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**The Renin-Angiotensin System, Cardiac  
Hypertrophy, and Salt-Sensitive Hypertension  
in Dahl Rats**

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program in Cellular Molecular Medicine Graduate Program  
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## ABSTRACT

We evaluated the activity of tissue (i.e., brain and cardiac) versus circulatory renin-angiotensin systems (RASs) during (1) the development of high salt-induced hypertension and cardiac hypertrophy; and (2) the prevention of salt-sensitive hypertension by chronic blockade of brain "ouabain". For protocol I, ACE mRNA and activity in the hypothalamus and pons, as well as in the left ventricle (LV) and right ventricle (RV); angiotensin I and II (Ang I, II) in the hypothalamus and pons, as well as in the ventricle and plasma, and plasma renin activity (PRA) were assessed in Dahl salt-sensitive (Dahl S) and salt-resistant (Dahl R) rats on high (1370  $\mu\text{mol/g}$ ) or regular salt (120  $\mu\text{mol/g}$ ) diet for 2 or 4-5 weeks. For protocol II, ACE mRNA and activity in the hypothalamus and pons, and in the LV and RV were measured in Dahl S rats on high salt with either icv Fab fragments binding brain "ouabain" or  $\gamma$ -globulins as control, or regular salt with icv  $\gamma$ -globulins; and in Dahl R rats on high or regular salt diet for 4-5 weeks. ACE mRNA levels were determined by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). ACE activity was measured by a fluorometric method. Ang I and II levels were assessed by radioimmunoassay (RIA) after high performance liquid chromatography (HPLC). High salt diet caused a gradual, but marked increase in blood pressure and LV weight in Dahl S but not Dahl R rats. On regular salt intake, Dahl S rats showed modest but significant increases in ACE mRNA and activity in the hypothalamus at 9 weeks of age as compared with Dahl R rats. In Dahl S rats ACE mRNA and activity in the hypothalamus and pons, as well as in the LV increased significantly after 2 wk of high salt diet, and increased markedly after 4-5 wk of high salt diet.

compared with Dahl S on control diet and Dahl R on either diet. ACE mRNA level increased approximately 3 fold in the hypothalamus, 2 fold in the pons, and 3 to 4 fold in the LV in Dahl S rats after 5 weeks of high salt diet compared to Dahl S rats on regular salt diet or Dahl R rats on either diet. However, high salt diet did not affect Ang I and II levels in either the hypothalamus or the pons in Dahl S and R rats. In the heart, Dahl S rats on regular salt diet showed significantly lower cardiac Ang I and Ang II than Dahl R rats. High salt intake did not cause significant changes in cardiac Ang I and II in either strain. On regular salt diet, PRA, plasma Ang I and II were all significantly lower in Dahl S vs R rats. High salt intake caused a decrease in PRA, a minor decrease in plasma Ang I but no change in plasma Ang II in Dahl R rats. In Dahl S rats, however, high salt did not cause further decreases of the already low PRA, plasma Ang I and II. Chronic blockade of brain “ouabain” with icv Fab fragments prevented the hypertensive effect of high salt intake as well as the increases in ACE mRNA and activity in the hypothalamus and pons, but did not block LV hypertrophy or the increases in ACE mRNA and activity in the LV of Dahl S rats. These results suggest that, in Dahl S rats, 1) the increases in brain ACE mRNA and activity induced by high salt depend on brain “ouabain”; 2) the increases in LV weight as well as in LV ACE mRNA and activity by high salt are largely independent of the development of hypertension; and 3) high salt intake induced increases in ACE mRNA and activity without parallel increase in Ang II, suggests that increased ACE expression may be relevant for other mechanisms (e.g. bradykinin).

**Key Words:** salt-sensitive hypertension, cardiac hypertrophy, angiotensin converting enzyme, angiotensin, brain, heart, Dahl rat

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## LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
ACTH	adrenocorticotrophic hormone
Ang I, II, III	angiotensin I, II, III
AT <sub>1</sub>	angiotensin II type-1 receptor
ATPase	adenosine triphosphatase
AVP	arginine vasopressin
BP	blood pressure
cAMP	3', 5'-cyclic adenosine monophosphate
Dahl R rat	Dahl salt-resistant rat
Dahl S rat	Dahl salt-sensitive rat
EDLS	endogenous digitalis-like substance
GCA	guanylyl cyclase A
HPLC	high performance liquid chromatography
HR	heart rate
hr	hour(s)
2K 1C	2-kidney 1-clip (renal hypertensive rat)
icv	intracerebroventricular
L(R)V	left (right) ventricle
LVH	left ventricle hypertrophy

MAP	mean arterial pressure (mmHg)
min	minute(s)
NO	nitric oxide
cNOS	constitutive nitric oxide synthase
OVLT	the organum vasculosum laminae terminalis
PAF	platelet-activating factor
PCR	polymerase chain reaction
PGK-1	phosphoglycerate kinase-1
PKC	protein kinase C
PLC	phospholipase C
PRA	plasma renin activity
PVN	the paraventricular nucleus
RAS(s)	the renin angiotensin system(s)
RIA	radioimmunoassay
mRNA	messenger ribonucleic acid
RSNA	renal sympathetic nerve activity
RT	reverse transcription
SFO	the subfornical organ
SHR-SP	spontaneously hypertensive rat-stroke prone
SON	supraoptic nucleus
TGF- $\beta_1$	transforming growth factor- $\beta_1$
WKY	Wistar Kyoto rat

## **STATEMENT OF THE CONTRIBUTION OF CO-AUTHORS**

This study was accomplished by cooperation in our laboratory. My main contribution to this study was collection of tissue samples and assessment of ACE mRNA and activity. Roselyn White performed the assessment for Ang I and II, Dr. Baoxue Yuan contributed for animal preparation and blood pressure recording.

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# INTRODUCTION

Essential hypertension is a common disease, characterized by devastating consequences such as stroke, myocardial infarction, end stage renal disease and death. Etiologically, essential hypertension has been recognized as a polygenic disease which occurs as a consequence of complicated interactions of a number of genetic changes and environmental factors (Hamet et al, 1997). Among environmental factors, salt intake has attracted great attention because sodium homeostasis has profound influences on the cardiovascular system of both normotensive and hypertensive subjects. High salt intake exaggerates the development of hypertension in humans and animals genetically predisposed to hypertension and is a major determinant of cardiac hypertrophy. The mechanisms involved remain elusive. The renin angiotensin system (RAS) plays an important role in the maintenance of normal cardiovascular homeostasis and in the pathogenesis of hypertension. For the past two decades, increasing evidence has demonstrated that not only the circulatory RAS but also local or tissue RASs may contribute to the development of hypertension and cardiac hypertrophy. The aim of this review is to summarize our view of high salt-induced hypertension and cardiac hypertrophy. Special attention will be paid to the local RAS, i.e., the brain and cardiac RAS, in the development of salt-induced hypertension and cardiac hypertrophy.

## **I. Salt intake, hypertension, and the brain RAS**

In 1960 Dahl (Dahl et al., 1960) first described a remarkable straight relation between the average salt intake and the prevalence of hypertension in five population groups. Since then evidence relating salt and blood pressure has extensively accumulated. It is now recognized that high salt can raise blood pressure as well as cause cardiac hypertrophy in both susceptible people and susceptible animals.

### **I. 1. *Salt intake and salt-sensitive hypertension***

Most epidemiological data, both across and within populations, support the notion that the level of salt intake is related to the rise in blood pressure especially in the second half of life (Elliott, 1991). In the INTERSALT Study of 10,079 men and women from 52 population samples in 32 countries, blood pressure plotted versus sodium excretion from age 20 through age 59 showed a significantly positive slope for each of 48 samples. The study showed that among individuals, a difference of 100 mmol salt intake per day is associated on average with a difference of 3-6 mmHg in systolic blood pressure. Among populations, a 100 mmol /day lower salt intake is associated with the attenuation of the rise in systolic blood pressure by 10 mmHg between the ages of 25 to 55 years. Only four isolated populations with habitual low salt intake failed to share this increase (Dyer et al., 1994). Most epidemiological data within populations also support the notion that the level of salt intake is related to the rise in blood pressure (BP) especially in the second half of life. A regression of BP on sodium excretion has been found in Japanese, Chinese, as well as in Western populations.

Randomized, controlled trials also provide compelling evidence for a causal relationship between salt intake and BP. In a meta-analysis of 56 trials, overall reductions of BP for a 100 mmol/d reduction in daily sodium excretion was 1.0 mmHg (0.5 to 1.6 mmHg) for systolic and 0.1 mmHg (-0.3 to 0.5 mmHg) for diastolic in normotensive trials, versus 3.7 mmHg (2.4 to 5.1 mmHg) for systolic and 0.9 mmHg (-0.1 to 1.9 mmHg) for diastolic in hypertensive patients. Greater reductions of BP were observed in hypertensive than in nonhypertensive subjects and in trials lasting >5 wk than in shorter trials (Midgley et al., 1996).

High salt intake raises blood pressure in both susceptible people and in susceptible animals. Dahl et al (1962) reported the development of two strains of rats with markedly different genetic propensities for development of hypertension during high salt intake. The Dahl salt-sensitive (Dahl S) strain developed hypertension when fed on high salt diet, but the Dahl salt-resistant (Dahl R) strain remained normotensive. The Dahl strain provides an excellent model of salt-induced hypertension. High salt intake also exaggerates the development of hypertension in the spontaneously hypertensive rat (SHR), another genetic model for salt-induced hypertension (Ely et al., 1986; Kurtz and Morris, 1985; Huang and Leenen, 1992a, 1994). In chimpanzees increased salt intake with the usual diet over 20 months resulted in 33 mmHg and a 10 mmHg elevation of systolic and diastolic blood pressure, respectively (Denton et al., 1995). This increase was completely reversed within 6 months of withdrawing the high salt intake. This study provides direct evidence for causation of salt-sensitive hypertension in the species phylogenetically closest to humans.

The mechanisms responsible for the increase in blood pressure with high salt intake are complex and only partially understood. A complicated interaction between

neuroendocrine factors and the kidney may underlie the propensity for salt retention and development of salt-dependent hypertension. Dahl and his colleagues (1972) originally showed that the genotype of the kidneys plays a critical role in regulating blood pressure in Dahl rats. Further studies demonstrated reduction of natriuretic capacity and renin release in isolated, blood-perfused kidneys of Dahl S rats (Tobian et al, 1977). A polymorphism of renin gene was found to cosegregate with part of the BP difference in Dahl S and R rats, one renin homozygous genotype of Dahl S rats was associated with an increase in BP of ~10 mmHg, two renin homozygous genotypes with a 20 mmHg increment in BP (Rapp et al., 1989). In Dahl R rats mutation of the 11 $\beta$ -hydroxylase caused reduced synthesis of the mineralocorticoid 18-hydroxy-11-deoxycorticosterone which linked to the regulation of BP (Cicila et al., 1993). By chromosomal mapping, the genetic locus containing the ACE gene was reported associated with BP regulation in SHRSP. The locus containing the ACE gene accounted for 15 mmHg of systolic and 13 mmHg of diastolic pressure difference in SHRSP after 12 day high salt (2% NaCl drinking) (Hilbert et al., 1991; Jacob, et al., 1991). In Dahl S rats, the ACE gene also cosegregated with BP (Deng and Rapp, 1992).  $\alpha_1$  Na,K-ATPase, the sole renal Na,K-ATPase isoform, has a special interest in salt-sensitive genetic hypertension because of its role in renal Na<sup>+</sup>-reabsorption and body fluid homeostasis. A mutation of an adenine<sup>1079</sup> to thymine transversion of this gene was demonstrated in Dahl S rats resulting in the substitution of glutamine<sup>276</sup> with leucine. This mutation was associated with a significant decrease in <sup>86</sup>Rb<sup>+</sup> net influx of  $\alpha_1$  Na,K-ATPase (Herrera and Ruiz-Opazo, 1990; Ruiz-Opazo et al., 1994). However, in Dahl rats alleles at the Na, K-ATPase  $\alpha_1$  locus failed to cosegregate with BP on high salt intake (Rapp and Dene, 1990). It is possible that

the  $\alpha_1$  Na,K-ATPase gene is only one of many genes involved in the regulation of BP, and the conditions of the study were inappropriate to demonstrate functional effects of this gene-mutation on BP change although some other functional differences such as ion transportation were found.

During the past two decades an increasing number of studies have demonstrated that the central nervous system contributes to salt-sensitive hypertension. High salt intake has been shown to stimulate rather than suppress sympathetic nervous system activity in both SHR and Dahl S rats (Chen et al, 1988; Koepke et al, 1988; Kotchen et al, 1991). In young, mildly hypertensive humans on high salt intake increased sympathetic activity was demonstrated by direct measurement of sympathetic nerve activity to the muscle circulation (Mark, 1990). In Dahl S rats overactivity of the sympathetic nervous system also contributes to the propensity to salt-sensitive hypertension (Mark, 1991). Recent studies in our laboratory demonstrated that increased sympathetic activity is mediated by both central "ouabain" and Ang II in both SHR and Dahl S rats (Huang and Leenen, 1996a; 1995; 1998).

## ***1.2. The brain RAS in salt-sensitive hypertension***

Historically, the RAS has been viewed as a circulatory system. During recent years, increasing evidence has accumulated that besides the circulatory RAS, endogenous or intrinsic tissue RAS might exist in a variety of tissues, e.g., kidney, adrenal glands, lung, arterial wall, heart, and in the brain. Although the existence of local RASs and their exact physiological and pathophysiological role are far from conclusive, accumulated evidence derived from biochemical and molecular biology studies have filled the gap and provide a novel view of

these systems . Protein and/or messenger RNA encoding all of the components of the RAS including angiotensin receptors have been co-localized within several tissues including brain, heart, kidney, adrenal glands, vasculature and reproductive tissues, leading to the suggestion that tissue RAS might function in a locally restricted manner.

### ***I. 2. 1. The circulatory RAS***

One hundred years ago, Robert Tigerstedt and Per Bergman discovered that the extracts from the renal cortex of rabbits when injected intervenously have a pressor effect and named the substance renin (Tigerstedt and Bergman, 1898). It was not until the late 1930s that it was proposed that renin might be an enzyme. For more than a half century, the circulatory RAS and its crucial role in the regulation of blood pressure and body fluid homeostasis has been well established (Braun-Menendez et al, 1940; Page and Helmer, 1949).

According to the classical concept, the liver-derived precursor angiotensinogen is cleaved in the circulating blood by the aspartyl protease renin, which is synthesized and released into the blood by the juxtaglomerular cells in the kidney, to the decapeptide angiotensin I, which does not have any known biological activity. Ang I is then converted by angiotensin converting enzyme located on the luminal side of the vascular endothelium, to the octapeptide, Ang II, which acts on high affinity angiotensin receptors. Circulating Ang II increases blood pressure by different mechanisms. Ang II per se is a strong direct vasoconstrictor peptide. It also interacts with the sympathetic nervous system both centrally

and peripherally, which leads to enhanced sympathetic activity and increases blood pressure (Zimmerman et al., 1984). In addition, Ang II is a stimulator of aldosterone secretion by the adrenal gland, leading to salt retention (Oparil and Haber, 1974). Recent studies have focused on the direct long-term growth-promoting effect of Ang II on cardiovascular structure (see section II.3.2).

Renin is recognized as the major rate-limiting factor in the cascade. In healthy children aged 1 wk to 13 years a significant correlation between Ang I, II and PRA but not ACE was observed after correction for age since PRA, Ang I and II but not ACE showed a age-related decrease (Fiselier et al., 1983). Similarly, in adults aged 35 to 64 years there was a strong association between PRA and plasma Ang II concentrations observed. ACE concentrations were associated with ACE genotypes but not plasma Ang II (Harrap et al., 1993). These studies indicate that renin but not ACE is rate-limiting for the production of circulatory Ang II. Among the various components of the RAS, however, ACE appears to be particularly important for the local synthesis of Ang II in tissues. ACE is a dipeptidyl peptidase which is responsible for the production of the active octapeptide Ang II from the decapeptide Ang I. Being present ubiquitously both peripherally (e.g., the lung, heart, kidney, testis) as well as centrally in the brain, ACE exists predominantly as an ectoenzyme of vascular endothelial cells converting Ang I to Ang II as well as inactivating bradykinin. ACE is widely expressed in the endothelial cells of all blood vessels (from artery to capillary) which can potentially control the local Ang II production in specific vascular beds where enough Ang I, the substrate for ACE is produced. Results with ACE inhibitors clearly demonstrate the importance of ACE activity for the production of Ang II. In normal male

Sprague Dawley rats ACE inhibitors perindopril or lisinopril administered in drinking water for 7 days caused dose-dependent decreases in Ang II and in the ratio of Ang II/Ang I, and increases in bradykinin in the plasma, as well as in the heart, and lung. The changes in Ang II and bradykinin were associated with the changes in ACE activity (Campbell et al., 1994). On the other hand, in male Wistar rats with LVH induced by chronic experimental aortic stenosis increased ACE mRNA and activity was observed in the hypertrophied LV, accompanying with a significant increase in the intracardiac fractional conversion to Ang II compared with the control ( $17.3 \pm 4.1\%$  vs  $6.8 \pm 1.3\%$ ) in the isolated buffer perfused beating hearts (Schunkert et al., 1990). Therefore, local increase in ACE could cause increased conversion of Ang I and Ang II production, either locally or in the circulation. In this manner, a second local control mechanism for the RAS could operate. Important progress has been made in recent years concerning the molecular biology and genetics of ACE and its possible involvement in cardiovascular diseases such as hypertension. Positional cloning has identified a locus which includes the ACE gene which cosegregates with blood pressure in crosses between SHRSP and WKY rats, especially when the rats were salt loaded (Hilbert et al., 1991; Jacob et al., 1991). In F<sub>2</sub> rats derived from Milan normotensive and Dahl S rats the linkage of the ACE locus to blood pressure has also been demonstrated, and the rats homozygous for the S allele at both the ACE and guanylyl cyclase A (GCA) loci had inordinately high BP (Deng and Rapp, 1992). In mice with an insertional mutation to inactivate ACE, heterozygous males but not females had decreased blood pressure (15-20 mmHg less than normal) although both male and female heterozygous had reduced plasma ACE activity. BP of both male and female homozygous mutants was reduced by ~35 mm

Hg associated with a marked decrease in serum ACE activity (Krege et al., 1995). The male-female difference of the ACE gene-mutation on BP is unknown. One explanation is that the mutation in the study was designed to inactivate both the somatic and testis forms of ACE, so the difference may stem from a function mediated by testis ACE. In mice with extra copies of the ACE gene plasma ACE activity increased progressively from 62% of normal in the one-copy ACE gene animals to 144% of normal in the three-copy animals, and ACE activity in the lung increased similarly (Krege et al., 1997). In this study the heart rates, heart weights, and renal tubulointerstitial volumes decreased significantly with increasing number of ACE gene copy. However, the BP in the mice with one to three extra copies of the ACE gene did not change significantly. Ang I and II were not measured in this study. One explanation for the absence of effects of increased ACE activity on BP is that increased ACE activity in the mice may be not accompanied by quantitatively similar increase in production of Ang II. Moreover, it is also possible that in the intact organism there are multiple systems maintaining BP homeostasis. Many of these systems may respond or adapt to changes of the genetically determined ACE level in a compensatory manner. The decreased heart weight with the increased ACE activity in the mice with extra copies of the ACE gene suggest that heart weight in the mice is independent of ACE activity and that multiple systems could be involved in the cardiac growth response in the intact organism. After the ACE cDNA was cloned, a frequent insertion/deletion (I/D) polymorphism, characterized by the presence/absence of a 287 bp fragment and corresponding to an Alu sequence in the 16 intron of the gene was found. The relationship between the ACE I/D polymorphism and plasma ACE level was studied in a sample of 80 healthy adults. The mean plasma ACE concentration

(measured by RIA) in I/I homozygotes, I/D heterozygotes, and D/D homozygotes were 299, 393, and 494  $\mu\text{g/L}$ , respectively. The effect of the gene was strictly codominant and accounted for half the variance of plasma ACE levels in humans (Rigat et al., 1990). However, the distribution of the ACE I/D genotypes did not significantly differ in groups of offspring contrasted for blood pressure and parental history of hypertension. In comparison of hypertensives and normotensives of Japanese origin, the distribution of ACE I/D genotypes was also similar in both groups (Jeunemaitre et al., 1992). In 170 young Caucasian adults different genotypes were associated with serum ACE concentrations, i.e., the homozygous for the ACE gene deletion had the highest serum ACE concentration (365  $\mu\text{g/L}$ ), whereas the homozygous for the ACE gene insertion had the lowest concentration (240  $\mu\text{g/L}$ ). However, plasma Ang II and aldosterone showed no significant differences with ACE genotype. Individual linear regression analysis on the plasma renin, ACE and Ang II showed that there were no significant correlations between plasma ACE and Ang II, but there was a significant association of PRA and Ang II, suggesting that in this population renin but not ACE is rate-limiting in the cascade (Harrap et al., 1993). These studies failed to find a linkage of the ACE I/D polymorphism with plasma Ang II and blood pressure, suggestive of multigenetic and multilevel control of BP in an intact organism.

### **I. 2. 2. *The brain RAS***

Bickerton and Buckley (1961) first demonstrated that Ang II not only acts in the periphery but also increases blood pressure by a direct action on the central nervous system. Today, AngII is regarded not only as a circulating hormone that plays an important role in

blood pressure control but also as a neuropeptide acting as a neurotransmitter. Central Ang II participates in the control of blood pressure and body fluid homeostasis through mechanisms distinct from those in the periphery. These mechanisms include enhancement of sympathetic outflow, blunting of baroreceptor reflex gain, stimulation of arginine vasopressin (AVP) and adrenocorticotrophic hormone (ACTH) release. Moreover, in the brain Ang II elicits two behaviors, thirst and NaCl appetite which are critical in body fluid and electrolyte regulation.

As angiotensin does not cross the blood-brain barrier, circulating Ang II has access to central sites of action only at areas devoid of a blood-brain barrier, namely the circumventricular organs. Binding sites for Ang II are more widely distributed than this (Bunnett and Synder, 1976) and are presumably occupied by Ang II produced by the brain's own RAS.

Ganten et al. (1971) and Fischer-Ferraro et al. (1971) first reported the presence of central renin activity and angiotensinogen. This finding was subsequently confirmed by several groups. In the following years all components of the RAS have been demonstrated in the brain by different techniques (Lewicki et al., 1978; Fuxe et al., 1980; Mendelsohn et al., 1984; Dzau et al., 1986; Chai et al., 1987). Using immunohistochemical techniques, immunoreactivity for Ang I, Ang II, renin as well as angiotensinogen was observed in the central nervous system (Healy and Printz, 1984). Brain angiotensinogen appears to be mainly localized in astrocytes and ependymal cells (Deschepper et al., 1986). The presence of brain renin independent of circulatory renin was confirmed by its activity in vivo and its inhibition by specific renin antibody (Hirose et al., 1978; Speck et al., 1981). By Northern

blot hybridization, low levels of renin mRNA and relatively high levels of angiotensinogen mRNA were detected in the brain tissue of mouse and rat, providing further definitive evidence for the local brain RAS (Dzau et al., 1986; Paul et al., 1988). By means of radioimmunoassays, renin activity has been shown to be widely distributed in the brain with particularly high concentrations in the pituitary, the choroid plexus, the hypothalamus, the cerebellum, and the amygdaloid nucleus (Genain et al., 1985).

