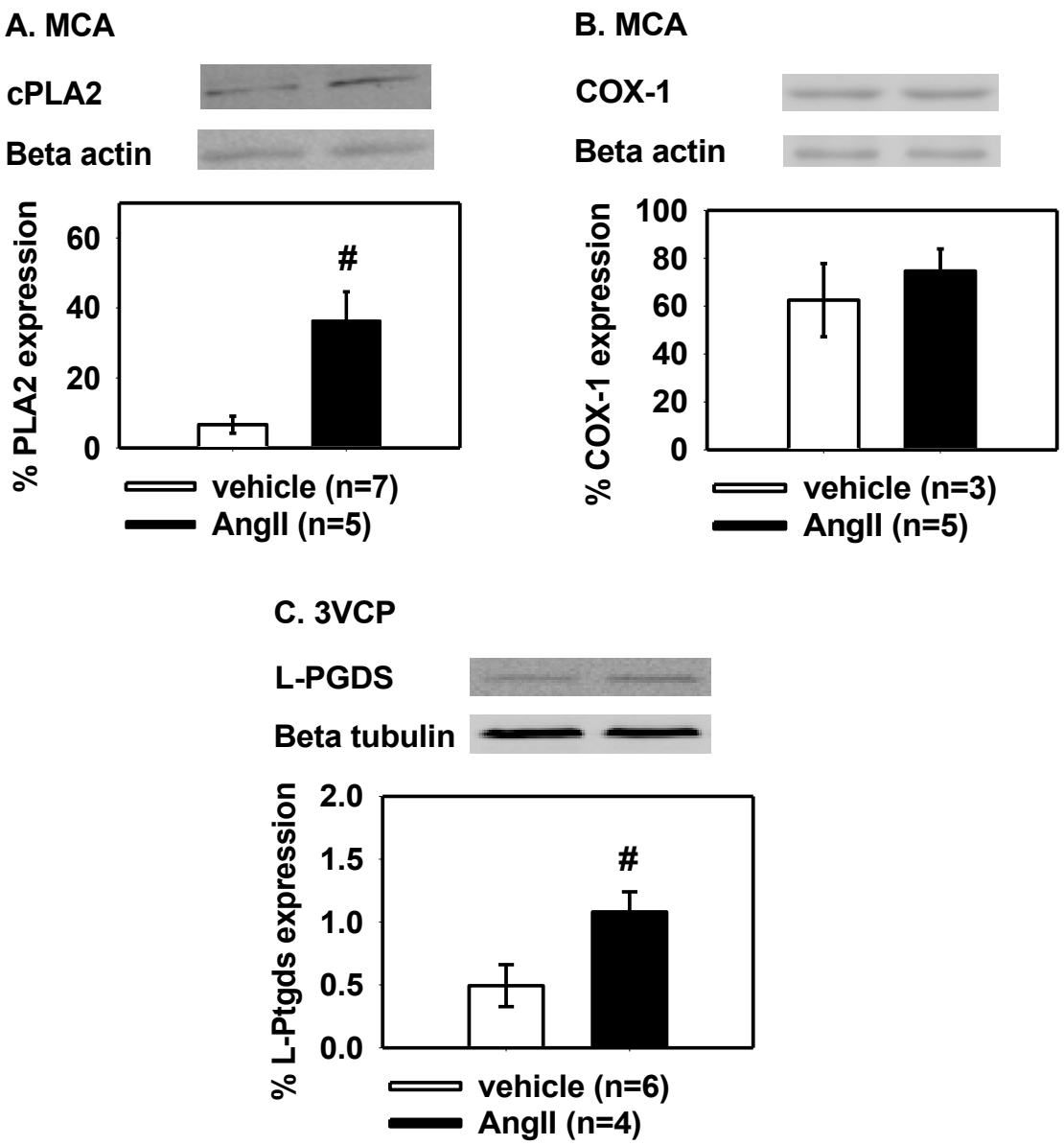


Figure 4-6: Phospholipase A2, cyclooxygenase-1 and lipocalin prostaglandin D synthase protein expression in 4-day AngII or vehicle treated rats. A) cPLA2 protein expression in 4 day AngII treated rats in middle cerebral artery (veh; n=7, AngII; n=5). B) COX-1 protein expression in 4 day AngII treated rats in middle cerebral artery (veh; n=3, AngII; n=5). C) Lipocalin type prostaglandin D synthase protein expression in 4 day AngII treated rats in CP in third ventricle (veh; n=6, AngII; n=4). ∗ Overexpression of proteins ($p<0.05$) in AngII infusion day 4 compared to control rats.

Enzyme	Groups	% Protein expression over control (Beta actin for MCA and Beta tubulin for brain regions)							
		MCA	OVLT	PVN	SFO	RVLM	NTS	3VCP	4VCP
PLA2	Control	**6.6±2.4**	3.3±0.7	**4.9±1.1**	3.3±0.5	2.1±0.2	4.0±0.2	4.2±0.9	20±3
	AngII	**36.4±8.2***	2.2±0.8	**4.2±0.4$**	4.2±0.5	2.2±0.4	3.3±0.2	5.4±1.1	27±5
COX-1	Control	62.5±15.4	0.077±0.02	0.075±0.01	0.17±0.05	0.08±0.07	0.01±0.03	1.04±0.2	4.8±2
	AngII	4.7±9.2	0.078±0.02	0.085±0.01	0.16±0.1	0.07±0.05	0.06±0.03	0.8±0.1	2.6±0.5
L-PGDS	Control	-	0.4±0.03	0.2±0.08	0.5±0.2	0.02±0.003	-	**0.5±0.2**	1.1±05
	AngII	-	0.2±0.03	0.3±0.04	0.6±0.1	0.01	-	**1.1±0.2***	0.3±0.2

Table 4-1: Phospholipase A2, cyclooxygenase-1, prostaglandin D synthase protein expression in 4-day angiotensin II compared to vehicle treated rats. Western blot analysis of protein of interest in samples from a) PVN, b) SFO, c) OVLT, d) RVLM, e) NTS, f) MCA, g) CP-third ventricle (3VCP) and h) CP-fourth ventricle (4VCP) is represented as the % protein expression over control. * Overexpression of proteins ($p<0.05$) in AngII infusion day 4 compared to control rats. $ Lower expression of proteins after AngII infusion compared to control rats on day 4 of HTN.

4. Discussion

The main findings of this study are: 1) acute COX inhibition *does not reverse* established AngII-salt HTN; 2) transient COX inhibition during the first phase of AngII-salt HTN development impairs the ultimate development of HTN in the second (neurogenic) phase; 3) AngII-salt HTN is associated with early transcriptional regulation of eicosanoid pathways, especially in the OVLT and the cerebral vasculature; and 4) altered expression of cPLA2 and L-PGDS proteins in the cerebral vessels and brain nuclei occur during the early phase of AngII-salt HTN.

In previous studies in our lab (discussed in Chapter 3) continuous treatment with ketoprofen attenuated AngII-salt HTN development, but only during the second phase of the HTN (8). Since this later phase of HTN is caused mainly by increased neurogenic pressor activity, we reasoned that eicosanoid products might drive that increased neurogenic pressor activity; and our results using ganglion blockade supported that idea. However, previous studies in experimental animals and humans indicate that NSAIDs like ketoprofen generally don't reduce BP in established HTN (see Chapter 1). Therefore, I wanted to test whether COX activity was required to maintain elevated SNA in the later phase of AngII-salt HTN. The results were clear: once HTN was established, blocking COX activity had no effect on BP or SNA (assessed from neurogenic pressor activity). I then went on to do experiments designed to determine when eicosanoids did act to increase SNA during AngII-salt HTN. I found that COX inhibition limited to the first week (early phase) of AngII-salt administration significantly attenuated later HTN development. Additionally, neurogenic pressor activity in short-term ketoprofen treated rats was lower than their untreated counterparts on day 10 of AngII. *Collectively these*

data indicate that COX products act during the early phase of HTN development to somehow increase SNA during the later phase. How might this delayed effect of eicosanoids be produced?

There is convincing evidence that short-term exposure to AngII can cause long-lasting changes in the brain pathways by which AngII regulates SNA and BP. One manifestation of this phenomenon is the ability of short-term increases in circulating AngII to "sensitize" responses to subsequent increases in circulating AngII occurring even weeks later (263). The mechanisms responsible for this "priming" action of AngII have not been identified, but could be similar to those responsible for the known influence of brain AngII and its metabolites on learning and memory (259). My data suggest that COX inhibitors may prevent this priming action in AngII-salt HTN. Interestingly, an analogous effect of COX inhibitors has been demonstrated in Alzheimer's disease (AD). Chronic COX-1 inhibition effectively delays the development of cognitive and other defects in AD, but has no benefit once the disease is established (106). COX-derived eicosanoids in the brain are able to induce long-lasting neuroplastic changes in numerous neuronal populations and pathways that after a period of time do not require continued eicosanoid action (12, 27). This phenomenon could explain the timing of the protective actions of COX inhibitors in both AD and HTN.

Eicosanoids could regulate sympathetic activity and blood pressure in AngII-salt HT in a variety of ways, but my studies focused on a possible role of eicosanoids in the brain. Eicosanoid products are produced throughout the brain in response to numerous physiological and pathophysiological stimuli, and are synthesized in various brain

regions by the pathways described in chapter 1 (Figure 1-3). Because eicosanoid products can act in the brain to increase SNA and BP, and also cause long-lasting adaptive changes in brain regulatory pathways, *I tested the hypothesis that discrete transcriptional and translational products of the brain eicosanoid pathway would be activated during the early development of AngII-salt HTN.*

Four days after starting AngII-salt treatment in rats eating a high salt diet, I found an increase in mRNA levels of cPLA2 in some cardio-regulatory brain regions and cerebral arteries. Since cPLA2 protein also was overexpressed in cerebral arteries, this could produce accelerated conversion of membrane phospholipids into AA, the major substrate for production of eicosanoids. AngII is known to stimulate membrane phospholipid metabolism in other tissues (153), and activate the AA cascade in the cerebral microvasculature (213). The possible importance of cPLA2 will be addressed further in Chapter 6. Downstream to cPLA2, however, there was no change in the transcript levels of COX-1, another key enzyme in eicosanoid synthesis. This latter finding is consistent with the fact that COX-1 is generally a constitutively active enzyme in the brain and elsewhere (241).

Expression of genes in the COX and lipoxygenase enzymatic pathways was evaluated to help identify specific eicosanoids that might act as brain mediators of elevated sympathetic activity and BP. Overall, other than cPLA2, very few changes in eicosanoid gene expression were found early in AngII-salt HTN, including genes for synthetic enzymes and known receptors. This could be taken as evidence against an important

role for brain eicosanoids in AngII-salt HTN, but also could indicate that the relevant eicosanoids generated by AngII-salt are part of a constitutively active pathway involved in normal brain physiology. A notable and surprising exception was my finding that transcription of L-PGDS, which is found primarily in the brain in meningioepithelial cells and oligodendrocytes (232), was strongly upregulated in the OVLT, a circumventricular organ that comes in contact with circulating angiotensin II through a “leaky” blood brain barrier. Because the leptomeninges, arachnoid trabecular cells and CP epithelial cells are the primary sources of L-PGDS production and secretion into the cerebrospinal fluid (14), the implications of expression of the L-PGDS gene in the OVLT are not clear. Nevertheless, when combined with the facts that 1) the L-PGDS product PGD2 is the most abundant eicosanoid in the brain, and 2) PGD2 injected into the brain causes a sympathetically mediated increase in BP (60), the dramatic increase in L-PGDS gene expression (along with cPLA2) led me to further investigate a possible role of brain eicosanoids, and especially L-PGDS, in the development of AngII-salt HTN. Those studies are described in Chapters 5 and 6.

