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Catecholamine and Corticosteroid Secretion and Gene Expression of the Synthesizing Enzymes in Adrenal Glands of SHRSP and WKY in Response to Cold Stress

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To clarify the role of adrenal glands in hypertensive animals, levels of catecholamines and corticosteroids in plasma and the mRNA expression of the associated enzymes were measured in 6 and 9-week-old stroke-prone spontaneously hypertensive rats (SHRSP) and normotensive Wistar Kyoto rats (WKY) following cold stress. With and without cold stress immersing in cold water at 4°C for 15 sec, catecholamines, adrenocorticotropic hormone (ACTH) and corticosteroids in plasma and mRNA expression in adrenal glands were measured using high performance liquid chromatography (HPLC), enzyme immunoassay (EIA) and DNA microarray, respectively. L-dopa, dopamine and adrenaline in plasma increased more in SHRSP than WKY at 6 and 9 weeks of age after cold stress. *Th, Ddc* and *Dbh* mRNAs were upregulated in the adrenal glands of SHRSP after cold stress, more apparent at 6 weeks than at 9 weeks of age. Corticosterone and aldosterone in plasma increased in both SHRSP and WKY, but this effect was more apparent in SHRSP after elevation of ACTH evoked by cold stress. Expressions of *cyp11a1* and *cyp21a1* mRNAs were upregulated in both SHRSP and WKY at 6 weeks of age after cold stress. We conclude that l-dopa, dopamine, and adrenaline were synthesized following induction of *Th*, *Ddc* and *Dbh* mRNAs. Corticosterone and aldosterone in plasma increased following the induction of *cyp11a1* and *cyp21a1* mRNAs which are stimulated along with ACTH elevation following cold stress in young SHRSP more than WKY. This difference may be related to the initiation and/or development of hypertension in SHRSP in normal condition and/or during stress.

Key words: Adrenal glands, hypertension, catecholamines, corticosteroids, DNA microarray, pituitary-adrenocortical axis

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INTRODUCTION

Stroke-prone spontaneously hypertensive rats (SHRSP) (Okamoto *et al*., 1974; Chiba and Ezaki, 2010) which were established from the spontaneously hypertensive rat (SHR) (Okamoto and Aoki, 1963; Burgi *et al*., 2010) strain by selective inbreeding for stroke proneness, exhibit severe high blood pressure. This blood pressure is usually more than 250 mmHg for systolic blood pressure (SBP) at 20 weeks of age. Many studies have been performed over the last four decades to clarify the causes of initiation and development of hypertension, but detailed mechanisms remain elusive. Hypotheses regarding the causes of hypertension in SHRSP often involve a role for the adrenal glands. Therefore, over the last decade, we have investigated the role of the adrenal glands in relation to hypertension by measuring catecholamines in plasma and urine in SHRSP and normotensive Wistar Kyoto rats (WKY) (Maeda *et al*., 1999; Higashino and Ooshima, 2002; Higashino *et al*., 2004). It has been reported that excessive amounts of noradrenaline and adrenaline are rapidly released into the bloodstream from sympathetic nerve endings and the adrenal medulla when SHRSP are active not only at night but also in the daytime and following cold stress. Kumai *et al*. (1999) also reported the same characteristics in SHR and SHRSP. The adrenal glands play a role in the sympathetic-adrenomedullary system and/or the pituitary-adrenocortical axis as described by Selye (1950), Nyhuis *et al*. (2010) and Redina *et al*. (2010). Some types of stress loadings are indispensable for elucidating the role of the adrenal glands when performing precise and accurate measurements in the animals. Multiple stress loading methods have been reported, including air-jet blowing (Yamazato *et al*., 2006), hypoxia (Tai *et al*., 2009), hypoglycemia (Adams *et al*., 2005), hyperbaric oxygenation (Nakada *et al*., 1984), restrained immobilization (Kubovcakova *et al*., 2006), isolation in caging (Horie *et al*., 1991; Gavrilovic *et al*., 2008), contusion (Takahashi, 2008), organic solvent inhalation (Gotohda *et al*., 2000) and cold stress (Baruchin *et al*., 1993; Cui *et al*., 2004). When certain types of stress are loaded to the body, the adrenal glands secrete two types of hormones to counter the stress. That is, one is catecholamines such as adrenaline produced by the medulla via the sympathetic-adrenomedullary system and the other is corticosteroids such as corticosterone and aldosterone produced by the cortex via the pituitary-adrenocortical axis (Selye, 1950; Nyhuis *et al*., 2010). When certain types of stress are loaded to the body, the adrenal glands secrete two types of hormones to counter the stress: Catecholamines such as adrenaline produced by the medulla via the sympathetic-adrenomedullary system and corticosteroids such as corticosterone and aldosterone produced by the cortex via the pituitary-adrenocortical axis (Selye, 1950; Nyhuis *et al*., 2010). It was hypothesized that these two types of hormones are tightly related to the initiation and development of hypertension. Therefore, we measured these two types of hormones and the corresponding mRNA expression levels of the synthesizing enzyme for each hormone following cold stress in young and slightly older SHRSP (6 and 9 weeks) and compared the levels to age-matched WKY. The data provide more information about the possible role of the adrenal glands in spontaneously hypertensive animals.

