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Role of the renin–angiotensin–aldosterone system in the enhancement of salt sensitivity caused by prenatal protein restriction in stroke-prone spontaneously hypertensive rats

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Abstract

We previously demonstrated that maternal protein restriction during pregnancy enhanced salt sensitivity and shortened life span in stroke-prone spontaneously hypertensive rats (SHRSP). The present study was conducted to investigate the participation of the renin–angiotensin–aldosterone system in the development of salt sensitivity in the offspring of dams fed a low-protein diet during pregnancy. We used SHRSP offspring from dams fed a 20% casein diet (CN) or a 9% casein diet (LP) during pregnancy. The CN and LP SHRSP offspring were further subdivided into tap-water-drinking and 1%-saline-drinking groups from the postnatal 10th week. A remarkable elevation in blood pressure in response to salt loading was observed in the LP SHRSP offspring. The protein levels of CYP11B2, an enzyme for aldosterone synthesis, were markedly elevated in response to salt loading in the kidneys of LP offspring. Treatment of the LP offspring with an aldosterone receptor antagonist prevented the blood pressure from elevating and lengthened the average life span in LP offspring in response to the drinking of 1% saline. No difference in the activity of angiotensin-converting enzyme or in the protein level of the angiotensin type 1 receptor was found between the CN and LP offspring in either the tap-water-drinking or saline-drinking conditions. In conclusion, the increment of aldosterone production in response to high-salt loading may contribute to the elevated salt sensitivity of the offspring of protein-restricted dams.

Keywords: Prenatal protein restriction; SHRSP; Salt sensitivity; Angiotensin; Aldosterone

1. Introduction

Cerebral stroke, a complex disorder caused by a combination of genetic and environmental factors, remains one of the most common diseases and is a major cause of death in Japan. Studies in human and animal models have demonstrated that improvements in nutritional factors are effective for preventing cerebral strokes and can thus lengthen life span [1,2]. Moreover, a growing number of epidemiological and experimental studies provide strong evidence that prenatal or postnatal undernutrition or overnutrition results in an increased risk of developing cardiovascular disease in later life [3–5]. In rodents, maternal protein restriction is the most commonly used rat model to investigate the fetal programming of hypertension and has clearly demonstrated the elevation of blood pressure in the offspring. However, the relationship between maternal undernutrition including protein restriction and the onset of stroke is unclear. We previously examined the effect of prenatal protein restriction on blood pressure and stroke incidence using stroke-prone spontaneously hypertensive rats (SHRSP), which are generally regarded as a useful genetic model of hypertension and stroke [6]. Under the high-salt condition, SHRSP offspring from dams that had been fed a low-protein diet during pregnancy had a marked elevation in blood pressure and a shortened life span resulting from the occurrence of strokes. Namely, maternal protein restriction induced salt-sensitive hypertension leading to the acceleration of the onset of stroke in SHRSP.

The renin–angiotensin–aldosterone system (RAAS) plays a pivotal role in regulating blood pressure and electrolyte homeostasis. Abnormal alterations of the RAAS have been shown to be involved in hypertension in the offspring of dams fed a low-protein diet [7,8]. A reduction of plasma angiotensin II (Ang II) levels as well as renin messenger RNA levels was shown in newborn rats born to dams fed a low-protein diet during pregnancy [9]. On the other hand, the

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offspring of dams fed a low-protein diet (9% casein) during pregnancy exhibited increases in angiotensin-converting enzyme (ACE) activity in the lung [10] and serum [11] and increases in angiotensin type 1 receptor (AT_1R) protein expression in the kidney, as well as decreases in angiotensin type 2 receptor (AT_2R) in the kidney [12]. A recent study in prenatally protein-restricted offspring showed hypermethylation of the promoter region of AT_1R in the adrenal glands [13]. The RAAS probably contributes to the hypertension induced by prenatal protein restriction.

It is well known that Ang II is a pivotal component of the RAAS, which has a variety of physiological actions, including vasoconstriction and inflammation as well as the stimulation of aldosterone secretion. A growing body of evidence indicates that aldosterone makes a major contribution to the pathogenesis of hypertension [14,15]. In the present study, we highlighted the role of the RAAS in the high salt sensitivity observed in prenatally protein-restricted SHRSP offspring. We also further evaluated the RAAS components involved in the development of salt sensitivity in SHRSP offspring of dams fed a low-protein diet during pregnancy.

2. Methods and materials

2.1. Animals

This study was carried out in accordance with the guidelines of the Experimental Animal Care Committee of the Kinki University Faculty of Agriculture, and the management of the rats was conducted in compliance with the "Guidelines for Care and Use of Laboratory Animals" (Kinki University). Inbred SHRSPs were bred in our laboratory for use in this study. The rats were maintained at a room temperature of 23°C±1°C and a humidity of 55%±5% under a 12-h light/dark cycle (lighting from 0700 to 1900).

