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Differential effect of chronic inhibition of calcium channel and angiotensin II type 1-receptor on aldosterone synthesis in spontaneously hypertensive rats

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Abstract

We have investigated the in vivo effect of chronic blockade of Ca^{2+}-channels and angiotensin II type 1 (AT_1)-receptors on aldosterone (Aldo)-synthesis in the adrenal glands of spontaneously hypertensive rats (SHR). Male SHR were administered Ca^{2+}-antagonist, amlodipine (10 mg/kg per day) or AT_1-receptor-antagonist, TCV-116 (1 mg/kg per day) from 7 until 11 weeks of age. Systolic blood pressure (SBP) and heart rate (HR) were significantly higher in SHR than Wistar-Kyoto (WKY) rats. Both treatments resulted in equivalent and significant reduction in SBP in SHR. Aldo-secretion in SHR, which was significantly higher than in WKY rats, was profoundly suppressed by TCV-116 compared with amlodipine. Both treatments resulted in thickening of the zona glomerulosa, which immunohistochemically contains Aldo, at the end of therapy. Competitive reverse transcription-polymerase chain reaction (RT-PCR) showed that CYP11A (P450scc) mRNA regulating the first step of Aldo-synthesis was significantly reduced from week 9 of age by amlodipine, and that CYP11B2 (P450aldo) mRNA regulating the last step of Aldo-synthesis was potently suppressed from 9 weeks of age by TCV-116. Our results indicate that chronic treatment with different antihypertensive agents directly modulates adrenocortical aldosterone synthesis in SHR in vivo via different mechanisms.

Keywords: Adrenal cortex; Zona glomerulosa; CYP11A and CYP11B2; Cytochrome P450scc and P450aldo

1. Introduction

The involvement of the renin–angiotensin system (RAS) in the development of hypertension and hypertension-related organ damage is well known [1]. Many investigators have shown that angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II (Ang II) type 1 receptor (AT_1) antagonists (AT_1a) reduce arterial blood pressure and ameliorate hypertension-associated organ injury [1,2]. These effects suggest that Ang II is a major factor involved in the above pathologic processes. Another key effector of RAS is aldosterone (Aldo), the primary mineralocorticoid, which acts on the distal nephron to regulate sodium resorption, potassium excretion, and intravascular volume [1,3,4]. These Aldo actions potentially contribute to the increase in arterial blood pressure. Moreover, recent studies have suggested direct effects for Aldo on end-organ injury [5]. Indeed, the Aldo antagonist, spironolactone, prevented the progression of cardiovascular and nephrosclerotic injuries [6,7] and Aldo substitution in ACEI-pretreated hypertensive rats resulted in the development of malignant nephrosclerosis [8]. In addition to the well-known action of Ang II (stimulation of Aldo production from the adrenal cortex), the reciprocal interaction that Aldo increases Ang II binding [9,10] and that Aldo induces upregulation of cardiac AT_1-receptor [11] have also been demonstrated. These findings indicate that Aldo as well as Ang II are closely involved in the development of hypertension and associated or-

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gan damage. However, the in vivo mechanisms that regulate Aldo synthesis in the adrenal cortex of hypertensive animals, e.g. spontaneously hypertensive rats (SHR), remain unknown at this stage.

Aldo synthesis in the adrenal zona glomerulosa is influenced by a variety of factors, such as extracellular Ang II, potassium ion (K^+) and adrenocorticotropin (ACTH). The potency of these factors in the physiological control of Aldo secretion depends on their combined activities and synergy [3,4]. The effect of Ang II on Aldo synthesis is mediated through AT_1-receptors [12,13] and this effect is induced even by subpressor doses of Ang II [14]. Furthermore, Ang II and K^+ synergistically increase cytosolic calcium ion (Ca^{2+}), depending on the influx of extracellular Ca^{2+}, which is followed by stimulation of adrenocortical Aldo secretion [3,15]. Although these agonists including Ang II, K^+ and ACTH activate different intracellular messenger systems, they eventually enhance the Ca^{2+} influx [16–19]. Regulation of Aldo production in the zona glomerulosa can be divided into two phases, (1) an acute phase that occurs within minutes and reflects cholesterol transfer to mitochondrial side-chain cleavage enzyme, cytochrome P450scc [3,20,21], the product of the CYP11A gene [22], which is followed by extramitochondrial conversion of pregnenolone into progesterone and then into 11-deoxycorticosterone by cytochrome P450c21 enzyme; and (2) a chronic phase that requires several hours and reflects increased expression of Aldo synthase, cytochrome P450aldo [3,4,20], the product of the CYP11B2 gene [23].