MATERIALS AND METHODS

Animals: Studies were performed with two rat strains at 6 and 9 weeks of age. Male WKY/Kpo and SHRSP/Kpo (Okamoto *et al*., 1974) were used as a normotensive wild-type control strain and as stroke-prone hypertensive model rats, respectively. WKY and SHRSP were purchased from the Animal Center of Kinki University School of Medicine. Strains and numbers of rats used in this study were as follows. That is, fifteen rats were used each in male 6 and 9-week-old WKY/kpo and SHRSP/kpo. All animals used in this experiment were handled with due care according to the guidelines established by the Japanese Association for Laboratory Animal Science which complies with international rules and policies. This study was performed under an approval (KAME-19-078 at April 1, 2010) of the Animal Care and Use Committee of Kinki University. Measures were taken to minimize pain and discomfort of the experimental animals.

Measurements of Systolic Blood Pressure (SBP) and Heart Rate (HR): SBP and HR were measured with a tail-cuff method (Yamakoshi *et al*., 1979) using a UR-5000 photoelectric detector (Ueda, Tokyo, Japan) after warming the body to 35°C for 5 min in a heater box. Three consecutive SBP and HR measurements were taken each time and averaged.

Analysis of body temperature, SBP and HR changes using a telemetric acquisition system: Continuous ambulatory body temperature, SBP and HR were monitored in unrestricted animals with a Dataquest A.R.T. system (Data Sciences International, St. Paul, MN, USA). A newly calibrated implant (TL11M2-C50-PXT, Data Sciences) was surgically placed in the descending aorta of rats that were anesthetized with sodium pentobarbital (50 mg kg^{-1}, intraperitoneally (i.p.)). The

output was relayed from the receiver through a consolidation matrix to a personal computer. After recovery from surgery for more than 5 days, body temperature, SBP and HR were monitored every 5 sec before and after cold stress.

Procedures for cold stress loading, isolation of the adrenal glands and taking blood samples: Cold stress loading was performed as follows. After measuring SBP and HR, rats were immersed in cold water at 4°C for 15 sec, and then their hair was wiped with a towel and blown with hot air. Rats in one group (n = 3) were anesthetized with 50 mg kg^{-1} sodium pentobarbital i.p. 25 min after cold stress and the adrenal glands were isolated from the body via a laparotomy procedure to detect mRNA expression using DNA arrays. Adrenal glands from other rats not exposed to cold stress were also isolated under pentobarbital anesthesia. To measure catecholamines, adrenocorticotropic hormone (ACTH) and corticosteroids, 0.3 mL of blood was taken from the tail vein of the rats each consisting 6, two times for catecholamine measurements before and 60 min after cold stress and three times for ACTH and corticosteroids measurements before and 30 and 60 min after cold stress after measuring the SBP and HR. All treatments were performed at 10:00 am to avoid the effects of circadian rhythms.

Catecholamine measurement: Fifty microliters of plasma from the blood obtained from a tail vein was acidified by adding one volume of 1.0 N perchloric acid (PCA) solution containing 10 ng of 3,4-dihydroxybenzylamine hydrobromide (DHBA) for recovery calculation. The top layer in the tube was filtered through an Ultrafree-MC filter (Millipore Corp., Bedford, MA, USA). After diluting the filtrates with 400 volumes of 0.1 N PCA solution, catecholamine metabolites were detected with a Neurochem-HPLC Coullochem electrode assay system (ESA, Inc., Bedford, MA, USA) and expressed as ng mL^{-1} of plasma.

ACTH, corticosterone and aldosterone measurement: Concentrations of ACTH, corticosterone and aldosterone were measured with EIA kits for ACTH (Peninsula Laboratories, San Carlos, CA, USA), corticosterone and aldosterone (Cayman Chemical, Ann Arbor, MI, USA) in the plasma taken before and 30 and 60 min after cold stress.

Tissue processing and RNA isolation: After the adrenal glands were harvested under sodium pentobarbital anesthesia (50 mg kg^{-1} i.p.) the tissues were stored in RNA later stabilization solution (QIAGEN GmbH, Hilden, Germany) at-20°C until use. Adrenal glands were then homogenized twice at a pitch speed of 22 strokes/sec for 2 min in a 2 mL plastic tube with 5 mm diameter glass beads with a Qiagen Tissue Lyser (Retsch Gmbh and Co., Haan, Germany). Total RNA was extracted with an RNeasy Mini kit (QIAGEN GmbH) according to the manufacturer's protocol. The RNA quality was checked with RNA Nano Chips (Agilent Technologies, Waldbornn, Germany) using an Agilent 2100 Bioanalyzer and high-quality RNA was used for microarray experiments. For DNA microarray analysis, RNA from adrenal glands obtained from three rats per group of WKY and SHRSP was used in each DNA array analysis.