Virgin female SHRSP weighing 150–180 g were allowed to mate with sibling males of the same strain (weight ≥210 g; blood pressure 175–185 mmHg). The date of conception (day 0) was confirmed by the presence of a vaginal plug with sperm. Pregnant rats were pair-fed a control diet (20% casein diet; CN) or a low-protein diet (9% casein diet; LP) during pregnancy. The composition of the synthetic diets was described previously [6]. Dietary manipulations were performed only during pregnancy.

After confirmation of delivery, the dams were switched to a commercial diet (Funabashi SP; Funabashi Farm Co., Ltd., Chiba, Japan), and the pups were weighed. On postnatal day 4, the offspring were culled to yield a uniform litter size of 6. The pups were weaned at 4 weeks of age, and the male pups of both groups were then separated from the dams and given *ad libitum* access to tap water and a commercial diet. To avoid possible gender differences, only male pups were used. The pups were weighed once every week.

2.2. Experiment 1: salt loading

The male SHRSP offspring of dams fed the CN or the LP were divided from the postnatal 10th week into tap water groups (CN-W, n=10; LP-W, n=9) and salt-loaded groups (CN-S, n=10; LP-S, n=11); the latter were thereafter given 1% saline solution as drinking water. All the offspring were fed a commercial diet *ad libitum*. Blood pressure was measured once a week from 10 weeks of age. After preliminary warming at 37°C, blood pressure was measured using the tail-cuff method (BP-98A; Softron Inc., Tokyo, Japan). Seven days before the animals were killed, they were placed in individual metabolic cages, and urine was collected 24 h a day during the last 3 days. Urinary albumin concentrations were determined using a commercial assay (Wako Pure Chemical Industries Ltd., Osaka, Japan). Male SHRSP offspring at 12 weeks of age were administered anesthesia by intraperitoneal injection of sodium pentobarbital (Nembutal 30 $mg \cdot kg^{-1}$; Abbott, North Chicago, IL, USA).

2.3. Experiment 2: spironolactone treatment

Male SHRSP offspring of dams fed the CN or the LP received daily subcutaneous injections of 10 $mg \cdot kg^{-1} \cdot day^{-1}$ spironolactone (Wako Pure Chemical Industries, Ltd., Osaka, Japan), an aldosterone receptor antagonist, from 10 weeks of age until their natural deaths. The CN-SS (SS means salt drinking and spironolactone treated) and LP-SS groups and two other groups of offspring of dams fed CN and LP but not treated with spironolactone (CN-S and LP-S) were fed commercial diets and 1% saline solution *ad libitum*. Body weight and blood pressure were measured at 12 weeks of age.

2.4. Determination of ACE activity

The rats from experiment 1 were used at 12 weeks of age. Blood was collected from the abdominal aorta of anesthetized rats into heparinized test tubes. The right kidney, the aorta and the mesentery were rapidly removed, gently flushed with saline containing heparin and extirpated immediately after they were killed. The tissues were then homogenized at 4°C in 0.05 mM Tris-HCl buffer (pH 7.8) with 0.5% Nonidet P40 and centrifuged at 20,000*g* for 20 min at 4°C. The supernatant was used for enzyme measurements after the protein concentration was determined and was stored at −80°C. ACE activity was measured within 3 days of sacrifice.

ACE activities was measured by a modification of the method of Horiuch et al [16] as described previously [17]. We measured the amount of hippuric acid derived from Hip-His-Leu (Peptide Institute, Osaka, Japan) using high-performance liquid chromatography (a Shimadzu LC-6A pump and SPD-10A spectrophotometric detector, a Cosmosil 5C18-AR-II column). The data were presented in terms of the amount of enzyme catalyzing the release of 1 μM of hippuric acid from Hip-His-Leu per minute at 37°C.

2.5. Protein extraction and Western blotting analysis

The left kidney and adrenal gland were rapidly removed and homogenized in 5 volumes of homogenizing buffer containing 10 mM $NaHCO_3$, 250 mM sucrose, 5 mM NaN_3 and 1% (vol/vol) protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). The homogenate was centrifuged at 1300 *g* and 4°C for 10 min, and the supernatant was centrifuged at 20,000*g* and 4°C for 20 min and then stored at −80°C.

The homogenized kidney and adrenal gland were denatured in sample buffer (1% mercaptoethanol, 0.15 M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 2% glycerol, 0.05% bromophenol blue) and boiled for 5 min, followed by 8% SDS-polyacrylamide gel electrophoresis. Twenty micrograms of proteins were loaded per lane. The total protein content in the homogenized kidney, adrenal gland and brain was determined by the Lowry method using bovine serum albumin as a standard. After the SDS-polyacrylamide gel electrophoresis, the separated protein samples were transferred onto polyvinylidene fluoride (PVDF) membranes using semidry blotting methods (1 mA/cm^2, 90 min). The transferred PVDF membranes were blocked with 10 mM sodium phosphate-buffered saline (pH 7.5) containing 0.1% Tween-20 and 5% skim milk. The primary antibodies used in immunoblotting were as follows: antirat AT_1R polyclonal antibody (1:500, N-10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), antirat AT_2R polyclonal antibody (1:100, C-18; Santa Cruz Biotechnology, Inc.) and antirat cytochrome p450 aldosterone synthase (CYP11B2) monoclonal antibody (1:100, MAB6021; Millipore Corporation, Billerica, MA, USA). The PVDF membranes were incubated with appropriate secondary antibodies conjugated to HRP (1:2000; BIO-RAD Laboratories, Inc., Tokyo, Japan). The signals were detected using enhanced chemiluminescence Western blotting reagents (GE Healthcare, Tokyo, Japan). The chemiluminescent signals obtained were recorded on X-ray film (hyper film; GE Healthcare). The bands were quantified by densitometry using NIH image 1.63 software.