CHAPTER 5

CENTRAL INHIBITION OF CYCLOOXYGENASE AND LIPOCALIN PROSTAGLANDIN D SYNTHASE BLOCKS THE DEVELOPMENT OF ANGIOTENSIN II-SALT HYPERTENSION IN THE RAT

1. Introduction

In previous Chapters, I showed evidence that cyclooxygenase products play a key role in the developmental stages of a neurogenic model of AngII-salt HTN (Figure 4-3). Specifically, administering either a non-selective, or a specific COX1, cyclooxygenase inhibitor during HTN development significantly attenuated the increase in blood pressure and also reduced neurogenic pressor activity (determined as the acute depressor response to ganglion blockade). Although many other previous reports implicate eicosanoids in angiotensin-dependent hypertension (155-157, 255), most of those reports suggest that the main tissue targets are blood vessels or the kidney. However, there is some evidence that COX1 in the brain participates in a sympathoexcitatory and pressor pathway (22, 23). So as part of my project I performed an analysis of eicosanoid pathway gene expression in cardioregulatory brain regions during the early phase of AngII-salt HTN. The analysis revealed no changes in COX1 or COX2 expression in the brain, but did highlight enzymes both upstream (cPLA2) and downstream (L-PGDS) of COX as potential regulated elements of the eicosanoid pathway during AngII-salt HTN development (Chapter 4). In the current study I tested the hypothesis that downstream products of cyclooxygenase activity in the brain contribute to AngII-salt HTN and that one mechanism for this effect is increased synthesis and release of L-PGDS. To that end, I did the following experiments: 1) measured brain COX activity, and CSF L-PGDS protein content, during the early phase of AngII-salt HTN development; and 2) tested the effect of selective brain blockade of COX activity, and LPGDS activity, on AngII-salt HTN.

2. Experimental protocols

2.1. Mean arterial pressure and cyclooxygenase activity in cardio-regulatory brain regions during the early stages of AngII-salt HTN development:

Rats were given high salt diet and either AngII (n=5) or saline vehicle (n=5) was infused subcutaneously for 4 days. BP was measured using a radio-telemeter implanted into the abdominal aorta through the femoral artery. On the fourth day of AngII infusion, the rats were euthanized and brains were collected, snap frozen on dry ice and stored at -80°C. Using a cryostat maintained at -15°C, 500µm thick coronal brain sections were prepared and brain punches from SFO, OVLT, PVN and RVLM were obtained. COX activity was measured in the punches as described in Chapter 2.

2.2. Brain cyclooxygenase inhibition in AngII-salt hypertensive rats:

To verify the involvement of brain COX pathways in HTN development, we used our standard AngII-salt HTN protocol. After three days of baseline control MAP recording (days C1, C2 and C3), a brain infusion cannula attached to an osmotic pump was implanted into the lateral ventricle (co-ordinates; -0.8mm AP, -1.5mm ML and 4.2mm DV). A mini-osmotic pump (Alzet, model 2004) delivered ketorolac at different doses (33, 3.3. or 0.6 nmol/h) or vehicle (DMSO) intracerebroventricularly (ICV) to different groups of rats throughout the “drug-alone” control period (days D1, D2, D3 and D4) and the remainder of the experimental protocol. After the 4-day drug-alone period, mini-osmotic pumps (Alzet, model 2ML2) for sc AngII infusion (150ng/kg/min) were implanted (days A1-A14). The control group received saline vehicle infusion. On days C2, D2 and A10, neurogenic pressor activity was determined in rats receiving ICV

ketorolac at 33 nmol/h and in vehicle treated rats, using the acute fall in blood pressure after injection of the ganglionic blocker hexamethonium (30mg/kg, ip).

2.3. Plasma ketorolac measurement using high performance liquid chromatography:

To determine an ICV infusion rate of ketorolac that would inhibit COX in the brain but not in peripheral tissues, we measured the drug concentration in blood during ICV infusion at three rates: 0.6nmol/h, 3.3nmol/h and 33nmol/h. Ketorolac was infused into the lateral ventricles of male Sprague Dawley rats using brain infusion cannula attached to an osmotic pump (Alzet, model 2004). After one week of infusion, blood was drawn from the abdominal aorta of anesthetized rats and plasma was collected. Ketorolac concentrations in plasma were measured as described in Chapter 2. Plasma concentrations were compared to the published IC50 of ketorolac, namely 0.27μM or 100ng/ml in plasma (33).

2.4. Western blot analysis for lipocalin prostaglandin D synthase expression in the cerebrospinal fluid during early stages of AngII-salt HTN:

Rats were made hypertensive using our standard AngII-salt HTN protocol (n=5/group), and CSF was collected from both hypertensive and normotensive control rats on day 4 (A4) of AngII or vehicle infusion respectively. CSF was collected and assayed for L-PGDS protein concentration as described in Chapter 2.

2.5. Central and peripheral L-PGDS inhibition:

AT56, a highly selective inhibitor of L-PGDS enzymatic activity (63), was used to test the role of L-PGDS in the development of AngII-salt HTN. Osmotic mini-pumps (Alzet, Model 2004) delivered AT56 centrally as described earlier (5.8nmol/h, ICV, n=7) or peripherally (5.8nmol/h, sc, n=4). The delivery rate of AT56 was chosen based on an amount reported earlier to produce effective L-PGDS inhibition (16). After three days of baseline control MAP recording (C1, C2 and C3) and 5 days of AT56 drug treatment alone (D1, D2, D3, D4 and D5) that continued for the rest of the protocol, AngII (150ng/kg/min sc) was infused for an additional 14 days (A1-A14, n=7/group). Neurogenic pressor activity was evaluated (as described earlier) on day 2 (C2), day 6 (D3) and day 17 (A10) of the protocol.

3. Results

3.1. Mean arterial pressure and cyclooxygenase activity in cardio-regulatory brain regions during the early stages of AngII-salt HTN development:

MAP and HR were not different between the groups during the control period. The AngII treated rats had a significantly higher MAP on A4 compared to vehicle infused rats (Figure 5-1A). In contrast there was no significant effect of AngII infusion on HR compared to the controls (Figure 5-1B).

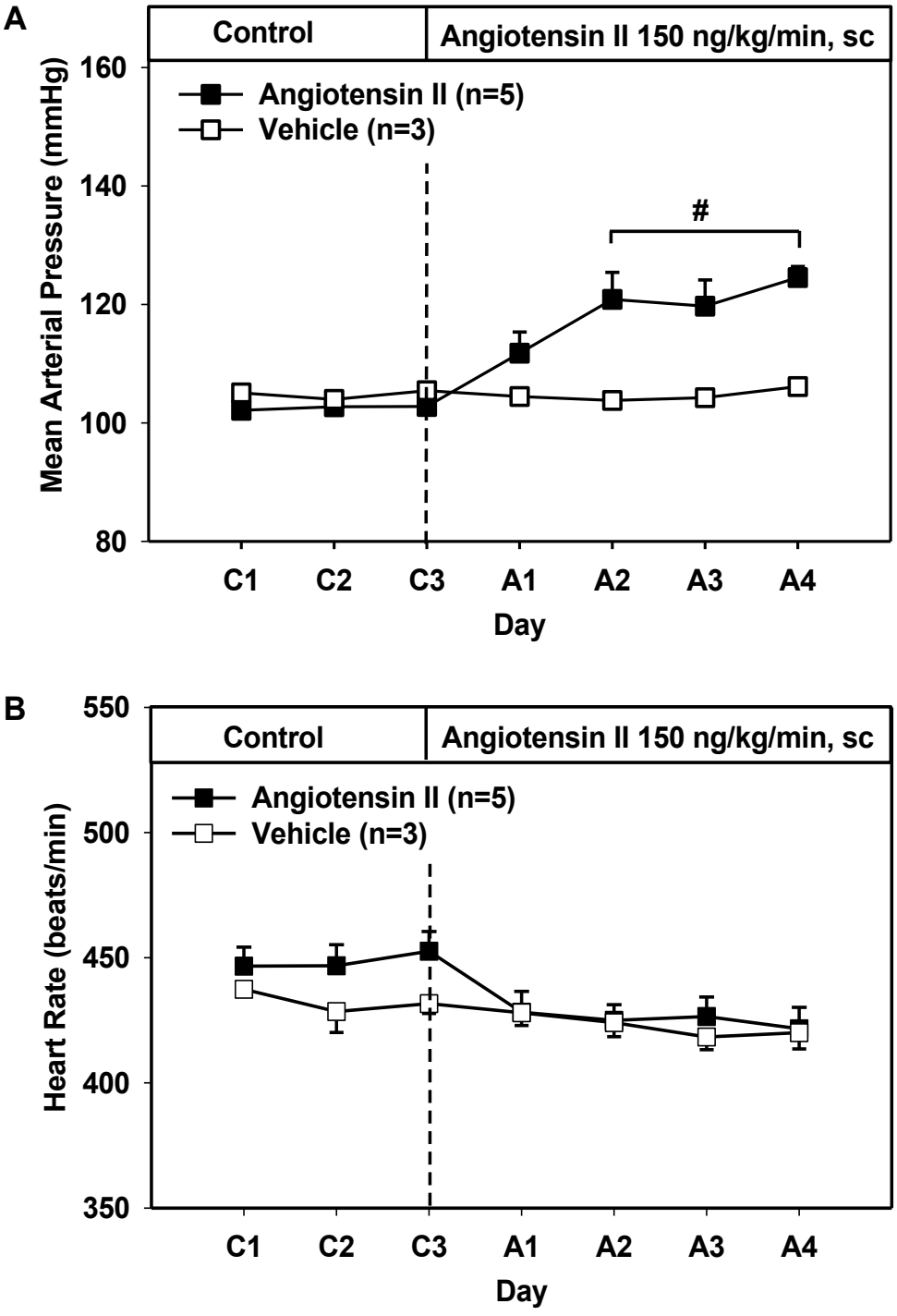


Figure 5-1: MAP and heart rate (HR) in AngII-salt HTN rats. Mean arterial pressure (A) and heart rate (B) in high salt (2% NaCl) fed rats before and after they were infused with AngII (n=5) or vehicle control (n=5). **#** = $p<0.05$ AngII versus vehicle treatment.

Total COX activity was measured in two hypothalamic CVOs (SFO and OVLT) and two brain regions known to regulate sympathetic activity (PVN and RVLM). There were no significant differences in COX activity on day A4 between vehicle treated and AngII treated rats in the RVLM (Figure 5-2A), SFO (Figure 5-2B) or OVLT (Figure 5-2C). However, total COX activity was significantly higher in the PVN of AngII-salt HTN rats compared to vehicle treated rats (Figure 5-2D).