Analysis of mRNA expression profiling with DNA oligonucleotide microarrays: To examine gene expression profiles in the adrenal glands, we synthesized RNA fragments labeled with cyanine 3-CTP (PerkinElmer, Boston, MA, USA) from 1 μg of DNase I-treated total RNA with a Low RNA Input Amplification kit (Agilent Technologies). We hybridized the synthesized RNA fragments to a Whole Rat Genome Microarray (4 x 44k formatted) (Agilent Technologies) in a rotor oven (Agilent Technologies) for 17 h at 65°C, followed by washing. The hybridized slides were scanned with an Agilent GenPix Scanner 4000 (Agilent Technologies). The data were extracted and the overall raw signal intensities on each array were normalized to the median value of all rat probes with BRB-Array Tool software ver. 3.7.0. (Biometric Research Branch) (Simon *et al*., 2007). A significance level ($p<0.01$) for each probe was set using Student's univariate t-test.

Annotation of differentially expressed genes: We performed a BLASTN search of the NCBI RefSeq database, employing corresponding 60-nucleotide probes (NCBI, GEO accession: GPL7294) to identify homologous genes with functional annotations (Altschul *et al.*, 1997). After running the BLASTN search, we defined those clones showing either a score higher than 50 or an E-value below 5e-05 as annotated clones.

Statistical analysis: Values were expressed as the Mean±SEM. Comparisons between means of data in each group were made with a Student's t-test for comparison between two groups and one-way analysis of variance (ANOVA) and Scheffe's multiple comparisons test (Pan and Kupper, 1999) for comparison between more than three groups. Differences were considered significant at $p<0.05$.

We conducted this study in the Department of Pharmacology, Kinki University School of Medicine, Osaka, Japan and performed the experiments in a period of April 1, 2008 to September 30, 2010 for 2.4 years.

RESULTS

SBP, HR, body weight and weight of adrenal glands: The values of SBP before cold stress were higher in 6 and 9-week-old SHRSP than values in the same aged WKY. The values of HR before cold stress were lower in SHRSP aged 9 weeks than those at 6 weeks of age. The body weights of both strains increased at 9 weeks of age compared with those at 6 weeks of age and those in 6-week-old SHRSP were lower than those of the same aged WKY. The weights of adrenal glands increased with age in both strains (Table 1). After cold stress, the SBP values in each group were unchanged compared with pre-cold stress values. HRs increased 30 min after cold stress in 6-week-old WKY, 9-week-old WKY and SHRSP to 412.2±4.9 from 376.1±4.4, 404.9±10.1 from 358.9±7.2 and 382.9±10.7 from 338.5±4.1 bpm from each preloading value, respectively.

Real-time measurements of body temperature, SBP and HR changes using a telemetric acquisition system: Because SBPs were not changed by cold stress when measured with a non-surgical tail-cuff method, SBPs were measured in real time using a telemetric acquisition instrument implanted in the abdominal cavity of the rat. A recording sample obtained from the two types of rats, WKY weighing 400 g at 14 weeks of age and SHRSP weighing 300 g at 13 weeks of age, is shown in Fig. 1. When a rat was placed in the cold water at 4°C, the body temperature decreased suddenly in both strains of rats but to a greater extent in SHRSP. Body temperature gradually returned to the preloading temperature over 20 min in WKY and 50 min in SHRSP. On the other hand, SBP was suddenly elevated just after cold stress and the value returned to the preloading value in 10 min in both types of rats. HR was also suddenly elevated just after the stress and was maintained for 40 min in both WKY and

Table 1: SBP, HR, body weight and adrenal gland weight in the four groups of rats

Parameter	6W WKY	6W SHRSP	9W WKY	9W SHRSP
SBP (mmHg)	118.6±1.6	149.6±4.4^{*6W}	129.1±1.6	179.9±5.5$^{*9W, 6S}$
HR (bpm)	376.1±4.4	399.0±4.8	358.6±7.2	338.5±4.1^{*6S}
Body weight (g)	172.8±3.1	122.2±1.5	299.5±3.6	220.6±1.9
Adrenal glands (mg)	18.7±2.0	13.6±0.5	26.8±1.2	26.4±0.8

SBP, HR and body weight were measured before cold stress, and adrenal gland weight was measured after adrenalectomy (n = 6). Significant differences at $p<0.05$; ^{*6W}: vs. 6-week-old WKY, ^{*9W}: vs. 9-week-old WKY, ^{*6S}: vs. 6-week-old SHRSP. 6W = 6-week-old; 9W = 9-week-old.