2.6. Statistical analysis

The data are presented as means±S.D. Data for single group comparisons were statistically analyzed using Student's t test. Data for multiple group comparisons were statistically analyzed using one-way analysis of variance (ANOVA). When required, directed pairwise comparisons of individual groups were performed using Tukey's test and the Bonferroni method. Kaplan-Meier plots of survival were analyzed using the log-rank test. All the statistical analyses were performed using a commercially available statistical package (SPSS version 12.0 for Windows; SPSS Japan, Inc., Tokyo, Japan). A probability value of P<.05 was considered statistically significant.

3. Results

3.1. Growth

Maternal mild protein restriction suppressed maternal weight gain at gestational day 21 (CN, 71±11 g; LP, 55±12 g; P<.01), but there were no differences in the birth weight of pups between the CN (male, 5.0±0.6 g; female, 4.8±0.6 g) and LP (male, 4.9±0.6 g; female, 4.5±0.4 g) groups. No difference was observed in the mean litter size at birth among groups. Thereafter, the growth of offspring was unaffected by maternal protein restriction; the body weights of the offspring in the CN and LP groups were almost the same at the postnatal 10th week (CN male, 207±20 g; LP male, 203±13 g) and thereafter.

3.2. Blood pressure after salt loading

At the age of 10 weeks, the offspring of the CN and LP groups were each further subdivided into tap-water-drinking and

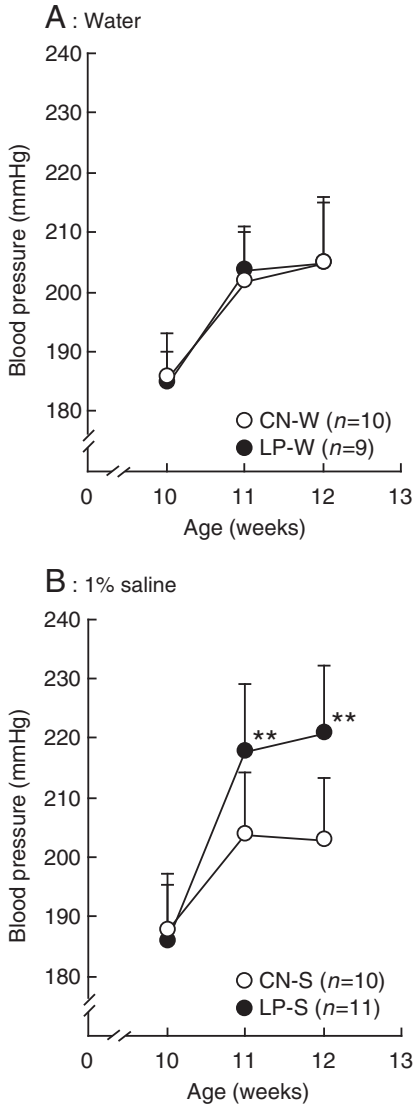

Fig. 1. The blood pressure of SHRSP exposed to LP diet *in utero* maintained on drinking water (A) or 1% saline solution (B) from 10 weeks of age. Offspring were exposed to a CN or LP diet *in utero* and were maintained on water or 1% saline solution from 10 weeks of age. To avoid potential gender differences, only male pups were studied. The systolic blood pressure was measured using the tail-cuff method. Data were found to be significantly different using two-way ANOVA. $^{**}P<.001$.

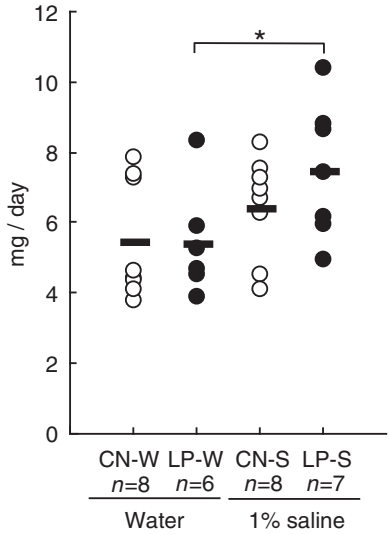

Fig. 2. Urinary albumin excretion of SHRSP exposed to LP diet *in utero* maintained on drinking water or 1% saline solution at 12 weeks of age. Offspring were exposed to a CN or LP diet *in utero* and were maintained on water or 1% saline solution from 10 weeks of age. To avoid potential gender differences, only male pups were studied. Data were found to be significantly different using two-way ANOVA. $^{*}P<.05$.