ACE is widely distributed in the brain, in association with the cerebral vasculature as in all other tissues but also in neurons demonstrated by biochemical assessment of enzymatic activity, and by quantitative autoradiography (Chai et al., 1987). High concentrations of the enzyme were detected in the forebrain circumventricular organs where the high concentrations of ACE occur primarily in association with plasma membrane of astrocytes (Chai et al., 1987; 1990). These are sites in which ACE may convert circulatory Ang I to Ang II which could then act locally on the high densities of Ang II receptors. Moderate to high levels of ACE are found in the paraventricular and supraoptic hypothalamic nuclei and the dorsal vagal complex corresponding to the distribution of Ang II immunoreactive cell bodies and fibres and Ang II receptors (Chai et al. 1987; 1990; Lind et al., 1985). ACE is also detected in the sites of the brain which are not generally associated with Ang II generation such as the basal ganglia and hippocampus (Chai et al., 1987;1990). ACE has a much wider distribution than previously thought mainly on endothelial cells, and it is also found in the neuroepithelial cells (e.g., subfornical organ, pallidonigral dendrites, and median eminence) (Bunnemann et al., 1992). Brain ACE is not a specific enzyme as its action is not confined to the conversion of Ang I acting as a peptidyl dipeptidase or dipeptidyl

carboxypeptidases (Erdős and Skidgel, 1986). Besides being identical with the kinin-degrading enzyme kininase II, brain ACE hydrolyzes opioid peptides, and other neuropeptides such as substance P by releasing C-terminal tripeptide (Erdős and Skidgel, 1986).

The biochemical demonstration of angiotensin in the brain has provided convincing evidence for an intrinsic RAS in the brain. Using high-pressure liquid chromatography followed by radioimmunoassay, Ganten et al. showed that Ang I and Ang II exist in brain extracts from nephrectomized rats (Ganten et al., 1983). This finding was confirmed by Phillips and Stenstrom (1985). Angiotensin peptides have also been identified from neuronal cell cultures which further supports the concept of an intrinsic brain RAS. Although Ang II is the principle bioactive peptide of the brain RAS, data about Ang III and Ang IV, the product peptides in the cascade, in mediating cardiovascular actions of the brain RAS is gradually emerging (Wright and Harding, 1992; Wright et al., 1996).

Specific Ang II receptors have been identified and localized in the central nervous system. Three of the four angiotensin receptors, namely the AT<sub>1</sub>, AT<sub>2</sub> and AT<sub>4</sub> receptors, have been described in the brain, with a predominance of AT<sub>1</sub> receptors involved in the central regulation of cardiovascular functions, neuroendocrine effects and body fluid homeostasis (Tsutsumi and Saveedra, 1991; Lenkei et al., 1995). The distribution of the receptors correlates fairly well with the localization of Ang II immunoreactivity. In rat brain high binding was observed in the septum, midbrain, hypothalamus, and medulla oblongata (Mendelsohn et al., 1984).

Central areas that mediate cardiovascular regulation and body fluid homeostasis in

which all components of the RAS have been detected including septal nuclei, the preoptic region, hypothalamic nuclei, the midbrain, and the nuclei of the medulla oblongata (Bunnemann et al., 1992).

### ***I. 2. 3. The brain RAS in the development of hypertension on regular salt intake***

There are several experimental lines of evidence that support a contribution of an overactive brain RAS to the development and maintenance of some forms of hypertension. In SHR and SHRSP rats an overactive brain RAS has been demonstrated in a large number of studies. Angiotensinogen content is higher in SHR at 4 wk of age than in WKY at the same age in the preoptic area, subfornical organ (SFO) and organum vasculosum laminae terminalis (OVLT) (Printz and Healy, 1983). In adult SHR, angiotensinogen is increased in the septum, preoptic nuclei and paraventricular nucleus (PVN) compared with WKY (Naruse et al., 1985; Printz and Healy, 1983). Ang II immunoreactivity in adult SHR is twice as much as in WKY in the supraoptic nucleus (SON) and PVN (Weyhenmeyer and Phillips, 1982). Ang II concentrations and turnover in the hypothalamus are higher in SHRSP than WKY (Ganten et al., 1983). Ang II levels in the hypothalamus and cerebellum are higher in SHR versus WKY at 2 to 20 weeks of age (Phillips and Kimura, 1986). In addition, Ang II receptors (Saavedra et al., 1986), affinity of brain Ang II receptors (Plunkett and Saavedra, 1985), and brain angiotensinogen mRNA (Yongue et al., 1991) were increased in SHR aged 4-14 wk, 10 wk, and 20 wk, respectively.

ACE activity in the brain of SHR has been reported increased. SHR at age of 14 wk

have higher cerebrospinal fluid ACE activity than normotensive age-matched WKY (Israel and Saavedra, 1987). Mizuno et al (1981) found that ACE activity was significantly higher in the hypothalamus of SHR aged 20-21 wk than in aged-matched WKY ( $10.4 \pm 2.6$  vs  $4.9 \pm 3.3$  units).

Ang II receptors in the brain of SHR are also increased. Autoradiography indicates a greater number of Ang II binding sites in the SFO in young and adult SHR than in age-matched WKY rats (Saavedra et al., 1986). Raizada et al. (1984) observed an increased number of Ang II receptors in neuronal cultures from 1 day old SHR.

However, ambiguous evidence for the functional involvement of the brain RAS in the development of hypertension has come from studies using ACE inhibitors, Ang II antagonists and antisense oligodeoxynucleotide to components of the RAS.

Central administration of antisense oligodeoxynucleotide to angiotensinogen mRNA in adult SHR caused a decrease in angiotensinogen in the hypothalamus and brain stem and lowered blood pressure to normotensive levels up to 24 h after administration. Peripheral administration at the same dose did not cause a significant change in blood pressure (Wielbo et al., 1995). In adult SHR central injection of antisense to  $AT_1$  receptor mRNA decreased blood pressure by 20 mmHg for up to 9 wk and slowed the development of hypertension in young SHR (Gyurko et al., 1993).

In adult SHR, acute injection of the  $AT_1$  receptor antagonist losartan into the anterior hypothalamus decreased BP by 8.7% in SHR aged 9 wk but not in age-matched WKY (Yang et al., 1992b). Injection of an Ang II peptide antagonist,  $(Sar^1, Ile^8)$  Ang II, into the RVLM in SHR also caused a significant decrease in BP compared to WKY rats (Chan et al.,

1994). However, DePasquale et al. (1992) reported that acute icv losartan did not cause a significant change in BP in SHR at 20 wk of age. Chronic icv losartan (1 mg/kg/d) for two weeks did not prevent either the development of hypertension or sympathetic hyperactivity in SHR aged 12 to 16 weeks on regular salt intake. At a high dose (10 mg/kg/d), icv or sc administration of losartan for two weeks caused a similar decrease in BP, suggestive for a depressor effect due to leakage of losartan out of the central nervous system and blockade of peripheral AT<sub>1</sub> receptors (Kawano et al., 1994). Consistent with this, our study (Huang and Leenen, 1996a) showed that chronic icv losartan (1 mg/kg/d) did not affect the development of hypertension or sympathetic activity in SHR on regular salt intake. The blockade of the brain RAS by chronic or acute icv losartan appears not be able to blunt the higher brain RAS activity in SHR, so to prevent the development of hypertension. The hyperactivity of the brain RAS may be involved in acute blood pressure regulation but not in the development of hypertension. It is also possible that at low doses chronic icv losartan may not reach certain areas in the brain to prevent the development of genetic hypertension..

Changes in angiotensin concentrations in the brain compatible with ACE inhibition were observed upon icv treatment with ACE inhibitors. After icv injection of captopril in nephrectomized rats, Ang I was significantly increased in the hypothalamus, while Ang II showed a tendency toward a decrease (Ganten et al., 1983). icv injection of ramiprilat, the active moiety of ramipril, lowered Ang II in the brainstem of SHR and decreased BP (Phillips and Kimura, 1986). Administration of the ACE inhibitor captopril by icv infusion attenuated the development of hypertension in SHR (Okuno et al., 1983). However, the effects of ACE inhibitors in the brain comprise more than a reduction in Ang II, since ACE

acts on several peptide substrates that are all present in the brain, including kinins, substance P and opioid peptides (Skidgel and Erdös, 1987; O'Sullivan and Harrap, 1995).

#### ***I. 2. 4. The brain RAS and high salt intake***

The circulatory RAS is inhibited by salt exposure (Hirawa et al., 1995). Circulatory Ang II is therefore unlikely involved in the high salt-induced hypertension. Schmieder et al. suggested that impaired suppression by high salt intake of the RAS, i.e., a relatively high level of Ang II associated with high salt intake may act as a stimulus for hypertension in hypertensive patients (Schmieder et al., 1996). However, direct evidence is emerging for the involvement of the brain RAS in the development of salt-induced hypertension.

Nishimura et al. found that renin mRNA increased by about two times in the hypothalamus of adult Wistar rats after 10 days high salt diet. After 8 wk of high salt intake, renin mRNA in the hypothalamus was not suppressed whereas renal renin mRNA was lowered by 1/3 of regular salt group. Renin mRNA levels in the hypothalamus were not suppressed either in the prehypertensive or in hypertensive stage in rats treated with DOCA or sodium, or both (Nishimura et al., 1997). The fact that the renin gene is constantly expressed in the hypothalamus during chronic high salt intake suggest that activated renin gene may be involved in overactivity of the brain RAS in high salt-induced hypertension. Mann et al. (1980) observed that central Ang II receptor sensitivity in SHR rats depends on the sodium status. They found that the increase in BP in Wistar rats to central Ang II injection could be abolished by feeding the rats a low-salt diet. In SHR on high salt intake for 6 days, the increases in BP to icv Ang II were significantly higher than in WKY on high

salt ( $47 \pm 4.5$  vs  $28 \pm 4.6$  mmHg). When SHR were on low salt, the pressor response induced by icv Ang II was similar to those in WKY rats. Specific  $^{125}\text{I}$ -Ang II binding in vitro to brain membranes was consistently lower in salt-depleted rats, suggesting salt intake modifies activity of the brain RAS via Ang II binding. In Dahl rats, high salt intake led to an upregulation of  $\text{AT}_1$  receptor in the brain in both strains, however, the increase in brain  $\text{AT}_1$  receptor expression on high salt was more pronounced in Dahl S (3-fold than Dahl S on regular salt) than in Dahl R rats ( $<1.5$ -fold vs Dahl R on regular salt) (Strehlow et al., 1999). In Sprague-Dawley rats, salt deprivation increased  $\text{AT}_{1\text{B}}$  receptor expression in decorticated brains by 164% compared with high salt intake, whereas high salt enhanced the expression of  $\text{AT}_{1\text{A}}$  receptors in the brain by 155% compared to low salt intake (Sandberg, et al., 1994). These data suggest that  $\text{AT}_{1\text{A}}$  and  $\text{AT}_{1\text{B}}$  receptors play reciprocal roles in the central mechanisms for the control of fluid homeostasis. Mizuno et al.(1983) measured ACE activity in the brain and found that high salt resulted in a significant rise in ACE activity in some areas of the brain such as midbrain in SHR and SHRSP compared to same strain on control diet. They suggested that increased ACE activity may contribute to the hypertension induced by high salt. In Sprague Dawley rats high salt tended to increase brain ACE but the changes were not significant (Jackson et al., 1986). However, Mendelsohn et al. (1982) showed that high salt increased ACE activity in the spinal cord but decreased it in the hypothalamus, striatum, and midbrain in male Sprague Dawley rats. Explanations for the inconsistent data may be due to the different rate, duration, route of salt intake, various strains, even technical limitations applied. However, whether changed ACE activity or renin expression is associated with changes in production of Ang II has not yet been determined.

Functional studies provide direct proof for the involvement of the brain RAS in salt-sensitive hypertension. Blocking  $AT_1$  receptors by microinjection of losartan to the anterior hypothalamus, the maximal decrease in MAP in response to losartan was significantly larger in 8% NaCl-fed SHR ( $23 \pm 1.8$  mmHg,  $11.2 \pm 0.77\%$ ) than in 1% NaCl-fed SHR ( $15 \pm 1.4$  mmHg,  $8.7 \pm 0.67\%$ ). In contrast, in WKY rats on either diet, losartan did not affect MAP or HR (Yang et al., 1992a). Chronic central administration of losartan prevents the enhancement of blood pressure in SHR by high salt diet (Huang and Leenen, 1996b). In Dahl S rats on high salt diet chronic blockade of central  $AT_1$  receptors by losartan prevents sympathetic hyperactivity, impairment of baroreflexes as well as the development of hypertension (Huang and Leenen, 1998). In Dahl-Iwai salt sensitive rats chronic central infusion of the  $AT_1$  receptor antagonist, CV-11974, also prevented the development of hypertension (Teruya et al., 1995). These results suggest that in these models the brain RAS is essential for sympathetic hyperactivity and the development of hypertension on high salt diet.

### ***I. 2. 5. The brain RAS and brain "ouabain" in salt-sensitive hypertension***

Recent studies have indicated that brain endogenous ouabain-like compound(s) ("ouabain") has a close relationship with the brain RAS in salt-sensitive hypertension. Although ouabain was discovered as a compound of plant origin more than one century ago, "ouabain" is also released from the adrenal cortex as well as from brain, especially in the hypothalamus and pituitary (Harris et al., 1991; Ludens et al., 1992; Leenen et al., 1994). Studies in hypertensive humans (Hamlyn et al., 1982) and animals (Tamura et al., 1985)

have shown that high salt intake causes an increase in plasma ouabain-like activity. Originally, endogenous “ouabain” from the adrenal cortex was considered to play a major role in cardiovascular regulation and in the pathogenesis of hypertension (Ludens et al., 1992). To evaluate the contribution of the adrenal glands, Leenen et al. (1993a) showed that in SHR adrenalectomy only caused minor decreases in peripheral and central “ouabain” and did not prevent the increases in both peripheral and central “ouabain” induced by high salt. These results clearly indicate that, at least in SHR, the adrenal glands are not essential for the maintenance of circulating and brain “ouabain” and the increases induced by high salt, the central nervous system may be the major source of both central and peripheral “ouabain”.

Intracerebroventricularly infused hypertonic saline causes similar sympathoexcitatory and pressor responses with icv infusion of ouabain, and these responses can be blocked by antibody Fab fragments (Huang et al., 1992b), which bind ouabain and related steroids with high affinity (Balzan et al., 1991). These results demonstrate that brain “ouabain” plays a major role in the sympathoexcitatory and pressor responses to increases in central sodium. In both salt-sensitive Dahl S rats and SHR, central “ouabain” plays a critical role in the development or exacerbation of hypertension. High salt intake elevates hypothalamic, pituitary, and pons “ouabain” in both Dahl S rats and SHR (Leenen et al., 1993b; 1994). Concomitant chronic icv infusion of antibody Fab fragments, prevents salt-induced sympathoexcitation and increase in BP (Huang and Leenen, 1994; 1995).

The brain RAS also plays a critical role in the development and maintenance of salt-sensitive hypertension in SHR and Dahl S rats. In order to clarify the relationship between the brain RAS and brain “ouabain”, the effects of blockade of brain “ouabain” or of the brain

RAS were compared. Chronic blockade of brain “ouabain” by icv administration of Fab fragments caused reduced sympathetic activity and BP, which is similar to that induced by blocking Ang II receptor with icv administration of losartan in SHR and Dahl S rats ( Huang and Leenen, 1996a; 1998). Moreover, chronic icv treatment with losartan blocks sympathoexcitatory and pressor responses to both Ang II and ouabain icv infusion in SHR and icv ouabain in Dahl S rats. In contrast, icv administration of Fab fragments to block brain “ouabain” does not attenuate but enhances the responses to icv Ang II (Huang and Leenen, 1996b; 1998). These data suggest that activation of the brain RAS is secondary to brain “ouabain” in the pathways leading to sympathetic hyperactivity and hypertension. But how brain “ouabain” activates the brain RAS, or which component of the brain RAS is involved, have not yet been studied so far.

## **II. Salt intake, cardiac hypertrophy and the cardiac RAS**

Although the role of high salt intake in aggravating hypertension has been extensively demonstrated, in recent years it has become clear that high salt intake is also a major determinant of the extent of LVH independent of blood pressure ( Du Cailar et al., 1989; Schmieder, 1988 ). It has become apparent now that high salt intake may cause cardiac trophic effects independent of increased pressure load although the mechanisms are poorly

understood.

## **II. 1. *Cardiac hypertrophy***

Cardiac hypertrophy, which refers to an increase in the heart mass or in the ratio of the heart weight to body weight, has generally been regarded as an adaptive response of the heart to increased hemodynamic workload to maintain normal cardiac function. On the other hand, however, cardiac hypertrophy is a major risk factor for heart diseases, including heart failure, myocardial infarction, and cardiac arrhythmias (Levy et al., 1990). Cardiac hypertrophy involves a sequence of events including initiating signals, coupling mechanisms, and regulation of gene expression. A number of factors are associated with initiating cardiac hypertrophy. These factors can be divided into three classes: mechanical stretch from pressure or volume overload, neural factors such as activation of the sympathetic nervous system, and endocrine factors such as thyroid hormone and Ang II (Morgan and Bader, 1991). As a result of these signals, membrane ion channels and enzymes are activated which cause increased intracellular contents of  $\text{Na}^+$ ,  $\text{Ca}^{++}$ ,  $\text{H}^+$ , cAMP, inositol phosphates, and diacylglycerol and enhanced activity of protein kinases A and C. Except for the hypertrophic initiating factors such as thyroid hormones acting via direct binding to nuclear receptors, most of the intracellular pathways finally converge to a common route---constitutive or inductive transcription of proto-oncogenes (Marban and Koretsune, 1990). These genes encode DNA binding proteins such as transcription factors, which further regulate RNA transcription and protein synthesis (Simpson et al., 1989).

## **II. 2. *Salt intake and cardiac hypertrophy***

Initial studies (Schmieder et al., 1988; Du Cailar et al., 1989) demonstrated that in mild hypertensive patients salt intake, estimated by 24-hr natriuresis, was directly correlated with posterior thickness and left ventricular mass. Stepwise multiple regression analysis confirmed that salt intake was a major determinant of left ventricular mass independent of systolic or diastolic pressure, body mass index, hematocrit and epinephrine. Subsequent clinical studies confirmed the role of salt intake in the development of LVH (Du Cailar et al., 1992; Liebson et al., 1993). In 91 never-treated hypertensive patients and 50 normotensive subjects, Du Cailar et al. (1992) showed that LV mass was positively correlated with urinary sodium excretion in both groups although hypertensive patients had a higher LV mass index than normotensive subjects for a given level of salt intake. Kupari et al. (1994) found that dietary salt intake is positively related to LV mass as an independent predictor in a population sample aged 36 to 37 years old by multiple linear regression, and that LV mass was clearly elevated only in persons with both BP and salt intake above the population medians, suggestive of a synergistic factor with blood pressure for cardiac hypertrophy.

Animal experiments also showed a positive correlation between salt intake and LVH. In young normotensive WKY rats on high salt diet (2% or 8% NaCl) for 4 to 10 wk, an 10% and 24% increase in LV mass was reported (Yuan and Leenen, 1991). A similar change was observed in normotensive Wistar rats on 1% NaCl in drinking water for 3 to 6 wk (Fields et al., 1991). However, in WKY rats, a longer high salt intake from age 10 to 16 wk and from 10 to 20 wk only resulted in 5-10 % increases in LV weight (Frohlich et al., 1993). The hypertrophic effects of high salt intake in rats seem to be age and strain dependent. The

preferential influence of high salt intake appears to be during the early developmental stage of the animals as shown in young WKY and Wistar rats (Yuan and Leenen, 1991). In two-kidney, one-clip (2K, 1C) rats, high salt increased and low salt decreased cardiac hypertrophy by 13% and 9%, respectively, without significant changes in blood pressure (Gallo et al., 1990). Our recent data also showed that in young SHR, 4 wk of 2% salt intake did not cause significant LVH but WKY had, while 4 wk of 8% salt intake caused a less increase in LV weight in SHR than in WKY, suggesting that young WKY was more susceptible to the development of cardiac hypertrophy than young SHR (Leenen and Yuan, 1998).

The precise pathogenetic mechanisms underlying the salt-induced cardiac hypertrophy are not yet understood. Studies have focused on three different mechanisms; the sympathetic nervous system, the renin angiotensin system and volume homeostasis.

Increased sympathetic activity can contribute to the development of cardiac hypertrophy. Infusion of norepinephrine into dogs leads to development of cardiac hypertrophy, independent of changes in blood pressure (King et al., 1987). Zierhut and Zimmer (1989) indicated that cardiac hypertrophy was induced by stimulation of  $\alpha$ - and  $\beta$ -adrenoceptors. However, a large body of data does not support the primary role of increased sympathetic activity in the development of salt-induced LVH. We previously showed that daytime LV norepinephrine turnover and tissue tyrosine hydroxylase activity were not increased but tended to decrease by high salt intake (Fields et al., 1991; Yuan and Leenen, 1991). Recently we also showed that chronic blockade of  $\alpha_1$ - $\beta$ -receptors did not prevent LVH in WKY (Song D et al, 1997). In addition, in normotensive humans high salt intake decreased the plasma and urinary catecholamine concentrations (Egan et al., 1991).

High salt diet may induce LVH by chronically expanding intravascular volume, which increases preload to the heart. Total blood volume is a determinant of LVH (Messerli et al., 1984). Du Cailar et al. (1992) demonstrated that dietary salt intake modulates the diastolic diameter (represents an increase in the end diastolic pressure by increased volume load) in normotensive subjects. Similar effects were observed in patients with mild essential hypertension by Schmieder et al. (1989). However, in more severe hypertensive patients salt intake determines the degree of the wall thickness and causes concentric LVH (Du Cailar et al., 1992; Schmieder et al., 1988). Furthermore, in Dahl S rats on high salt intake an increase in LV weight was associated with an increase in total peripheral resistance but not cardiac output (Pfeffer et al., 1984), and in Wistar rats on high salt no increases in intravascular volume were noted, and right atrial pressure remained unchanged (Fields et al., 1991), suggesting that cardiac hypertrophy induced by high salt intake was independent of volume load.