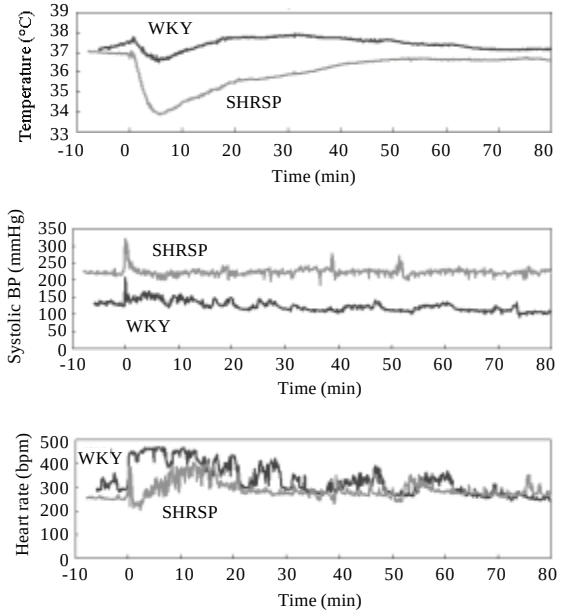

Fig. 1: Sample data of body temperature, SBP and HR detected with a telemetric data acquisition system. Three types of data were obtained every 5 sec. from 10 min before to 80 min after cold stress. A male 13-week-old SHRSP weighing 300 g and a male 14-week-old WKY weighing 400 g were used. Both rats were cold stressed by immersing in cold water at 4°C for 15 sec

SHRSP. Therefore, it might not be observed the differences in the SBP or HR values before and 30 and 60 min after cold stress in WKY and SHRSP with a non-surgical tail-cuff method.

Catecholamine values in plasma before and after cold stress: Concentrations of the first catecholamine metabolite from tyrosine, l-dopa, in the plasma of both of 6 and 9-week-old SHRSP increased dramatically compared with preloading concentrations (Fig. 2A). On the other hand, no change was observed in the values in WKY at 6 and 9 weeks of age before and after cold stress (Fig. 2A). Dopamine concentrations increased more in 9-week-old SHRSP after cold stress than preloading, but this was not seen in WKY (Fig. 2B). On the contrary, the noradrenaline concentration decreased more in 6-week-old WKY and SHRSP and 9-week-old SHRSP after cold stress compared with preloading (Fig. 2C). Adrenaline concentrations in the plasma were significantly increased in 6 and 9-week-old SHRSP 30 min after cold stress compared with preloading, but not in WKY (Fig. 2D). Of particular interest, no differences were seen in the concentrations of these four catecholamines before cold stress in any group.

ACTH, corticosterone, and aldosterone levels in plasma before and 30 and 60 min after cold stress: ACTH concentrations in plasma increased significantly in both WKY and SHRSP at 6 weeks of age 60 min after cold stress compared with preloading concentrations. In 9-week-old WKY and SHRSP, ACTH levels also increased 30 and 60 min after cold stress, although the degrees of increase tended to be lower compared with 6-week-old rats (Fig. 3A).

Corticosterone concentrations in plasma increased similarly as ACTH. The values in 6-week-old WKY and SHRSP increased 30 and 60 min after cold stress compared with preloading concentrations and the values increased to similar levels between the two groups of rats. The values in both strains of rats at 9 weeks of age increased 60 min after cold stress and reached similar levels in both strains, but the changes were less dramatic than changes in 6-week-old rats (Fig. 3B).

Aldosterone concentrations in plasma increased after cold stress in 6 and 9-week-old WKY and SHRSP, similar to the ACTH increase. The values increased at 60 min in WKY and 30 and 60 min after cold stress in SHRSP at 6 weeks of age and the increase was greater 30 min after cold stress in SHRSP than in WKY. The