1%-saline-drinking groups. At this point, postnatal 10th week, systolic blood pressure elevated to over 180 mmHg in the offspring of the CN (male, 187±6 mmHg; $n=20$) and LP (male, 186±5 mmHg; $n=20$) groups, but no difference was observed between them (Fig. 1A, B). In the tap-water-drinking condition, the blood pressure was further elevated to 205 mmHg at 12 weeks of age, and no difference was observed between the CN-W and LP-W groups (Fig. 1A). In response to saline drinking, the blood pressure of the LP-S group (221±11 mmHg) was significantly elevated ($P<.001$) compared with that of the CN-S group (203±7 mmHg) at 12 weeks of age (Fig. 1B).

3.3. Urinary albumin excretion

Albumin excretion in urine was not significantly different between the CN-W and LP-W groups with tap water drinking at 12 weeks of age (Fig. 2). Saline drinking did not affect urinary albumin excretion in the CN-S group but significantly increased it in the LP-S group ($P<.05$).

3.4. ACE activity after salt loading

No differences were found in ACE activity in the plasma, kidney, aorta or mesentery between the tap-water-drinking CN-W and LP-W groups (Fig. 3). Saline drinking did not affect the ACE activities in the plasma or tested tissues of the offspring. Moreover, maternal protein restriction did not affect ACE activity in the plasma, kidney, aorta or mesentery.

3.5. AT_1R and AT_2R protein expression in the adrenal gland, kidney and brain

The protein levels of AT_1R and AT_2R in the kidney and adrenal gland of the four groups were examined by Western immunoblot analyses. No difference was observed in the levels of AT_1R in the kidney or adrenal gland between the CN-W and LP-W groups at 12 weeks of age (Fig. 3A, B). Saline drinking did not affect AT_1R expression.

In both the adrenal gland and kidney, the LP-W group showed down-regulation of AT_2R in the tap-water-drinking condition (Fig. 4C, D). The effect of maternal low protein was more prominent in the kidney than in the adrenal grand. Salt loading was associated with marked suppression of the AT_2R levels in CN-fed groups. However, in both organs, the AT_2R levels in the saline-drinking LP group (LP-S) were significantly higher than those of CN-S group, which were similar to those of the tap-water-drinking CN-W group.

3.6. Renal–aldosterone synthesis

In the tap-water-drinking group, the renal CYP11B2 protein expression levels in the LP-W group at 12 weeks of age were similar to those observed in the CN-W group (Fig. 5). Salt loading was associated with marked elevation of the renal CYP11B2 protein