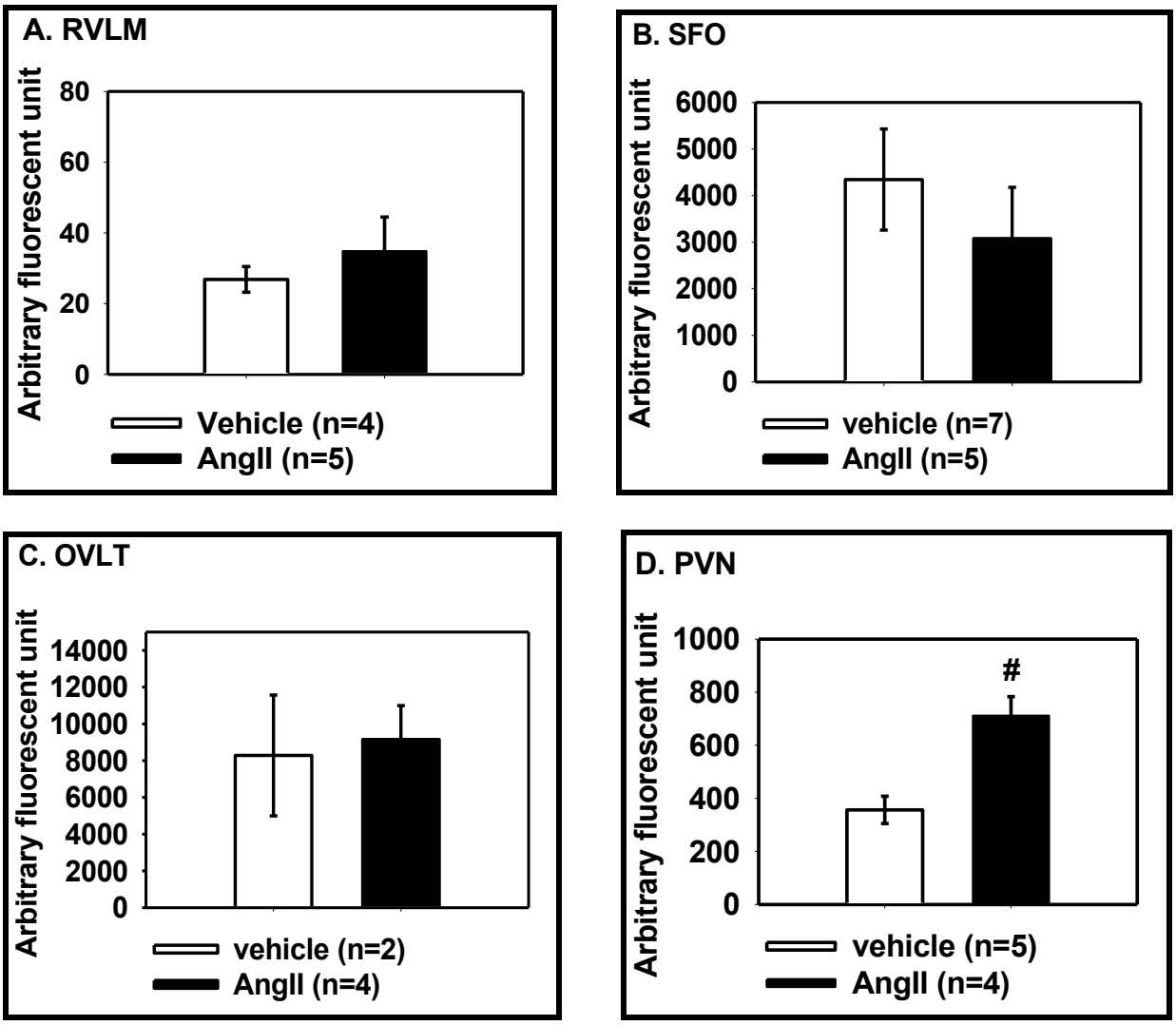


Figure 5-2: Change in cyclooxygenase activity in cardio-regulatory brain regions on day 4 of AngII-salt HTN. RVLM (Veh; n=4, AngII; n=5) (A), SFO (Veh; n=7, AngII; n=5) (B), OVLT (Veh; n=2, AngII; n=4) (C) and PVN (Veh; n=5, AngII; n=4) (D). # =$p<0.05$ versus control.

3.2. Brain cyclooxygenase inhibition in AngII-salt hypertensive rats:

During the control period (C1, C2 and C3), baseline MAP was not different between the groups (Figure 5-3A). MAP also was not affected by any ICV infusion rate of ketorolac or vehicle administration (D1, D2, D3 and D4). In the control group (ICV vehicle with sc vehicle), MAP did not change from day A1 (105 ± 2mmHg) through day A14 (103 ± 2mmHg). In the AngII infused rats receiving ICV vehicle, MAP increased significantly from 110 ± 3mmHg on day A1 to 140 ± 10mmHg on day A14 ($p<0.05$). In the groups that received ICV ketorolac alone (33nmol/h or 0.6nmol/h), MAP did not change throughout the study. In the AngII-infused rats receiving the two higher rates of ICV ketorolac (3.3 and 33nmol/h), MAP rose for the first few days then declined to values not different from those seen in the control groups, i.e. ICV ketorolac with sc vehicle and ICV vehicle with sc vehicle. In rats receiving the lowest rate of ICV ketorolac infusion (0.6nmol/h), MAP increased less during AngII infusion than in the control group, but the difference was not statistically significant. There were no significant differences in HR between the groups at any time (figure not shown). Finally, there were no differences in neurogenic pressor activity (figure 5-3B) between the 7 groups during the control period (day C2) or during drug control period (day D2). However, after 10 days of AngII infusion (day A10), the hexamethonium-mediated fall in MAP (mmHg ± SE) in the vehicle treated AngII-salt rats was 78 ± 10mmHg. In comparison, the fall was 48 ± 5mmHg ($p<0.05$), 45 ± 9mmHg ($p<0.05$), and 57± 9mmHg ($p>0.05$) in AngII-salt rats treated with 33, 3.3 and 0.6mmol/h ICV ketorolac respectively that was not significantly different from the fall in MAP in rats not receiving AngII: ICV vehicle, 47 ± 1mmHg; ICV ketorolac (33nmol/h), 43 ± 8mmHg; and ICV ketorolac (0.6nmol/h), 36 ± 4mmHg.

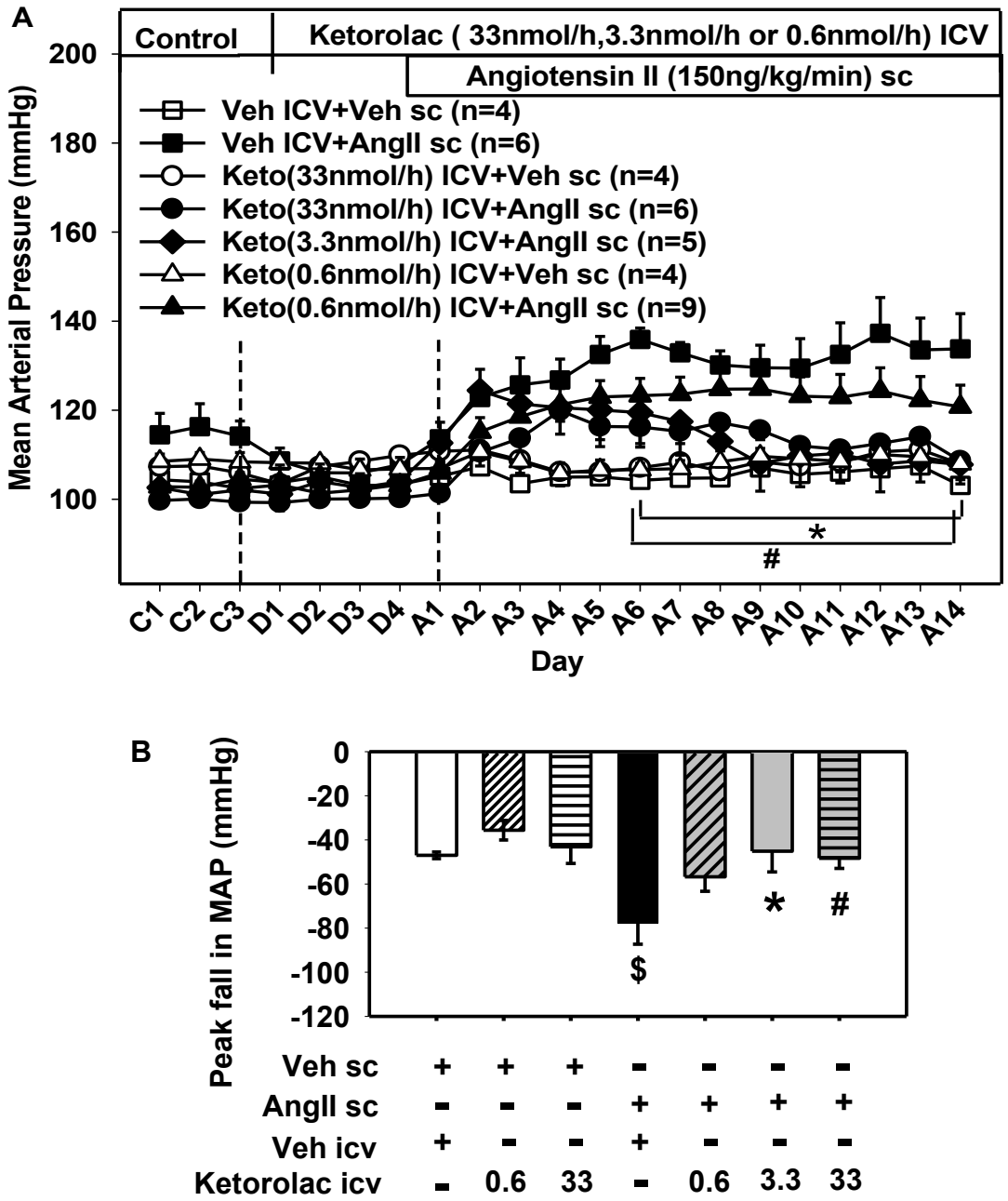


Figure 5-3: Effect of chronic COX inhibition on MAP and neurogenic pressor activity. MAP (A) and neurogenic pressor activity (B) in the rats on 2% salt diet and infused with AngII. # = $p<0.05$ keto 33nmlo/h + AngII vs veh + AngII, *=$p<0.05$ keto 3.3nmol/h + AngII vs. veh + AngII and $=$p<0.05$ all three control groups (veh + veh, keto 33nmol/h + veh, keto 0.6nmol/h + veh) vs. veh + AngII.

3.3. Plasma ketorolac measurement using high performance liquid chromatography:

Three infusion rates of ketorolac, 33nmol/h (n=4), 3.3nmol/h (n=5) and 0.6nmol/h (n=9), were studied after measuring their MAP (Figure 5-3A). After 14 days of ICV infusion in these rats the plasma concentration of ketorolac were 173±30ng/ml, 45±6ng/ml and undetectable, respectively in comparison to the concentration (100ng/ml) of ketorolac required to inhibit COX activity by 50% (IC50) in plasma (33).

3.4. Lipocalin-prostaglandin D synthase content in CSF during the early stages of AngII-salt HTN in rats:

L-PGDS protein content in CSF was significantly higher in the AngII-salt HTN rats compared to the vehicle treated rats (Figure 5-4).

3.5. Central and peripheral L-PGDS inhibition:

MAP was not affected by either ICV or sc administration of AT56 alone compared to vehicle treated rats (Figure 5-5A). During the first two days of AngII infusion, MAP rose similarly in all three groups of rats. By day A14, however, MAP rose to a significantly higher level in vehicle treated rats (124 ± 5mmHg) compared to rats given AT56 either ICV (105 ±5mmHg) or sc (108 ± 1mmHg). There were no significant differences in HR between the groups at any time (Figure 5-5B). Neurogenic pressor activity (Figure 5-5C) was not different between the groups on day C3 or day D3, but was significantly lower in both AT56 treated groups compared to the control rats ($p<0.05$) on day A10.

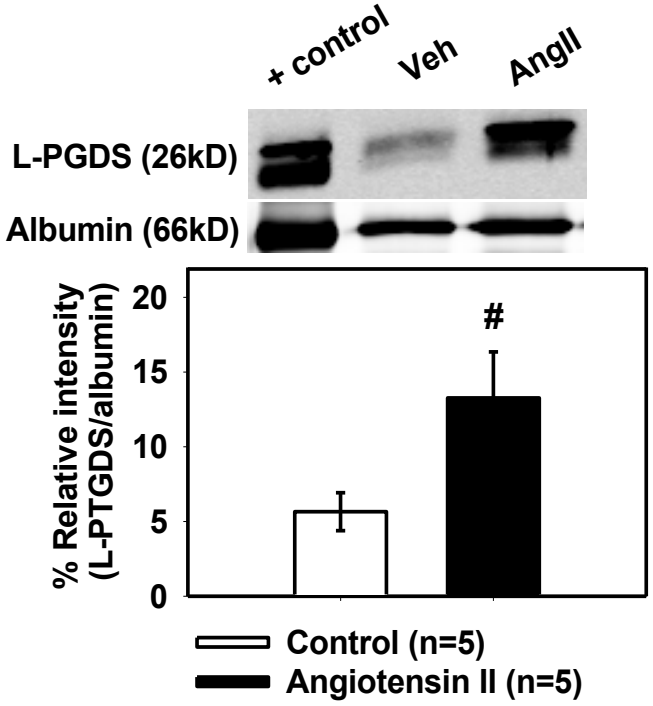


Figure 5-4: Lipocalin-prostaglandin D synthase expression in cerebrospinal fluid during early stages (A4) of AngII-salt HTN development. A representative western blot is shown in the upper panel. In the lower panel, the L-PGDS and albumin bands were scanned and the intensity of L-PGDS was normalized to the band intensity value of albumin. Data are expressed as relative intensity and # =$p<0.05$ AngII versus vehicle treated rats (n=5 in each group).