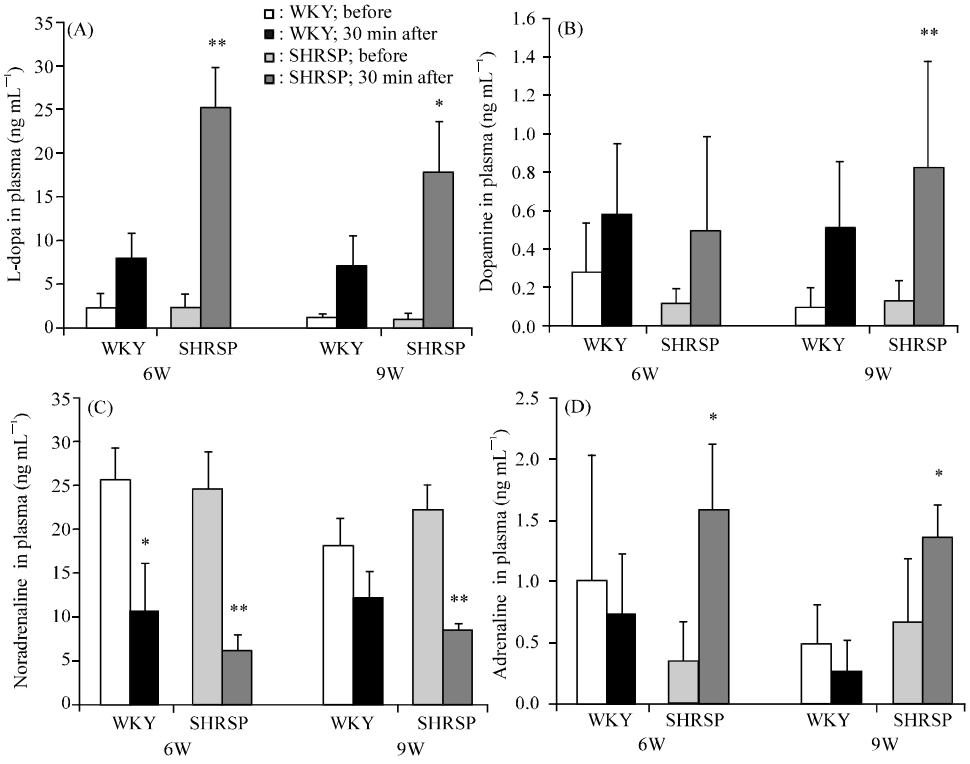

Fig. 2: Catecholamine levels in the plasma of WKY and SHRSP at 6 and 9 weeks of age before and 30 min after cold stress (A) L-dopa, (B) Dopamine, (C) Noradrenaline, (D) Adrenaline, Asterisks represent significant differences between values before and after cold stress in each rat group (n = 6), *: $p<0.05$, **: $p<0.01$ vs. preloading in the same group by Student's t-test

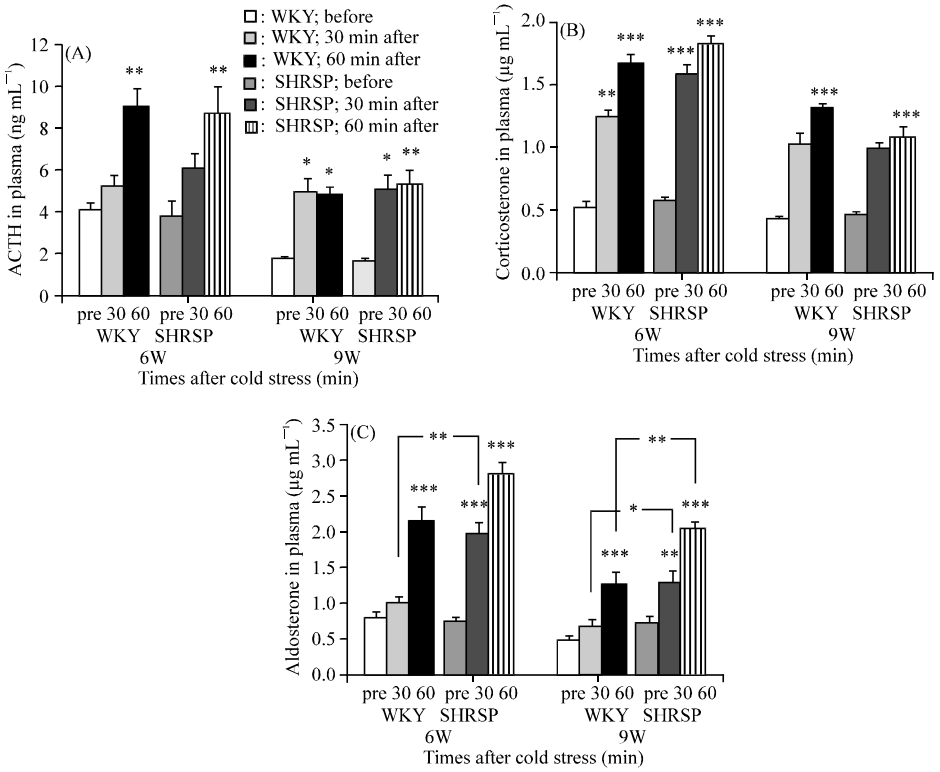

Fig. 3: ACTH and corticosteroid levels in the plasma of WKY and SHRSP at 6 and 9 weeks of age before and 30 and 60 min after cold stress. (A) ACTH, (B) corticosterone and (C) aldosterone. Asterisks represent significant differences between values before and after cold stress in each rat group (n = 6), *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs. preloading in the same group for values between two groups by Student's t-test

values in 9-week-old rats increased at 60 min in WKY and 30 and 60 min after cold stress in SHRSP, but the increase was greater 30 and 60 min after cold stress in SHRSP than in WKY (Fig. 3C). There was no difference between the concentrations of ACTH and the two steroid hormones in any group preloading.