The above steps are possible target sites for the regulation of Aldo synthesis. Many in vitro findings indicate that both AT_1-action due to Ang II and Ca^{2+} influx are major determinants that potentiate Aldo synthesis and secretion in the zona glomerulosa of the adrenal cortex. However, there is little in vivo information regarding the mechanisms that regulate Aldo synthesis in SHR and the effects of antihypertensive agents on adrenocortical Aldo synthesis in these rats. Therefore, we here investigated the effects of chronic blockade of Ca^{2+}-channel and AT_1-receptor on Aldo synthesis in the adrenal glands of SHR, with special emphasis on the enzyme mRNAs responsible for Aldo synthesis. The aim of our study was to elucidate the in vivo differential action of Ca^{2+}-antagonist (Ca^{2+}a) and AT_1a in modifying adrenocortical Aldo synthesis in SHR.

2. Materials and methods

2.1. Materials

SHR and Wistar-Kyoto (WKY) rats (5-weeks-old) were purchased from Japan SLC (Shizuoka, Japan). All rats were housed in climate-controlled metabolic cages with a 12:00 and 12:00 h light–dark cycle. The animals received normal food containing 0.24% sodium (MF, Oriental Yeast, Tokyo, Japan), with water provided ad libitum. The primary antibodies used for immunohistochemistry were rabbit anti-aldosterone-3-CMO (Biogenesis, NH) polyclonal antibodies, which were cross-reactive with rat Aldo. Oligonucleotides for reverse transcription-polymerase chain reaction (RT-PCR) were synthesized on a model 380B DNA synthesizer (Applied Biosynthesis, Foster City, CA). The location of oligonucleotides of primer pairs was as follows: CYP11A, 1078–1097 and 1689–1708 [22]; and CYP11B2, 862–879 and 1142–1159 [23]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University Medical School.

2.2. Experimental protocol

At 7 weeks of age, SHR were divided at random into three groups. Rats in the first group were treated with oral amlodipine, a Ca^{2+}a, (10 mg/kg per day in 0.3 ml of saline; SHR + Aml; $n = 20$). Rats in the second group were treated with TCV-116, an AT_1a, (1 mg/kg per day in 0.3-ml of saline; SHR + TCV; $n = 20$). The third (control) group was treated with 0.3-ml of saline (SHR + vehicle; $n = 20$). All treatments were administered once daily by gavage. Another control group of WKY rats was treated orally with 0.3-ml of saline (WKY + vehicle; $n = 20$).

Systolic blood pressure (SBP) and heart rate (HR) were measured weekly, at 9:00 am, in conscious, restrained, and warmed rats by tail-cuff plethysmography (UR-5000, Ueda Seisakusyo, Tokyo). Once a week, urine was collected over a 24-h period and used for the measurement of urinary sodium (u-Na), potassium (u-K) and Aldo (u-Aldo) excretion. u-Na and u-K were determined on an autoanalyzer while u-Aldo was determined with a SPAC-S-aldosterone RIA kit (Daiichi Radioisotope, Tokyo). Five rats in each group were killed by decapitation at 7, 8, 9 and 11 weeks of age, and the adrenal glands dissected, removed and prepared for histological examination and extraction of RNA. Trunk blood samples were collected and stored at -30°C until assayed for electrolytes (sodium, potassium and chloride), creatinine, and total cholesterol on an autoanalyzer system. Plasma Aldo concentrations (p-Aldo) were measured with a SPAC-S-aldosterone RIA kit (Daiichi Radioisotope) and plasma renin activity (PRA) by RIA for Ang I (Dinabot Radioisotope, Tokyo).