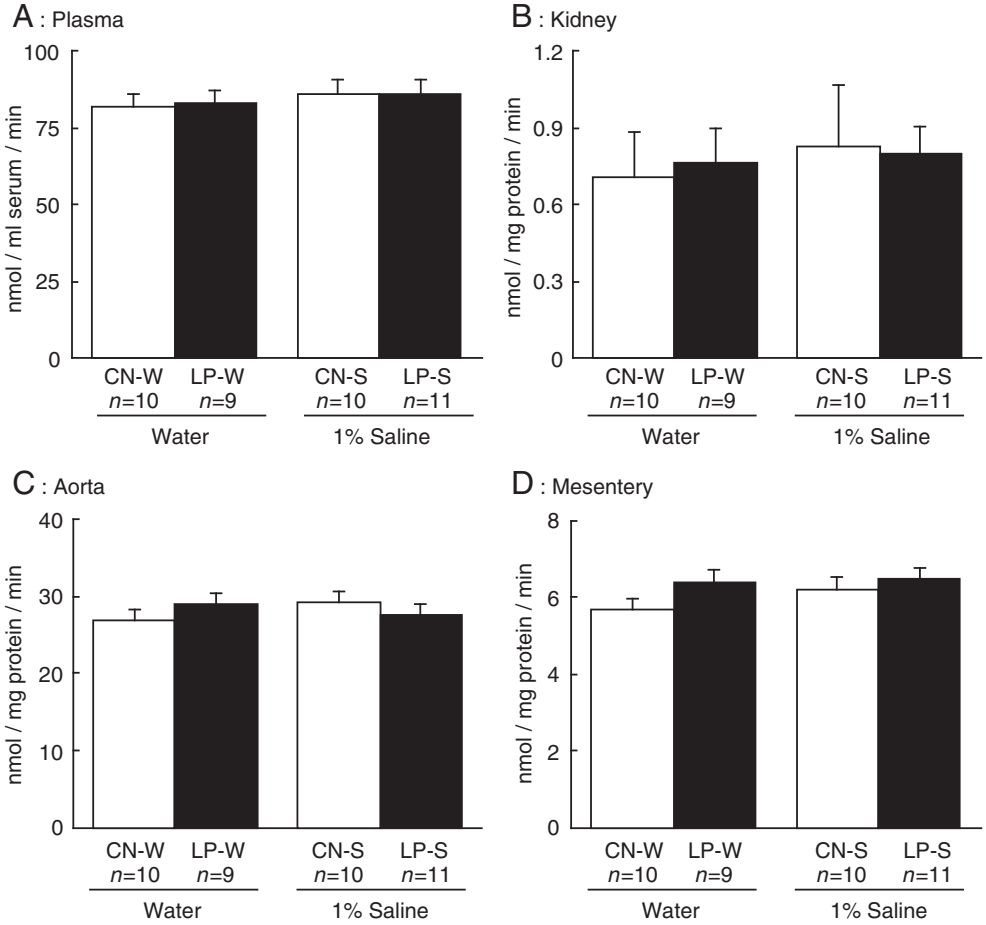

Fig. 3. ACE activities in the plasma (A), kidney (B), aorta (C) and mesentery (D) of SHRSP exposed to LP diets *in utero* maintained on drinking water or 1% saline solution at 12 weeks of age. The ACE activities of plasma or tissues were measured using the synthetic substrate Hip-His-Leu. The offspring were exposed to a CN or LP diet *in utero* and maintained on water or 1% saline solution from 10 weeks of age. To avoid potential gender differences, only male pups were studied. Data are presented as means±S.D. Data were found to be significantly different using two-way ANOVA.

expression in both the CN-S and LP-S groups. The elevation rate in the saline-drinking LP-S group was significantly higher ($P<.01$) than that in the saline-drinking CN-S group.

3.7. Effect of spironolactone administration on LP-exposed SHRSP offspring of dams fed 9% casein diet during pregnancy

With the initiation of 1% saline drinking, the CN and LP SHRSP offspring were treated daily with spironolactone (10 $mg \cdot kg^{-1} \cdot day^{-1}$) from 10 weeks of age to their natural death. Table 1 shows the blood pressure and body weight in the spironolactone-treated or untreated CN and LP SHRSP offspring at 12 weeks of age. Without spironolactone treatment, blood pressure was significantly increased in the saline-drinking LP-S group compared with the saline-drinking CN-S group. However, spironolactone treatment canceled the effect of LP on blood pressure (CN-SS and LP-SS). There were no differences in the average life span after salt loading between the CN-SS (108±73 days) and LP-SS (72±27 days) groups under saline-drinking and spironolactone-treated condition.

4. Discussion

We previously demonstrated that maternal protein restriction in SHRSP led to salt-sensitive hypertension and a reduced life span [6]. The aim of the current study was to investigate the involvement of RAAS in the development of the high salt sensitivity observed in LP SHRSP offsprings. To investigate the effect of maternal mild protein restriction on salt sensitivity, the CN and LP SHRSP offsprings were further subdivided into a tap-water-drinking group and a 1%-saline-drinking group from the postnatal 10th week. One of the main findings of the present study was that the aldosterone receptor antagonist suppressed the elevation of blood pressure in response to salt loading in the LP SHRSP offsprings. The protein level of CYP11B2, an enzyme for aldosterone synthesis in the kidney, was markedly elevated in response to salt loading in the LP SHRSP offspring. However, no differences in the ACE activity or AT_1R were found between the CN and LP offsprings in the water-drinking condition or saline-drinking condition. AT_2R protein expression was lower in LP offspring than CN offspring in the tap-water-drinking groups but was higher in LP offspring than CN offspring in saline-drinking groups.

Prenatal protein nutrition is recognized as an important determinant of blood pressure levels in later life. Prenatal mild (9% casein) and severe (6% casein) protein restriction during the gestation period have been the most commonly used animal models for fetal programming of hypertension [5,18,19]. SHRSP shows an age-related increase in blood pressure to approximately 250 mmHg after maturation, which results in a 99% mortality rate from stroke. There were no differences in blood pressure between the CN and LP SHRSP offspring under the tap-water-drinking condition, the same result as in our previous study [6]. Blood pressure showed an age-related increase in SHRSP in the present study and was higher than 180 mmHg after the postnatal 10th week irrespective of maternal