mRNA expression levels of catecholamine synthesizing enzymes in adrenal glands: The mRNA expression levels of catecholamine synthesizing enzymes in the adrenal glands were measured with the DNA microarray method. Expression levels of mRNA for tyrosine hydroxylase (Th), a rate-limiting enzyme that converts tyrosine to l-dopa in the first step of catecholamine synthesis, were upregulated similarly 30 min after cold stress in WKY and SHRSP at 6 weeks of age, but this upregulation was not seen in 9-week-old WKY and SHRSP (Fig. 4A). Expression levels of mRNA for dopa decarboxylase (*Ddc*), which converts l-dopa to dopamine, were upregulated 30 min after cold stress in 6-week-old SHRSP, but not in the same age WKY or 9-week-old rats of either strain (Fig. 4B). Expression levels of mRNA for dopamine β-hydroxylase (*Dbh*), which converts dopamine to noradrenaline, were upregulated 30 min after cold stress in 6-week-old SHRSP and WKY, but this was not seen for the expression levels of Dbh mRNA after cold stress in 9-week-old WKY or SHRSP (Fig. 4C). There were no differences in the expression levels of these three catecholamine synthesizing enzyme mRNAs in any group at the time of preloading. Although we were interested in detecting phenylethanolamine-N-methyltransferase (PNMT) mRNA expression in the adrenal glands because this enzyme is associated with the conversion from noradrenaline to adrenaline, we could not detect this mRNA with the DNA microarrays used in this study.

mRNA expression levels of corticosteroid synthesizing enzymes in adrenal glands: mRNA expression levels of corticosteroid synthesizing enzymes in the adrenal glands were measured with the DNA microarray method at the same time of detection of catecholamine synthesizing enzyme mRNAs. Expression levels of mRNA for cyp11a1, a rate-limiting enzyme that converts cholesterol to pregnenolone in the first step of corticosterone synthesis, were upregulated 30 min after cold stress in 6-week-old SHRSP, but not in 9-week-old WKY and SHRSP (Fig. 5A). Expression levels of mRNA for

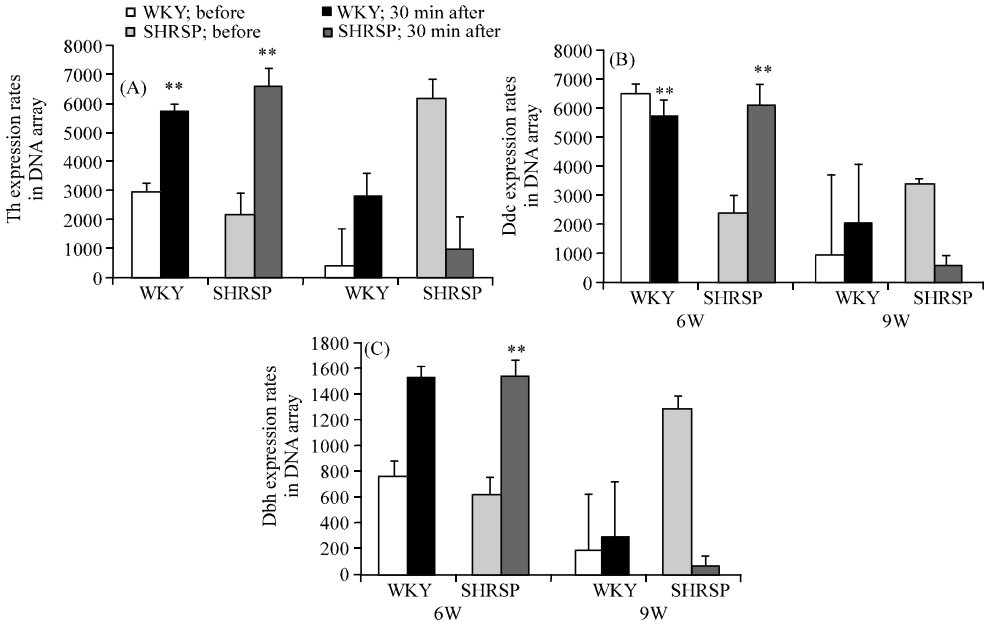

Fig. 4: mRNA expression levels of catecholamine synthesizing enzymes in the adrenal glands of WKY and SHRSP at 6 and 9 weeks of age before and after cold stress, (A) *Th* mRNA, (B) *Ddc* mRNA and (C) *Dbh* mRNA , Asterisks represent significant differences between values before and after cold stress in each rat group (n = 3), *: $p<0.05$, **: $p<0.01$ vs. preloading in the same group by Student's t-test