2.3. Histological examination

For tissue examination by light microscopy, a portion of each adrenal gland was fixed with 10% buffered

para-formaldehyde, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin-eosin (HE). The width (μm) of the adrenal cortex (on at least three portions of each adrenal gland), as viewed by light microscopy, was measured with an objective micrometer (OB-M, Olympus Optical, Tokyo) [24]. The distribution of Aldo-immunoreactivity was evaluated by immunoperoxidase assay using Vectastain kit (Vector Laboratories, Burlingame, CA) as previously described by our laboratory [24] on 4-μm thick cryostat sections fixed with acetone. Non-specific protein binding sites were blocked by incubation of sections with 10% normal goat serum in Tris-buffered saline for 20 min. Nonspecific staining was blocked by incubation of the sections with avidin and biotin for 15 min each. Endogenous peroxidase activity was inhibited by a 20-min incubation with methanol containing 0.3% H_2O_2. The slides were then stained for Aldo in a humidity chamber for 1 h at 37°C, washed twice with phosphate-buffered saline (PBS), stained with biotin-labeled goat anti-rabbit IgG which was not cross-reactive with rat IgG for 60 min, and then incubated with avidin and biotinylated horseradish peroxidase. The slides were washed once more in PBS before color reaction, which was accomplished by incubation in freshly prepared 3,3-diaminobenzidine reagent until staining was complete. The reaction was stopped by placing each slide in water. After another wash in PBS, Mayer's hematoxylin was added as a counterstain.

2.4. Extraction of RNA from adrenal cortex and analysis by competitive RT-PCR

The adrenal cortex was dissected out and minced followed by extraction of total RNA with RNeasy Midi Kits (Quiagen, Santa Clarita, CA). The RNA was quantified by measuring absorbance at 260 nm and stored at −20°C until assay.

For quantification of CYP11A and CYP11B2 mRNA, competitive RT-PCR analysis was performed as previously described by our laboratory [2,24]. First, mutant cDNAs were generated by a PCR MIMIC Construction Kit (Clontech Lab., Palo Alto, CA). The mutant fragments for G3PDH, which was used as an internal control, was 556 bp, and mutants for CYP11A and CYP11B2 were 400 bp each. The fragments were reamplified with four sets of specific primers to determine their abilities to act as competitors for the native mRNAs. The products obtained were as follows, G3PDH, 596 bp; CYP11A, 442 bp; and CYP11B2, 436 bp, which were purified using Chrome Spin Column (Clontech), and diluted to 100 amol/μl with ultrapure glycogen. Extracted RNA was subjected to RT using a GeneAmp RNA PCR Kit (Perkin Elmer Cetus, Norwalk, CT). RT was performed with 10 ng of RNA per reaction using random hexamer (2.5 μM), reverse transcriptase (2.5 U/μl) and deoxynucleotide triphosphate (dNTP; 1 mM) at 42°C for 55 min, 99°C for 5 min, and 42°C for 5 min. The resultant cDNA was resuspended in 50 μl deionized, autoclaved water for competitive PCR. The linear portion of the relationship between native cDNAs and competitive mutant cDNAs was determined for G3PDH, CYP11A and CYP11B2. In a preliminary analysis, a fixed amount (0.5 ng) of cDNA derived from a control was co-amplified with 10-fold serial dilutions ($1–10^{-6}$) of the competitive mutant cDNAs using the three primer sets and the preceding kit. For fine-tuned competitive PCR, another re-amplification was performed using 2-fold serial dilutions ($2^{-1}–2^{-7}$) of one of the dilution steps of the preliminary PCR as a starting point (G3PDH, 10^{-3}; CYP11A, 10^{-3}; CYP11B2, 10^{-4}). Competitive PCR was performed using the preceding PCR kit and a thermal cycler (Perkin Elmer Cetus) under the following conditions, 35 cycles of denaturation at 94°C for 1 min; annealing at 55°C for 1 min and extension at 72°C for 1 min. Aliquots of PCR products of cDNA and competitive mutant cDNA were electrophoresed on 1.5% agarose gels, visualized after ethidium bromide staining, and photographed using an instant positive/negative film (337; Polaroid, Cambridge, MA). Negatives were analyzed by scanning densitometry (Scanning Imager 300-SX, Molecular Dynamics, Sunnyvale, CA), and the relative integrated density of each band was digitized by multiplying the absorbance by the surface area. Finally, the ratios between the densitometric readings of the negative cDNA and mutant cDNA-PCR products were plotted with a logarithmic scale on the ordinate against the logarithmic dilutions of mutant cDNA on the abscissa. After establishing the working ranges in which linear relationship existed, cDNA from all individual samples were subjected to competitive PCR analysis, using CYP11A and CYP11B2 primers. Control analysis was performed using G3PDH primers.

2.5. Statistical analysis

All data are expressed as mean ± S.E.M. and statistically analyzed by analysis of variance (ANOVA). P values of < 0.01 were accepted as statistically significant.