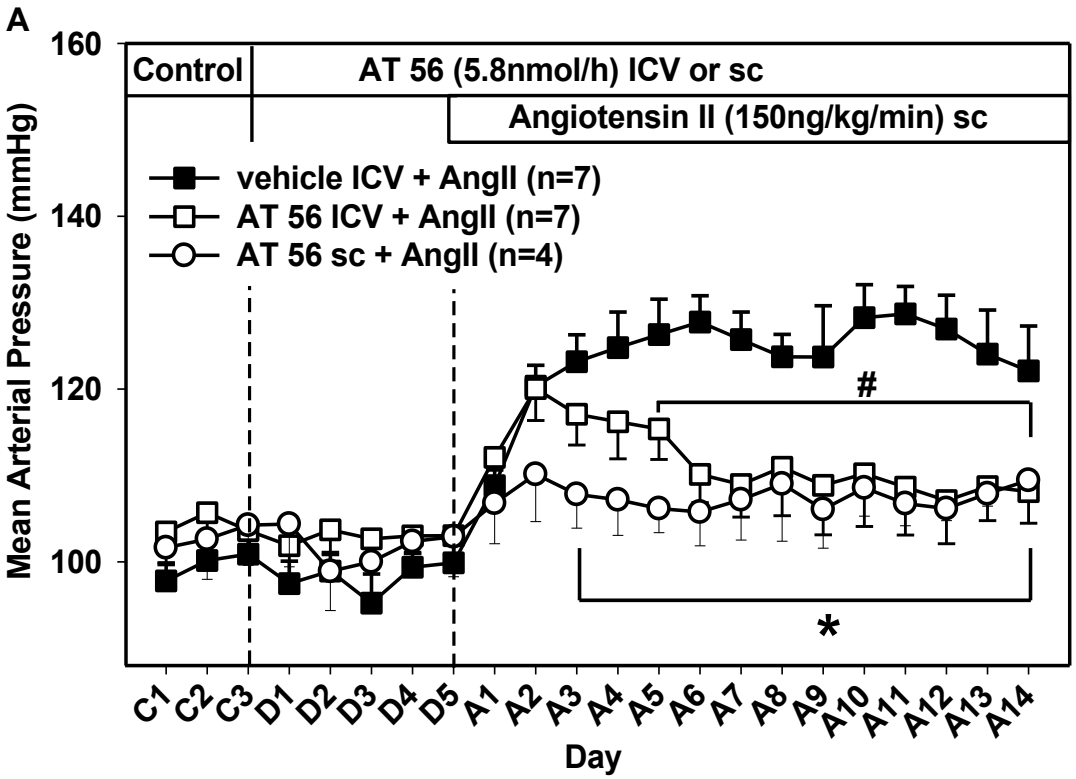
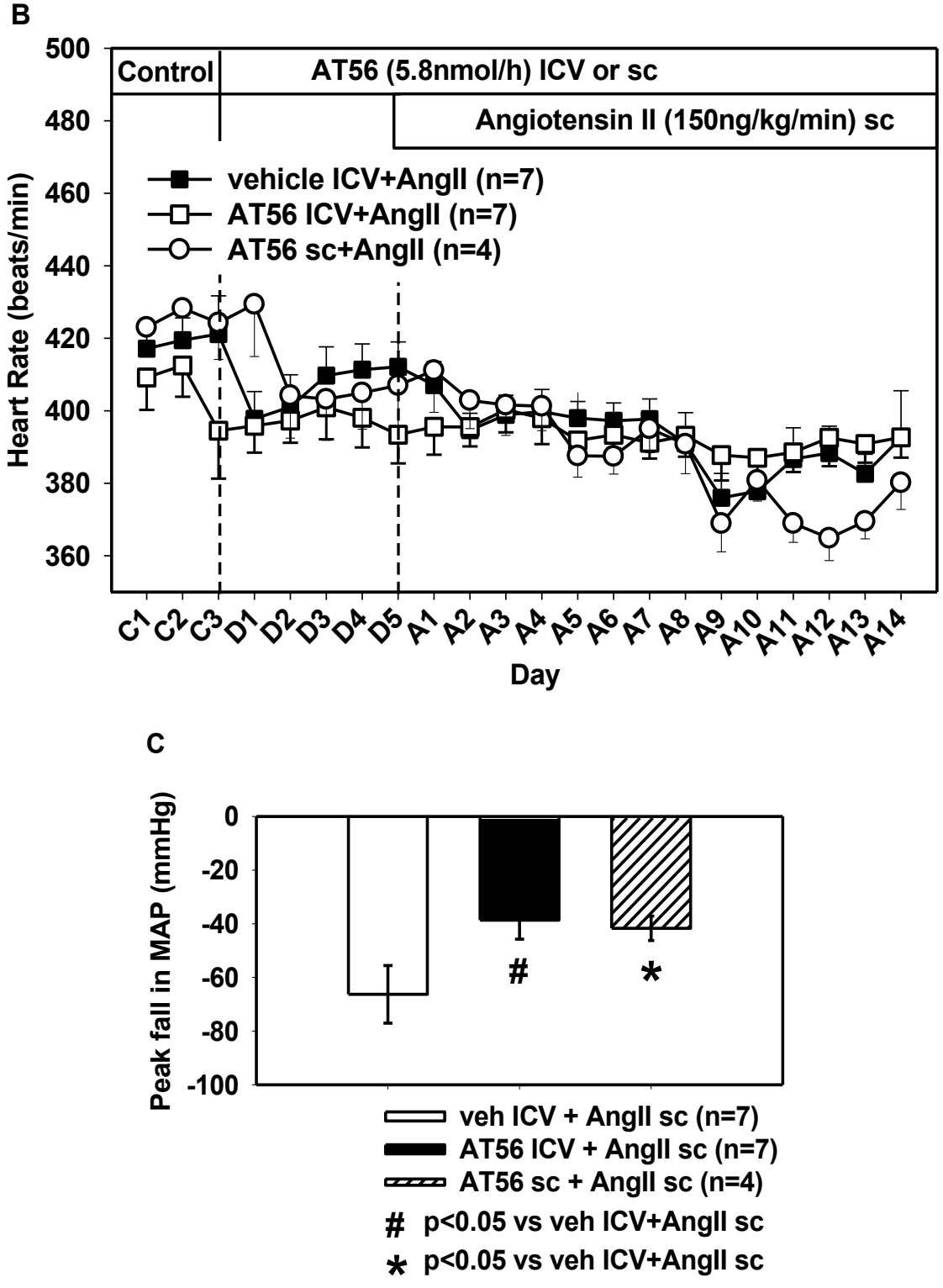


Figure 5-5: The effect of chronic L-PGDS inhibition with AT56. Effect on the mean arterial pressure (A), heart rate (B) and depressor response to ganglionic blockade (C) in rats infused with AngII and fed a high salt diet. # = $p<0.05$ Vehicle ICV + AngII sc versus AT56 ICV/ AngII sc treated rats. * = $p<0.05$ Vehicle ICV/AngII versus AT56 sc + AngII sc treated rats.

Figure 5-5 (Cont'd)



4. Discussion

Cao et al (22) showed that COX-1 activity specifically in the brain is critical for AngII-mediated HTN in mice. In previous studies we found evidence that COX-1 activity is vital for increased neurogenic pressor activity, increased SNA and HTN development in an AngII-salt model in rats (8). AngII-salt HTN in rats is characterized by two stages, an early non-neurogenic phase (1-5 days), and a second neurogenic phase (6-14 days) (28, 31). Interestingly, I demonstrated that inhibiting COX activity only during the early phase (first 3-5 days of a 14 day infusion) of AngII administration is sufficient to attenuate subsequent neurogenic HTN development (Chapter 4, Figure 4-3). In an attempt to localize the tissue(s) where COX products influenced neurogenic hypertension development, I investigated COX gene expression (Chapter 4, Figure 4-4 and Figure 4-5) and protein expression (Chapter 4, Figure 4-6 and Table 4-1) in various brain regions and peripheral tissues during this early phase of the rat model, but observed no significant changes. Therefore, in the first part of the investigation described in this Chapter 1 measured the direct enzymatic activity of COX in our AngII-salt HTN model.

Circumventricular organs with a deficient blood brain barrier like the SFO and OVLT contain AT1 receptors and facilitate signaling in the brain in response to circulating AngII (17, 180). Critical AngII signaling through central circuits beginning at either the SFO or OVLT and passing through the hypothalamic PVN and brainstem RVLM has been reported in AngII HTN models both in the absence (118, 249) and presence (137, 176, 177) of high salt intake. Therefore, I measured COX enzymatic activity in these

regions during the early phase of AngII-salt HTN. I found an increase in COX activity only in the PVN. It is not clear if this increase occurred in neurons or other cells in the PVN. Generally COX-1 in the brain is found primarily in microglia and is expressed constitutively (192). There is evidence though that COX-1 enzymatic activity in glial cells can be directly stimulated via G-protein based signaling pathways (13, 223). In addition, however, COX-1 expression is increased in spinally projecting PVN neurons during both stress (103) and corticotropin releasing factor (206) induced sympathetic activation. PGE2 is a major product of COX-1 and acute injection of PGE2 into the PVN causes elevated SNA and BP in rats (120, 153). Collectively these results suggest that during the early phase of AngII-salt HTN increased COX-1 activity could generate PGE2 or other products in the PVN and thereby increase SNA and BP. But because no direct links between circulating AngII, COX activity in the PVN, SNA and blood pressure have been reported, we performed additional experiments exploring the role of brain COX in HTN development.

In my previous studies on COX activity in AngII-salt HTN, I used systemic administration of COX inhibitors and thus could not rule out that possibility that the antihypertensive effects I found were due at least in part to blocking the formation of eicosanoids in the vasculature, kidney or other peripheral organs. In order to determine whether COX activity specifically in the brain is required for the development of AngII-salt HTN, I tested the effect of selective COX inhibition in the brain using chronic ICV infusion of the COX1/COX2 inhibitor ketorolac. I chose ketorolac because of its relatively high water solubility, making it easier to deliver high concentrations of drug into the CSF. Chronic

central COX inhibition with ICV ketorolac had no effect on BP or HR in normotensive rats, but attenuated HTN development and reduced neurogenic pressor activity in the second phase of AngII-salt HTN. I infused different doses of ketorolac ICV and measured plasma ketorolac concentration in an attempt to insure maximal inhibition of COX in the brain with minimum inhibition of peripheral COX (due to diffusion of ketorolac out of the brain into the systemic circulation). Although all ICV infusion rates of ketorolac successfully ameliorated AngII-salt HTN, it's important to note that rates of 3.3 and 0.6 nmol/h produced plasma concentrations much lower than the reported IC50 required for inhibition of COX (100 ng/ml). This suggests that these infusion rates inhibited COX only in the brain and not in peripheral tissues. Although a limitation to this study is that I did not directly confirm COX inhibition in the brain of ketorolac treated rats, the results still strongly suggest that *COX-derived eicosanoids in the brain* are necessary for the increase in neurogenic pressor activity and BP in AngII-salt HTN.

I previously reported increased expression of L-PGDS, an enzyme downstream from COX in the eicosanoid pathway, in the CP during the early stage of AngII-salt HTN (Figure 4-6C). L-PGDS is synthesized by CP lining the ventricles of brain and is secreted into the cerebrospinal fluid (232, 235). COX converts arachidonic acid to PGH2 and L-PGDS catalyzes the conversion of PGH2 to PGD2; this occurs predominantly in the brain. Apart from PGD2 synthesis, L-PGDS acts as a transporter of lipophilic molecules (235, 257), and thereby is part of the superfamily of secretory proteins called lipocalins. Brain L-PGDS is important in sleep induction (46, 132, 233, 236), temperature regulation (118, 164) and inflammation (47, 56, 150), but has not

previously been linked to BP regulation. However, acute ICV injection of PGD2 causes a modest, sympathetically mediated increase in BP (76, 112, 118). Therefore, I performed two studies to test the hypothesis that brain L-PGDS contributes to the development of AngII-salt HTN. First I measured L-PGDS in the CSF of rats during the early phase of AngII-salt HTN, and second I explored the effects of chronically blocking L-PGDS in the AngII-salt model using chronic ICV and sc infusion of the drug AT56, a highly selective L-PGDS blocker.