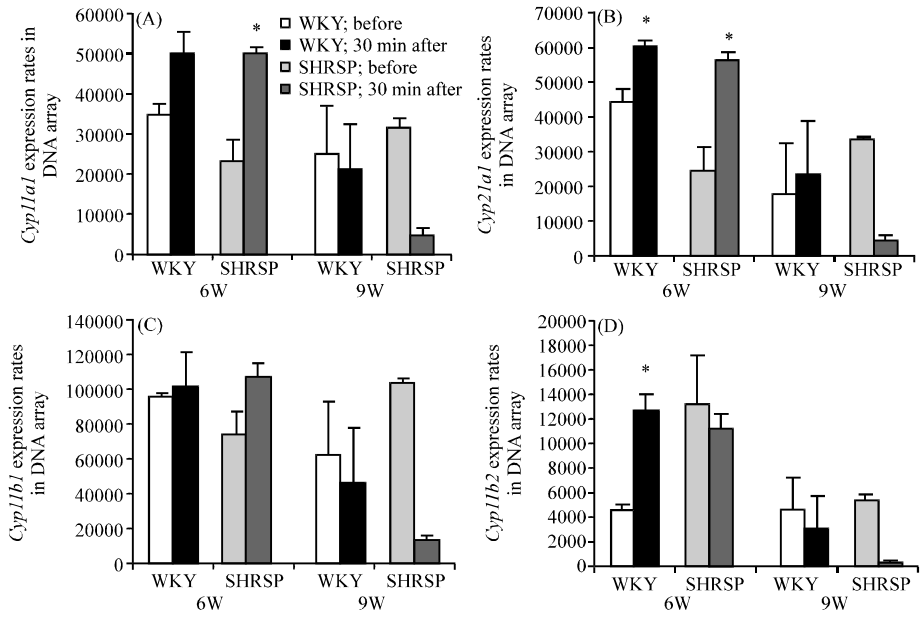

Fig. 5: mRNA expression levels of corticosteroid synthesizing enzymes in the adrenal glands of WKY and SHRSP at 6 and 9 weeks of age before and after cold stress. (A) *cyp11a1* mRNA, (B) *cyp21a1* mRNA and (C) *cyp11b1* mRNA, D: *cyp11b2* mRNA, Asterisks represent significant differences between values before and after cold stress in each rat group (n = 3), *: $p<0.05$ vs. preloading in the same group by Student's t-test

cyp21a1, which converts progesterone to 11-deoxycorticosterone during corticosterone synthesis, were upregulated 30 min after cold stress in 6-week-old WKY and SHRSP, but no changes were observed in 9-week-old WKY or SHRSP (Fig. 5B). Expression levels of mRNA for cyp11b1, which converts 11-deocycorticosterone to corticosterone, did not change 30 min after cold stress in 6-week-old or 9-week-old WKY and SHRSP compared with preloading values (Fig. 5C). Expression levels of mRNA for cyp11b2, which converts corticosterone to aldosterone, were upregulated 30 min after cold stress in 6-week-old WKY to the level of SHRSP at preloading, but no change was observed in 9-week-old rats of either strain (Fig. 5D). There was no difference in the expression levels of mRNA for these four corticosteroid-synthesizing enzymes in any group at preloading. On the other hand, the data for 9-week-old SHRSP were significantly different between preloading and cold stress. The reason of these differences in this study remains unclear.

DISCUSSION

Continuous brother-sister mating of six generations of WKY with a slightly higher blood pressure created SHR at Kyoto University School of Medicine in Japan around 1963. Using this strain with high blood pressure, many types of fundamental studies about essential hypertension such as pathophysiology and the effects of food have been performed by Hoffmann (1986) and Laurent *et al.* (2005). As descendants of SHR, SHRSP (Okamoto *et al.*, 1974) were created by continuous brother-sister mating in a closed colony of SHR after about one decade of mating. SHRSP are stroke-prone spontaneously hypertensive rats in which the SBP elevates with age to more than 250 mmHg and strokes such as cerebral hemorrhage, thrombosis and subarachnoid hemorrhage occur with a high incidence. So far, the causes of hypertension and stroke remain elusive despite multiple attempts by many researchers to clarify them.

Adrenal glands release two types of hormones: Catecholamines such as adrenaline and corticosteroids such as corticosterone and aldosterone, both with tonic actions. Adrenal glands are considered key organs related to the cause of hypertension. In our previous study to examine the role of the adrenal glands in hypertension using DNA microarray with three types of substrains, SHR, SHRSP and malignant type of SHRSP (M-SHRSP) (Okamoto *et al.*, 1986), we did not find any positive data regarding the expression of mRNAs for hormone synthesizing enzymes (Ashenagar *et al.*, 2010). Therefore, we investigated the pathophysiological role of adrenal glands in this study by measuring two different types of hormones with special reference to the gene expression of hormone synthesizing enzymes following cold stress. The rats were cold stressed with a stronger but shorter duration that was different from the usual method of exposing rats to an air conditioned atmosphere at 4°C for 4-5 h. In our current study, to give a stronger stress for a shorter period, rats were immersed in cold water at 4°C for 15 sec and then wiped with a dry towel. Changes in body temperature, SBP and HR detected with the telemetric acquisition system shown in Fig. 1 demonstrated the effects of stronger but shorter cold stress.