Intracellular mechanisms such as  $\text{Na}^+\text{-H}^+$  exchange,  $\text{Na}^+\text{-K}^+\text{/Ca}^{++}$  exchange may be involved in the development of cardiac hypertrophy. Endogenous “ouabain” can inhibit  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , therefore, it has a close relationship with  $\text{Na}^+\text{-H}^+$  or  $\text{Na}^+\text{-K}^+\text{/Ca}^{++}$  exchanges. LV mass index in hypertensives with high plasma “ouabain” was greater than in hypertensives with normal plasma “ouabain”, suggesting a close relationship between endogenous “ouabain” and cardiac hypertrophy (Manunta et al., 1999). High salt intake increases both central (in the hypothalamus and pituitary) and peripheral (including plasma and LV) “ouabain” in SHR and WKY, but the increases in SHR are higher than in WKY (Ienen et al., 1993b). In parallel, in vitro ouabain induced both some early response and late response genes similar

to those of hypertrophic stimuli by pressure overload; the induction of these genes was initiated by an increase in net influx of  $\text{Ca}^{++}$  through partial inhibition of Na/K-ATPase leading to activation of PKC-dependent pathways (Huang et al., 1997). A recent study by Gu et al. (1998) indicated that sodium has a direct effect on induction of hypertrophy of cultured myocardial myoblasts by showing an increase in cellular protein synthesis, a decrease in cellular protein degradation and an increase in cell mean diameter after exposure to the medium with a sodium concentration of 10% above normal. A study by Yu et al. (1998) reported that high salt intake induced widespread fibrosis and increased TGF- $\beta_1$  in the heart and kidney in both normotensive and hypertensive rats. These results suggest a specific effect of dietary salt on fibrosis, possibly via TGF- $\beta_1$  pathways.

## **II. 3. *The cardiac RAS in the development of cardiac hypertrophy***

### **II. 3. 1. *The cardiac RAS***

In the early 1970s, evidence was presented which suggested that the heart might have its own intrinsic RAS. In dog heart renin activity was demonstrated (Hayduk et al. 1970) and conversion of Ang I to Ang II was noted in isolated perfused rabbit heart preparation (Needleman et al., 1975). With the advent of molecular biological techniques, the existence of the cardiac RAS has become much more firmly established.

Biochemical, immunohistochemical and molecular biological techniques have demonstrated the existence of a cardiac RAS independent of the circulating RAS (for reviews, see Lindpaintner and Ganten, 1991; Dostal and Baker, 1999). All components of the RAS have been identified in the heart at both mRNA and protein levels (Boer et al., 1994;

Iwai et al., 1995; Lindpaintner and Ganten, 1991; Passier et al., 1996; Pieruzzi et al., 1995). In normal cardiac tissue very low renin mRNA was determined by quantitative reverse transcriptase polymerase reaction (RT-PCR) (Boer et al., 1994; Pieruzzi et al., 1995; Passier et al., 1996). Comparing renin concentrations in the heart with its concentration in the plasma in normal and nephrectomized pigs, Danser et al (1994 ) found that the levels of renin in cardiac tissue (expressed per g wet weight) were similar to those in plasma in normal pigs. Both in cardiac tissue and in plasma, renin fell to undetectable levels after nephrectomy, suggesting that under physiologic conditions renin in cardiac tissue is primarily of kidney origin. Although low cardiac renin mRNA and activity were also detected in rats and humans, there is no universal agreement that sufficient renin is produced for cardiac Ang II production in physiological conditions. Angiotensinogen mRNA levels in porcine cardiac tissue are very low, 25% of the levels in the plasma, which is compatible with its diffusion from plasma into interstitium, suggesting plasma origin of angiotensinogen in cardiac tissue under physiological condition (Danser et al., 1994; Passier et al., 1996). On the other hand, substantial concentrations of angiotensinogen mRNA were found in rat heart (Lindpaintner et al., 1987). Angiotensinogen has been localized in human ( Sawa et al., 1992) and dog (Lee et al., 1996) hearts, as well as in cultured neonatal and adult rat cardiac myocytes (Sadoshima et al., 1993) and fibroblasts (Dostal et al., 1995). ACE has been demonstrated by autoradiography (Yamada et al., 1991), as well as by measurement of its activity in cardiac homogenates (Schunkert et al., 1990). In the atria ACE activity was reported to be higher than in the ventricles. Cardiac ACE mRNA is also readily detectable and limited normally to the endothelial cells (Falkenhahn et al., 1995). mRNA for Ang II receptors has also been

detected in cardiac tissue (Fareh et al., 1996; Meggs, et al., 1993; Sechi et al., 1992; Zhang et al., 1995). The number of Ang II receptors and the ratio between AT<sub>1</sub> and AT<sub>2</sub> subscript differ depending on whether assessments were made in a whole heart or in cardiomyocytes or other cell types present in normal cardiac tissue, as well as on the developmental stage of cardiac tissue or cardiac cells used (Fareh et al., 1996; Meggs et al., 1993; Sechi et al., 1992). Finally, Ang I and II have been localized in the heart of pig (Danser et al., 1994) and rat (Ruzicka et al., 1995). In normal rats, levels of the cardiac Ang I (~60 - 70 pg/g) and II (~4 - 6 pg/g) were similar to plasma Ang I and II (Ruzicka et al., 1993). The presence of Ang I and II in cultured cardiac myocytes (Dostal et al., 1992), fibroblasts (Dostal et al., 1992), and microvascular endothelial cells (Fischer et al., 1997) suggests that these cell types can independently contribute to Ang II generation. Ang II levels in media were reported in cultured neonatal rat cardiac myocytes (2.01 fmol/10<sup>6</sup> cells per 48 hr), fibroblasts (3.16 fmol/10<sup>6</sup> cells per 48 hr) (Dostal et al., 1992), and microvascular endothelial cells (3.16 fmol/10<sup>6</sup> cells per 5 hr) (Fischer et al., 1997). In isolated perfused rat heart, addition of losartan to the renin/angiotensinogen perfusion to block AT<sub>1</sub>-mediated Ang II-uptake did not cause significant change in the cardiac Ang II levels (de Lannoy et al., 1998), suggesting of intracellular Ang II formation . Assessment of in vivo angiotensin production showed that in normal pigs the cardiac renin and angiotensin levels were directly correlated with their respective plasma levels, and the levels in both the heart and plasma were undetectable after nephrectomy (Danser, 1996). Similarly, in Sprague-Dawley rats a 78% decrease in cardiac Ang II level was observed 48 hr after nephrectomy (Campbell et al., 1993). It appears that cardiac angiotensin production depends, at least under normal conditions, on the uptake of

renal renin from the circulation.

### **II. 3. 2. *The cardiac RAS in the development of cardiac hypertrophy***

The cardiac RAS has been linked to enhanced cardiac growth both in vivo ( Suzuki et al., 1993) and in vitro ( Liu et al., 1998; Naftilan et al., 1989). Angiotensin II has both indirect and direct effects on cardiac tissue. The indirect actions include central nervous system effects such as increased sympathetic outflow and stimulation of thirst, maintenance of vascular tone, and regulation of aldosterone synthesis and release. The direct cardiac growth-promoting effects of Ang II have recently become established. In isolated cardiac cells Ang II induces hyperplasia of fibroblasts (Fujisaki et al., 1995) and hypertrophy of myocytes (Miyata and Haneda, 1994). Ang II also enhances collagen production of cardiac fibroblasts (Brilla et al., 1994). These actions are most probably mediated by AT<sub>1</sub> receptors (Suzuki et al., 1993). Stimulation of AT<sub>1</sub> receptors, mediated by activation of protein kinase C (PKC) or phosphorylation of nuclear proteins, results in an increase in protein synthesis in myocytes (Sadoshima et al., 1993). In vitro pretreatment of CV-11974 ( an active metabolite of the AT<sub>1</sub> receptor blocker TCV-116) in cultured neonatal myocytes inhibited the activation of mitogen-activated protein (MAP) kinase by 60%, partially suppressed the induction of *c-fos* gene, and attenuated the stretch-induced increase in phenylalanine incorporation in cells (Kojima et al., 1994). However, in isolated beating adult rat hearts subjected to increased systolic load LV *c-fos* and *c-mys* mRNA levels, and the rate of phenylalanine incorporation into cardiac proteins was increased, and AT<sub>1</sub> blockade with losartan did not prevent these hypertrophic responses (Thienelt et al., 1997).

Much data have been accumulated with regards to the cardiac RAS and its involvement in the development of cardiac hypertrophy. Components of the cardiac RAS, including renin, angiotensinogen, ACE, and angiotensin AT<sub>1</sub> receptors, have been found upregulated in different models of pressure and volume overload-induced hypertrophy and heart failure (Boer et al., 1994; Iwai et al., 1995; Passier et al., 1996; Pieruzzi et al., 1995; Schunkert et al., 1990; Suzuki et al., 1993).

In cardiac volume overload by aortocaval shunt cardiac renin mRNA (Boer et al., 1994) increased associated with a major rise in cardiac renin activity (~ 2-fold) at 1 day after surgery (Ruzicka et al., 1993). Although cardiac renin mRNA increased further at 1 wk after surgery (Boer et al., 1994; Pieruzzi et al., 1995), cardiac renin activity remains at the same level as at 1 day (Ruzicka et al., 1993). Iwai et al. (1995) reported a similar trend of changes in cardiac renin mRNA at 1 and 7 days although the changes were not statistically significant. In cardiac pressure overload there are no data available yet on cardiac renin mRNA and activity. In myocardial infarction loss of viable myocardium results in cardiac pressure/volume overload. In this model Passier et al. (1996) observed 4-, 14-, and 8-fold increases in renin mRNA in the LV in rats at 2, 4 and 7 days after coronary artery ligation compared with sham operated rats. Using *in situ* hybridization dense renin mRNA labeling was observed around the infarcted area of the LV at the same time points.

Cardiac angiotensinogen mRNA was significantly increased during postinfarction remodelling. During the initial period of cardiac volume overload cardiac angiotensinogen mRNA or cardiac angiotensinogen were not significantly changed (Boer et al., 1994; Iwai et al., 1995). Passier et al. (1996) also demonstrated that cardiac angiotensinogen mRNA

expression during the initial period after coronary artery ligation did not change. Whether or not cardiac angiotensinogen mRNA and cardiac angiotensinogen levels increase in pressure overload is still unknown.

Similarly, there were no increases in cardiac  $AT_1$  or  $AT_2$  mRNA expression or changes of ratio of  $AT_1$  to  $AT_2$  receptors assessed at 1, 7, and 40 days after the shunt surgery (Pieruzzi et al., 1995, Iwai et al., 1995). No changes in gene expression in Ang II receptors were observed in the pressure overload model by aortic banding (Wolf et al., 1996). However, after coronary artery ligation, angiotensin II receptor changes were reported. LeFroy et al. (1996) demonstrated that at 7 days after ligation, Ang II binding in the infarcted area of the LV was markedly increased compared to the non-infarcted area of the LV. Using competitive reverse transcriptase polymerase chain reaction, nuclear runoff and binding assay, Nio et al. (1995) showed that both  $AT_1$  mRNA and  $AT_2$  mRNA increased substantially and was associated with significantly increased numbers of these receptors in the infarcted myocardium.

The most consistent change is an increase in cardiac ACE mRNA and ACE activity associated with cardiac hypertrophy, irrespective of the model. At 5 weeks after the banding of the ascending aorta, a fourfold increase in ACE mRNA of the hypertrophied LV and a significant increase in ACE activity of the LV were observed in the absence of changes in lung ACE mRNA and circulating ACE activity (Schunkert et al., 1990). In the same animal model at 12 weeks a 1.7-fold increase in LV weight and a 1.6-fold increase in RV weight were associated with 2-fold increases in ACE mRNA and activity in both hypertrophied ventricles whereas pulmonary ACE mRNA and activity were markedly decreased (Pfeifer et al., 1998).

In two-kidney, one-clip hypertensive rats LV ACE activity increased by twofold at 2 and 4 weeks after renal artery clipping but not at 12 weeks while a twofold increase in ACE activity of the RV at 4 and 12 weeks was observed (Challah et al., 1995). However, although these studies show elevated ACE mRNA and activity in the hypertrophied left ventricle induced by pressure overload, there is no evidence yet for associated increases in cardiac Ang II in response to cardiac pressure overload. In hypoxia-induced RV hypertrophy, marked increases in ACE expression and activity in the hypertrophied RV after 8 and 14 days of hypoxia were demonstrated while ACE activity and expression were decreased in the non-hypertrophied left ventricle, suggestive of differential effects of hypoxia on RV versus LV ACE expression and activity (Morrel et al., 1997). In volume overload-induced cardiac hypertrophy due to aortocaval shunt increased ACE mRNA in both LV and RV was reported (Iwai et al., 1995; Lear et al., 1997). In this model, increases in the mRNA level (i.e. for renin and ACE) were associated with increased activity at the protein level (i.e. renin activity and Ang II) (Boer et al., 1994; Ruzicka et al., 1995).

Increased generation of Ang II provides direct evidence for overactivity of the cardiac RAS. In rats with cardiac volume overload by aortocaval shunt for 1 day cardiac Ang II increased by 100%. After 1 wk of shunt surgery, cardiac Ang II remained increased by 65% whereas only a small increase in plasma Ang II (Ruzicka et al., 1995). Furthermore, quinapril, an ACE inhibitor with high affinity for cardiac ACE, fully prevented the increase in cardiac Ang II, whereas another ACE inhibitor, enalapril with low affinity for cardiac ACE, did not (Ruzicka et al., 1995). In rats with postmyocardial infarction a marked increase in Ang II was found in the infarcted area of the LV as well as in the noninfarcted area of the LV

at 6 hr and 1 day after coronary artery ligation, which returned to normal by 3 days in the noninfarcted area and after 1-2 wk in the infarcted area of the LV (Leenen et al., 1999). In cardiac pressure overload, however, whether overactivity of the cardiac RAS is associated with increased Ang II generation, has not been evaluated yet.

In rats with banding the abdominal aorta subpressor dose of ramipril (10  $\mu\text{g}/\text{kg}$ ) for 6 wk caused same complete regression of cardiac hypertrophy without decrease in blood pressure as in the group treated with the antihypertensive dose of ramipril (1 mg/kg) (Linz et al., 1989). Chronic treatment with same subpressor dose of ramipril (10  $\mu\text{g}/\text{kg}$ ) for one year in same model prevented LVH with myocardial fibrosis without effect on BP as in the 1 mg/kg antihypertensive dose group (Linz et al., 1992). In SHR at established stage of hypertension aged 15 wk administration of imidapril (2 and 5 mg/kg/day) by gavage for 8 wk, LV weight reduced markedly with a significant decrease in myocardial collagen content compared to control (Yokota et al., 1998). TCV 116 ameliorated LVH by reducing LV wall thickness, transverse diameter of myocytes, relative amount of LV myosin heavy chain, and interstitial fibrosis as well as prevented the increase in BP of SHR (Kojima et al., 1994). Long-term (15 wk) administration of the specific AT<sub>1</sub> receptor blocker BMS-186295 did not regress LVH in rats with persistent systolic pressure overload by ascending aortic stenosis (Weinberg et al., 1997). Several explanations may account for the discrepant observations. For one thing, it remains to be determined which responses to hemodynamic overload have relevance to hypertrophic growth regulation of the heart, although different responses exist. Activation of PKC pathway is involved in the neonatal myocyte growth responses, but may not be responsible for cardiac hypertrophy in the adult heart. Moreover, the developmental stage

may be related to the change in the molecular mechanisms of cardiac hypertrophy. It is no wonder that the different gene programs are expressed at different stages, i.e., in cultured neonatal myocytes opposed to terminally adult cardiomyocytes in vivo. The involvement of the cardiac RAS in cardiac hypertrophy remains uncertain especially in the adult hearts.

### **II. 3. 3. *The cardiac RAS and high salt intake***

Little information is available about changes in the cardiac RAS with high salt intake. Kreutz et al. (1995) reported that in SHRSP aged 16 wk high salt intake induced hypertension and cardiac hypertrophy in SHRSP but not WKY, while cardiac ACE mRNA increased to two to three-fold, and ACE activity increased to four-fold in both strains. Furthermore, although ACE mRNA in the LV was significantly greater in SHRSP than in WKY, its activity was similar in both strains on either diets. These results indicate that left ventricular ACE activity was upregulated by high salt in both strains, independently from the development of hypertension, since in WKY blood pressure and left ventricular weight remained unchanged despite increased ACE activity on high salt intake. The greater increase in ACE mRNA of SHRSP but similar ACE activity in both SHRSP and WKY rats suggests that high salt induces ACE mRNA expression differentially in the two strains and that post-transcription down-regulation occurred in SHRSP. However, Ang II concentrations were not measured. The study in our laboratory showed that 8% salt intake for 4 wk caused a 25% increase in LV weight in young WKY but was not associated with an increase in cardiac Ang II (Leenen and Yuan, 1998). Nickenig et al. (1998) reported in SD rats that 8% salt diet caused an increase in aortic AT<sub>1</sub> receptor mRNA and AT<sub>1</sub> receptor density to approximately 160% compared

with control levels. Wang and Du (1998) showed that in male Wistar rats high salt intake increased AT<sub>1</sub> receptor mRNA levels both in the aorta and mesenteric resistance arteries. Nonpressor dose of Ang II infusion completely suppressed the increase of AT<sub>1</sub> receptor expression. In contrast, using radioligand binding assay and quantitative RT-PCR, Strehlow et al.(1999) recently demonstrated that an 8% salt diet decreased aortic AT<sub>1</sub> receptor mRNA and receptor density in both Dahl S and R rats. They suggested that regulation of vascular AT<sub>1</sub> receptor expression is influenced by various other events that are currently only partly understood. In Dahl Iwai salt-sensitive rats at age of 6 wk, however, 8% high salt intake for 4 wk increased blood pressure and the LV weight but did not change cardiac AT<sub>1</sub> and AT<sub>2</sub> receptors (Sumida et al., 1998). In parallel, AT<sub>1</sub> receptor antagonists, TCV 116 or losartan, did not prevent cardiac hypertrophy with minor effects on BP in 4% salt-loaded Dahl S rats (Sugimoto et al., 1996; Sugimoto et al., 1994). One possible explanation for these studies is that the cardiac RAS may be suppressed in Dahl S rats on high salt and the cardiac Ang II production may not be sufficient to initiate intracellular signaling of cardiomyocyte hypertrophy. However, in these studies cardiac Ang II was not measured. Alternatively, other mechanisms may play a critical role in the development of LVH in Dahl S rats on high salt intake.

### **III. Hypotheses and Objectives**

In Dahl S rats, high salt intake exaggerates the development of hypertension and cardiac hypertrophy. So far, however, whether the brain and cardiac RASs are actually

overactive in Dahl S rats, and if so, which component is involved, has not been evaluated. In addition, whether blockade of hypertensive response of high salt by blocking brain “ouabain” with antibody Fab fragments can prevent cardiac hypertrophy in Dahl S rats, has not been studied.

We hypothesized that (1) the development of hypertension and cardiac hypertrophy induced by high salt may be associated with the overactivity of the local RAS, i.e., the brain and cardiac RASs; and (2) cardiac hypertrophy induced by high salt may be independent of the development of hypertension in this model.

To assess the activity of the brain and cardiac vs the circulatory RASs in the development of salt-sensitive hypertension and cardiac hypertrophy, parameters of the brain and cardiac as well as the circulatory RASs were evaluated in Dahl S and Dahl R rats after 2 or 4-5 weeks on high or regular salt intake. Specifically, in the hypothalamus and pons as well as in the ventricles, ACE mRNA and activity, and Ang I and Ang II were measured. In addition, plasma renin and Ang I and II were measured to assess the circulatory RAS.

To assess the hypothesis that cardiac hypertrophy induced by high salt is independent of the development of hypertension, Dahl S rats were placed on high salt plus chronic infusion of Fab fragments or  $\gamma$ -globulins, and on regular salt plus  $\gamma$ -globulins, as well as Dahl R rats on either high or regular salt diet for 4-5 weeks, ACE mRNA and activity in the hypothalamus and pons, as well as in the LV and RV were measured. ACE mRNA levels were determined by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (Lear et al., 1997), ACE activity was measured using a fluorometric method (Morrel et al., 1997). Ang I and II levels were assessed by RIA after HPLC (Ruzicka et al., 1995).

## **ARTICLE I**

# **High Salt Intake and the Brain Renin-Angiotensin System in Dahl Salt-Sensitive Rats<sup>1</sup>**

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**Running Title:** Brain ACE and Ang II in Dahl rats

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## Abstract

**Objective** To assess changes in the activity of the brain renin angiotensin system during (1) the development of salt-sensitive hypertension; and (2) the prevention of salt-sensitive hypertension by blocking brain “ouabain”.

**Methods** For protocol I, ACE mRNA and activity and angiotensin I and II (Ang I, II) levels were assessed in the hypothalamus and pons of Dahl salt-sensitive (Dahl S) and salt-resistant (Dahl R) rats on regular (120  $\mu\text{mol/g}$ ) or high (1370  $\mu\text{mol/g}$ ) salt diet from 4 to 6 or 4 to 9 weeks of age. For protocol II, ACE mRNA and activity were assessed in the hypothalamus and pons in Dahl S on high salt treated with icv Fab fragments blocking brain “ouabain” or  $\gamma$ -globulins, or on regular salt, and in Dahl R on high or regular salt. ACE mRNA was assessed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay and Ang I and II by RIA after HPLC.

**Results** High salt diet caused a gradual, but marked increase in blood pressure in Dahl S but not Dahl R rats. Dahl S rats showed small but significant increases in ACE mRNA in the hypothalamus on regular salt diet. In Dahl S rats on high salt diet for 2 or 5 weeks ACE mRNA levels significantly increased in both hypothalamus and pons, compared with Dahl R rats on either diet or Dahl S rats on regular salt diet. After 5 weeks of high salt diet ACE mRNA levels in the hypothalamus in Dahl S rats were almost three fold higher and in the pons two fold higher than in Dahl R rats on either diet or Dahl S on regular salt diet. High salt diet also increased ACE activity of the hypothalamus and pons in Dahl S but not Dahl R. Ang I levels in the hypothalamus and pons were similar in both groups of rats and there were no significant changes caused by high salt diet in Dahl S and R rats. On regular salt intake

Ang II levels in the hypothalamus of Dahl S rats showed a modest but significant decrease as compared with Dahl R rats on regular salt diet, and were similar in the pons of the two strains. High salt intake did not affect Ang II levels in either hypothalamus or pons in Dahl S and R rats. Chronic blockade of brain “ouabain” by icv Fab fragments prevented the increases in BP, ACE mRNA and activity in the hypothalamus and pons by high salt intake in Dahl S rats.

**Conclusions** These results indicate that high salt intake increases BP, ACE expression and activity in the hypothalamus and pons of Dahl S rats without a parallel increase in Ang II levels. Effects of high salt intake on ACE mRNA and activity appear to be secondary to activation of brain “ouabain”.