In the first experiment, consistent with my hypothesis, I found significantly elevated CSF content of L-PGDS protein in rats with early phase AngII-salt HTN. Relatively little is known about the factors regulating L-PGDS synthesis and secretion into CSF (71), and there are no published data at all on AngII, although AT1 receptors are found on the CP and leptomeninges (33, 67). One possibility is that AngII-salt could work by increasing oxidative stress in the CP, since the latter has been shown to drive L-PGDS expression (273).

In the second experiment, I found that both ICV and sc administration of the L-PGDS inhibitor AT56 markedly reduced BP and neurogenic pressor activity during the late (neurogenic) phase of AngII-salt HTN. Unfortunately, limited data were available to guide my selection of dose for AT56, so I can't be certain how completely L-PGDS activity in the brain was inhibited. Likewise I cannot rule out that the effects observed were due to blockade of peripheral L-PGDS. However, PGD2 is a vasodilator in the systemic circulation and inhibition of peripheral L-PGDS would be expected to cause

vasoconstriction and increased BP (121, 271). Despite these caveats, when considered with my other findings, the results from this experiment support the idea that increased brain L-PGDS activity is important in the development of AngII-salt HTN.

My data here in rats provide strong support for the recent findings of Cao et al. in mice that COX1 in the brain plays an important role in AngII mediated HTN. A particularly notable similarity is that both our results and those of Cao et al show that brain COX activity is important *only during the early stage* of HTN development. As noted in Chapter 4, this suggests that COX-derived eicosanoids are "priming" a later increase in neurogenic pressor activity and HTN rather than causing them directly. There are, however, also some important differences between our findings and those of Cao et al. Their data in mice indicate the COX-1 activity and eicosanoid generation are increased only in the SFO and not in other brain regions; we found COX activity to be increased in the PVN but not in the SFO. Their data support PGE2 acting on the EP1 receptor as the main downstream signaling mechanism causing HTN; we did not specifically investigate PGE2 or its receptors, but our results implicate (for the first time) an alternative downstream eicosanoid pathway enzyme (L-PGDS) and product (PGD2) in AngII-salt HTN. These disparities could reflect differences in species, AngII infusion rates, and/or salt intake between our studies and require further investigation.

In conclusion, the results presented in this chapter provide insight into the central signaling mechanisms driving neurogenic AngII-salt HTN. We found an increase in COX activity in the PVN during early HTN development stages with no change in COX

expression indicating the possibility of more conversion of substrate into eicosanoid products through increases in COX activity. The importance of the COX pathway is further supported by successful attenuation of HTN development with brain-selective COX inhibition. Downstream of COX, L-PGDS was discovered to be critical in increasing neurogenic pressor activity and BP during AngII-salt HTN. The final studies in my dissertation were performed to explore in more detail how L-PGDS contributes to AngII-salt HTN and are described in Chapter 6.

CHAPTER 6

BRAIN PROSTAGLANDIN D2 AND EICOSANOID RECEPTOR CHARACTERIZATION DURING DEVELOPMENT OF ANGIOTENSIN II-SALT HYPERTENSION IN THE RAT

1. Introduction

In the central nervous system L-PGDS is predominantly synthesized in the leptomeninges and CP and later, secreted into the CSF (222, 237). Urade et al reported dominant mRNA expression of L-PGDS in leptomeninges, CP and oligodendrocytes of adult rat brain (237). L-PGDS (also known as beta trace protein in humans) is the second most abundant protein (next only to albumin) that is secreted into the CSF (105). The two important functions of L-PGDS are: synthesis of PGD2, a lipid mediator that regulates physiological processes like sleep (234), pain (118) and body temperature (13); and a transporter of lipophilic molecules viz., bilirubin, biliverdin, and retinol. Due to this transport property L-PGDS is considered a member of the lipocalin family of lipid transporters (232).

Inhibiting L-PGDS enzymatic activity results in low brain PGD2 levels and suppression of sleep (189, 234). The CSF concentration of PGD2 in rats fluctuates in a circadian fashion: from 903 ± 162 pg/ml at night to 503 ± 78 pg/ml during the day (222). PGD2 affects sleep by acting on D-type eicosanoid receptors (DP1R) (189) on arachnoid trabecular cells on the ventral surface of rostral forebrain (158). PGD2 also binds to DP-2 receptors (DP2R), otherwise known as chemo-attractant receptor homologue molecule expressed on T helper 2 cells (CRTH2). DP2R are mainly localized on type 2 lymphocytes, basophils and eosinophils (206). Recently, however, DP2R on neurons have been shown to mediate communication between microglia and neurons in the spinal cord (118).

A role for brain L-PGDS or PGD2 in the pathogenesis of hypertension has not been previously reported, although, Hirawa et al (103) found higher levels of L-PGDS in the plasma and urine of patients with essential hypertension. As described in earlier chapters of my dissertation, I found increased transcript levels of L-PGDS in the OVLT and CP, and increased L-PGDS protein expression in the CSF, during the developmental stage of our AngII-salt HTN rat model. Furthermore, I showed that inhibition of L-PGDS in the brain significantly attenuates AngII-salt HTN and the associated increase in neurogenic pressor activity. Those results, however, do not establish whether the enzymatic or transport function of L-PGDS is most important for HTN development. Because most known physiological effects of L-PGDS in the brain are mediated by PGD2, I hypothesized that *PGD2 levels in the brain increase during the development of AngII-salt HTN.* DP1Rs are expressed in the arachnoid and CP (158) but expression in brain cardio-regulatory regions has not been reported. Therefore I performed experiments designed to localize DP1Rs in circumventricular organs (SFO, OVLT), and other BP regulating brain regions like the PVN and RVLM, and examine changes in their expression during the development of AngII-salt HTN.

2. Experimental protocols

2.1. AngII-salt HTN and tissue collection for eicosanoid measurement:

Tissues were obtained from high salt (HS) (2%NaCl) fed rats that were infused with AngII (150 ng/kg/min, sc; n=5) or saline (n=5) for 4 days. BP was measured using a radio-telemeter catheter implanted into the abdominal aorta through the femoral artery. On the fourth day of AngII infusion (A4), CSF from cisterna magna of these rats was

collected using the protocol that is described in the methods section in Chapter 2. The rats were then euthanized and brains were collected. Brains were snap frozen on dry ice and stored at -80°C. Using a cryostat maintained at -15°C, 500µm thick coronal brain sections were prepared and brain punches from SFO, OVLT, PVN and RVLM were obtained.

2.2. Ultra-performance liquid chromatography-tandem mass spectrometry for measurement of AA and prostaglandins:

The substrate for prostaglandin synthesis (AA) and the resultant eicosanoids, in particular PGD2 and its metabolites PGJ2, delta-12-PGJ2 and PGE2 levels were measured in brain punches and CS using ultra high performance liquid chromatography coupled with mass spectrometry as described in Chapter 2.

2.3. Immunofluorescence detection of DP1 receptor in early stages of AngII-salt HTN:

Two groups of rats (AngII infused or vehicle infused) were prepared using our standard AngII-salt HTN protocol as described in Chapter 2, but I did not measure BP in these animals. After four days of AngII (n=5) or vehicle (n=5) infusion, brain tissue was collected. Using a microtome, 10µm thick sections coronal brain sections were prepared. After immunofluorescent staining, the neurons and DP1 receptor expression were imaged in different regions like SFO, OVLT, PVN, RVLM and CP using confocal microscopy as described in Chapter 2.

3. Results

3.1. Blood pressure and heart rate:

The blood pressure in high salt fed rats after 4-days of AngII infusion was consistently higher compared to vehicle infused control rats (data not shown).

3.2. Eicosanoid levels in cardio-regulatory brain regions during the early stage of AngII-salt HTN development:

During the early stage of hypertension development there were higher levels of AA in the CP lining the third ventricle in HTN versus control rats (Figure 6-1A). PGD2 was significantly higher in CP (Figure 6-1B), CSF (Figure 6-1C) and RVLM (Figure 6-2A, 2B, 2C). No differences were found in other brain regions. There also were no differences in the levels of PGE2, or the PGD2 metabolites PGJ2, delta-12-PGJ2 and 15-deoxy-PGJ2 in the various brain regions or CSF of AngII-salt HTN rats compared to the control rats.

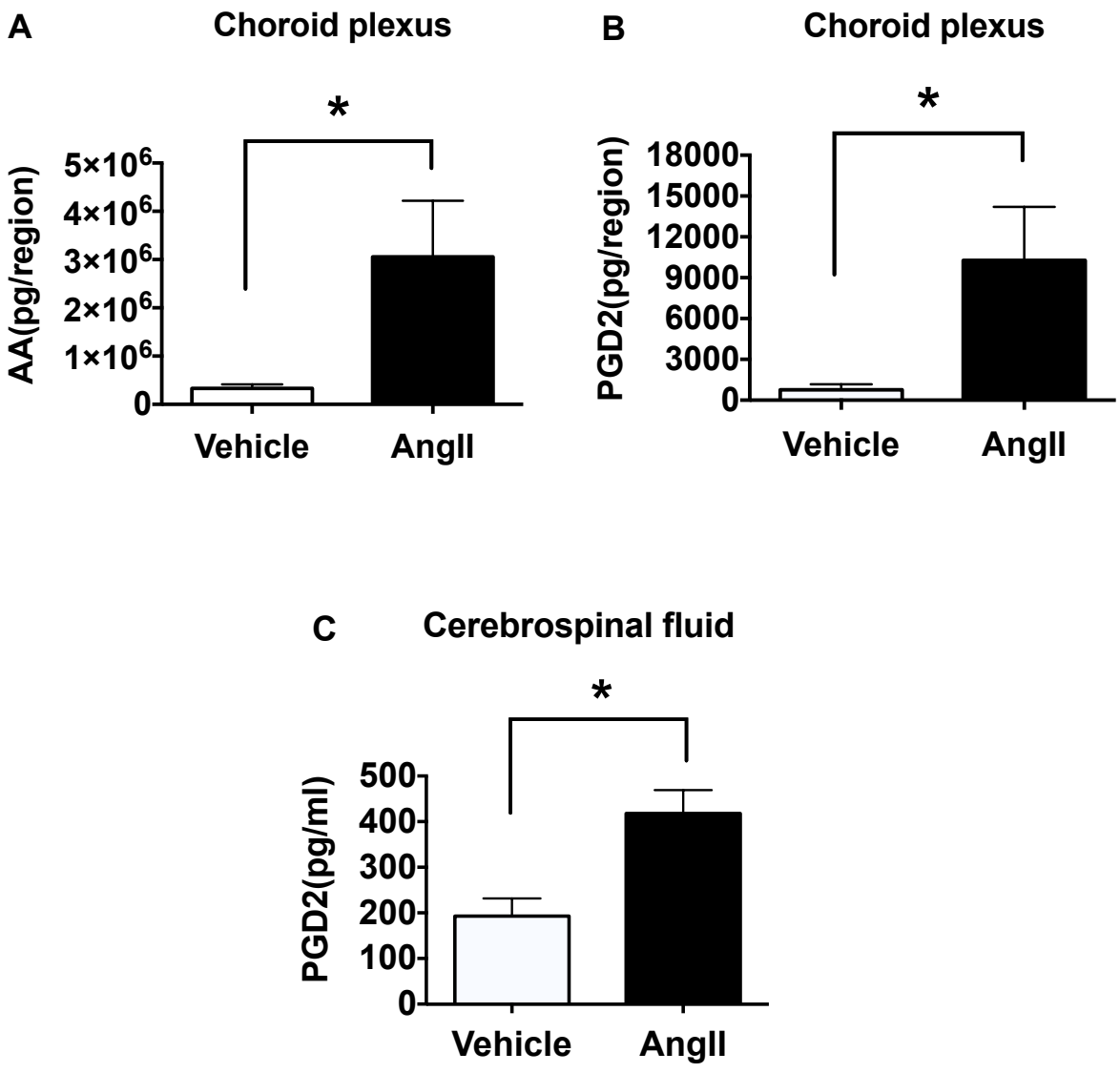


Figure 6-1: Ultra performance liquid chromatography-tandem mass spectrometric analysis of lipid mediators. Measured AA (A) and PGD2 (B) levels in the CP lining the third ventricle and PGD2 levels in the cerebrospinal fluid (C). t test *=$p<0.05$ vs. vehicle. (n=5 in each group).