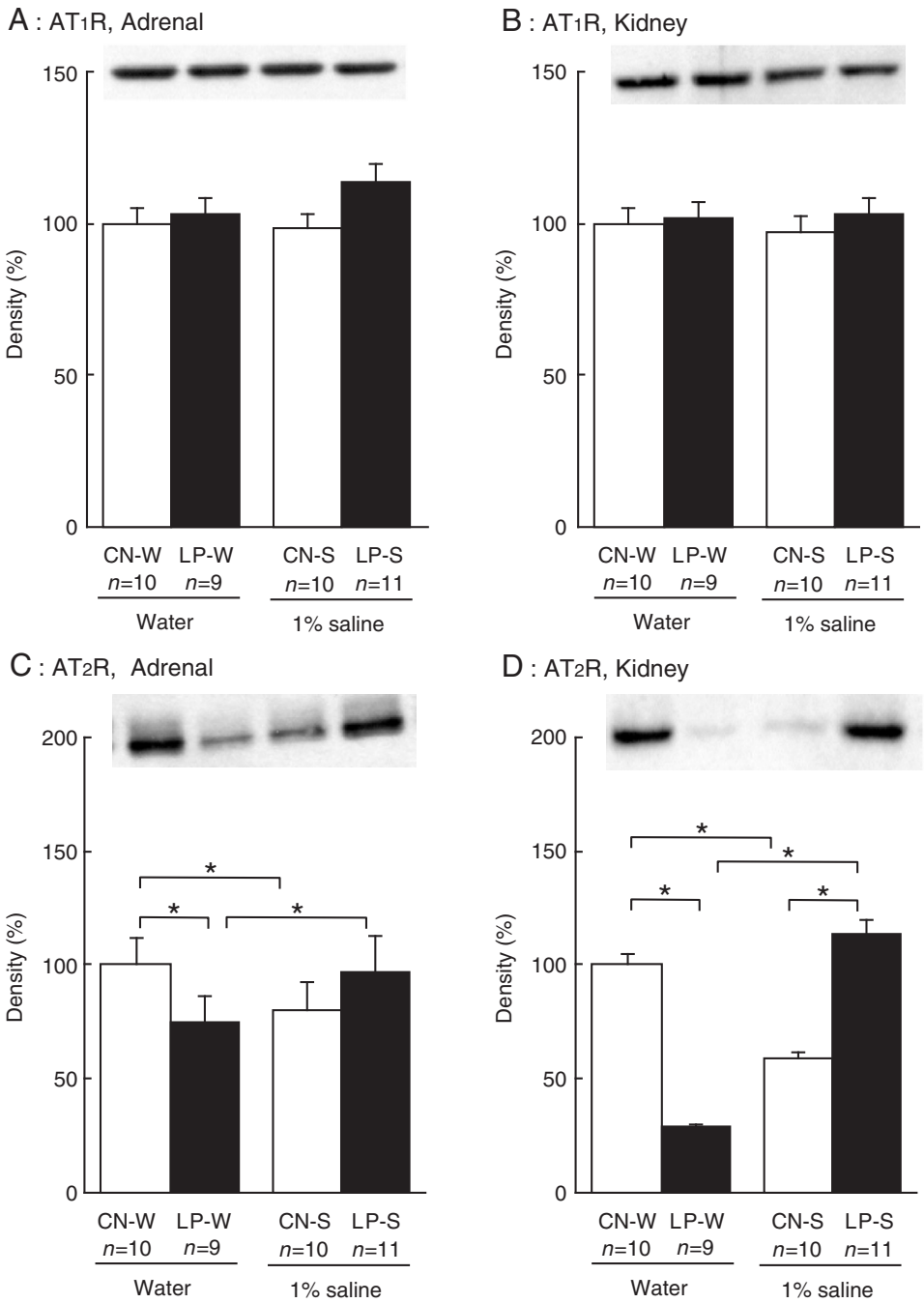

Fig. 4. Renal and adrenal AT_1R and AT_2R protein expression in SHRSP exposed to LP diets *in utero* maintained on drinking water or 1% saline solution at 12 weeks of age. Offspring were exposed to a CN or LP diet *in utero* and were maintained on water or 1% saline solution from 10 weeks of age. To avoid potential gender differences, only male pups were studied. Data were found to be significantly different using two-way ANOVA. *$P<.05$.

protein nutrition. In the case of normotensive rats, others have reported that maternal protein restriction results in a modest elevation in blood pressure of approximately 15–20 mmHg (120–140 mmHg) in adult offspring [19–21]. The lack of influence of prenatal protein restriction on blood pressure in tap-water-drinking SHRSP may be attributable to the innate hypertension of SHRSP.

Salt was loaded from 10 weeks of age. As a result, a remarkable elevation in blood pressure was observed in response to salt loading in the SHRSP offspring with mild protein restriction (9% casein) *in utero*. Lines of evidence show that salt intake is related to the development of hypertension and cardiovascular diseases [22]. Dietary sodium acts as a direct trigger for cardiovascular diseases in salt-sensitive patients and salt-sensitive animal models such as Dahl rats [23] and SHRSP [14,24]. Previously, we demonstrated that high salt intake reduced the life span of LP SHRSP offspring [6]. In the present study, increased blood pressure and albuminuria were concomitantly observed in LP SHRSP offspring after only 2 weeks of salt loading. It can be speculated that high salt intake by SHRSP with prenatal protein restriction causes relatively rapid cardiovascular damage through a mechanism independent of the chronic effect of the elevated blood pressure.