3. Results

3.1. Serial changes in SBP and HR

SBP of SHR + vehicle group was significantly higher than in WKY group throughout the study (Fig. 1A).

Treatment with Aml and TCV produced significant and equivalent reductions in SBP throughout the experiment, relative to SHR + vehicle rats. The HR was generally stable in all groups, although that of SHR tended to be high and that of SHR + Aml group tended to decrease throughout the experiment (Fig. 1B). None of the rats died throughout the experimental period.

3.2. Serial changes in urine volume (UV), u-Na and u-K excretion, and blood chemistry

Treatment with Aml increased UV, relative to WKY rats (Fig. 2, left panel), while TCV treated rats showed a significant decrease in UV relative to SHR + Aml group. u-Na and u-K excretion did not change significantly throughout the experiment in each group (Fig. 2, middle and right panels). As shown in Table 1, serum sodium, potassium, chloride and creatinine concentrations did not differ significantly among the four groups of rats throughout the experiment. Serum total cholesterol level in SHR + vehicle was significantly lower than in WKY rats and, at the age of 11 weeks, that of SHR + Aml group became significantly higher than other SHR groups.

3.3. Serial changes in p-Aldo, PRA, p-Aldo/PRA and u-Aldo levels

As shown in Fig. 3, at 7–8 weeks (the prehypertensive phase) p-Aldo level in SHR rats was less than in WKY rats. After the age of 9 weeks, i.e. the established hypertensive phase, p-Aldo in SHR, however, increased relative to that in WKY rats. Treatment with TCV resulted in a significant reduction of p-Aldo level compared with SHR + Aml and SHR + vehicle groups. The daily u-Aldo level in SHR was significantly higher than in WKY rats, but was significantly suppressed by TCV-116 treatment. PRA levels were similar in SHR + vehicle and WKY + vehicle groups throughout the experiment. However, TCV treatment markedly increased PRA levels at age of 8–11 weeks and Aml increased PRA particularly at the age of 11 weeks (Fig. 3). The p-Aldo/PRA ratio, indicating adrenocortical responsiveness of Ald secretion, was not different between SHR and WKY rats. Both treatments lowered this ratio but the effect of TCV-116 treatment was more potent and prolonged than amlodipine treatment.

3.4. Serial changes in body and organ weights

The rate of change of body weight (BW) throughout the experiment tended to be higher in SHR than WKY rats (Fig. 4). Treatment with amlodipine or TCV-116 did not influence BW. The adrenal weight (AdW) of SHR was significantly lower than that of WKY rats but neither of the antihypertensive treatment altered AdW. Heart and kidney weights were significantly higher in SHR than in WKY rats. Although kidney

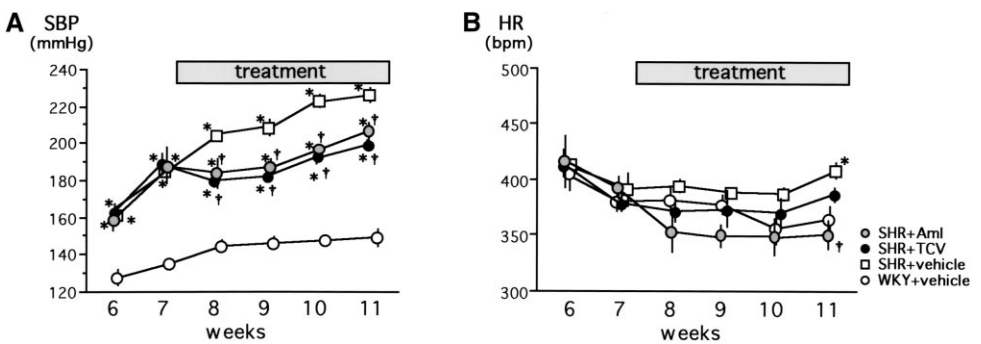

Fig. 1. Effects of chronic inhibition of Ca^{2+}-channel and AT_1-receptor on SBP and HR. Data are mean ± S.E.M. ($n = 5$); *, $P < 0.01$ vs. WKY + vehicle; and †, $P < 0.01$ vs. SHR + vehicle.