**Key Words:** hypertension, angiotensin converting enzyme, angiotensin, brain, Dahl rats

## Introduction

The renin angiotensin system (RAS) in the hypothalamus and brainstem is involved in the central regulation of blood pressure and body fluid homeostasis [1]. The brain RAS also contributes to the development and maintenance of certain forms of hypertension in particular salt-sensitive hypertension [2-4]. In spontaneously hypertensive rats (SHR), sympathetic hyperactivity and exaggerated hypertension in response to a high salt diet are prevented by chronic blockade of central AT<sub>1</sub> receptors with losartan [3]. In Dahl salt-sensitive (Dahl S) rats on high salt intake chronic icv treatment with losartan prevents sympathetic hyperactivity, impairment of baroreflexes as well as the development of hypertension [2]. In Dahl-Iwai salt sensitive rats chronic central infusion of CV-11974, another AT<sub>1</sub> receptor antagonist, also prevented the development of hypertension [4]. These results suggest that in these models the brain RAS is essential for sympathetic hyperactivity and the development of hypertension on high salt intake. However, whether the brain RAS is actually overactive in Dahl S rats, and if so, which component is involved, has so far not been evaluated. The sympatho-excitatory and hypertensive effects of high salt can also be prevented by blocking brain "ouabain" with antibody Fab fragments in both Dahl S rats [5] and SHR [6]. Studies in our laboratory [3, 7] suggest that high salt appears to increase brain "ouabain" first, and the latter subsequently activates the brain RAS. However, how brain "ouabain" activates the brain RAS, or which component of the RAS is activated, has so far not been studied.

In the present study, we therefore examined the effects (1) of high salt intake; and (2) of blockade of the effect of high salt by Fab fragments, on components of the RAS in the

hypothalamus and pons in Dahl S vs salt resistant (Dahl R) rats. These two brain regions were chosen because of their importance in cardiovascular regulation and the presence of functional AT<sub>1</sub> receptors. Angiotensin converting enzyme (ACE) is a dipeptidyl peptidase which is responsible for the production of the active octapeptide Ang II from the decapeptide Ang I. ACE is present ubiquitously both peripherally (e.g., the lung, heart, kidney, testis ) as well as centrally in the brain. In the periphery, high salt intake can increase ACE mRNA and activity in eg the heart [8]. In the present studies, we therefore assessed effects of high salt intake on ACE expression and activity in the brain and its relation to Ang I and Ang II levels.

## Methods

**General protocol** Male Dahl rats were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed two per cage on a 12-hour light-dark cycle at 24°C and allowed a 3-5 day acclimatization period on normal rat chow and tap water before entering the study. At 4 weeks of age, rats were randomly placed on either regular or high salt diet (rat chow containing 120 or 1370  $\mu\text{mol Na}^+$ /g food, Harlan Sprague Dawley Inc., Madison, WI). For protocol I, the diets were provided for 2 or 4-5 weeks. Two sets of animals were used, one set for ACE mRNA and activity assays, another for Ang I and II assays. For protocol II, the diets were provided for 4-5 weeks. 18 Dahl S rats were divided into 3 groups, 2 groups treated with high salt and either icv Fab fragments or  $\gamma$ -globulins, the third with regular salt plus  $\gamma$ -globulins; 12 Dahl R rats were divided into 2 groups and placed on high or regular salt. At 6 or 8-9 weeks of age, the rats were anesthetized with halothane-oxygen and polyethylene catheters (PE-50) were inserted into the left carotid artery. The following morning blood pressure and heart rate were recorded in conscious, unrestrained animals. For ACE mRNA and activity assays, rats were killed by decapitation; for angiotensin assays, rats were killed with 2 mol/L KCl (1 ml/rat). Then the brain was excised and frozen on dry ice immediately. The hypothalamus and pons were dissected according to the method from Paxinos and Watson [9].

Chronic treatment with antibody Fab fragments by intracerebroventricular (icv) cannulas was carried out as described previously [3]. Briefly, at 4 weeks of age, with rats under sodium pentobarbital anesthesia (65 mg/kg IP), a 23-gauge stainless steel cannula was implanted into the right lateral ventricle and fixed to the skull of the rats as a guide cannula

(0.4 mm posterior and 1.3 mm lateral to bregma and 3.5 mm from dura). The upper end was connected to an osmotic minipump ( model 2002, Alza Corp ) that was filled with either antibody Fab fragments ( Digibind, Glaxo Welcome Inc), or  $\gamma$ -globulins ( Sigma Chemical Co ) as a control ( 200  $\mu\text{g}/\text{d}$  for both ). At 6 weeks of age, with rats under halothane anaesthesia, the original pumps were replaced with new pumps with original compounds for icv infusion for another 2 weeks.

**ACE mRNA assay** Total RNA was extracted from the hypothalamus and pons by the guanidium thiocyanate method [10]. Total RNA (4  $\mu\text{g}$ ) of the hypothalamus or pons was reverse transcribed into cDNA in a 40  $\mu\text{l}$  reaction assay with 10 U each of avian myeloblastoma virus reverse transcriptase (AMV-RT, Promega-Fisher, Ottawa, Ontario), 0.4 mM of each deoxyribonucleotide, 4 mM DTT and 50 ng random primer in 1x AMV- RT buffer at 42°C for 120 min.

ACE mRNA levels were determined by quantitative RT-PCR assay using an internal standard as described previously [11] with some modifications. In short, a 934 bp fragment from ACE cDNA was co-amplified with an 810 bp ACE internal standard (obtained from a PCR reaction using a composite primer ACE3 with ACE2 [11]) in the same PCR reaction with the primers ACE1 and ACE2. The PCR reaction for ACE mRNA assay was performed in a total volume of 50  $\mu\text{l}$  containing the cDNA from 400 ng total RNA, 1x reaction buffer, 2.5 mM  $\text{MgSO}_4$ , 0.16  $\mu\text{M}$  of each primer, 0.25 mM of each deoxyribonucleotide, and 1 U Vent DNA polymerase (New England Biolabs, Boston, MA). The PCR profile consisted of an initial 1 min 94°C incubation period, followed by 36 cycles of 94°C 36 sec, 68.3 °C 36 sec, and 72°C 45 sec, then incubated at 72°C for 5 min for elongation. To control for the varying

reverse transcription efficiency, all samples were assayed for phosphoglycerate kinase-1 (PGK-1) mRNA by quantitative RT-PCR as described before [11]. For each sample, six different concentrations of ACE internal standard (100 to 3200 copies) and four different concentrations of the PGK-1 internal standard (2000 to 16000 copies) were used. The PCR products were detected with laser-scanning densitometry (Gel Doc 1000, BIO-RAD), and ACE mRNA levels were determined as described previously [11].

The primers for ACE mRNA assay were ACE1: 5'-AGT GAG GGC AGT GGC TAC GA-3'; ACE2: 5'-GGC AGA GTG GAT GGG AAC AG-3'; and ACE3: 5'-AGT GAG GGC AGT GGC TAC GAC AGG AGT GGT GGA GTC TCA G-3'. The primers for PGK-1 mRNA assay were: PB3: 5'-ACC ATC CAG CCA GCA GGT AT-3' and PB5: 5'-GTG AAG GGG AAG CGG GTC GT-3'.

***ACE activity assay*** ACE activity was measured by use of a fluorometric method described by Morrell [12] with some modifications. Tissue was homogenized in ice-cold 10-fold excess of Tris (50 mM, pH 7.4) buffered saline (150 mM NaCl). The homogenate was centrifuged at 1000g for 15 min at 4°C. An aliquot of the resulting supernatant was diluted 1:4. 5 µl of dilution were then incubated with 100 µl of 5 mM Hip-His-Leu substrate, 50 mM Tris, pH 7.5, 150 mM NaCl at 37°C for 60 min. Blank controls were performed in the same fashion except homogenate was not added. The reaction was terminated by addition of 750 µl 280 mM NaOH. 50 µl Phthalaldehyde (1% in methanol) was then added for 10 min. This step was stopped with 100 µl 2 N HCL. All samples were measured in duplicate including blank controls, and His-Leu standards were prepared for every assessment. The fluorescence of the samples was measured at an emission wavelength of 500 nm with an excitation wavelength

of 360 nm (LS 50B fluorescence spectrophotometer, Perkin-Elmer). The results were expressed as mU/g of tissue/min, where 1 mU represents the generation of 1 nMol His-Leu/min.

**Ang I and II assay** Ang I and II levels in the hypothalamus and pons were measured as previously described in detail [13]. Briefly, the hypothalamus and pons were boiled in 1 M acetic acid for 15 min, then homogenized for 25 sec and centrifuged for 15 min at 7000g. The supernatants were applied to Sep Pak C18 cartridges, and Ang I and II levels determined by radioimmunoassay after separation of Ang I and II by high-performance liquid chromatography [13]. Recovery of Ang I and II determined by spiking of the brain tissue was in the 85-90% range.

**Statistical analysis** Values are expressed as means  $\pm$ SEM. Differences between groups were evaluated by ANOVA. The Bonferroni test was used to locate significant differences for post-hoc analysis if applicable.  $P < 0.05$  was considered statistically significant.

## **Results**

### ***Blood pressure and heart rate***

In Dahl S rats on regular salt diet, blood pressure was mildly elevated at 6 wk of age, and significantly increased at 9 wk of age compared to Dahl R rats (Table 1). In Dahl R rats, high salt intake did not affect blood pressure. In contrast, in Dahl S rats on high salt diet, blood pressure was modestly increased after 2 wk of high salt intake, and severe hypertension had developed after 5 wk of high salt intake (Table 1). High salt intake caused minor increases in heart rate which were only significant in Dahl S rats (Table 1).

### ***ACE mRNA and activity in the hypothalamus and pons***

In Dahl S rats on regular salt diet, ACE mRNA and activity in the hypothalamus showed a small but significant increase as compared to Dahl R rats on the same salt diet at 9 weeks of age (Fig. 1). In Dahl R rats, ACE mRNA levels in both hypothalamus and pons were similar on regular or high salt intake. In contrast, high salt intake increased ACE mRNA in Dahl S rats in both the hypothalamus and pons, as well as enhanced ACE activity in the hypothalamus and pons of Dahl S but not Dahl R (Fig. 1 and 2). At 9 wk of age ACE mRNA levels had increased about three-fold in the hypothalamus, and two-fold in the pons as compared with Dahl R rats on high salt intake and Dahl S rats on regular salt diet (Figures 1 and 2). ACE activity in the hypothalamus of Dahl S increased about two-fold, and less but still significantly increased in the pons compared with Dahl R (Fig. 1).

### ***Ang I and II in the hypothalamus and pons***

In both the hypothalamus (Fig. 3) and pons (Table 2) of Dahl S and R rats, Ang I levels were similar and high salt intake did not cause significant changes. On regular salt

intake, however, in Dahl S rats Ang II in the hypothalamus was significantly lower than in Dahl R rats (Fig. 3). High salt intake did not cause significant changes in Ang II levels in the hypothalamus of Dahl S or Dahl R rats (Fig. 3). In the pons Ang II levels were similar in the two strains on both regular and high salt intake (Table 2).

***Blockade of brain “ouabain” with Fab fragments***

Chronic icv treatment with the antibody Fab fragments prevented the increase in BP in Dahl S rats on high salt compared with the group of Dahl S on high salt plus  $\gamma$ -globulins and Dahl S on regular salt intake ( Fig. 4 ). Interestingly, the treatment also prevented the increases in ACE mRNA and activity of the hypothalamus ( Fig. 4 ) and pons (Fig. 5) in Dahl S rats on high salt intake.

## Discussion

The present study provides the following primary findings. First, high salt intake causes marked increases in ACE mRNA and activity levels in both the hypothalamus and pons of Dahl S but not Dahl R rats. These changes parallel the changes of blood pressure in Dahl S vs R on high vs regular salt diets. Secondly, Ang II levels in the hypothalamus and pons do not increase with the enhanced ACE expression and activity in Dahl S rats on high salt intake. Thirdly, chronic blockade of brain "ouabain" prevents the increase in BP as well as the increases in ACE mRNA and activity in both the hypothalamus and pons of Dahl S rats.

High salt intake causes sympathetic hyperactivity, impairs baroreflexes and exaggerates the development of hypertension in rat strains genetically predisposed to hypertension, such as SHR [6,14] and Dahl S [5]. The brain RAS appears to play a major role, since central blockade by AT<sub>1</sub> receptor antagonists abolishes sympathetic hyperactivity in both models, hypertension in Dahl S rats, and the exaggerated hypertensive response in SHR to high salt [2-4]. Very little is known about the biochemical components of the brain RAS in Dahl S rats during the development of hypertension. ACE is widely distributed in the brain, and high concentrations are found in the forebrain circumventricular organs and paraventricular hypothalamic nucleus [15-17]. ACE is not only an important enzyme for Ang II formation but is also associated with other functions such as degradation of bradykinin and other peptides. The present study shows that ACE mRNA and activity in the hypothalamus of Dahl S rats on regular salt at 9 weeks of age are significantly higher than Dahl R rats on the same diet. Moreover, in Dahl S rats on high salt, ACE mRNA levels as well as ACE activity significantly increased in the hypothalamus and pons as compared with

Dahl S rats on regular salt as well as Dahl R rats on either salt diet. These results are consistent with a study by Mizuno et al. [18] showing that ACE activity is significantly higher in the hypothalamus of SHR and high salt intake increases ACE activity in the midbrain.

In contrast to ACE mRNA and activity, Ang II levels in the hypothalamus of Dahl S rats on regular salt diet were lower than in Dahl R rats and did not increase in Dahl S on high salt intake despite the large increases of ACE expression and activity. Several explanations are possible for this apparent dissociation of ACE activity and Ang II in Dahl S rats. First, the absence of an increase in "steady state" Ang II levels could be caused by increased Ang II turnover by metabolism or cellular internalization after binding to its receptor. Alternatively, Ang II levels may only increase in some nuclei/regions and not in the whole hypothalamus or pons. Secondly, a decrease in the production of the substrate for ACE, angiotensin I, (by e.g. a decrease in renin activity or angiotensinogen levels) could also cause Ang II levels to remain constant despite an increase in ACE activity. However, this is rather unlikely, since Ang I levels were not affected by high salt intake. Thirdly, it is possible that increases in brain ACE mRNA and ACE activity produced by high salt intake are not functionally related to the brain RAS, but to other systems such as bradykinin which may also be involved in the development of hypertension [20]. Further biochemical/molecular biological characterization of the brain RAS is required to identify the reason(s) for the apparent dissociation between ACE and Ang II levels.

Several explanations are possible for the apparent dissociation between the dependence of the hypertension on the brain RAS as indicated by the effectiveness of icv

losartan [2] and the absence of an increase in Ang II levels in the whole hypothalamus or pons. First, increased Ang II turnover by increased Ang II-AT<sub>1</sub> receptor internalization may leave steady-state levels of Ang II unchanged and mask enhanced AT<sub>1</sub>-receptor stimulation. Secondly, increases in Ang II in some specific nuclei may not be reflected in levels in the whole hypothalamus or pons. Thirdly, high salt intake leads to an increase in AT<sub>1</sub> receptor numbers in hypothalamic nuclei such as the PVN and SON [19], and this may increase the binding of Ang II which leads to enhanced functional responses as well as a decrease in steady state of Ang II.

Besides the brain RAS, the sympathoexcitatory and hypertensive effects of high salt in Dahl S rats and SHR are also mediated by an increase in brain “ouabain” [5,6]. Chronic blockade of brain “ouabain” with icv Fab fragments prevents salt-induced sympathetic hyperactivity and hypertension in a similar pattern as blockade of the brain RAS by icv losartan [3]. Blockade of brain “ouabain” does not blunt excitatory responses of blood pressure, heart rate and renal sympathetic nerve activity to acute icv injection of Ang II, whereas blockade of brain AT<sub>1</sub>-receptors by losartan blocks responses to both ouabain and Ang II icv [3]. These findings suggest that the two systems interact and are involved in the same pathways mediating the sympathoexcitatory and pressor effects of high salt. It appears that high salt activates brain “ouabain”, then the brain “ouabain” activates the brain RAS. It is unknown, however, how the two systems interact with each other, or which components of the brain RAS are activated by brain “ouabain”. The present study demonstrates that the increases in ACE expression and activity in the hypothalamus and pons of Dahl S rats on high salt intake are prevented by chronic blockade of brain “ouabain” with antibody Fab

fragments. These results suggest that activation of the brain RAS, as reflected in the increases in ACE expression and activity is indeed secondary to increased brain "ouabain". It is conceivable that brain "ouabain" inhibits  $\text{Na}^+$  pumps and increases intracellular  $\text{Ca}^{++}$  leading to increased ACE expression and activity.

In summary, the present study demonstrates that high salt intake substantially activates ACE gene expression and activity in the hypothalamus and pons in Dahl S but not Dahl R rats and this activation correlates with the development of hypertension. However high salt intake does not affect steady state Ang II levels in the whole hypothalamus and pons of Dahl rats. The increases in ACE expression and activity in the hypothalamus and pons of Dahl S rats can be prevented by blockade of brain "ouabain", suggesting that high salt intake increases ACE expression and activity through pathways involving brain "ouabain".

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## Legends for the Figures

**Fig. 1.** Top: agarose gel electrophoresis of ACE RT-PCR using 1920 to 60 copies (lane 2 to lane 7) of 810 bp internal standard (low band). The upper band is the 934 bp PCR product of ACE cDNA. Lane 1:  $\lambda$  Hind III marker. Middle and bottom: effects of high salt intake for 2 or 5 weeks on ACE mRNA and activity in the hypothalamus of Dahl S and R rats. Values are expressed as means $\pm$ SEM (n=6 per group). ACE mRNA levels were standardized by PGK-1 mRNA level.

<sup>b</sup> $p < 0.05$  vs. same strain on control diet; \*  $p < 0.05$  vs. Dahl R on same diet.

**Fig. 2.** Effects of high salt intake for 2 or 5 weeks on ACE mRNA and activity in the pons of Dahl S and R rats. Values are expressed as means $\pm$ SEM (n=6 per group). ACE mRNA levels were standardized by PGK-1 mRNA level.

<sup>b</sup> $p < 0.05$  vs. same strain on control diet; \*  $p < 0.05$  vs. Dahl R on same diet.

**Fig. 3.** Effects of high salt diet for 2 or 5 weeks on Ang I and Ang II levels in the hypothalamus of Dahl S and Dahl R rats. Results are expressed as means  $\pm$  SEM (n=6 per group). \*  $p < 0.05$  vs. Dahl R on the same diet.

**Fig. 4.** Effects of chronic blockade of brain “ouabain” by icv Fab fragments on blood pressure and hypothalamus ACE mRNA and activity in Dahl S and R rats. Results are expressed by mean $\pm$ SEM (n=6 per group).

@  $p < 0.05$  vs. same strain on high salt plus Fab fragments or control diet; \*  $p < 0.05$  vs. Dahl R on the same diet.

**Fig. 5.** Effects of chronic blockade of brain “ouabain” by icv Fab fragments on the pons ACE mRNA and activity in Dahl S and R rats. Results are expressed by mean $\pm$ SEM (n=6 per group).

@  $p < 0.05$  vs. same strain on high salt plus Fab fragments or control diet; \*  $p < 0.05$  vs. Dahl R on the same diet.

**Table 1. Effects of high salt intake for 2 or 5 weeks on blood pressure and heart rate in Dahl S vs R rats**

Strain/Diet	2 weeks of high salt diet	5 weeks of high salt diet
	<i>Mean Arterial pressure, mmHg</i>	
Dahl R		
Control	106±5	110±5
High	104±4	107±4
Dahl S		
Control	118±5	128±5 <sup>*</sup>
High	136±6 <sup>*</sup>	199±8 <sup>a</sup>
	<i>Heart rate, beats/min</i>	
Dahl R		
Control	416±20	379±19
High	446±21	386±18
Dahl S		
Control	406±19	390±20
High	454±22 <sup>b</sup>	442±21 <sup>*b</sup>

Values represent means±SE, n=5 per group. <sup>a</sup>  $p < 0.01$  vs. same strain on control diet; <sup>b</sup>  $p < 0.05$  vs. same strain on control diet; \*  $p < 0.05$  vs. Dahl R on same diet.

Control: 120  $\mu\text{mol Na}^+/\text{g}$  food.

High: 1370  $\mu\text{mol Na}^+/\text{g}$  food. The diet was provided from 4 wk of age of rats.

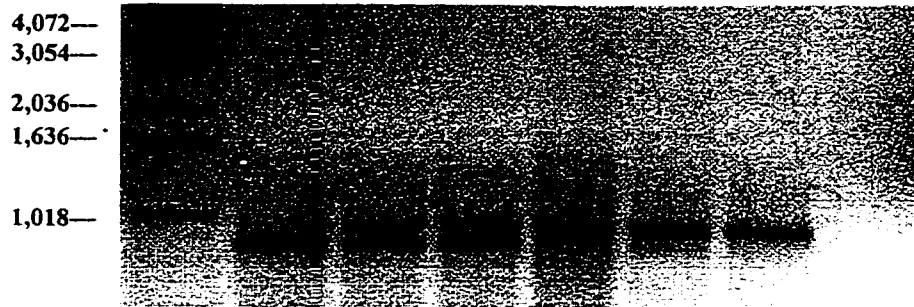
**Table 2. Effects of high salt intake for 2 or 5 weeks on Ang I and II levels in the pons of Dahl S and R rats**

Strain /diet	2 weeks of high salt diet		5 weeks of high salt diet	
	<i>Ang I and II (pg/g)</i>			
	Ang I	Ang II	Ang I	Ang II
Dahl R				
Control	51±9	12±2	46±15	18±4
High	47±11	18±3	43±8	11±3
Dahl S				
Control	31±9	19±5	39±10	13±5
High	47±10	14±5	46±15	17±3

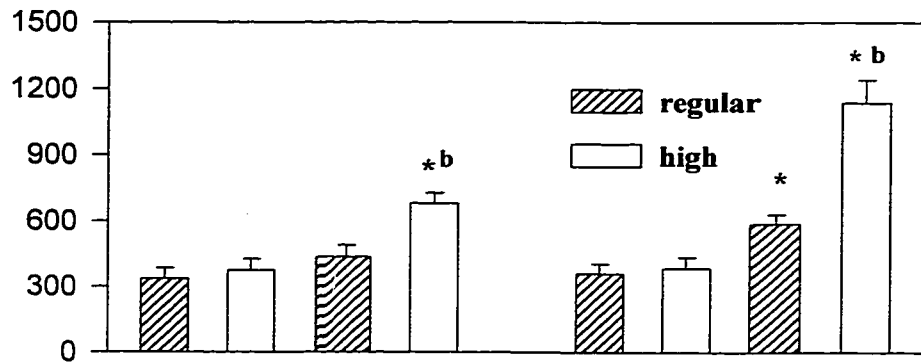
Values are expressed as mean ±SEM; n=5-6 per group. Control: 120 μmol Na<sup>+</sup>/g food; High: 1370 μmol Na<sup>+</sup>/g food.