In the adrenal glands of SHRSP, higher amounts of catecholamines such as dopamine, noradrenaline and adrenaline (Maemura *et al.*, 1982; Schober *et al.*, 1989; Uchida *et al.*, 1995) and higher expression of *Th* mRNA (Kumai *et al.*, 1999) have been reported. In addition, adrenaline is increased in urine (Yamori *et al.*, 1985; Horie *et al.*, 1991) and plasma of SHRSP compared to WKY (Cui *et al.*, 2004) following cold stress at 4°C for 4 h and a greater ACTH response was observed following restraint stress (Imaki *et al.*, 1998). However, there are no reports regarding corticosteroid secretion following any type of stress in SHRSP.

In this study, catecholamine plasma level changes evoked by cold stress appeared to be larger in SHRSP than WKY at both 6 and 9 weeks of age, although there were no differences in the levels of l-dopa, dopamine, noradrenaline, or adrenaline between the two rat strains before cold stress. l-dopa and adrenaline levels in plasma increased significantly in SHRSP at 6 and 9 weeks of age, and the dopamine level increased in SHRSP at 9 weeks of age but not in WKY at either age. The mRNA expression of three catecholamine synthesizing enzymes was elevated in both rat strains at 6 weeks of age but not at 9 weeks of age. On the other hand, noradrenaline levels in plasma decreased in all groups after cold stress, probably due to consumption of noradrenaline when converted to adrenaline in the adrenal glands. From these findings, it was not surprising that mRNA for the synthesizing enzymes for l-dopa (*Th*), dopamine (*Ddc*) and adrenaline (*Dbh*) were also induced by cold stress, especially in young SHRSP compared with WKY. In this study, phenylethanolamine N-methyltransferase (PNMT), the enzyme to convert noradrenaline to adrenaline, mRNA could not be detected unfortunately because the DNA fragment derived from PNMT was not incorporated in DNA microarrays used for our experiments. There is a report that PNMT mRNA rose up to 7.0-fold with acute or chronic stress in rats (Wong *et al.*, 2004). Therefore, the same data might be obtained as rising up of PNMT

mRNA in either SHRSP or WKY after cold stress. Some discrepancies may have occurred between higher catecholamine levels in SHRSP than WKY but no differences in mRNA expression levels of the synthesizing enzymes in the two strains. Schober *et al.* (1989) reported an increased pool of secretory catecholamines in the adrenal glands of SHRSP. The observation might be consistent with this report. Ooshima *et al.* (2009) reported that decrease of catechol-O-methyltransferase (COMT), an enzyme which inactivates catecholamines, might be an important factor reading to the development of hypertension in the kidney of SHRSP.

On the other hand, regarding ACTH and corticosteroids that are produced in response to cold stress, ACTH, corticosterone and aldosterone increased significantly in both SHRSP and WKY, at both 6 and 9 weeks of age. Aldosterone levels increased more in SHRSP than WKY at both 6 and 9 weeks of age 30 and 60 min after cold stress. Expression levels of mRNA for *cyp11a1* and *cyp21a1*, which are associated with corticosteroid production, were upregulated in SHRSP at 6 weeks of age, although both *cyp21a1* and *cyp11b2* mRNAs were also elevated in 6-week-old WKY. ACTH is probably released from the anterior pituitary via the pituitary-adrenocortical axis into the bloodstream prior to induction of these enzyme mRNAs following cold stress. We observed several discrepancies between hormone increases and mRNA expression of the associated synthesizing enzymes and between the two rat strains and/or ages.

In the absence of other data, it is possible that the same mechanism that regulates catecholamine production in SHRSP may regulate corticosteroid production and release.

Although the precise mechanism was not identified in this comprehensive study, hyperactivity of both catecholamine and corticosteroid responses without any difference in synthesizing enzyme mRNA expression in the adrenal glands of SHRSP following cold stress, especially in young rats compared with WKY, was elucidated. These differences may partially contribute to the initiation and development of hypertension in SHRSP in normal condition and/or during stress.

CONCLUSIONS

We obtained the following conclusion from the experiments to clarify the role of adrenal glands in spontaneously hypertensive rat model (SHRSP). That is, (1) l-dopa, dopamine and adrenaline were synthesized following induction of *Th*, *Ddc* and *Dbh* mRNAs, (2) corticosterone and aldosterone in plasma increased following the induction of *cyp11a1* and *cyp21a1* mRNAs, which are stimulated along with ACTH elevation following cold stress in young SHRSP more than WKY. This difference, therefore, may be related to the initiation and/or development of hypertension in SHRSP in normal condition and/or during stress.

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