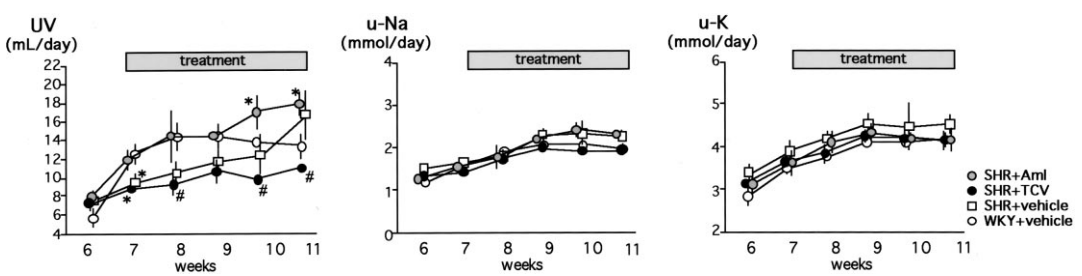

Fig. 2. Effects of chronic inhibition of Ca^{2+}-channel and AT_1-receptor on UV and u-Na and u-K excretion. Data are mean ± S.E.M. ($n = 5$); *, $P < 0.01$ vs. WKY + vehicle; and #, $P < 0.01$ vs. SHR + Aml.

Table 1
Sequential changes of blood chemistry data[a]

	8 Weeks				9 Weeks				11 Weeks			
	SHR + Aml	SHR + TCV	SHR + vehicle	WKY + vehicle	SHR + Aml	SHR + TCV	SHR + vehicle	WKY + vehicle	SHR + Aml	SHR + TCV	SHR + vehicle	WKY + vehicle
Serum sodium (mmol/l)	136.8 ± 0.6	139.3 ± 0.6	138.8 ± 1.3	137.3 ± 0.3	138.5 ± 0.3	138.3 ± 1.3	139.3 ± 0.5	136.5 ± 1.0	140.2 ± 1.2	140.0 ± 1.2	138.4 ± 0.8	139.2 ± 0.5
Serum potassium (mmol/l)	5.05 ± 1.4	5.95 ± 0.2	5.85 ± 0.1	5.85 ± 0.1	6.45 ± 0.07	6.13 ± 0.2	6.08 ± 0.3	5.83 ± 0.1	6.18 ± 0.1	6.34 ± 0.3	5.76 ± 0.2	6.00 ± 0.1
Serum potassium (mmol/l)	103.8 ± 0.8	105.3 ± 0.3	105.3 ± 0.8	104.8 ± 0.6	104.0 ± 0.4	104.0 ± 0.4	104.5 ± 0.3	104.0 ± 0.4	103.0 ± 0.6	106.0 ± 1.0	105.2 ± 0.7	104.2 ± 0.2
Serum creatinine (mg/dl)	0.20 ± 0.01	0.20 ± 0.01	0.21 ± 0.03	0.30 ± 0.01	0.23 ± 0.03	0.20 ± 0.01	0.28 ± 0.03	0.28 ± 0.02	0.34 ± 0.02	0.30 ± 0.03	0.30 ± 0.01	0.34 ± 0.02
Total cholesterol (mmol/l)	61.5 ± 1.7[b]	63.0 ± 0.9[b]	63.8 ± 0.8[b]	86.8 ± 0.8	68.8 ± 1.8[b]	66.3 ± 2.2[b]	65.0 ± 2.1[b]	81.3 ± 6.5	72.2 ± 2.2[b,c]	60.8 ± 2.0[b,d]	58.8 ± 2.8[b]	87.6 ± 2.0

[a] Data are mean ± S.E.M. ($n = 5$).
[b] $P < 0.01$ vs. WKY + vehicle.
[c] $P < 0.01$ vs. SHR + vehicle.
[d] $P < 0.01$ vs. SHR + Aml.