The major roles of RAAS are the regulation of vascular contractility and electrolyte balance. Recent studies have shown that prenatal protein restriction affects postnatal programming of hypertension

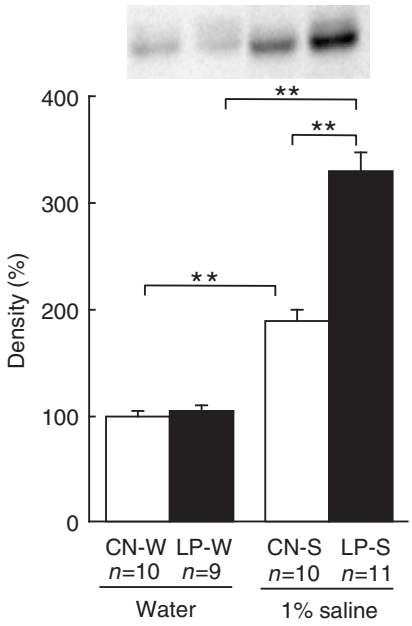

Fig. 5. Renal CYP11B2 protein expression in SHRSP exposed to LP diets *in utero* maintained on drinking water or 1% saline solution at 12 weeks of age. Offspring were exposed to a CN or LP diet *in utero* and were maintained on water or 1% saline solution from 10 weeks of age. To avoid potential gender differences, only male pups were studied. Data were found to be significantly different using two-way ANOVA. $^{*}P<.05$, $^{*}P<.001$.

through alterations of RAAS [8,11,12]. Maternal protein restriction during pregnancy has been linked to elevated serum and pulmonary ACE activities [11]. In the case of hypertension induced by prenatal protein restriction in normotensive rats, AT_1R protein and AT_1a messenger RNA were up-regulated in the kidney and were likely to contribute to elevated blood pressure [12,25]. In our results, AT_1R expression and ACE activities in plasma and tissue were not affected by prenatal protein restriction, and the activity was the same between the CN and LP groups. It may be due to the interplay of the genetic background in SHRSP, which shows a high level of AT_1R expression [26], tissues ACE activity [17] and Ang II concentration [27]. The RAAS activation in various tissues contributes to the pathogenesis of hypertension and vascular injury in SHRSP. Thus, in the SHRSP strain, additional activation in AT_1R and ACE may not be elicited by prenatal protein restriction.

It is well recognized that RAAS components exist in local organs such as the kidney, brain and blood vessels and that these organs are capable of producing aldosterone locally. CYP11B2 is known as the key enzyme for aldosterone synthesis. We detected CYP11B2 in the kidneys of both the CN and LP SHRSP offspring by immunoblotting. The increment of CYP11B2 expression in association with saline drinking was found to be more dramatic in LP SHRSP offspring than in CN offspring. In addition, the plasma aldosterone levels were decreased by high salt intake in the CN offspring, but this decrease did not occur in the LP SHRSP offspring (supplemental data). The plasma aldosterone level was significantly increased in the saline-drinking LP group compared with the saline-drinking CN group (supplemental data). These results indicate that the suppression of plasma aldosterone by salt intake is lifted by prenatal protein restriction during fetal life, which means that the activity of aldosterone to regulate renal function is not maintained even under salt loading. These findings are consistent with the finding that normotensive offspring from dams fed a 6% casein diet during pregnancy showed increased blood pressure under high salt intake with higher plasma aldosterone levels than the control group [28]. In an epidemiological study, Reynolds et al. [29] have reported that low birth weight in men and women is associated with increased plasma aldosterone levels and that blood pressure is related to a single polymorphism in the gene encoding aldosterone synthase (CYP11B2). Moreover, urinary aldosterone levels decreased after salt loading in normotensive individuals but not in hypertensive individuals [30]. We assumed that the enhanced salt sensitivity in the LP SHRSP offspring was linked with abnormal aldosterone production under the high-salt condition.

Table 1
Effects of spironolactone on blood pressure and body weight in saline-drinking controls and low-protein-exposed SHRSP offspring at 12 weeks of age

	Untreated saline drinking		Spironolactone-treated saline drinking	
	CN-S (n=6)	LP-S (n=7)	CN-SS (n=6)	LP-SS (n=7)
Body weight (g)	251±13	242±14	244±13	242±8
Blood pressure (mmHg)	215±5	230±6**	217±11	217±8

Data are means±S.D.
** $P<.01$ versus untreated saline drinking CN-S rats.

Salt-sensitive hypertension is related not only to the elevation of blood pressure but also to the induction of end-organ damage and is also considered to be a high risk factor for cardiovascular diseases [31–33]. Our results demonstrated that SHRSP offspring exposed to a low-protein diet during fetal period showed high susceptibility to cardiovascular damage in response to salt loading. Urinary albumin excretion was increased in the LP SHRSP offspring after 2 weeks of salt loading. In addition, we previously demonstrated the occurrence of a shortened life span in saline-drinking LP SHRSP offspring [6]. These data implied that high salt intake directly elicited renal injury independent of high blood pressure in LP SHRSP offspring. Under high-salt conditions, Ang II and aldosterone cause direct damage to target organs and play pathophysiological roles in hypertension and cardiovascular diseases [15,32]. Administration of spironolactone lengthened the average life span of both the CN- and LP groups (data not shown); no significant difference was found in the life span between the CN-SS and LP-SS groups. In addition, the elevation of blood pressure by LP was suppressed in the LP-SS group. These data implied that high salt intake directly elicited renal injury independent of high blood pressure in LP SHRSP offspring. These observations are consistent with the major role aldosterone plays as a hormonal mediator of vascular injury. Rocha et al. [15] established that the administration of an aldosterone receptor antagonist resulted in marked reductions in the extent of renal damage and the expansion of life span in salt-loaded SHRSP in the absence of lowered blood pressure.