**Fig. 1 (Fig 1 in Article I)** Effects of high salt intake on ACE mRNA and activity in the hypothalamus of Dahl S and R rats

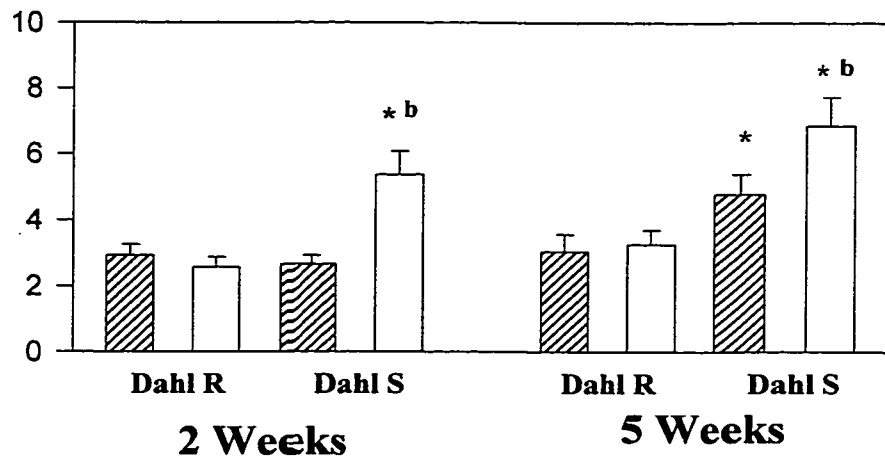
**Quantitative RT-PCR for ACE mRNA**



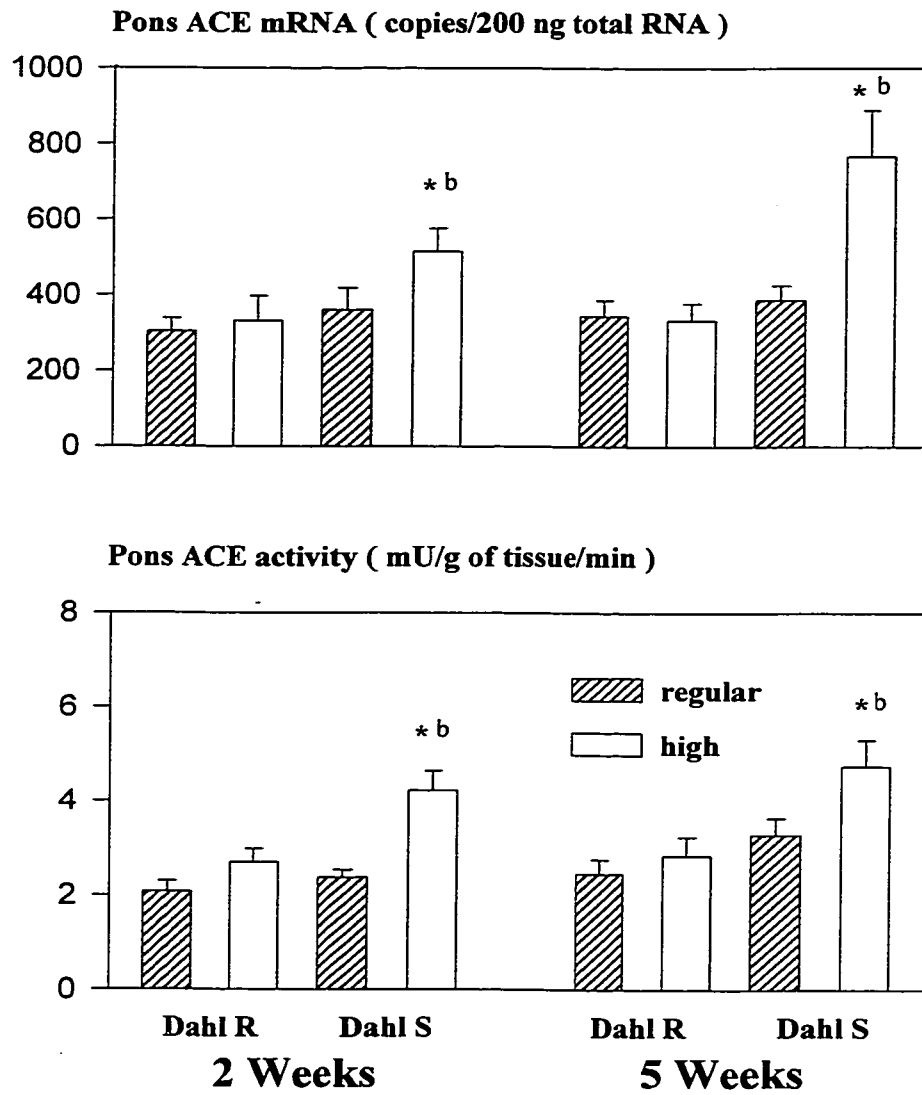
**Hypothalamus ACE mRNA (copies/200 ng total RNA)**



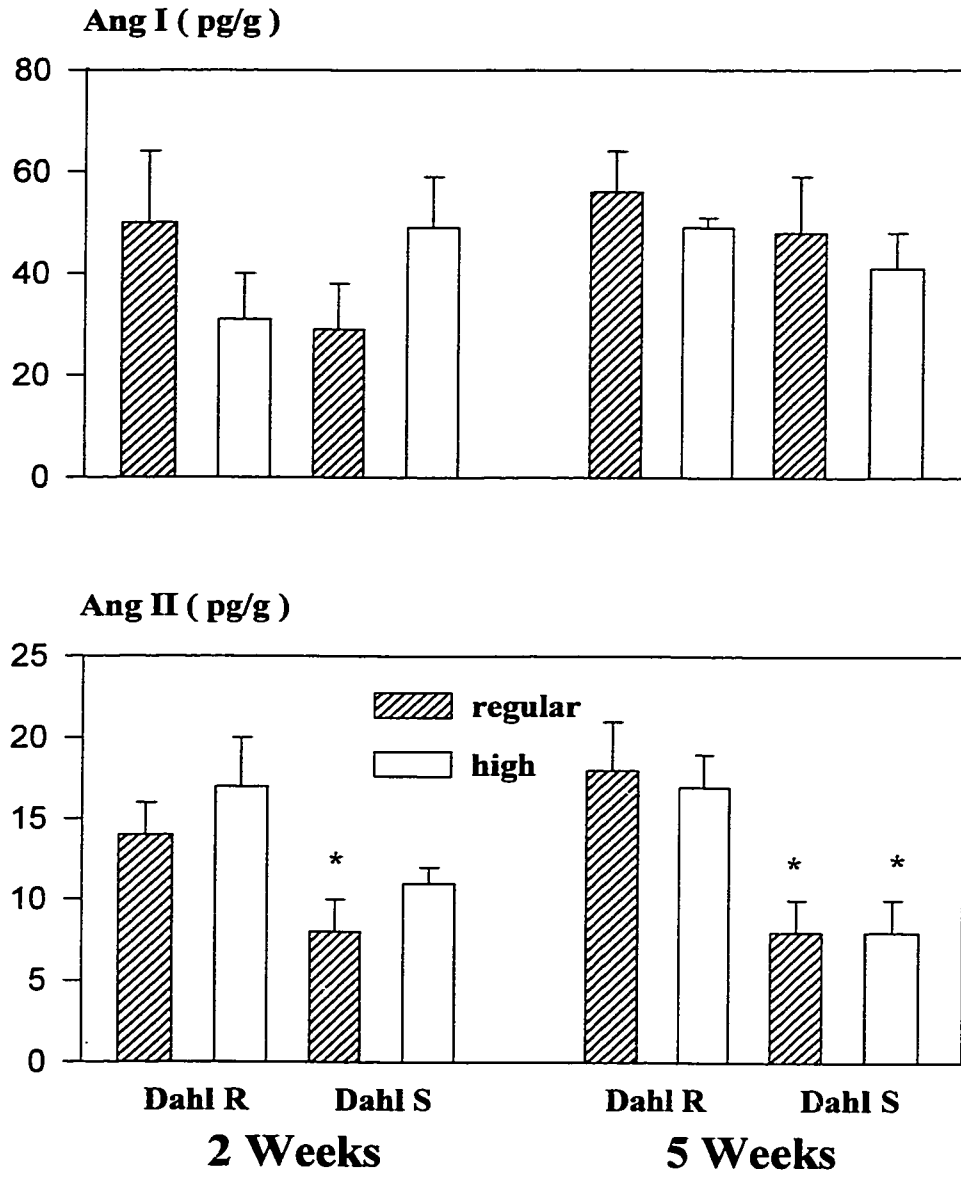
**Hypothalamus ACE activity (mU/g of tissue/min)**



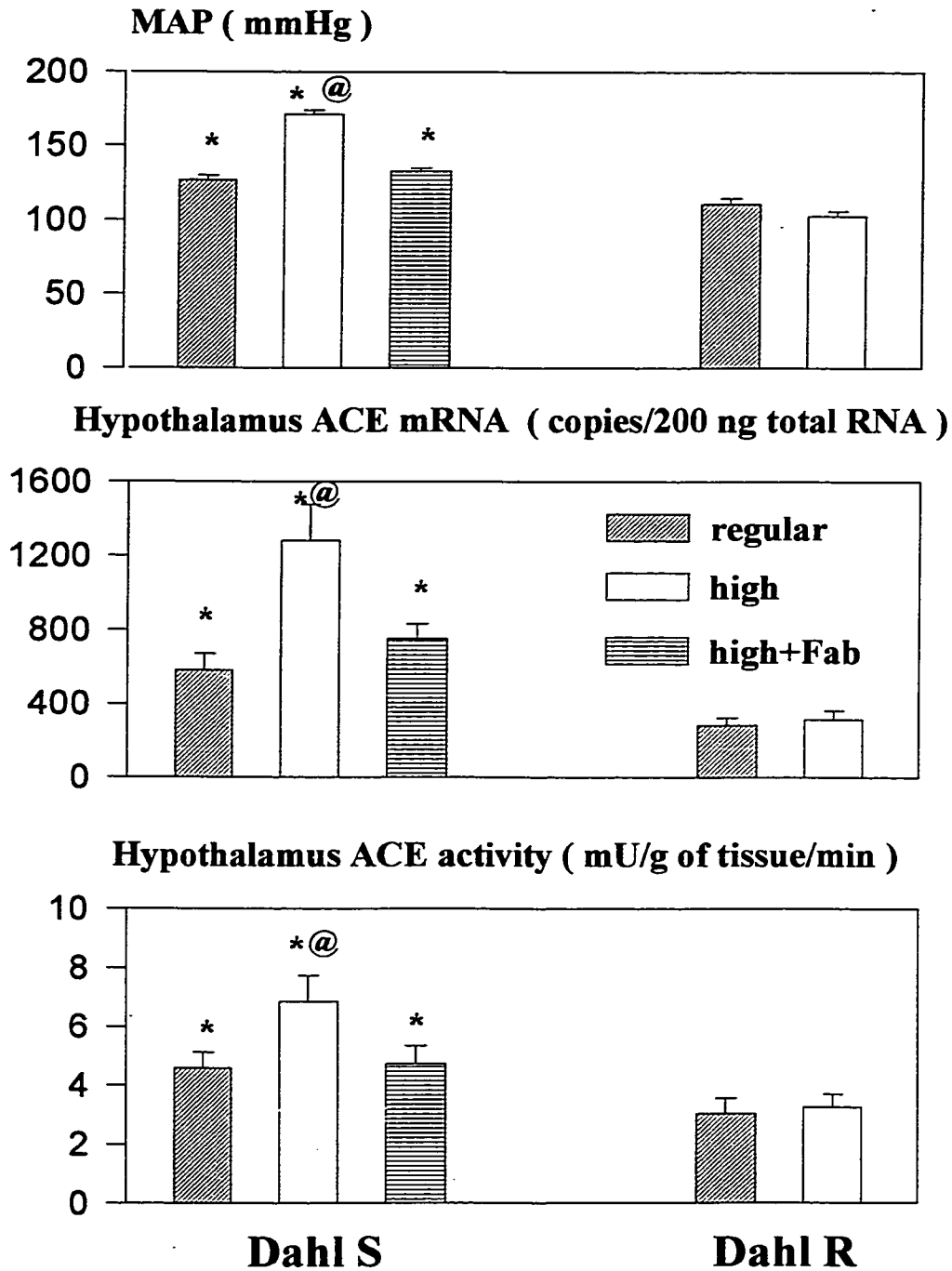
**Fig. 2. (Fig. 2 in Article I) Effects of high salt intake on ACE mRNA and activity in the pons of Dahl S and R rats**



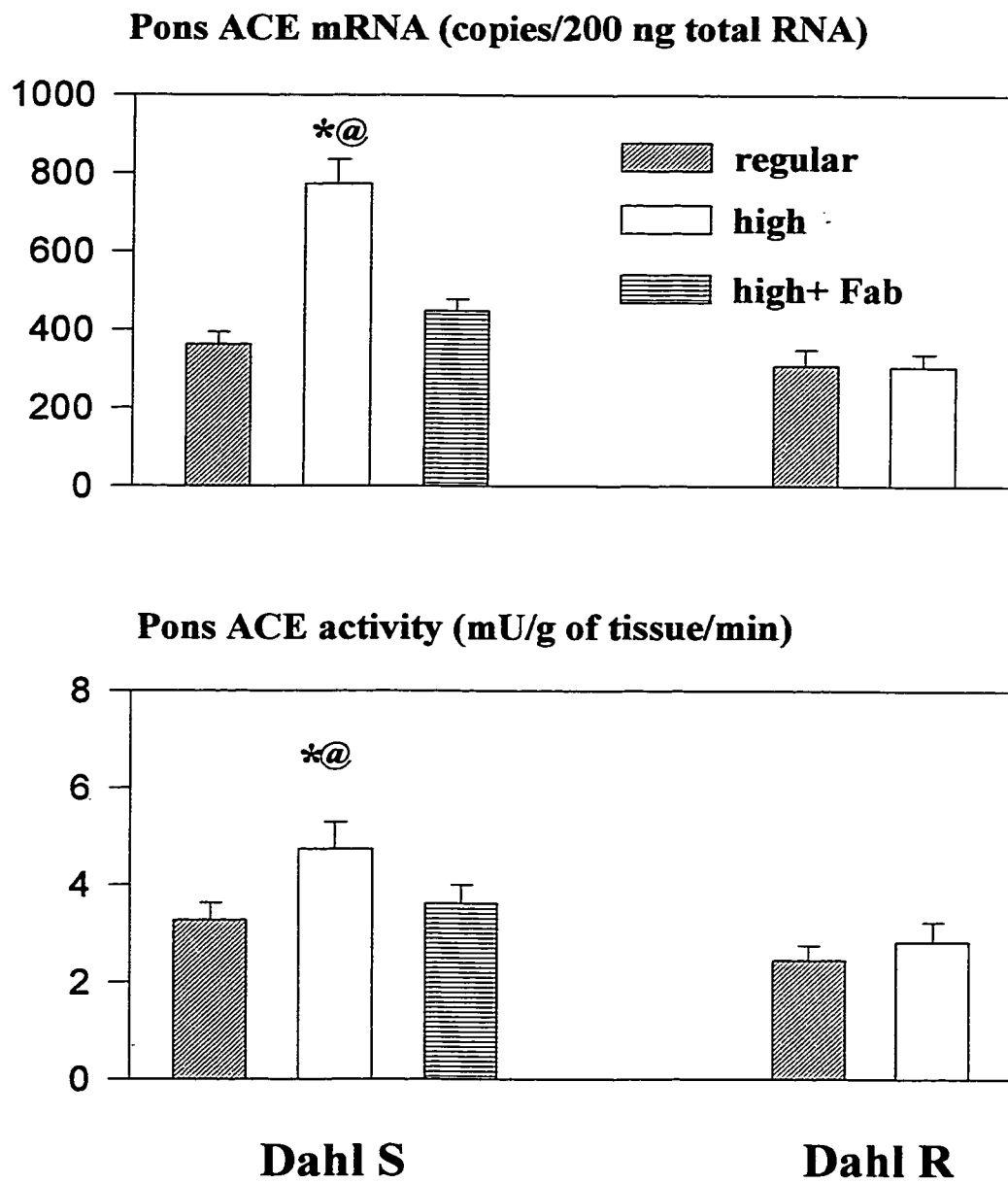
**Fig. 3 (Fig. 3 in Article I)** Effects of high salt intake on Ang I and II in the hypothalamus of Dahl S and R rats



**Fig. 4 (Fig 4 in Article I)** Effects of blockade of brain "ouabain" on blood pressure, hypothalamus ACE mRNA and activity of Dahl S and R rats



**Fig. 5. (Fig. 5 in Article I)** Effects of blockade of brain "ouabain" on ACE mRNA and activity in the pons of Dahl S and R rats



## **ARTICLE II**

### **Cardiac Hypertrophy and Cardiac RAS in Dahl Rats on High Salt<sup>1</sup>**

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**Running Title:** Cardiac hypertrophy and RAS in Dahl rats

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## Abstract

On high salt intake, Dahl salt-sensitive rats develop cardiac hypertrophy disproportionate to the degree of hypertension. In the present studies, we assessed whether the cardiac hypertrophy induced by high salt depends on the development of hypertension, and leads to overactivity of the cardiac renin angiotensin system (RAS). Cardiac ACE mRNA and activity, cardiac and plasma angiotensin I and II (Ang I, II), as well as plasma renin activity (PRA) were assessed in Dahl salt-sensitive (Dahl S) and salt-resistant (Dahl R) rats on high (1370  $\mu\text{mol/g}$ ) or regular salt (120  $\mu\text{mol/g}$ ) diet for 2 to 5 weeks. In Dahl S rats ACE mRNA and activity of the LV increased markedly after 4-5 wk of high salt diet compared with Dahl S on control diet and Dahl R on either diet. Chronic icv treatment with Fab fragments blocking brain "ouabain" prevented the hypertension by high salt in Dahl S rats but did not affect the increases in LV weight or in LV ACE mRNA and activity. On regular salt diet, Dahl S rats demonstrated significantly lower cardiac Ang I and Ang II than Dahl R rats. High salt intake did not cause significant changes in cardiac Ang I and II in either strain. On regular salt diet, PRA, plasma Ang I and II were also significantly lower in Dahl S vs R. High salt intake lowered PRA in Dahl R to the low levels in Dahl S. Plasma Ang I showed a modest decrease by high salt in Dahl R, but plasma Ang II did not decrease. In Dahl S rats, high salt did not cause further decreases of the already low PRA, plasma Ang I and II. These data indicate a low activity of both circulatory and cardiac RAS in Dahl S versus R rats. The cardiac hypertrophy induced by high salt in Dahl S appears not to depend on the increase in BP, and is associated with a marked increase in cardiac ACE mRNA and activity but not in cardiac Ang II, suggesting that the increase in ACE mRNA and activity

may be relevant for non-angiotensinergic mechanisms.

**Key Words:** salt-sensitive hypertension, angiotensin converting enzyme, angiotensin, heart,

Dahl rat

## Introduction

Whereas the role of high salt intake in exaggerating the development of hypertension has been extensively demonstrated, in recent years it has become apparent that high salt intake is also a major determinant of the extent of left ventricular hypertrophy (LVH) independent of blood pressure in humans<sup>1</sup> as well as rats.<sup>2-4</sup> In normotensive Wistar rats high salt intake induces cardiac hypertrophy without increases in blood pressure or sympathetic activity.<sup>2,4</sup> In Dahl salt-sensitive (S) rats high salt intake causes cardiac hypertrophy disproportionate to the level of hypertension,<sup>5</sup> whereas in Dahl salt-resistant (R) rats high salt intake does not increase cardiac weight. It appears that salt-sensitivity or salt-resistance may apply both to blood pressure and to heart structure, and that the regulation of heart structure may be independent of hemodynamic changes under certain circumstances. In Dahl S rats, high salt intake causes hypertension by an increase in sympathetic activity. These effects of high salt can be reversed by blockade of brain "ouabain" with icv antibody Fab fragments,<sup>6</sup> which bind endogenous ouabain-like compound(s) ("ouabain") with high affinity. Whether the cardiac hypertrophy of Dahl S rats induced by high salt intake can be prevented by preventing the development of hypertension, or is at least partly independent of the increase in blood pressure, has not yet been assessed.

Schmieder et al suggested that impaired suppression by high salt intake of the renin-angiotensin system (RAS) acts as a stimulus for myocardial hypertrophy in hypertensive patients.<sup>7</sup> The cardiac RAS has been linked to enhanced cardiac growth both in vitro and in vivo.<sup>8,9</sup> Components of the cardiac RAS, including renin, angiotensinogen, angiotensin converting enzyme (ACE), and angiotensin AT<sub>1</sub> receptors, have been found upregulated in

different models of pressure and volume overload-induced hypertrophy and heart failure. Cardiac ACE mRNA and activity have consistently been found to be enhanced in different models of pressure-overload induced LVH<sup>10,11</sup> as well as volume overload-induced ventricular hypertrophy.<sup>12</sup> Studies on the effects of high salt intake on the activity of the cardiac RAS are limited. Recently we reported that in WKY rats and SHR high salt intake for 2 weeks did not affect cardiac Ang II.<sup>3</sup>

The objectives of the present study were to assess whether 1) high salt intake causes cardiac hypertrophy at least in part independent of the hypertension by chronic blockade of brain “ouabain” with Fab fragments; and 2) high salt intake suppresses the activity of the circulatory RAS but may be associated with hyperactivity of the cardiac RAS. For this, parameters of the circulatory and cardiac RAS were evaluated in Dahl S and Dahl R rats after 2 to 5 weeks on high or regular salt intake. Specifically, responses of cardiac ACE mRNA and activity, and Ang I and II, and of plasma renin and Ang I and II to a high salt diet were assessed.

## Methods

**Preparation of animals** Male Dahl S and R rats (Harlan Sprague Dawley, Indianapolis, IN) were housed two per cage on a 12:12-h light-dark cycle at 24°C with standard laboratory food and tap water ad libitum for 3-5 days before entering the study. At 4 weeks of age rats were randomly placed on regular or high salt diet (120 or 1370  $\mu\text{mol Na}^+/\text{g}$  food, Harlan Sprague Dawley Inc., Madison, WI). In protocol I, two sets of each 24 Dahl S and 24 Dahl R rats were randomized to receive high vs regular salt diets from 4 to 6 or 4 to 9 wk of age. One set was used for cardiac ACE mRNA and activity measurement, another for PRA, Ang I and II assays. In protocol II, 18 Dahl S rats were divided into 3 groups, 2 groups on high salt diet with concomitant administration of either Fab fragments or  $\gamma$ -globulins, the third group on regular salt diet and  $\gamma$ -globulins. In parallel, 12 Dahl R rats were placed on high or regular salt diet. The diets were provided from 4 to 8 wk of age. At the end of study, cardiac ACE mRNA and activity were assessed.