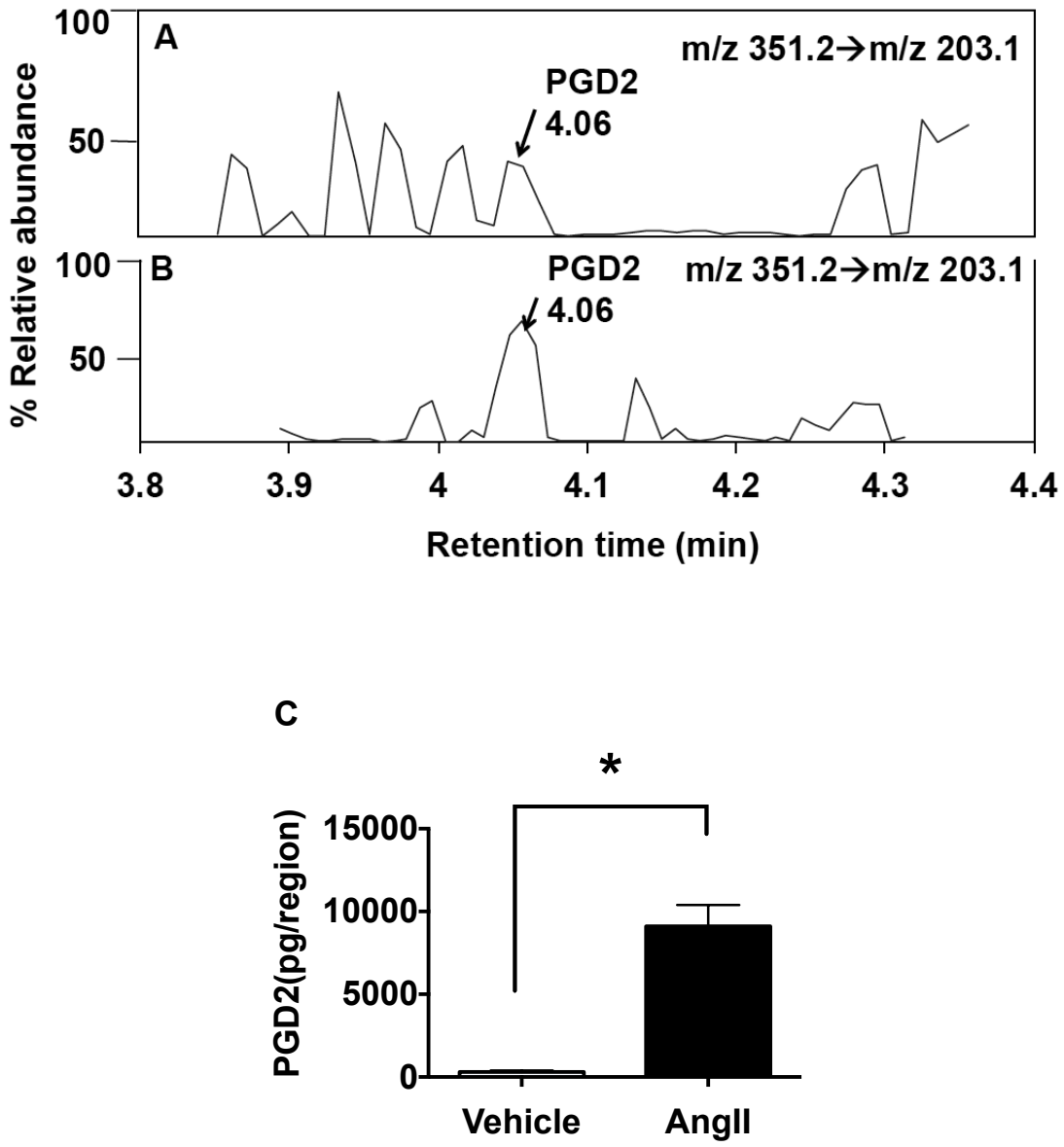


Figure 6-2: Representative chromatogram with PGD2 levels in RVLM.

With a retention time of 4.06 minutes and m/z of 351.2→203.1, PGD2 peaks were detected in the RVLM of vehicle treated (A) and angiotensin II treated (B) rats which was higher in the RVLM (C). t test *=$p<0.05$ vs. vehicle. (n=5 in vehicle treated rats and n=4 in AngII-salt treated rats).

3.3. Immunofluorescence detection of DP1 receptor in early stages of AngII-salt HTN:

Control rats showed significant expression of DP1R in the RVLM (Figure 6-3). In AngII infused rats there was a decrease in DP1R expression that was statistically significant (Figure 6-4). Another area that expressed DP1R was CP (Figure 6-5A) but there was no difference in the expression in AngII infused rats (data not shown). In the cardio-regulatory regions of the brain like SFO (Figure 6-5B), OVLT (Figure 6-5C) and PVN (Figure 6-5D) there was no detectable DP1R expression in control or AngII treated rats.

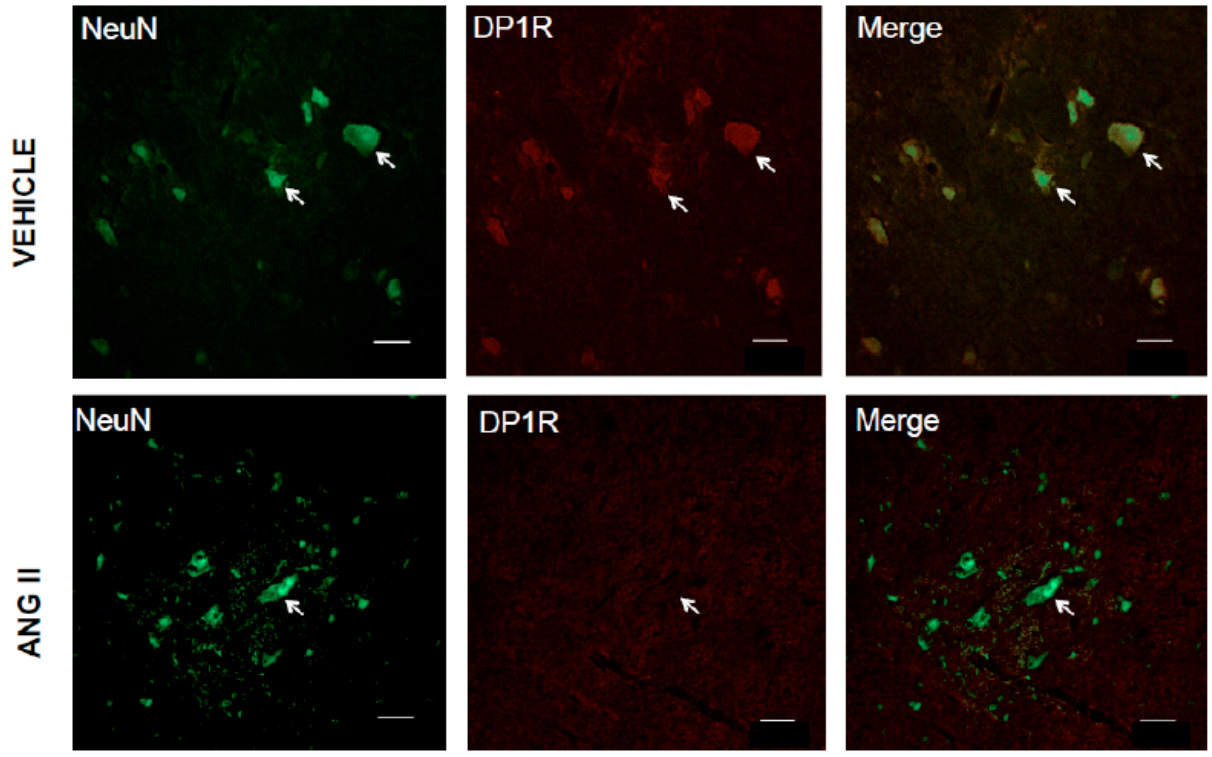


Figure 6-3: Double immunofluorescent staining for neuronal cell bodies and the G-protein coupled receptor DP1R in early stages of HTN development. Arrows point at the neurons and the DP1 receptors with NeuN and DP1 receptor staining respectively. . The DP1R in the RVLM of 4-day vehicle treated rats as shown in the top panel, were localized on the NeuN immunoreactive neurons. 4 days of angiotensin II treatment decreased the DP1R expression on the NeuN immunoreactive RVLM neurons (as shown in the bottom panel). Scale bar=30μm. [For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation]

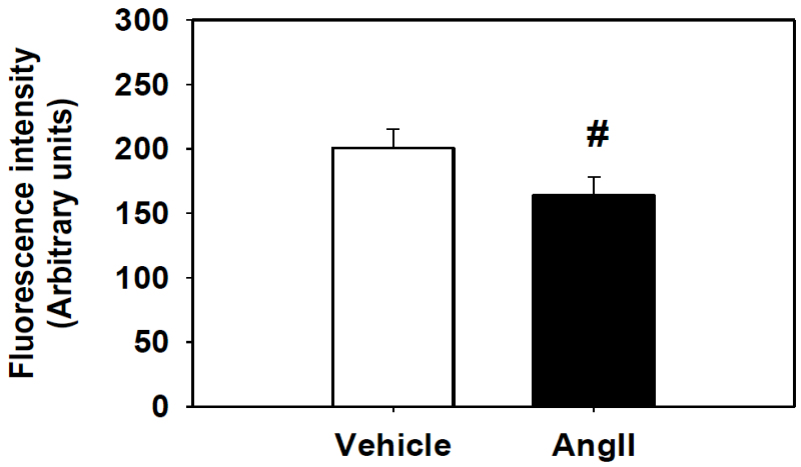


Figure 6-4: DP1 receptor expression in RVLM of 4-day AngII-salt HTN rats. DP1R expression was lower in the RVLM neurons of 4-day angiotensin II treated rats compared to the vehicle treated rats. Paired t test # =$p<0.05$ vs. vehicle.

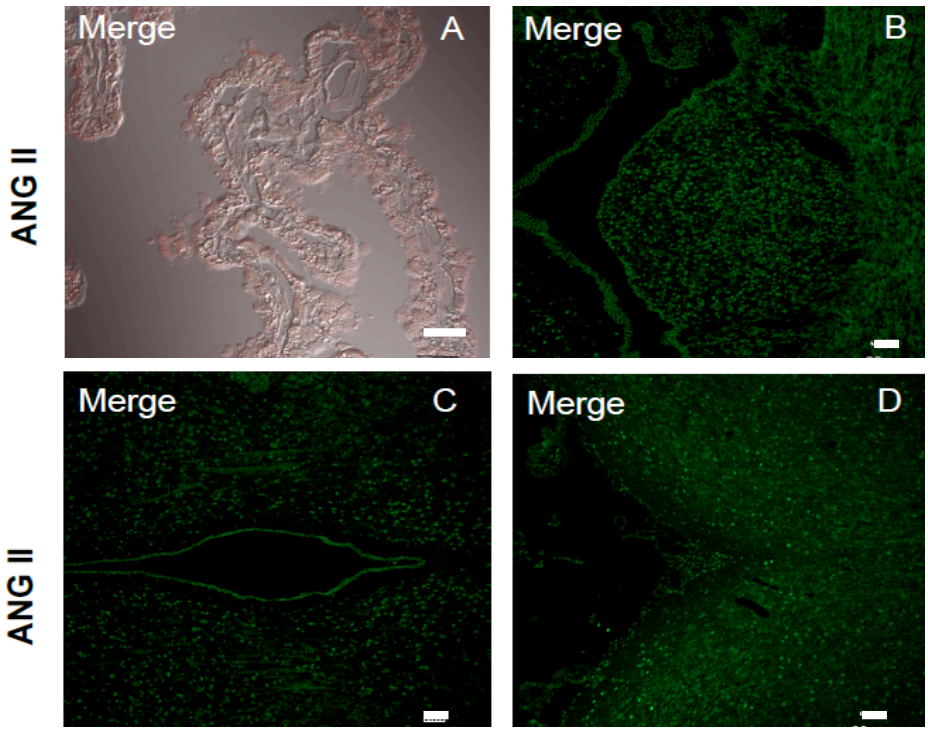


Figure 6-5: DP1R expression in CP, SFO, PVN and OVLT of AngII treated rats. CP lining the brain ventricles expressed DP1R as shown in the DIC image of AngII HTN rat Scale bar=30μm in a 40X image (A) and the expression was not different from the control rats (fig not shown). DP1R were not seen in SFO **(**B), PVN (C) or OVLT (D) of AngII or vehicle (fig not shown) treated rats. Scale bar=60μm in a 20X image.