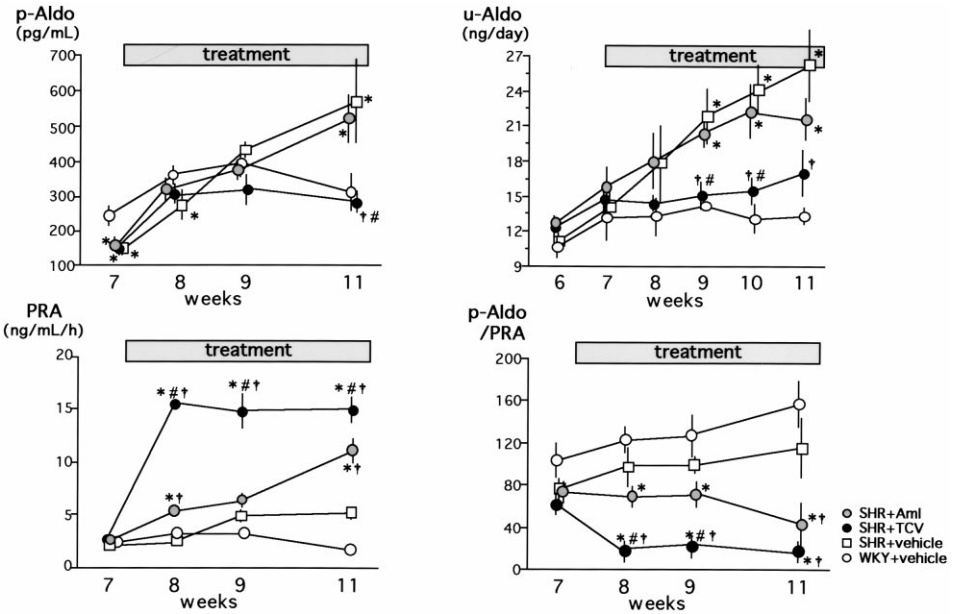

Fig. 3. Effects of chronic inhibition of Ca^{2+}-channel and AT_1-receptor on p-Aldo, PRA, the ratio of p-Aldo/PRA and u-Aldo excretion. Data are mean ± S.E.M. ($n = 5$); *, $P < 0.01$ vs. WKY + vehicle; #, $P < 0.01$ vs. SHR + Aml; and †, $P < 0.01$ vs. SHR + vehicle.

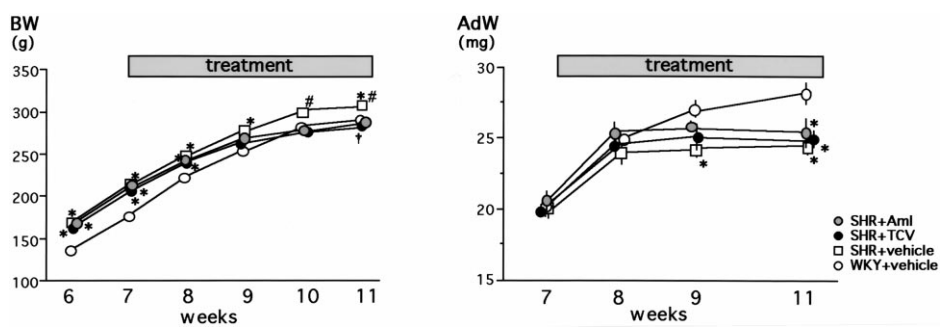

Fig. 4. Effects of chronic inhibition of Ca^{2+}-channel and AT_1-receptor on BW and AdW. Data are mean ± S.E.M. ($n = 5$); *, $P < 0.01$ vs. WKY + vehicle; #, $P < 0.01$ vs. SHR + Aml; and †, $P < 0.01$ vs. SHR + vehicle.

weight was not different between SHR treated by either treatment, TCV-116 treatment for 4 weeks significantly reduced the heart weight of SHR (SHR + vehicle, 1.17 ± 0.2 g; SHR + TCV, 1.01 ± 0.01 g; $p < 0.01$).

3.5. Histological and immunohistochemical findings

The structure of zona glomerulosa was compared in the four experimental groups by light microscopy. Fig. 5a and c are representative HE stained sections, and Fig. 5b and d are representative immunohistochemically stained sections for Aldo in the adrenal cortex of 11-weeks-old SHR. No histological changes were noted in this area throughout the observation period in all four groups. The expression of Aldo was predominantly localized in the zona glomerulosa of the adrenal cortex. Changes in the width of zona glomerulosa are shown in Table 2. Although the mean thickness of this zone did not differ significantly among the four groups, the relative width (%) of zona glomerulosa was significantly greater at the age of 11 weeks in both SHR + Aml and SHR + TCV groups, compared with WKY + vehicle.