For chronic administration of antibody Fab fragments, intracerebroventricular (icv) cannulas were placed at 4 weeks of age, as described in details previously.<sup>13</sup> The upper end of the icv cannula was connected to an osmotic minipump (model 2002, Alza Corp) that was filled with either antibody Fab fragments (Digibind, Glaxo Welcome Inc), or  $\gamma$ -globulins (Sigma Chemical Co) as a control (200  $\mu\text{g}/\text{d}$  for both). At 6 weeks of age, under halothane anaesthesia the original pumps were replaced with new pumps with original compounds for icv infusion for another 2 weeks.

At the end of each protocol, rats were anaesthetized and instrumented with left carotid artery catheters (PE50). After a 20 hr recovery blood pressure and heart rate were

recorded in conscious, unrestrained animals. For ACE mRNA and activity assays rats were then killed by decapitation, the heart rapidly excised and the LV and the right ventricle (RV) were dissected. For PRA, Ang I and II assays, 1-1.5 ml of blood was collected from the arterial line after blood pressure was recorded and rats were then killed with 2 mol/L KCl (1 ml/rat). The heart was removed immediately for Ang I and II assay.

**Cardiac ACE mRNA assay** Total RNA from the ventricles was isolated by the guanidium thiocyanate method. 4 µg of total RNA of each sample were reverse transcribed into cDNA with 10 U each of avian myeloblastoma virus (AMV) reverse transcriptase (AMV-RT, Promega-Fisher, Ottawa, Ontario) in a 40 µl reaction volume containing 0.4 mM of each deoxyribonucleotide, 4 mM DTT and 50 ng random primer in 1xAMV RT buffer at 42°C for 120 min.

Cardiac ACE mRNA levels were measured by using the quantitative RT-PCR method with internal standard described in detail previously<sup>12</sup> with some modifications. Briefly, a 934 bp fragment of ACE cDNA from reverse transcription reaction was co-amplified with an 810 bp ACE internal standard in a standard PCR reaction with primers ACE1 and ACE2. The PCR reaction was performed in a total volume of 50 µl containing the cDNA from 400 ng total RNA, 1 x reaction buffer, 2.5 mM MgSO<sub>4</sub>, 0.16 µM of each primer, 0.25 mM of each deoxyribonucleotide, and 1 U Vent DNA polymerase (New England Biolabs, Boston, MA). The PCR conditions were denaturation at 94°C 1 min, 36 cycles of 94°C 36 sec, 68.5°C 36 sec and 72°C 45 sec, followed by incubation at 72°C for 5 min. To control varying reverse transcription efficiency all samples were also assayed for phosphoglycerate kinase-1 (PGK-1) mRNA levels. PGK-1 mRNA levels were also measured by quantitative RT-PCR using PGK-

1 internal standard as previously described.<sup>12</sup> For each sample, six different concentrations of ACE internal standard (100 to 3200 copies) were used to measure ACE mRNA level, and four different concentrations of PGK-1 internal standard (2000 to 16000 copies) for PGK-1 mRNA assay. The mRNA level was determined as described before.<sup>12</sup>

**Cardiac ACE activity assay** Cardiac ACE activity was measured using a modified fluorometric assay according to Morrel et al.<sup>14</sup> Tissues were homogenized at 4°C in Tris (0.05 M, pH 7.4) buffered saline (0.15 M NaCl; 10ml / gl), then centrifuged at 1000 g for 15 min at 4°C. An aliquot of the resulting supernatant was diluted 1:4, 5 µl of each sample were added to 100 µl of 5 mM Hip-His-Leu substrate, 50 mM Tris, pH 7.5, 150 mM NaCl and incubated at 37°C for 60 min. Then 750 µl of 0.28 N NaOH were added to stop the reaction. Following addition of 50 µl of 1% O-Phthalaldehyde (1 mg in 100 µl methanol) for 10 min, the reaction was stopped with 100 µl 2N HCl. All samples were measured in duplicate including blanks (consisting of all reagents except homogenate). Standard solutions were prepared for every measurement. The fluorescence at 500 nm was measured using an excitation wavelength of 360 nm with a fluorescence spectrometer (Perkin-Elmer LS 50B).

**Plasma and Cardiac Ang I and II and PRA assays** Ang I and II levels were measured as previously described in details.<sup>15</sup> Briefly, the ventricles were boiled in 1M acetic acid for 15 min, then homogenized for 25 sec and centrifuged at 7000 g for 15 min. The supernatants were applied to Sep Pak C18 cartridges, and Ang I and II levels were determined by radioimmunoassay after separation of Ang I and II by high-performance liquid chromatography. Blood samples were centrifuged at 3000 g for 5 min, and plasma was removed for assessment of Ang I and II. PRA was assessed by an antibody-trapping

technique.

***Statistical analysis*** All data were expressed as mean $\pm$ SEM. Differences between diets and strains were evaluated by ANOVA and Bonferroni post-hoc test. Significance was accepted for  $p < 0.05$ .

## **Results**

### ***Blood pressure and cardiac weight***

In Dahl R rats, high salt intake did not affect blood pressure or LV weight ( Table 1). In Dahl S rats, high salt diet caused a gradual and progressive increase in BP and a marked increase in LV weight. High salt intake caused a minor increase in RV weight, only significant in Dahl S at 9 wk of age. In Dahl R rats high salt intake did not significantly affect body weight whereas in Dahl S rats after 5 wk of high salt diet body weight was significantly lower than control.

### ***ACE mRNA and activity in the left and right ventricles***

In Dahl R rats high salt intake did not affect ACE mRNA levels or ACE activity in the left ventricle ( Fig. 1). High salt intake caused a 2-fold increase in ACE mRNA in Dahl S after 2 weeks, and a 4-fold increase after 4-5 weeks. In Dahl S rats high salt intake also progressively increased ACE activity of the LV over time (Fig. 1).

In the right ventricle, ACE mRNA levels were similar in both strains on regular salt diet and high salt intake did not significantly increase ACE mRNA in either strain (Table 2).

### ***Plasma and Cardiac Ang I and II, and plasma renin activity***

On regular salt diet, PRA was significantly higher in Dahl R versus S rats. High salt intake caused a marked decrease in PRA in Dahl R, but no change in Dahl S rats after both 2 and 4-5 weeks (Fig. 2). On regular salt intake, plasma Ang I and II were markedly higher in Dahl R vs S rats with rather low levels in Dahl S rats. High salt intake caused a modest decrease in plasma Ang I in Dahl R with no effect on the low levels in Dahl S. High salt did not change plasma Ang II significantly in either strain (Fig.2)

On regular salt intake, cardiac Ang I and II were significantly higher in Dahl R versus S rats. High salt intake did not change cardiac Ang I and II in Dahl R or Dahl S rats (Fig. 3).

***Blockade of brain "ouabain"***

Chronic icv treatment with Fab fragments prevented an increase in blood pressure in Dahl S rats on high salt intake as compared with the group of Dahl S on high salt intake plus  $\gamma$ -globulins and Dahl S on regular salt intake (Fig. 4 ). In contrast, the increase in LV weight was not prevented by chronic treatment with the Fab fragments (Fig.4), nor were the increases in ACE mRNA and activity (Fig. 5 ). In Dahl S rats on high salt with Fab fragment treatment, LV weight was close to that of Dahl S on high salt with  $\gamma$ -globulins, and significantly higher than that in Dahl S on regular salt diet. Similar increases in ACE mRNA and activity were observed in Dahl S on high salt with Fab fragments or  $\gamma$ -globulins, as compared to Dahl S on regular salt diet (Fig.5 ). Body weight was not affected by the treatment with Fab fragments (data not shown).

## Discussion

The present study provides several new findings regarding the development of high salt-induced LVH and the cardiac RAS in Dahl S rats. First, high salt intake markedly increases ACE mRNA and activity in the hypertrophied LV of Dahl S rats. Second, chronic blockade of brain “ouabain” with Fab fragments prevents hypertension but not the LVH nor the increases in LV ACE mRNA and activity in Dahl S rats on high salt. Thirdly, on regular salt diet, cardiac Ang I and Ang II contents are markedly lower in Dahl S rats as compared to Dahl R rats, and high salt does not affect cardiac Ang I or II in either strain.

Increases in cardiac ACE mRNA and activity have consistently been reported to be associated with pressure overload induced cardiac hypertrophy, irrespective of the model.<sup>10,11,16</sup> In hypoxia-induced RV hypertrophy, ACE expression and activity markedly increased in the hypertrophied RV but decreased in the non-hypertrophied LV, suggestive of differential effects of hypoxia on RV versus LV ACE expression and activity.<sup>14</sup> In volume overload-induced cardiac hypertrophy due to aortocaval shunt ACE mRNA increased in both the LV and RV.<sup>12</sup> In this model, increases at the mRNA level (i.e. for renin and ACE) were associated with increased activity at the protein level (i.e. renin activity and Ang II).<sup>15</sup>

The present study is the first to demonstrate that ACE mRNA and activity increase markedly in the hypertrophied left ventricle of Dahl S on high salt intake. A 2-fold increase in ACE mRNA level of the LV after 2 weeks and a 4-fold increase after 4-5 weeks on high salt diet were found. ACE activity of the LV in Dahl S on 2 week high salt was more than 2-fold higher after 2 weeks of high salt and increased further after 4-5 weeks on high salt diet. These results indicate that high salt intake increases ACE mRNA and activity of the LV in

Dahl S rats but not Dahl R rats. To evaluate the functional role of changes in ACE expression and activity, both plasma and cardiac Ang I and II were evaluated. On regular salt intake, in Dahl S rats both Ang I and II in the heart were lower than in Dahl R rats. However, high salt intake did not affect cardiac Ang I or II levels in either strain.

On regular salt diet, PRA, plasma Ang I and II were also significantly lower in Dahl S vs R rats. High salt intake reduced PRA and plasma Ang I but not Ang II in Dahl R rats. In Dahl S rats, high salt intake did not cause further decreases of the already low PRA, Ang I and II. To the best of our knowledge, no studies have so far assessed circulatory or cardiac Ang I and II in Dahl rats on regular or high salt intake. The present study provides direct evidence of low activity of the RAS in Dahl S vs R, as assessed by the biologically most relevant parameter, Ang II, both in the circulation and in the heart on both regular and high salt intake.

There are several possible explanations for the distinct discrepancy between the increase in ACE expression and activity, and the persistent low Ang II levels in the heart of Dahl S rats on high salt intake. The most likely explanation is that the formation of Ang II is not only determined by ACE but also by other components of the RAS such as angiotensinogen and renin. Cardiac Ang I levels were very low in Dahl S vs R on both regular and high salt intake. This suggests that although increased ACE mRNA was associated with increased ACE enzymatic activity, at these low Ang I levels ACE is not rate-limiting and therefore an increase in enzymatic activity does not lead to increased Ang II formation. Why ACE gene expression then would increase is another relevant issue. Since ACE gene expression increases in all models of cardiac hypertrophy (as far as studied), this

gene appears to be part of the genetic program activated during the development of cardiac hypertrophy. Rather than contributing to enhanced activity of the cardiac RAS, increased ACE expression and activity might functionally contribute through other systems such as the breakdown of bradykinin or other peptides to mediate the formation of cardiac hypertrophy.

Previous studies suggested that salt-sensitivity may apply both to blood pressure and to heart structure, and cardiac hypertrophy induced by high salt intake can be independent of an increase in blood pressure.<sup>23</sup> Young WKY rats showed a graded hypertrophic response to 2 and 8% salt intake without an increase in blood pressure. In contrast, young SHR did not respond to 2% salt diet and the increase in LV weight caused by 8% salt diet was less than that induced in WKY rats, despite the clear further increase in blood pressure in SHR.<sup>3</sup> Yu et al.<sup>17</sup> recently showed that in both SHR and WKY 8% salt intake leads to similar LVH and widespread cardiac fibrosis although the increase in BP of WKY rats was minor compared to SHR. In normotensive strains of rats, high salt intake caused the most LVH in WKY, but had less effect on the heart of Wistar rats, and no effect in Dahl R rats at all. In hypertensive strains of rats, at similar blood pressure levels Dahl S rats exhibited more LVH than SHR. This difference was associated with a greater constitutive nitric oxide synthase activity of the LV in SHR which might inhibit the cardiac growth response.<sup>18</sup> The present study shows that in Dahl S rats, high salt intake causes clear hypertension and induces a marked increase in LV weight. Chronic blockade of brain "ouabain" by the Fab fragments icv blocked the hypertensive effect of high salt intake, which is consistent with our previous studies.<sup>6</sup> Interestingly, icv Fab fragments affected neither the LV hypertrophy nor the

increases in LV ACE mRNA and activity induced by high salt intake in Dahl S rats. These results provide strong further evidence that, at least in Dahl S rats, the early phase of salt-induced cardiac hypertrophy is largely independent of the development of hypertension. Mechanisms contributing to this trophic effect of high salt intake have not yet been established. High salt intake appears not to increase cardiac Ang II. <sup>(3, present study)</sup> However, this does not exclude a role for the cardiac RAS through eg overexpression of AT<sub>1</sub> receptors by high salt. <sup>19</sup> In addition, sodium may cause cardiac hypertrophy either directly or indirectly by enhanced release of ouabain-like compounds. <sup>21</sup>

In conclusion, our data demonstrate that the transcription of the cardiac ACE gene and its activity are increased by high salt intake in Dahl S but not Dahl R rats. In contrast, cardiac Ang I and II are decreased in Dahl S compared to Dahl R and remain low on high salt intake. Blockade of brain "ouabain" prevents the hypertensive action of high salt but not the growth of cardiac mass neither the increases in cardiac ACE mRNA and activity in Dahl S rats. Cardiac hypertrophy in Dahl S rats on high salt is therefore 1) largely independent of the development of hypertension; and 2) is not associated with increased cardiac Ang II, and the increase in ACE mRNA is likely relevant for other factors such as bradykinin.

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## Legends to the Figures

**Fig. 1.** Top: agarose gel electrophoresis of ACE RT-PCR using 1920 to 60 copies (lane 2 to lane 7) of 810 bp internal standard (low band). The upper band is the 934 bp PCR product of ACE cDNA. Lane 1:  $\lambda$  Hind III marker. Middle and bottom: effects of high salt intake on ACE mRNA and activity in the left ventricle of Dahl rats. Values are expressed as mean $\pm$ SEM (n=5-6 per group). ACE mRNA levels were standardized by PGK-1 mRNA level. ACE activity was expressed as mU/g of tissue/min, where 1 mU represents the generation of 1 nmol His-Leu/min.

\* $p$ <0.05 vs. Dahl R on same salt diet, <sup>b</sup> $p$ <0.05 vs. same strain on control diet.

**Fig. 2.** Effects of high salt intake on plasma renin activity and plasma Ang I and II levels of Dahl S and R rats. Values represent mean $\pm$ SEM (n=5-6 per group).

\* $p$ <0.05 vs. Dahl R on same salt diet. <sup>b</sup> $p$ <0.05 vs. same strain on control diet.

**Fig. 3.** Effects of high salt on ventricular Ang I and II levels of Dahl S and R rats. Values are expressed as mean $\pm$ SEM (n=5-7 per group). \* $p$ <0.05 vs. Dahl R on same salt diet.

**Fig. 4.** Effects of chronic icv infusion of Fab fragments inhibiting brain "ouabain" on blood pressure and LV weight of Dahl rats. Values are expressed as mean  $\pm$ SEM (n=6).

\* $p$ <0.05 vs. Dahl R on same salt diet. <sup>@</sup> $p$ <0.05 vs. same strain on both high and regular salt diets. <sup>b</sup> $p$ <0.05 vs. same strain on regular salt diet.

**Fig. 5.** Effects of chronic icv infusion of Fab fragments on ACE mRNA and activity of the LV in Dahl rats. ACE activity was expressed as mU/g of tissue/min, where 1 mU represents the generation of 1 nMol His-Leu/min. Values are mean $\pm$ SEM (n=6).

\* $p$ <0.05 vs. Dahl R on same salt diet. <sup>b</sup> $p$ <0.05 vs. same strain on regular salt diet.

**Table 1. Effects of high salt intake on blood pressure, heart weight and body weight of Dahl S and R rats**

	2 weeks high salt	4-5 weeks high salt
<u>Mean Arterial Pressure, mmHg</u>		
Dahl R		
Control	106±2	105±4
High	103±2	109±3
Dahl S		
Control	108±2	105±2
High	123±2 <sup>*b</sup>	140±5 <sup>*b</sup>
<u>LV Weight, mg/100g BW</u>		
Dahl R		
Control	256±6	223±3
High	266±3	241±3
Dahl S		
Control	270±3	229±3
High	304±5 <sup>*</sup>	379±13 <sup>*b</sup>
<u>RV Weight, mg/100g BW</u>		
Dahl R		
Control	62±2	53±2
High	65±2	59±2
Dahl S		
Control	65±2	52±1
High	71±2	66±3 <sup>b</sup>
<u>Body Weight, g</u>		
Dahl R		
Control	178±3	270±6
High	164±4	249±7
Dahl S		
Control	199±4	328±9
High	191±2	267±8 <sup>b</sup>

Values are expressed as mean±SEM; n=5-6 per group. LV, RV and BW represent left and right ventricle and body weight, respectively. <sup>\*</sup>p<0.05 vs. Dahl R on same diet; <sup>b</sup>p<0.05 vs. same strain on control diet.

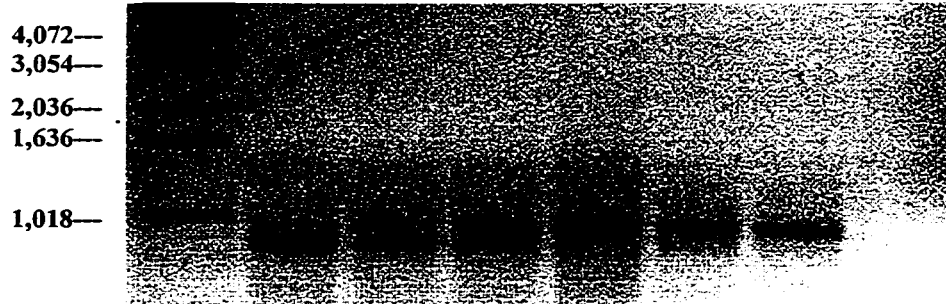
**Table 2. Effects of high salt intake on ACE mRNA levels in the right ventricle (RV) of Dahl S and R rats**

	2 weeks high salt	4-5 weeks high salt
	<i>ACE mRNA in the RV, copies / 200 ng RNA</i>	
Dahl R		
Control	149±45	95±34
High sodium	185±35	171±54
Dahl S		
Control	159±44	126±47
High sodium	209±40	237±45

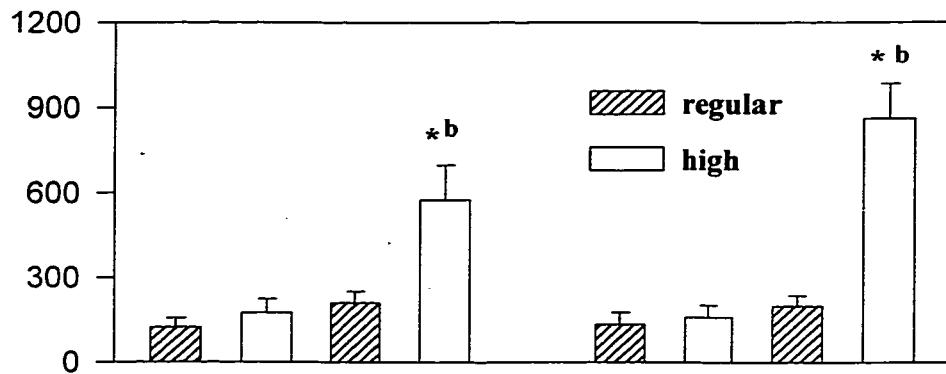
Values are expressed as mean±SEM. n=5-6 /group.