Discussion

There are two main findings of this study. First, during the early stage of development of AngII-salt HTN there is an increase in PGD2 levels in the CP and CSF: brain sites where the enzyme L-PGDS is synthesized and secreted, respectively. Surprisingly, PGD2 concentrations also were increased in the RVLM, but not in the SFO, OVLT or PVN. Second, I found that DP1Rs are expressed on neurons in the RVLM, but not in the SFO, OVLT or PVN; and that their expression is decreased during the development of AngII-salt HTN. These findings suggest the existence of a heretofore-unknown brain signaling mechanism-involving PGD2 that may contribute to the pathophysiology of HTN.

In my experiments, AngII-salt HTN was associated with an early increase in AA levels in the CP (Figure 6-1A), suggesting increased activity of cPLA2. Combined with my observation that neither cPLA2 gene expression nor protein concentration was increased in the CP at the same time period during AngII-salt HTN development (Chapter 4), I conclude that cPLA2 is functioning here as a constitutive. Similar to L-PGDS, cPLA2 in the brain is found predominantly in the CP, oligodendrocytes and astrocytes (135), but its role in brain physiology remains largely unknown (53). Although AngII is a potent stimulus for membrane phospholipid metabolism in other tissues (153), and activates the AA cascade (including PGD2 synthesis) in the cerebral microvasculature (213), this is the first report of increased choroid AA production in AngII HTN. As noted earlier, the CP is the predominant site of synthesis of PGD2 and the CSF acts as a reservoir of secreted PGD2 (14, 222, 237). I found increased PGD2

levels in the CP and CSF in AngII-salt treated rats compared to control rats, indicating increased synthesis and secretion into CSF. I speculate that a coordinated increase in AA levels and L-PGDS enzymatic activity in the CP is required to generate elevated levels of PGD2 in response to AngII-salt treatment. Most importantly, when combined with my data showing that AngII-salt HTN is attenuated in animals receiving an inhibitor of L-PGDS (AT56) in the brain (Chapter 5), the current results support the conclusion that *PGD2 signaling in the brain is a critical part of AngII-salt HTN development.* This raises the question of *where* and *how* PGD2 acts in the brain to facilitate HTN development.

Current thinking is that PGD2 initiates signaling in the brain by accessing target tissues both from the CSF (sleep) and from local tissue synthesis (from L-PGDS) (232), but details on the latter are sparse. Administering PGD2 into the subarachnoid space of rats was shown to elicit powerful neuronal activation in brain regions involved in sleep regulation (e.g. ventrolateral preoptic area) and modest activation of the PVN, but to have no effect on neurons in the RVLM (204). As noted in Chapter 1, injection of PGD2 into the brain causes an acute, sympathetically mediated increase in BP. To my knowledge, however, no one has previously measured PGD2 concentrations in the CVOs, or cardioregulatory regions like the PVN or RVLM, in experimental models of HTN. A new finding of my study was increased PGD2 levels in the RVLM (but not in SFO, OVLT or PVN) during AngII-salt HTN development (Figure 6-2C). This observation led me to conclude that *PGD2 could act in the RVLM to increase SNA and neurogenic pressor activity in AngII-salt HTN*.

As an aside, Cao et al revealed elevated COX1-derived PGE2 levels in the SFO during the early stages of AngII HTN in mice (22). I also found a small increase in PGE2 levels in the SFO early in AngII-salt HTN, but this difference was not statistically significant. Disparity between my results and those of Cao et al may be due to use of different infusions rates of AngII or to species differences.

I also measured the PGD2 metabolites PGJ2 and delta-12-PGJ2 in the CP, CSF and cardio-regulatory brain regions. No changes in the levels of these metabolites were observed in AngII-salt HTN rats. This suggests that conversion of PGD2 into these metabolites does not play a role in mediating HTN development. Another metabolite of PGD2 is 15deoxy delta-PGJ2. It acts as a ligand for the transcription factor peroxisome proliferator activated receptor gamma and could inhibit activation of RAS, sympathetic outflow and brain inflammation, especially in PVN (271). However, my assay was unable to detect 15deoxy delta-PGJ2 in any of the samples, so I'm unable to make any conclusions about its role in AngII-salt HTN.

The next question I addressed was how PGD2 might alter SNA and neurogenic pressor activity in AngII-salt HTN. As reviewed earlier, PGD2 binds to two different receptors, DP1R and DP2R (166). I investigated the DP1 receptors because it has been linked to most actions of PGD2 in the brain, especially sleep. DP1R are localized in arachnoid trabecular cells of the rostral forebrain and CP (158). There is also evidence of localization of DP1R mRNA in the CP and leptomeninges of rat brain (257). But to my

knowledge no one has reported DP1R in cardioregulatory brain regions. While confirming sites identified in earlier reports, my study shows for the first time localization of DP1R on neurons of the RVLM. Combined with my finding that PGD2 concentrations are elevated in the RVLM early during AngII-salt HTN development, these results support the hypothesis that *PGD2 acting at DP1R on RVLM neurons contributes to AngII-salt HTN.* My additional observation that DP1R in the RVLM were downregulated with AngII treatment (Figure 6-4) is consistent with an AngII-salt mediated increase in PGD2 signaling providing the feedback stimulus for receptor downregulation.

How might AngII and salt cause an increase PGD2 in the RVLM? One possibility is that AngII-salt treatment could increase PGD2 levels in the CSF (via the pathway outlined earlier) and PGD2 could then diffuse into the RVLM. This raises the question though of why I did not observe elevated PGD2 levels in other brain regions. Alternatively, AngII-salt stimulated neuronal signals from CVOs to the RVLM could increase the activity of L-PGDS and generation of PGD2 within the RVLM. However, I did not observe an increase in L-PGDS gene expression in the RVLM of rats early in AngII-salt HTN (Chapter 4, Figure 4-4D). That does not rule out the possibility of changes in enzymatic activity. Finally, AngII could act on cerebral blood vessels coursing through the RVLM to release PGD2, which could then diffuse into the brain parenchyma. Again, however, this raises the question of why increases in PGD2 were not seen in other vascularized brain regions.

How might PGD2 act in the RVLM to increase neurogenic pressor activity and cause HTN? As described in Chapter4, my hypothesis is that eicosanoid products are acting in the brain not to directly increase SNA, but rather to produce slowly developing, adaptive changes in brain circuits that modulate SNA over the long-term (i.e. “prime” later sympathoexcitation). Therefore, presumably the same cellular mechanisms proposed to be responsible for eicosanoid-mediated neuroplasticity (27) and neuronal adaptive responses (66) in other neural responses (stress, learning) could operate in the RVLM. A more detailed understanding at the cellular level of how long-term, adaptive changes occur in neurons in cardioregulatory brain regions is emerging (26).

Finally, even though there is abundant evidence linking increased neuronal activity in the RVLM to the pathophysiology of AngII HTN (132), Pedrino and colleagues (181) recently reported that increased resting sympathoexcitatory discharge from RVLM neurons is not required for the maintenance of AngII-salt HTN in anesthetized rats. Thus, I need to consider the possibility that PGD2 may not influence AngII-salt HTN by actions in the RVLM.

The data in this study reveal a novel association between PGD2 signaling via the DP1R in the brain and the development of AngII-salt hypertension. More detailed studies are required to determine if this information can be used to develop new, practical therapies for treating HTN or other conditions involving increased SNA.

CHAPTER 7

DISCUSSION

As reviewed in Chapter 1, eicosanoids have long been implicated in the pathogenesis of hypertension but actions of eicosanoids on the cardioregulatory regions of the brain have received relatively little attention. Studies from Wilcox and colleagues suggested that the most important brain eicosanoids involved in AngII-dependent HTN are thromboxanes acting on thromboxane (TP) receptors (70, 255). More recently Cao et al (4) proposed a very different scheme linking brain eicosanoids to the development of HTN based on experiments in mice. They suggested that during the early phase of HTN development, AngII increased PGE2 generation in the SFO, which then acted on EP-1 receptors to increase BP and neurogenic pressor activity.

My work reveals a completely new paradigm for understanding how eicosanoids act in the brain during AngII HTN development. In particular, I propose a previously unsuspected role for L-PGDS and PGD2. Figure 7-1 is a summary of my data and a hypothetical scheme based on those data to explain how exposure to AngII and high salt diet might produce HTN. Briefly, I propose that in rats fed a high salt diet, peripheral AngII acts on brain AT-1 receptors to cause direct sympathoexcitation via established pathways originating in the OVLT and SFO. In addition, however, AngII acts on other AT-1 receptors to initiate eicosanoid-based signaling. In cerebral vessels and CP, cPLA2 is stimulated to produce AA, which is then converted by COX1 to PGH2. L-PGDS is induced in the CP and secreted into the CSF, and as a result converts PGH2 to PGD2 in both CP and CSF. Possibly by diffusion from the CSF, PGD2 levels also increase in the RVLM.

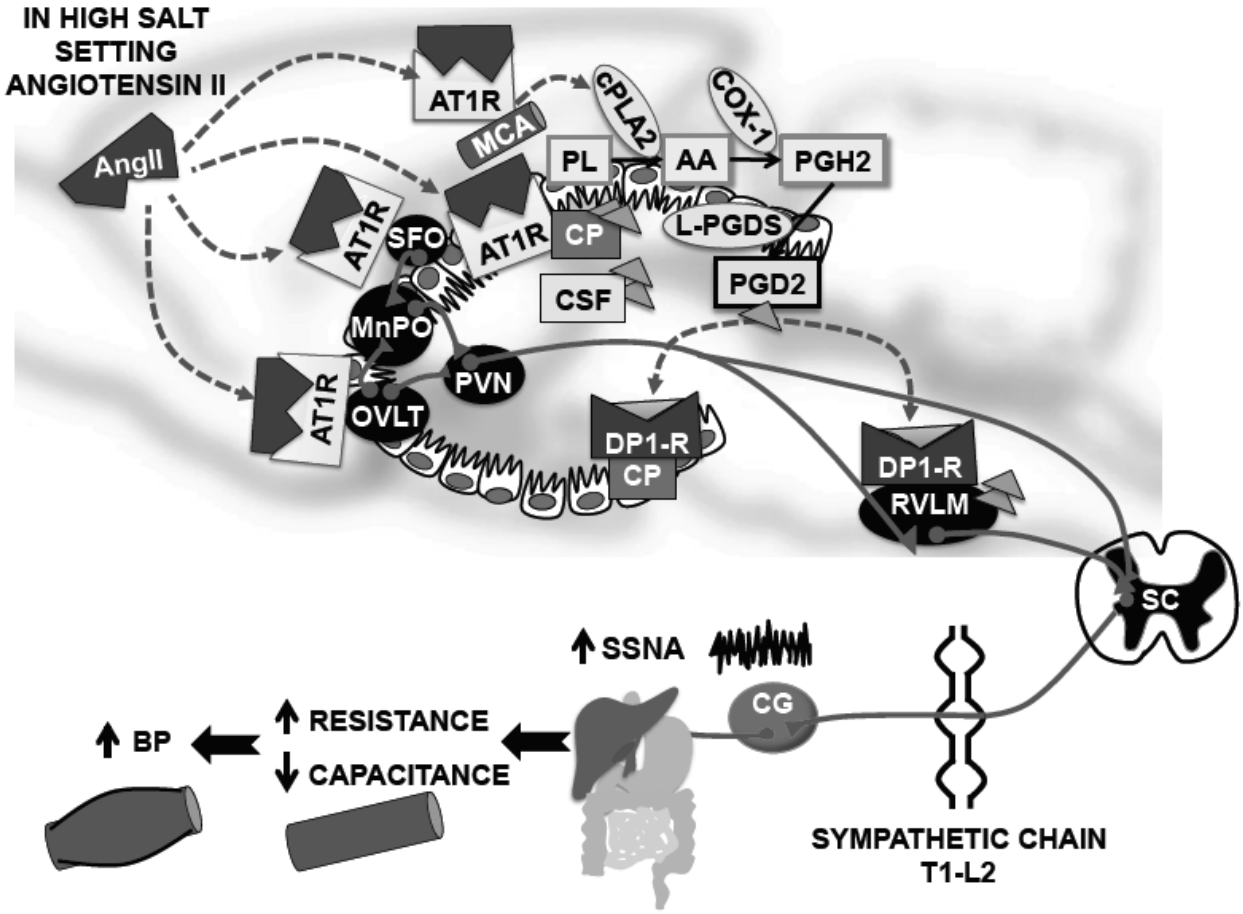


Figure 7-1: A hypothetical signaling pathway by which circulating AngII and high salt intake may cause increased splanchnic SNA and HTN. Acronyms are as defined in the text. CG = celiac ganglion

PGD2 binds to DP1R in the RVLM and causes a slowly developing change in either neuronal excitability or synaptic function that ultimately produces higher splanchnic SNA and HTN.