CYP11B2 expression and aldosterone synthesis are mainly regulated by Ang II via AT_1R [34]. In our results, ACE activity and AT_1R expression did not differ between the CN and LP groups after their offspring were given either saline solution or tap water. It can be assumed that ACE and AT_1R are not involved in the abnormal aldosterone regulation in response to the salt loading seen in the prenatal protein-restricted SHRSP offspring.

Ang II evokes vasodilatation and the inhibition of cell growth, which are mediated by AT_2R [35]. Increased AT_2R expression was observed in numerous models of kidneys that were damaged due to protein overloading [36] and renal ablation [37]. In addition, Vazquez et al. [38] demonstrated that a time-dependent increase in AT_2R expression occurred after renal ablation. In the present study, AT_2R was increased in the saline-drinking LP SHRSP offspring exhibiting albuminuria. This increased AT_2R expression might result in renal damage in the saline-drinking LP group. Microalbuminuria is both a sign of early renal damage and a marker of inflammation [39]. Some evidence has suggested that AT_2R up-regulation is associated with the

inflammatory response [40,41], but the precise role of AT_2R in the development of inflammation is still not clear.

The results of the present study showed a decrease in the AT_2R protein expression in the LP SHRSP offspring under tap-water-drinking conditions without elevated blood pressure. We assume that the AT_2R-mediated regulation of chronic blood pressure seems to be minimally influenced in the water-drinking SHRSP. Our data show that chronic depression of AT_2R does not act on rising blood pressure in prenatal protein-restricted SHRSP offspring of rat strains that have an overexpression of AT_1R. There was no change in basal blood pressure in the AT_2R knockout mice [42] and rats administrated AT_2R antisense [43]. AT_2R is coupled to tyrosine phosphatase and inhibits the tyrosine kinase activities in AT_1R signaling, leading to the inhibition of AT_1R activation [44–46]. A similar phenomenon was seen in the hypertensive model of intrauterine severe protein restriction (6% casein), in which reduced AT_2R protein expression and increased AT_1R protein expression were observed in the kidneys of offspring at 4 or 16 weeks of age [25]. Mesquita et al. [47] demonstrated that low-protein-exposed progeny showed a lower expression of AT_1R and AT_2R in the kidney but a high expression in the adrenal. While the down-regulation of AT_2R was commonly observed in the prenatal protein-restricted normotensive model [20,25,47] and in our model, different results were obtained with regard to the amount of AT_1R observed. RAAS components show different levels of expression in the process of prenatal and postnatal development. The AT_1R expression was also linked to the progression of hypertension and growth status [48]. The expression level of AT_1R differs in each tissue [49]. The discrepancy may be due to the differences in age at the time the measurements were taken and/or the level of protein restriction (9% vs. 6% casein) during pregnancy. The underlying relationship between the decreased AT_2R and salt sensitivity in LP SHRSP offspring needs to be further elucidated.

We speculate that the depression of AT_2R is related to the inflammatory action that occurs after high salt intake. AT_2R knockout mice develop more renal injury and increased proteinuria as compared with wild-type mice [50]. AT_2R signaling is coupled with the production of endothelial nitric oxide synthase and cGMP via the bradykinin B_2 receptor [51]. A high salt diet induced impairment of endothelial nitric oxide synthase activation and an increase in oxidative stress, as the results aggravated endothelial functions [52]. However, the physiological roles of AT_2R are still controversial. We need to further study the relationship between the reduction of AT_2R and salt sensitivity.

In conclusion, an increment of aldosterone production was found in prenatal protein-restricted SHRSP offspring that exhibited elevated blood pressure and urinary albumin excretion under a high-salt condition. These blood pressure elevations in response to high salt intakes were suppressed by the spironolactone treatment administered to prenatal protein-restricted SHRSP offspring. We did not assume that high salt sensitivity was induced only by aldosterone. Other factors may be attributed to the development of salt sensitivity in our model. This may imply that there are other factors that mediate the effect of prenatal protein restriction on the development of cardiovascular diseases in SHRSP offspring exposed to a low-protein diet *in utero*. The effect of maternal protein restriction during pregnancy on the ACE activity and AT_1R expression counteracted the genetic up-regulation of the ACE activity and AT_1R expression in SHRSP. On the other hand, we found decreased AT_2R expression in the water-drinking LP group but did not confirm the effects of prenatal protein restriction on salt-sensitive hypertension in SHRSP. Thus, further study is needed to clarify the mechanisms governing the increase in salt sensitivity that occurs due to prenatal protein restriction.

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