**Fig. 6 (Fig. 1 in Article II)** Effects of high salt intake on LV ACE mRNA and activity of Dahl S and R rats

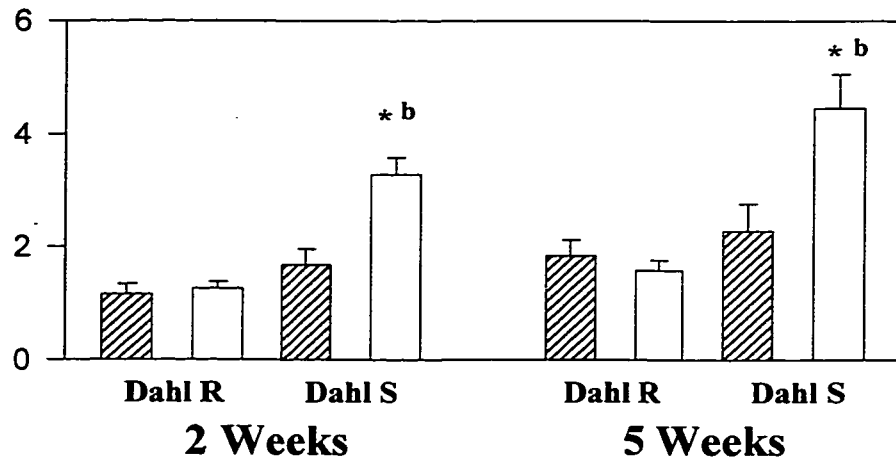
**Quantitative RT-PCR for ACE mRNA**



**LV ACE mRNA (copies/200 ng total RNA)**

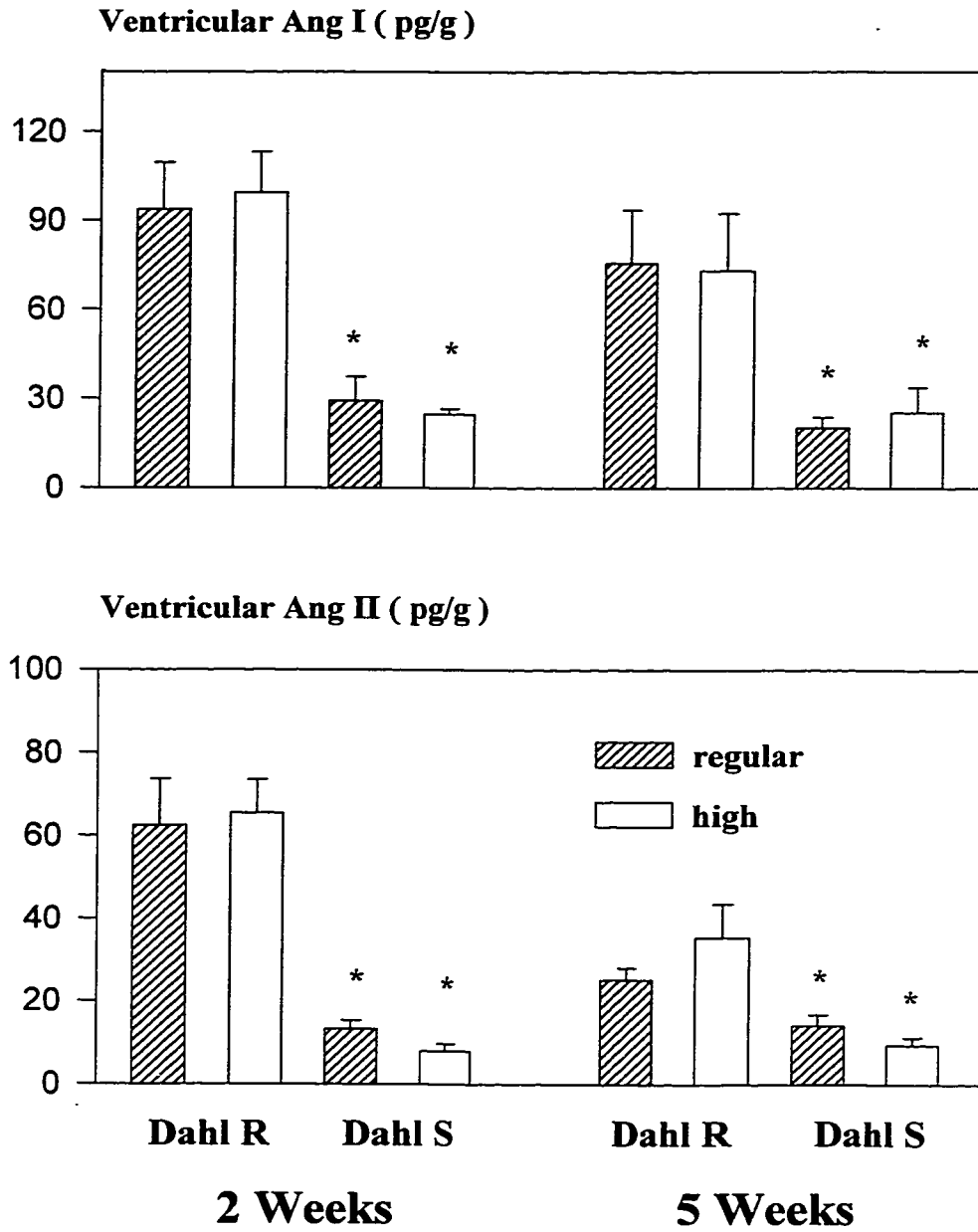


**LV ACE activity (mU/g of tissue/min)**

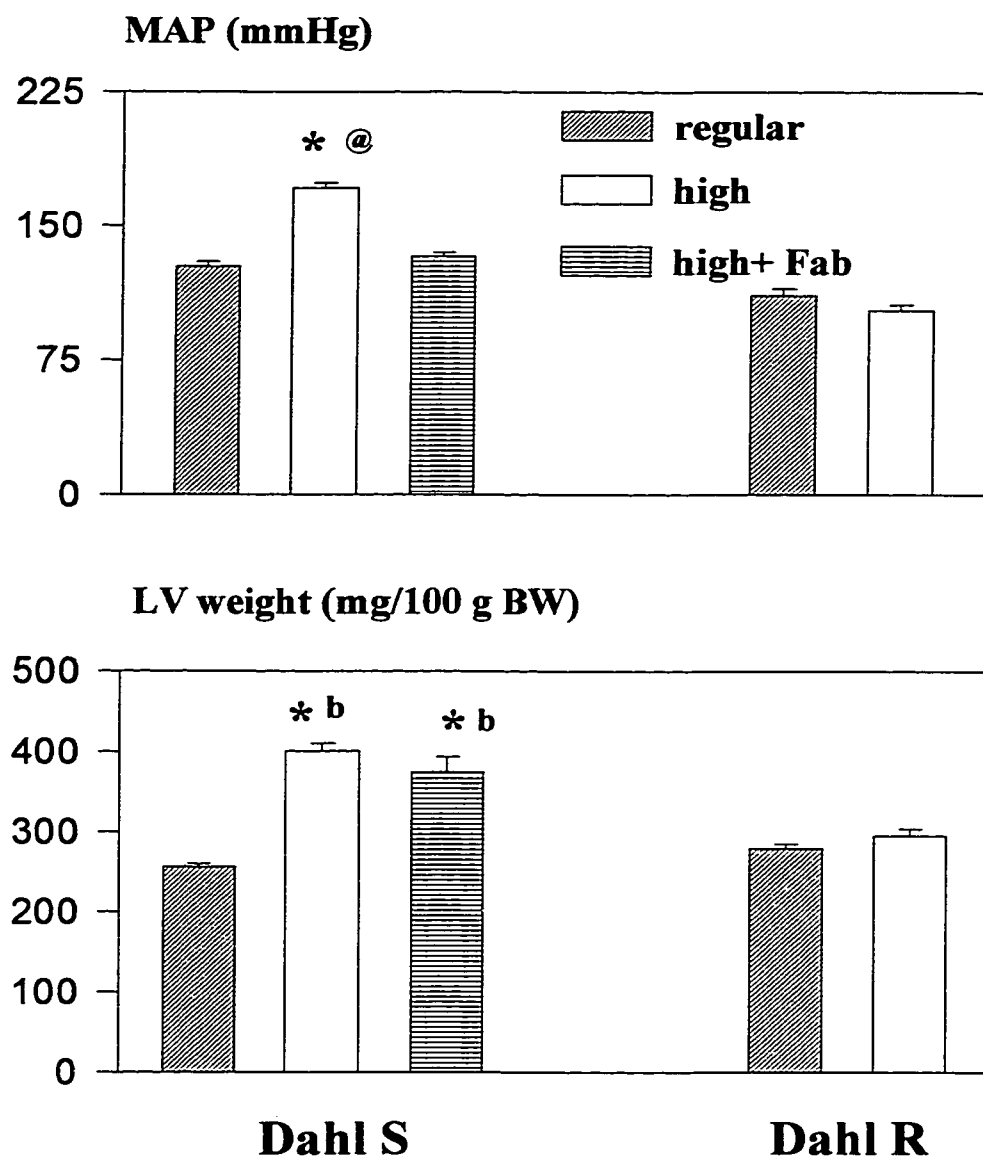




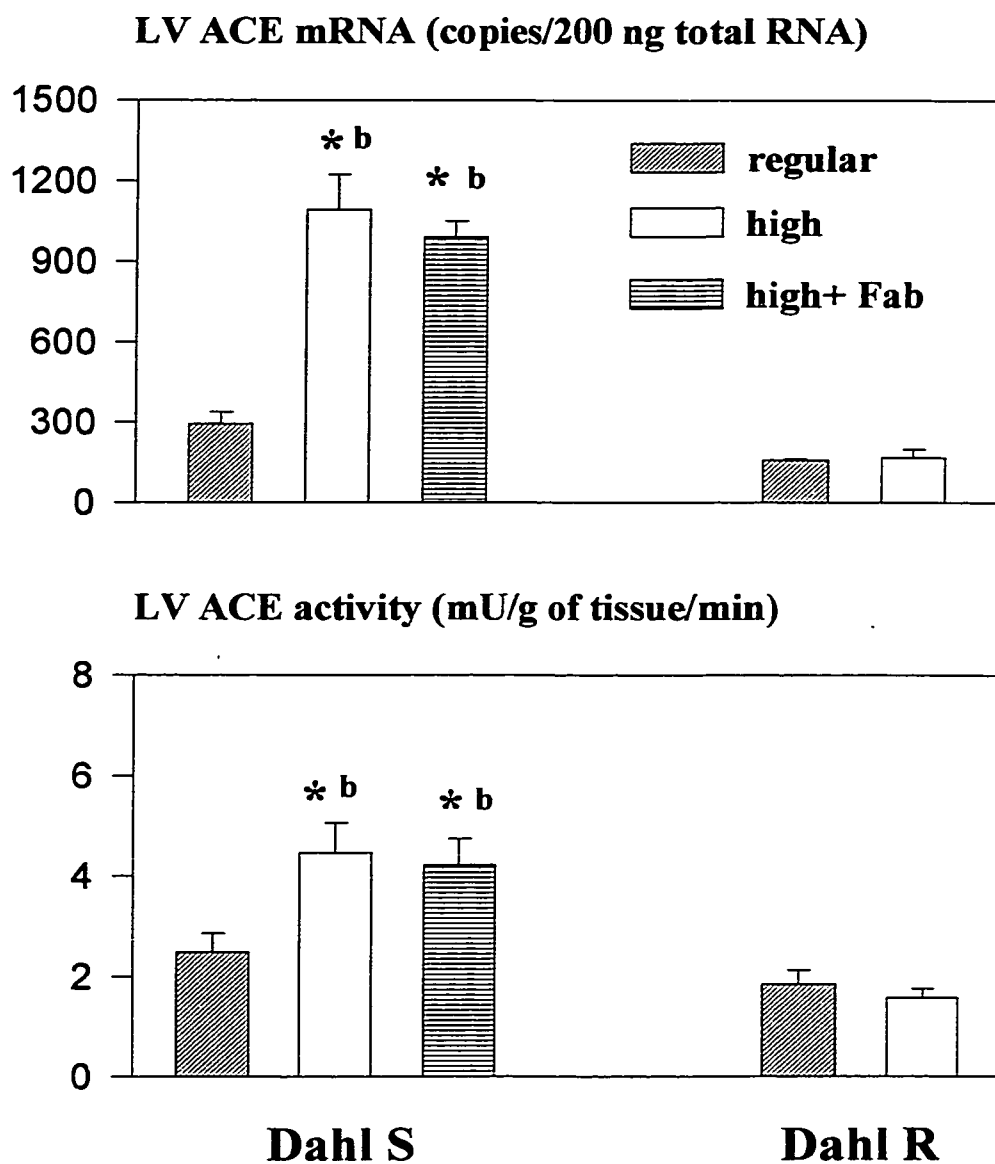
**Fig. 8 (Fig. 3 in Article II)** Effects of high salt intake on ventricular Ang I and II of Dahl S and R rats



**Fig. 9 (Fig. 4 in Article II)** Effects of blockade of brain "ouabain" on blood pressure and LV weight



**Fig. 10 (Fig. 5 in Article II) Effects of blockade of brain "ouabain" on LV ACE mRNA and activity of Dahl S and R rats**



## **DISCUSSION**

The present study provides several new findings regarding the tissue and circulatory RASs during the development of high salt-induced hypertension and cardiac hypertrophy in Dahl S rats. First, high salt intake markedly increases ACE mRNA expression and activity in the hypothalamus and pons, as well as in the hypertrophied LV of Dahl S but not Dahl R rats. Second, on regular salt intake, cardiac Ang I and Ang II, as well as hypothalamic Ang II are significantly lower in Dahl S rats than in Dahl R rats, but high salt does not affect brain and cardiac Ang I or II in either strain. Thirdly, on regular salt, PRA, plasma Ang I and II levels all are significantly lower in Dahl S rats compared to Dahl R rats. High salt intake lowers PRA and plasma Ang I but not Ang II in Dahl R rats. In Dahl S rats, high salt does not cause further decreases of the already low PRA, Ang I and II. Finally, blockade of brain “ouabain” with icv antibody Fab fragments blocks the hypertensive effect of high salt intake in Dahl S rats and prevents the increases in ACE mRNA and activity in the hypothalamus and pons, but does not prevent the LVH neither the enhancements of LV ACE mRNA and activity in Dahl S rats by high salt intake.

### **I. Effects of high salt intake on the brain RAS and BP**

#### ***I.1. Salt intake and the brain RAS and BP***

Numerous studies indicate that high salt intake potentiates the development of

hypertension in rats genetically predisposed to hypertension as well as profoundly influences the structure of the LV. In agreement with the original study by Dahl et al. (1962) and a large number of other studies (Huang and Leenen, 1994, 1998; Sugimoto et al., 1996; Sumida et al., 1998), the present study demonstrates that high salt intake causes gradual increases in BP over time in Dahl S rats but not in Dahl R rats.

High salt diet causes sympathetic hyperactivity, impairment of baroreflexes and exaggerates the development of hypertension in rat strains genetically predisposed to hypertension, such as SHR (Huang and Leenen, 1995; 1996) and Dahl S (Huang and Leenen, 1994). In SHR and Dahl S rats, the brain RAS appears to play a major role, since blockade by AT<sub>1</sub> receptor antagonists abolishes sympathetic hyperactivity in both models, hypertension in Dahl S rats, and the exaggerated hypertensive response in SHR to high salt (Huang and Leenen, 1998; Huang and Leenen, 1996; Teruya et al., 1995).

In Dahl S rats little is known about the biochemical components of the brain RAS and their changes during the development of salt-sensitive hypertension. ACE is widely distributed in rat brain, and high concentrations are found in the forebrain circumventricular organs and paraventricular hypothalamic nucleus (Chai et al; 1987; Pickel et al., 1986; Whiting et al., 1991). In SHR ACE activity is more than 2-fold higher in the hypothalamus than in other areas (Mizuno et al., 1981). The present study also found that ACE activity in the hypothalamus is much higher than in the medulla and cortex (239±30, 66±12, 37±5 mU/g of tissue/min, respectively). Although changes in peripheral ACE expression and activity have been reported, little is known about central ACE expression in salt-sensitive hypertension. The present study shows that at 9 weeks of age ACE mRNA

and activity in the hypothalamus of Dahl S rats on regular salt diet are significantly higher than in Dahl R rats on regular salt diet. Moreover, high salt diet significantly increased ACE mRNA and activity in the hypothalamus and pons in Dahl S rats as compared with that in Dahl S rats on regular salt as well as in Dahl R rats on either diet. At 9 wk of age ACE mRNA levels had increased about 3-fold in the hypothalamus, and 2-fold in the pons as compared with Dahl S rats on regular salt diet and Dahl R rats on high salt diet. ACE activity in the hypothalamus of Dahl S on high salt increased about 2-fold, and less but still significantly increased in the pons compared with Dahl R rats. The magnitude of these changes is parallel with the increase in blood pressure. To the best of our knowledge, this is the first report of brain ACE mRNA expression in a genetic hypertensive animal model.

In adult SHR ACE activity in the cerebrospinal fluid is higher than WKY (Israel and Saavedra, 1987). Mizuno et al. (1981) demonstrated that 1% salt solution drinking for 4 wk increased ACE activity by about 2-fold in the midbrain in SHR, WKY, and SHRSP aged 20-21 wk compared with same strain on control salt intake, and increased ACE activity by more than 3-fold in the striatum only in SHRSP. However, high salt drinking did not affect ACE activity in the hypothalamus in WKY, SHR and SHRSP. Congruent to the increase in ACE activity in the midbrain of SHR by high salt, the present study shows that high salt intake increases ACE mRNA and activity in the pons of Dahl S rats, but it also markedly increases ACE mRNA and activity in the hypothalamus of Dahl S rats. One explanation for the differences may be due to different strains. In fact, in the same study high salt intake increased ACE activity of the striatum in SHR-SP by 3-fold, but did not affect ACE activity of the striatum in SHR (Mizuno et al., 1983). It is no wonder that Dahl S rats

have distinct response to high salt from SHR. Moreover, we used young Dahl rats aged 8-9 wk in contrast with SHRSP at about 25 wk of age. It is possible that ACE expression to high salt is different at different stages of development, i.e., in young Dahl S rats ACE mRNA and activity increased by high salt intake but not in older SHR or Dahl S rats.

The regulation of ACE expression remains largely unclear. In cultured endothelial cells, ACE activity is increased by cAMP analogues, methylxanthines (Krulowitz and Fanberg, 1986), calcium ionophore A23187, and sodium ionophore monensin (Dasarathy and Fanburg, 1991), as well as by glucocorticoid and thyroid hormones (Hubert et al., 1991). The latter is consistent with increased ACE activity during hyperthyroidism in humans (Yotsumoto et al., 1982). Both PKC and cAMP-dependent mechanisms appear to increase ACE activity in cultured endothelial cells, but only activators of PKC increase ACE activity in the medium (Villard et al., 1998). ACE expression/activity is also regulated by NO. An upregulation of the cardiac and vascular ACE activity was observed in rats with long-term blockade of NO synthesis (Takemoto et al., 1997). On the other hand, increased NO or NO releasing compounds were able to inhibit ACE activity (Ackemann et al., 1998). In hypertensive rats this feedback regulation between NOS and ACE was demonstrated after long-term ACE inhibition where inhibited ACE expression and activity was associated with an increase in NOS expression and vascular NO release (Wiemer et al., 1997). In Sprague-Dawley rats with 3 wk of 17 $\beta$ -estradiol replacement therapy, reduced ACE mRNA levels and activity were observed in the kidney, lung and aorta compared to the control, suggesting that estrogen downregulates ACE expression and activity in tissues (Gallagher et al., 1999). On the other hand, infusion of Ang II for 3 days caused a decrease in pulmonary ACE mRNA

with a parallel decrease in pulmonary ACE activity (Schunkert et al., 1993), indicating that pulmonary ACE expression is subjected to negative feedback by Ang II. Ang II downregulated pulmonary ACE mRNA and activity, but did not affect serum or testicular ACE (Schunkert et al., 1993), suggesting that ACE expression and activity are differentially regulated in different tissues.

In experimental models of hypertension and cardiac failure, increased ACE expression and activity has been observed consistently in association with cardiac hypertrophy (as reviewed in the Introduction and will be discussed later). In some inflammatory states such as sarcoidosis elevated ACE content is due to excessive production of ACE in the activated macrophages. In rats with intense proteinuria ACE mRNA in the renal cortex was increased by 2 fold. By in situ RT-PCR and immunohistochemistry, ACE expression was mainly localized in the proximal tubules (Largo et al., 1999). It appears that ACE expression (both gene and protein) is upregulated in most pathophysiological conditions such as hypertension, inflammation, heart failure.

Although the exact mechanisms for increased ACE expression in Dahl S rats by high salt intake remains unknown, high salt intake activates numerous cellular events (e.g., intracellular  $Ca^{++}$  changes) and second messenger pathways. In hypertensive subjects on low- (UNaV<50 mEq/d) and high-(UNaV>200 mEq/d) salt diets for 2 months, elevated cytosolic  $Ca^{++}$  was associated with high salt, and these changes occurred predominantly in salt-sensitive subjects. Nifedipine blunted the pressor response to salt loading >50% and reversed salt-induced ionic changes (Resnick et al., 1994). In Dahl S rats fed on 8% salt diet a marked increase in cellular  $Ca^{++}$  was found as compared with Dah R rats on high salt ( $280\pm 38$  vs

81±22 nmol/l, respectively), correlated with severe hypertension in Dahl S but not Dahl R rats (Vasdev et al., 1988). These data demonstrated that salt sensitivity involves cellular calcium accumulation from the extracellular space. Kawaguchi et al.(1990) studied the effects of platelet-activating factor (PAF) on ACE in cultured pulmonary artery endothelial cells. In the absence of PAF, the cells converted 1 nmol/dish of <sup>125</sup>I-Ang I to Ang II. ACE activity was increased to 2.5 nmol/dish in the presence of 1x10<sup>-6</sup> M of PAF. Furthermore, PAF elevated Ca<sup>++</sup> influx in a dose-dependent manner as well as increased phospholipase C (PLC) activity and IP3 release. Administration of neomycin abolished the increase in Ca<sup>++</sup> and IP3 release induced by 10<sup>-6</sup> M of PAF by about 60-70%. ACE activity was also inhibited up to 70% in the presence of 10<sup>-6</sup> M of PAF by 50 M of neomycin (Kawaguchi et al., 1990). ACE activity was also increased more than 2 times after exposure of 1x10<sup>-8</sup> M endothelin-1 in cultured pulmonary artery endothelial cells. Endothelin-1 stimulated Ca<sup>++</sup> influx and PLC activity in a dose-dependent manner. Ca<sup>++</sup> influx and ACE activity were suppressed 60-70% in the presence of endothelin-1 (10<sup>-10</sup> to 10<sup>-6</sup> M) by 50 M neomycin (Kawaguchi et al., 1991). These results clearly indicate that increased ACE activity by PAF and endothelin-1 in endothelial cells is mediated by increased cytosolic Ca<sup>++</sup>. Therefore increased intracellular Ca<sup>++</sup> by long-term exposure of high salt may be involved in activation of ACE expression because high salt intake increases endogenous “ouabain” resulting in inhibition of Na<sup>+</sup> pump and redistribution of cellular Ca<sup>++</sup>.

The present study also demonstrates that in Dahl S and R rats Ang I levels in the hypothalamus and pons are similar and high salt intake does not cause significant changes. Ang II in the hypothalamus of Dahl S rats on regular salt diet is significantly lower than in

Dahl R rats. High salt intake does not cause significant changes in Ang II levels in the hypothalamus of either Dahl S and R rats. In the pons, Ang II levels are similar in the two strains on both high and regular salt diet. These data demonstrate that Dahl S rats have a low activity of the brain RAS as indicated by low Ang II. Although ACE mRNA and activity are increased in the hypothalamus and pons in Dahl S rats by high salt intake, Ang II levels are not affected by high salt intake.

Little information is available about central Ang I and II in salt-sensitive hypertension. On regular salt diet, Ang II levels in the hypothalamus and cerebellum are higher in SHR than in WKY at 2 to 20 wk of age (about 3 times vs WKY at 2 wk; more than 2 times vs WKY at 20 wk in the hypothalamus) (Phillips and Kimura, 1988). In adult SHR Ang II immunoreactivity in the SON and PVN is twice as much as in WKY (Weyhenmeyer and Phillips, 1982). Brain Ang II concentration and turnover are also higher in SHRSP vs WKY. Because assessment of absolute concentrations of angiotensins does not allow a precise quantitative measurement of the biological activity, turnover of angiotensins was measured in this study by blockade of Ang II formation in the brain with the ACE inhibitor captopril. Accumulation of Ang I and decrease of Ang II indicates an active turnover of the peptides. In the case, an increase of 99% for Ang I and a decrease of 36% for Ang II were observed in ACE inhibitor treated SHRSP but not in WKY (Ganten et al., 1983). However, whether high salt intake increases central angiotensins in SHR remains unknown. In Dahl rats there are no other data about central Ang I and II on either diet.

Several explanations are possible for the apparent dissociation of ACE mRNA and activity and Ang II in Dahl S rats. For example, it is conceivable that the absence of an

increase in steady-state Ang II levels could be caused by increased Ang II turnover by metabolism (Ganten et al., 1983). Cellular internalization after binding to its receptor also can cause a decrease in steady-state Ang II (Sasamura et al., 1997). Moreover, a decrease in the production of the substrate for ACE, angiotensin I, (e.g. by decreased renin activity or angiotensinogen levels) could cause Ang II levels to remain constant despite an increase in ACE activity. However, this is rather unlikely, since Ang I levels were not affected by high salt intake.