7.1. Function of choroid plexus in angiotensin II-salt hypertension development

One unique aspect of my hypothesis is the postulated role of the CP in HTN development. In the central nervous system, CP lines the lateral ventricles, third ventricles and fourth ventricles. CP is made up of a monolayer of ependymal cells that are the choroid epithelial cells with basal lamina, large central spherical nucleus and villi on the luminal surface. It plays an important role in the synthesis of CSF (19). Importantly, AT-1 receptors are found in the CP (73, 229) and previous work has suggested that the CP is a target of AngII signaling in HTN (52) but mainly to affect the ability of the CP to regulate CSF sodium concentration (5). On the other hand, my data indicate that the CP is a source of eicosanoids that regulate SNA.

Binding of AngII to AT-1 receptors increases intracellular calcium and thereby causes cPLA2 to translocate to the intracellular endoplasmic reticulum and nuclear membranes (14). The 85kD enzyme cPLA2 is present in different organs including brain and is localized in platelets, macrophages, neutrophils, endothelial cells and vascular smooth muscle cells (131). Cytosolic PLA2 mRNA is reported in pia mater both on the brain surface as well as in the inner core of the CP (135). In response to injury there is an increase in cPLA2 expression in the astrocytes that act as neuron support cells providing cell-to-cell communication in the brain (220). Phosphorylation regulates the

catalytic activity of cPLA2 at the phospholipid membrane (39). One caveat to my observation of increased cPLA2 activity in AngII-salt HTN is the lack of data on the levels of phosphorylated cPLA2 in hypertensive rats compared to the controls. However, increased production of AA in the CP after AngII administration is strong evidence of increased functional cPLA2 activity there, either in the vasculature or other cell types.

Arachidonic acid release from the membrane phospholipids (PL) by the action of cPLA2 is acted upon by the two isozymes of cyclooxygenase viz., COX-1 and COX-2, localized in the endoplasmic reticulum (ER) and nuclear envelope, respectively (75). The positioning of COX-1 near cPLA2 in the ER could provide ready access to free AA in the CP for eicosanoid synthesis. Since I observed a COX-1 dependent increase in BP in my model, it is logical to hypothesize that COX-1 mediates the synthesis of downstream eicosanoid products in the brain near the site of AA release--the choroid plexus--during early stages of AngII-salt HTN. My data indicate that this likely does not require additional COX protein to be produced.

7.2. Possible role of L-PGDS in the development of AngII-salt hypertension

CP is the major site of L-PGDS synthesis (15). The CP promptly responds to peripheral inflammatory stimuli with induction of genes like interleukin 1 and tumor necrosis factor α, but L-PGDS (normally constitutive) also is strongly induced (147). The mRNA levels of L-PGDS are increased in the CP after 6, 12 and 24 hours, but return to baseline after 72 hours (147). This could be why I did not see increased L-PGDS transcript levels in

the CP in 4-day AngII salt treated rats. L-PGDS protein expression on the other hand was higher in the CP lining the third ventricle when compared to the control rats suggesting a differential transcriptional and translational regulation of L-PGDS in the CP during early developmental stages of AngII-salt HTN. L-PGDS in the brain is localized in subcellular structures like rough endoplasmic reticulum, Golgi apparatus, arachnoid trabecular cells and oligodendroglial cells (231). Interestingly, similar to the close association of cPLA2 with COXs, L-PGDS is also co-localized with COXs in choroid plexus, leptomeninges and perivascular microglial cells (14). Given the increase in L-PGDS expression in the CP (Figure 4-6C) along with increased PGD2 levels in CP (Figure 6-1B) and CSF (Figure 6-1C), I postulate that synthesis of PGD2 in CP and CSF is due to increased L-PGDS activity during early development of AngII-salt HTN. This implies that the mechanism by which the competitive inhibitor of L-PGDS, AT56, reduces both PGD2 levels (Figure 6-3) in the brain and AngII-salt HTN development is via reduced PGD2 formation from L-PGDS in the CP. A limitation of my study is that I did not measure PGD2 levels in the CP or CSF in AT56 treated rats (I measured them only in the RVLM). The importance of L-PGDS in AngII-salt HTN development is bolstered by the observed disparity in the regulation of L-PGDS in males compared to the female rats. Kittikulsuth and co-workers (130) recently reported that female rats are protected against development of AngII-salt hypertension compared to male rats. This protection is likely caused by gonadal sex hormone estrogen (E2). The L-PGDS promoter contains E2 binding sites (40) and E2 decreases L-PGDS m-RNA levels in the brain (161). It is therefore tempting to speculate that regulation of L-PGDS by E2 could be the cause of the relative resistance of female rats to AngII-salt HTN.

7.3. Possible role of PGD2 acting on DP1Rs in the development of AngII-salt hypertension

The main function of L-PGDS is synthesis of PGD2 from the precursor PGH2. PGD2 is the most abundant prostaglandin in the brain (234). It is present in CSF and rapidly transported out through a variety of lipid and organic anion transporters (222). In AngII-salt HTN rats I found increased PGD2 levels in the CP, CSF and RVLM. So does PGD2 signaling in the brain alter blood pressure? As reviewed earlier, most PGD2 actions in the brain are mediated via DP1Rs. Because I found DP1R expression in the RVLM (Figure 6-4), I predicted that paracrine signaling by PGD2 through DP1Rs in the RVLM could mediate AngII-salt HTN development. Some support for this idea came from my finding that DP1Rs are downregulated in the RVLM during the early stage of AngII-salt HTN (perhaps as a response to increased local agonist concentration). Two important caveats to this idea, however, are that: 1) I did not demonstrate that PGD2 in the brain can by itself cause neurogenic HTN; and 2) I did not show that blocking DP1Rs in the RVLM attenuates development of AngII-salt HTN.

Although not included in my dissertation, I did two preliminary studies in an attempt to address both of these concerns. First, I infused PGD2 ICV for 14 days into rats on high salt diet and measured BP via telemetry. I expected to see a gradual rise in BP and neurogenic pressor activity after a delay of 3-5 days, but instead there were no changes in either variable when compared to vehicle-infused control rats. Very surprisingly though, when I measured the concentration of PGD2 in the CSF of these rats at the end of the study I found equally low levels of PGD2 in the two groups of rats. Thus, it is likely

that this experiment was not a valid test of my original hypothesis. Second, I induced AngII-salt HTN with our standard protocol in rats treated ICV with either vehicle or the DP1R antagonist BW A868C. I hypothesized that the DP1R antagonist would block the development of HTN in a manner similar to what I observed with ketorolac and AT56. Instead the rats receiving the DP1R antagonist actually became more hypertensive than the vehicle treated rats, and exhibited greater neurogenic pressor activity. The results of this study certainly do not support my hypothesis concerning DP1R in AngII-salt HTN. However, I did not do experiments to confirm that the infusion rate of BW A868C I used was in fact effective in blocking DP1R in the RVLM, so these results also are not definitive.

In light of this uncertainty about the role of the DP1R in AngII-salt HTN, an alternative explanation may be an action of PGD2 on the other main receptor subtype, DP2R, also known as chemoattractant receptor homologous molecule expressed on T helper cells (CRTH2). DP2Rs are present at low levels in the brain and are predominantly localized on T helper 2-lymphocytes, eosinophils and basophils (102). However recently it was shown that PGD2 derived from COX-1 in microglial cells could affect neuronal function by actions on DP2R, at least in the spinal cord (118). Therefore, a role for DP2Rs in PGD2 signaling during development of AngII-salt HTN is possible and could be tested with a pharmacological DP2R antagonist or with DP2R knockout approaches.

7.4. Importance of RVLM as the site of action of PGD2 to increase BP

Potentiated RVLM neuronal activity is believed to cause both experimental hypertension and human essential hypertension (132). As noted by Mueller (165): "*The increased*

output of the RVLM in hypertension is likely dependent on increased excitatory input from other brain regions, as well as an increase in sensitivity to excitatory input. Interestingly, the increase in sensitivity to excitation may occur together or separately from the increased tonic excitatory input depending on the model or risk factor studied."

In this dissertation I propose that in AngII-salt HTN there may be both increased excitatory input from the OVLT and SFO, and increased sensitivity to excitatory input in the RVLM; and that the latter is caused in part by PGD2 derived mainly from the CP. Neurons expressing the Gs subtype DP1 receptors are cyclic AMP dependent. When stimulated, DP1-Rs could activate protein kinase A and increase intracellular calcium. An increase in calcium is known to activate the calmodulin-mediated myosin light chain kinase (CAM kinase II) that has been associated with memory formation in male mice (159). CAM kinase II expressed by the dendritic spine has been shown to modulate neural plasticity (212). The differential expression of CAM kinase II with PGD2 signaling has not been reported but there could be a modulation of synaptic transmission through CAM kinase II that could cause enhanced synaptic transmission during HTN development.

Mueller also highlights the evidence that increased sensitivity of RVLM neurons to excitatory inputs is the result of slowly developing but persistent structural and/or functional changes in synaptic transmission within the RVLM (i.e. neuroplasticity) (165). This is consistent with my finding, and that of Cao et al (22), that increased production of eicosanoid products is essential for the development but not the maintenance of AngII-salt HTN, suggesting that eicosanoids work by gradually changing neuronal sensitivity rather than by direct actions on neuronal activity.

7.5 Future directions

The data presented in my dissertation support sequential roles for cPLA2, COX-1, L-PGDS and PGD2 in potentiating the development of AngII-salt HT. However, it does not exclude the possibility that the critical actions of L-PGDS in neurogenic HTN are independent of PGD2 formation. Therefore, more experiments need to be performed to confirm a cause-and-effect relationship between increased brain PGD2 and neurogenic HTN. Specifically, it needs to be determined whether the main function of L-PGDS is to act as a lipid carrier or to synthesize PGD2. This could be investigated by additional studies on the ability of PGD2 itself to cause chronic HTN, and by further investigation of DP1R and DPR2 antagonists or knockout animals. The contribution of PGD2 signaling at the molecular level in different cell types like neurons, astrocytes and microglia also needs to be further explored in order to develop a complete understanding of how PGD2 in the brain affects SNA and BP. Finally, much additional work is necessary to confirm that the RVLM is the principal site of action of COX products in mediating neurogenic HTN. This would likely require tissue-specific genetic targeting strategies because of the difficulty of chronically and specifically accessing the RVLM with drugs in conscious animals.

7.6. Overall significance, perspectives and therapeutic implications

In light of suggested roles for brain L-PGDS in sleep (189), obesity (43) and insulin resistance (51), and the known associations of these conditions with sympathetic overactivity and essential HTN, my findings reveal an up-to-now unknown signaling pathway in the brain that could explain why these physiological and pathological

conditions often occur together. Nevertheless, with regards to HTN it will be challenging to capitalize on my findings to generate novel therapies. This is because the new mechanism I discovered appears to operate only during the initiation of HTN and is not required once HTN is established. Current therapy of HTN is focused only on lowering BP once the condition is established. On the other hand, it's possible that a more refined understanding of how L-PGDS or PGD2 affects brain pathways regulating SNA could lead to strategies for reversing those changes and thereby modulating SNA for therapeutic benefit.

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