It is also possible that increases in brain ACE mRNA and activity produced by high salt diet are not functionally related to the brain RAS, but to other systems such as bradykinin. ACE is not only an important enzyme for Ang II formation but is also associated with other functions such as degradation of bradykinin. Administration of exogenous bradykinin into the cerebroventricular space increases BP and HR in rats (Lindsay et al., 1989). These effects were mediated via activation of the sympathetic nervous system (Qadri et al., 1999). However, micronuclear injection of bradykinin into the nucleus hypothalamicus anterior caused decreases in BP and HR, whereas microinjection into the hypothalamus dorsalis and rostralventrolateral medulla increased BP and HR (Diz, 1985; Privitera et al., 1994). The final effects of the peptide appeared to depend on the injection site, possibly because of differential interactions with the sympathetic and parasympathetic components of the central nervous system. A limitation of these studies is that pharmacological doses may not reflect the physiological action of the endogenous peptide. Using antisense oligodeoxynucleotides targeting the components of the kallikrein-kinin system to block the system, Madeddu et al. (1996) showed that bradykinin play an important role in central regulation of blood pressure

with opposite effects of central Ang II. In 9 wk-old SHR acute icv injection of antisense oligodeoxynucleotides targeting kininogen or B2 receptor mRNAs increased BP by ~20 mmHg whereas in age-matched WKY rats BP increased by less than 10 mmHg. Microinjection of antisense oligodeoxynucleotides to kininogen or B2 receptor mRNA into the nucleus tractus solitarius increased BP and prevented the vasodepressor effect induced by intranuclear microinjection of bradykinin (Madeddu et al., 1996). These results indicate that brain bradykinin plays a role in the central regulation of BP. Increased ACE expression and activity may lead to increased degradation of brain bradykinin which may either lower or increase BP depending on where this effect is predominant. The increases in brain ACE expression and activity, thus, may be involved in the development of hypertension via enhanced degradation of bradykinin. Further biochemical/molecular biological characterization of the brain RAS or brain kinin system is required to identify the reasons for the apparent dissociation between ACE mRNA production and Ang II levels.

Another apparent dissociation is reflected in the dependence of the hypertension on the brain RAS as indicated by the effectiveness of icv losartan (Huang and Leenen, 1998) without an increase in Ang II levels in the hypothalamus and pons. Several explanations are possible here as well. First, the brain RAS may mediate the hypertension without increasing its activity since same level of central Ang II may cause hypertension in genetic hypertensive rats by not in control strain. Upregulation of brain AT<sub>1</sub> receptor by high salt in SD rats was reported (Schmid et al., 1997). An increase in AT<sub>1</sub> receptor number or affinity for Ang II, as observed in SHR (Saavedra et al., 1986), and recently in Dahl S rats (Veerasingham and Leenen, 1998; Strehlow et al., 1999), and thereby to enhanced functional

responses. Secondly, increased Ang II turnover may prevent increases in Ang II production from being reflected in increased steady-state levels. Thirdly, Ang II levels may only increase in some nuclei/regions but not in the whole hypothalamus or pons.

### ***I. 2. Effect of blockade of brain “ouabain” on BP and the brain RAS***

Besides the brain RAS, the sympathoexcitatory and hypertensive effects of high salt intake in Dahl S rats and SHR are also mediated by an increase in brain “ouabain” (Huang and Leenen, 1994; 1996b). In SHR and Dahl S rats, the increase in BP by high salt intake is associated with increases in sympathoexcitation and decreases in sympathoinhibition leading to an increase in sympathetic neuronal activity (Koepke et al., 1988; Huang and Leenen, 1994, 1992b). High salt intake induces an increase in hypothalamic, pituitary and pons “ouabain” concentration in Dahl S rats and SHR (Leenen et al., 1993, 1994). In SHR, chronic blockade of brain “ouabain” with icv Fab fragments binding ouabain and related steroids prevents salt-induced sympathetic hyperactivity and hypertension in a similar pattern as blockade of the brain RAS by icv losartan (Huang and Leenen, 1996a). In similar doses Fab fragments administered iv are ineffective in preventing salt-induced hypertension suggesting that the actions of ouabain are central. Blockade of brain “ouabain” does not blunt excitatory responses of blood pressure, heart rate and renal sympathetic nerve activity (RSNA) to acute icv injection of Ang II, whereas blockade of brain AT<sub>1</sub> receptors by losartan blocks responses to both ouabain and Ang II icv (Huang and Leenen, 1996a). These findings suggest that the two systems interact and are involved in the same pathways mediating the sympathoexcitatory and pressor effects of high salt. It appears that high salt activates brain

“ouabain”, then the brain “ouabain” activates the brain RAS. It is unknown, however, how the two systems interact with each other, or which components of the brain RAS are activated by brain “ouabain”.

The present study demonstrates that the increases in ACE expression and activity in the hypothalamus and pons of Dahl S rats on high salt intake are prevented by chronic blockade of brain “ouabain” with antibody Fab fragments. These results suggest that activation of the brain ACE expression and activity is indeed secondary to increased brain “ouabain”, and that increased ACE expression and activity may be relevant for the increase in BP. There is no data yet how brain “ouabain” regulates brain ACE mRNA or activity.

Endogenous “ouabain” may directly inhibit the plasmalemmal  $\text{Na}^+\text{-K}^+$  ATPase activity in a variety of cell types. As a result of inhibition of  $\text{Na}^+$  pump, cytosolic  $\text{Na}^+$  concentration rises, which thereby affects all sodium gradient-dependent processes, albeit to only a small extent. Most important is the secondary redistribution of  $\text{Ca}^{++}$ , mediated by  $\text{Na}^+ - \text{Ca}^{++}$  exchange, causing a slight increase in cytosolic free  $\text{Ca}^{++}$  concentration. When a new steady state of cytosolic  $\text{Ca}^{++}$  is achieved,  $\text{Ca}^{++}$  store in the endoplasmic and/or sarcoplasmic reticulum (ER/SR) is substantially augmented. Thus the extra  $\text{Ca}^{++}$  store is then available for mobilization whenever the cells are activated. Cytosolic  $\text{Ca}^{++}$  is the key “second messenger” functioning in virtually all cells controlling numerous physiological processes such as contraction, secretion, and excitability (For review, see Blaustein 1993). It is conceivable that brain “ouabain” may inhibit  $\text{Na}^+$  pumps and increase intracellular  $\text{Ca}^{++}$  leading to increased ACE expression and activity in the brain. Increased cytoplasmic  $\text{Ca}^{++}$  appears to mediate the induction of ACE activity by platelet-activating factor (Kawaguchi et al., 1990).

## **II. Effects of high salt intake on cardiac hypertrophy and the cardiac RAS**

### **II. 1. *Salt intake and cardiac hypertrophy***

Previous studies have identified salt intake as a major determinant of LVH independent of blood pressure (Du Cailar et al., 1992; Schmieder et al., 1988). Studies have shown that increasing salt intake by providing 0.9% saline as the sole source of drinking water in normotensive rats causes LVH without increasing blood pressure, cardiac filling pressure, and resting cardiac sympathetic activity (Fields et al., 1991; Meggs et al., 1988). Increasing salt intake by food also increases LV weight in normotensive rats depending on age, time and strain. In young WKY rats, high salt diet induces a marked LVH ( up to 25%) while in young Wistar a moderate LVH (14%) without hypertrophic response in Dahl R rats (Yuan and Leenen, 1991; Leenen and Yuan, 1998; Pfeffer et al., 1984). Consistent with previous studies, the present study demonstrates that in young Dahl R rats high salt intake does not affect blood pressure or LV weight after 2 or 5 wk of salt loading.

In contrast, in young SHR 2% salt diet did not significantly increase left ventricle mass and was associated with minor hemodynamic changes; while 8% salt diet did increase left ventricle mass with increased blood pressure. Thus, left ventricle mass in young SHR increased only when blood pressure increased too (Leenen and Yuan, 1998). In Dahl S rats, Pfeffer and colleagues (1984) showed that high salt intake caused a graded cardiac hypertrophy depending on dietary salt. The present study is also congruent with previous studies (Pfeffer et al., 1984; Hayakawa and Raji, 1997; Huang and Leenen, 1994) that in Dahl S rats high salt intake induces LVH which is disproportionate to the increases in blood pressure.

## **II. 2. *Effects of blockade of brain “ouabain” on BP and salt-induced LVH***

Previous studies suggested that salt-sensitivity may apply both to BP and to heart structure, and cardiac hypertrophy induced by high salt intake can be independent of an increase in BP (Frohlich et al., 1993; Leenen and Yuan, 1998). Young WKY rats showed a graded hypertrophic response to 2 and 8% salt intake without an increase in BP. In contrast, young SHR did not respond to 2% salt diet and the extent of LVH induced by 8% salt diet was less than that in WKY rats, despite the clear further increase in BP in SHR rats (Leenen and Yuan, 1998). Yu et al. (1998) recently showed that in both SHR and WKY rats 8% salt intake leads to similar LVH and widespread cardiac fibrosis although the increase in BP of WKY rats was minor compared to SHR rats. They also showed that tissue fibrosis was associated with an increase in transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) expression, suggesting that TGF- $\beta_1$  pathway is involved in the development of salt-induced LVH and that high salt intake may be an important direct pathogenetic factor for LVH. In normotensive strains of rats, high salt intake induced the most LVH in WKY rats, but had less effect on the heart of Wistar rats, and no effect in Dahl R rats at all. In hypertensive strains of rats, at similar blood pressure levels Dahl S rats exhibited more LVH than SHR rats. This difference was associated with a greater constitutive nitric oxide synthase (cNOS) activity of the LV in SHR than in Dahl S rats (Hayakawa and Raji, 1997). Compared with their normotensive counterparts, aortic cNOS activity was increased 106% in SHR but reduced by 73% in Dahl S rats. In vitro and in vivo studies suggest that nitric oxide (NO) is an endogenous inhibitor of cardiovascular smooth muscle growth that may modulate cardiovascular remodelling. Reduced cNOS activity can lead to less NO production and a greater LVH in Dahl S rats

(Hayakawa and Rajj, 1997).

The present study demonstrates that in Dahl S rats, high salt intake causes severe hypertension and marked LVH. Chronic blockade of brain “ouabain” by antibody Fab fragments icv prevents the hypertensive response to high salt consistent with our previous studies (Huang and Leenen, 1994). Interestingly, chronic infusion of icv Fab fragments affects neither LVH nor the increases in the LV ACE mRNA and activity induced by high salt intake in Dahl S rats. These results provide strong further evidence that, at least in Dahl S rats, the early phase of salt-induced cardiac hypertrophy is largely independent of the development of hypertension.

### ***II.3. Salt intake and the cardiac vs circulatory RASs***

An increase in the activity of the cardiac RAS has been implicated in the development of cardiac hypertrophy. Among the components of the cardiac RAS, increased cardiac ACE mRNA and activity have been reported consistently in different models of pressure overload (Bruckschlegel et al., 1995; Pfeifer et al., 1998; Schunkert et al., 1990) and ACE mRNA in volume overload (Iwai et al., 1995; Lear et al., 1997). Right ventricular ACE expression and activity are increased during hypoxic pulmonary hypertension (Morrel et al., 1997). Whether or not such increases in ACE by pressure overload cause increases in cardiac Ang II has not yet been assessed. Few data are available on changes in cardiac Ang II during the development of cardiac hypertrophy.

The mechanisms for high salt-induced cardiac hypertrophy remain unknown. We previously showed that in rats cardiac hypertrophy induced by high salt is independent of

cardiac pressure overload or sympathetic activity ( Fields, et al., 1991; Song et al., 1997). Schmieder et al. suggested that impaired suppression by high salt of the RAS acts as a stimulus for myocardial hypertrophy (Schmieder et al., 1996). However, studies on the effects of high salt intake on the activity of the cardiac RAS are limited. In WKY rats 2 wk of high salt intake did not affect cardiac ACE activity, whereas a significant increase was found in WKY rats with combined DOCA-salt (Michel et al., 1994). In contrast, in adult WKY and SHR-SP high salt for 2 wk increased ACE mRNA and activity in both strains, but increased BP and LV weight only in the SHR-SP (Kreutz et al., 1995). In this study, cardiac Ang II was not measured, plasma Ang II showed no decrease on high salt in either strain. In young SHR and WKY rats, high salt for 2 wk did not affect cardiac Ang II (Leenen and Yuan, 1998).

The present study demonstrates that ACE mRNA and activity increase markedly in the hypertrophied left ventricle of Dahl S on high salt intake. A 3-fold increase in ACE mRNA level of the LV after 2 weeks and a 4-fold increase after 4-5 weeks on high salt diet were found. ACE activity of the LV in Dahl S on 2 week high salt was more than 2-fold higher than in Dahl S on regular salt diet, increased further after 5 week high salt. These results indicate that increased ACE mRNA and activity also occur in the hypertrophied LV of Dahl S rats on high salt intake. Dahl R rats showed no increase in BP, LV weight or cardiac ACE in response to high salt, indicating a differential response in the salt-sensitive vs salt-resistant strain. In contrast, adult WKY rats also showed no increases in BP and LV weight, but did show a clear increase in LV ACE mRNA and activity in response to high salt intake (Kreutz et al., 1995). These findings indicate that even salt-resistant strains can exhibit

different cardiac responses, and that increased cardiac ACE can occur independent of cardiac hypertrophy.

To evaluate the functional role of changes in ACE expression and activity, we assessed cardiac Ang I and II. On regular salt intake, in Dahl S rats both Ang I and II in the heart were decreased when compared with Dahl R rats. However, high salt intake did not affect cardiac Ang I and II levels in either strain. These results are consistent with our previous study showing that high salt intake does not affect cardiac Ang II in WKY and SHR (Leenen and Yuan., 1998).

There are several possible explanations for the distinct discrepancy between ACE expression and activity, and Ang II levels in the heart of Dahl S rats. The most likely explanation is that the formation of Ang II is not only determined by ACE but also by other components of the RAS such as angiotensinogen and renin. The present study shows that PRA and plasma Ang I and II in Dahl S rats are all rather low and significantly reduced compared to Dahl R rats. In the present study, cardiac renin ( mRNA or activity ) was not assessed. However, cardiac Ang I levels were also very low in Dahl S vs R on both regular and high salt intake. This suggests that although increased ACE mRNA was associated with increased ACE enzymatic activity, ACE is not rate-limiting (Fiselier et al., 1983; Harrap et al., 1993) and that therefore an increase in enzymatic activity does not lead to increased Ang II formation. The present results are consistent with a recent study showing that in transgenic mice overexpression of cardiac ACE (40- to 100-fold) did not change the production of cardiac Ang II. However, ultrastructural changes and a hypertrophic pattern of gene expression were observed, suggesting that ACE is not rate-limiting for Ang II production in

this model and that the hypertrophic responses in the mice may be independent of the cardiac RAS (Pinto et al., 1998). Why ACE gene expression then would increase is another relevant issue. Since ACE gene expression increases in all models of cardiac hypertrophy (as far as studied), this gene appears to be part of the genetic program activated during development of cardiac hypertrophy. Rather than contributing to enhanced activity of the cardiac RAS, increased ACE expression and activity might functionally contribute through other systems such as the breakdown of bradykinin to mediate the formation of cardiac hypertrophy (see page 106-107).

On regular salt diet, all components of the circulatory RAS, i. e. PRA, plasma Ang I and II were significantly lower in Dahl S vs R rats. Both 2 weeks and 4-5 weeks of high salt intake reduced PRA in Dahl R to the low levels in Dahl S, plasma Ang I showed a more modest decrease by high salt in Dahl R but high salt caused no decrease in plasma Ang II possibly due to already low Ang I. In Dahl S rats, high salt intake did not cause further decreases of the already low PRA, Ang I and II. Put together, these data indicate on regular salt intake a low activity of both the circulatory and cardiac RAS in Dahl S vs R rats. High salt intake does not affect the activity of the cardiac RAS as assessed by cardiac Ang I and II in either strain despite a marked enhancement of ACE expression in Dahl S, and decreases the activity of the circulatory RAS only in Dahl R.

Little information is available on the effects of high salt intake on the activity of the circulatory RAS in Dahl S vs R. In Dahl S rats, high salt suppressed PRA after 4 weeks, but increased PRA above baseline after 8 weeks possibly due to the secondary renal vascular lesions (Von Lutterotti et al., 1992). Another study showed that on regular salt diet, plasma

renin concentration in Dahl S was lower than in Dahl R without differences in plasma angiotensinogen concentration (Franco-Saenz et al., 1997). An earlier study showed that on regular salt diet, PRA was significantly lower in Dahl S than in Dahl R rats. Low salt diet markedly increased PRA in Dahl R but caused only a minor increase in Dahl S (Rodriguez-Sargent et al., 1981). These data demonstrate that Dahl S rats have low PRA and salt intake appears to have minor or no effects on PRA. To the best of our knowledge, no studies have so far assessed circulatory or cardiac Ang I and II in Dahl rats on regular or high salt intake. The present study provides direct evidence of low activity of the RAS in Dahl S vs R rats, as assessed by the biologically most relevant parameter, Ang II, both in the circulation and in the heart on both regular and high salt intake.

The mechanisms for salt-induced cardiac hypertrophy have not yet been established. Although it is suggested that impaired suppression of the cardiac RAS by high salt may be involved in the development of cardiac hypertrophy, overactivity of the cardiac RAS appears not likely to be involved in salt-induced cardiac hypertrophy in Dahl S rats as indicated by consistently low cardiac Ang II (Leenen and Yuan, 1998, present study). In addition, in Dahl S rats LV AT<sub>1</sub>, AT<sub>2</sub> receptors measured by radioligand binding assay were not affected by high salt intake (Sumida et al., 1998). Moreover, the AT<sub>1</sub> receptor blocker, TCV 116, did not prevent cardiac hypertrophy in Dahl S rats on high salt diet (Sugimoto et al., 1996). Therefore, at least in Dahl S rats, the cardiac RAS may be not involved in the development of cardiac hypertrophy induced by high salt.

Increased ACE expression and activity might contribute to other non-angiotensin mechanisms such as degradation of bradykinin. A large body of data indicates that

bradykinin has an antitrophic function in cardiac remodelling (for reviews, see Remme, 1997; Gohlke et al., 1997). ACE inhibitors inhibit LVH in vivo. A component of this effect has been attributed to tissue accumulation of bradykinin. In vitro bradykinin increased [<sup>3</sup>H] phenylalanine incorporation by 23±3% in adult and by 36±10% in neonatal cardiomyocytes, similar to the hypertrophic response to Ang II. In cardiomyocytes cocultured with endothelial cells, however, bradykinin abolished Ang II-induced hypertrophy but not in myocytes in absence of endothelial cells (Ritchie et al., 1998). These results suggest that whereas bradykinin has a direct hypertrophic effect on cardiomyocytes it prevents hypertrophic response in the presence of endothelial cells. Other studies (Ishigai, et al., 1997) indicated that the protective effects of bradykinin in cardiac hypertrophy are associated with augmented NO release. Perindoprilat, an ACE inhibitor, blocked the progression of cardiac hypertrophy and increased NO release. Hoe-140, an kinin B<sub>2</sub> antagonist, abolished the effects of perindoprilat. Furthermore, a significant correlation of protein/DNA content and nitrite/nitrate content was observed (Ishigai, et al., 1997). This study suggests that bradykinin prevents cardiac hypertrophy by increasing NO release. Therefore, it is possible that excessive degradation of bradykinin by increased ACE expression and activity leads to reduced protective effects and contributes to the development of cardiac hypertrophy by high salt.

In addition, high salt intake may cause cardiac hypertrophy either directly (Gu et al., 1998) or indirectly by enhanced release of endogenous “ouabain” (Kometiani et al., 1998). In vitro ouabain caused hypertrophic growth of neonatal rat cardiac myocytes, and induced several early or late-response genes that are markers of cardiac hypertrophy (Huang et al.,

1997). In hypertensive humans elevated endogenous “ouabain” is associated with an increase in LV mass (Manunta et al., 1999). High salt intake increased plasma and LV “ouabain” in both SHR and WKY rats (Leenen et al., 1993). It is conceivable that increased endogenous “ouabain” is involved in the development of LVH.

### **III. Summary**

1. High salt intake induces a gradual and significant increase in blood pressure in Dahl S but not Dahl R rats. After 2 wk of high salt diet, mean arterial pressure in Dahl S rats is increased significantly compared to Dahl R rats. It is further increased to severe hypertension in Dahl S rats on 5 wk of high salt intake.

2. In Dahl S rats, high salt intake causes a marked rise in the LV weight especially after 5 wk of high salt diet, as well as a minor rise in RV weight, but only significant after 5 wk of high salt intake. In Dahl R rats, high salt intake does not cause cardiac hypertrophy.

3. In Dahl S rats on regular salt diet, ACE mRNA in the hypothalamus showed a modest but significant increase as compared to Dahl R rats at 9 weeks of age. High salt intake causes a significant increase in ACE mRNA and activity in the hypothalamus and pons, as well as in the LV in Dahl S but not Dahl R rats. After 5 weeks on high salt, ACE mRNA levels have increased about three-fold in the hypothalamus as well as in the LV, and two-fold in the pons as compared with Dahl R rats on high salt diet and Dahl S rats on

either diet.

4. Ang I in the hypothalamus and pons, as well as Ang II in the pons are similar in both strains. Ang II in the hypothalamus of Dahl S rats, however, is significantly lower than Dahl R rats. High salt intake does not cause significant changes in Ang I and II levels in the hypothalamus and pons in both strains.

On regular salt intake, cardiac Ang I and II are significantly higher in Dahl R versus S rats. High salt intake does not change cardiac Ang I and II in Dahl R or Dahl S rats.

5. On regular salt diet, PRA of Dahl R rats is significantly higher in Dahl R vs S rats. High salt intake causes a clear decrease in PRA in Dahl R, but only a minor nonsignificant decrease in Dahl S rats. On regular salt intake, plasma Ang I and II are markedly higher in Dahl R vs S rats with rather low levels in Dahl S rats. High salt intake causes a modest decrease in plasma Ang I in Dahl R with no effect on the low levels in Dahl S rats. High salt does not change plasma Ang II significantly in either strain.

6. In Dahl S rats on high salt intake, chronic blockade of brain "ouabain" with antibody Fab fragments prevents the increase in BP, as well as the increases in ACE mRNA and activity in the hypothalamus and pons in Dahl S rats, but does not prevent LVH as well as the increases in ACE mRNA and activity in the LV.

## CONCLUSIONS

This study demonstrates that high salt intake increases the expression of the ACE gene and its activity in the hypothalamus and pons, as well as in the hypertrophied hearts in Dahl S but not Dahl R rats. Cardiac Ang I and II in Dahl S rats on regular salt diet are significantly lower than in Dahl R rats, but high salt intake does not affect cardiac Ang I and II in both strains. In the brain, high salt intake does not cause significant changes in Ang I and II in either the hypothalamus and pons in both strains. The increase in ACE mRNA and activity in the brain and heart may contribute, however, to increased Ang II synthesis in specific nuclei of the brain or specific compartments of the heart, or may be relevant for other mechanisms such as degradation of bradykinin.

Blockade of brain “ouabain” with antibody Fab fragments prevents the increases in BP as well as ACE mRNA and activity in the hypothalamus and pons, but does not prevent LVH or the increases in ACE mRNA and activity in the LV in Dahl S rats on high salt diet. These results suggest that 1) LVH of Dahl S rats induced by high salt is at least partly independent of BP, and 2) increased brain ACE mRNA and activity depends on brain “ouabain”.

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