3.6. Quantitation of adrenocortical expression of CYP11A and CYP11B2 mRNAs

To quantify the level of adrenocortical expression of CYP11A and CYP11B2 by competitive RT-PCR, we first determined the linear range of the ratios of co-amplified mutant cDNAs and native cDNAs reverse transcribed from adrenocortical RNA as described in Section 2 (Fig. 6). For quantitation of native cDNA samples, we selected the following logarithmic dilutions of mutant cDNAs: $2^{-5}(3.125 \times 10^{-3}$ amol/μl) for

Table 2
Sequential changes of width of zona glomerulosa[a]

	8 Weeks				9 Weeks				11 Weeks			
	SHR + Aml	SHR + TCV	SHR + vehicle	WKY + vehicle	SHR + Aml	SHR + TCV	SHR + vehicle	WKY + vehicle	SHR + Aml	SHR + TCV	SHR + vehicle	WKY + vehicle
Width of zona glomerulosa (μm)	80.7 ± 2.2	79.5 ± 2.5	80.5 ± 2.5	78.1 ± 1.5	80.9 ± 2.6	83.6 ± 2.1	82.3 ± 1.6	79.0 ± 0.9	84.4 ± .3.8	84.7 ± 1.5	84.3 ± 1.3	82.2 ± 1.1
Relative width in adrenal cortex (%)	8.33 ± 0.3	8.52 ± 0.3	7.71 ± 0.3	7.52 ± 0.2	8.40 ± 0.3	9.14 ± 0.3	8.60 ± 0.2	8.54 ± 0.2	9.46 ± 0.4[b]	9.61 ± 0.2[b]	9.33 ± 0.4	8.14 ± 0.3

[a] Data are mean ± S.E.M. ($n = 5$).
[b] $P < 0.01$ vs. WKY + vehicle.

G3PDH, 2^{-3}(1.25×10^{-3} amol/μl) for CYP11A and 2^{-4}(6.25×10^{-4} amol/μl) for CYP11B2. For competitive PCR reactions, 2 μl of these dilutions were added to 2 μl of each cDNA (0.25 ng/μl). The resultant PCR products were quantified by densitometric scanning as described in Section 2. At each time point, the relative change in cDNA/mutant ratio was standardized to the level of cDNA/mutant in SHR + vehicle group (Fig. 7). CYP11A and CYP11B2 mRNA levels at the age of 8 weeks were not different significantly among the four groups; however, at the age of 9 weeks, amlodipine significantly reduced CYP11A level, and at the age of 11 weeks, both amlodipine and TCV-116 significantly reduced CYP11A level. On the other hand, only TCV-116 treatment resulted in a significant reduction of CYP11B2 level between the age of 9 and 11 weeks. G3PDH levels did not change throughout the observation period in the four groups (Fig. 6).

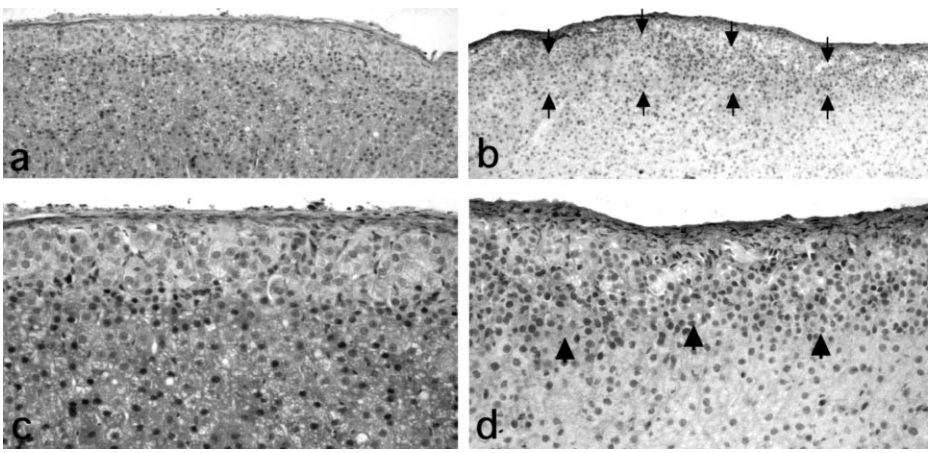

Fig. 5. (a and c) Representative histological findings in the adrenocortical zona glomerulosa of 11-week-old SHR. No major differences are evident histologically through the observation period in all groups. (b and d) Aldo peptide expression is localized predominantly in the zona glomerulosa of the adrenal cortex (arrows). (a and c, HE stain, magnification, × 50 and × 100, respectively; b and d, immunohistochemical staining for Aldo, magnification, × 50 and × 100, respectively).

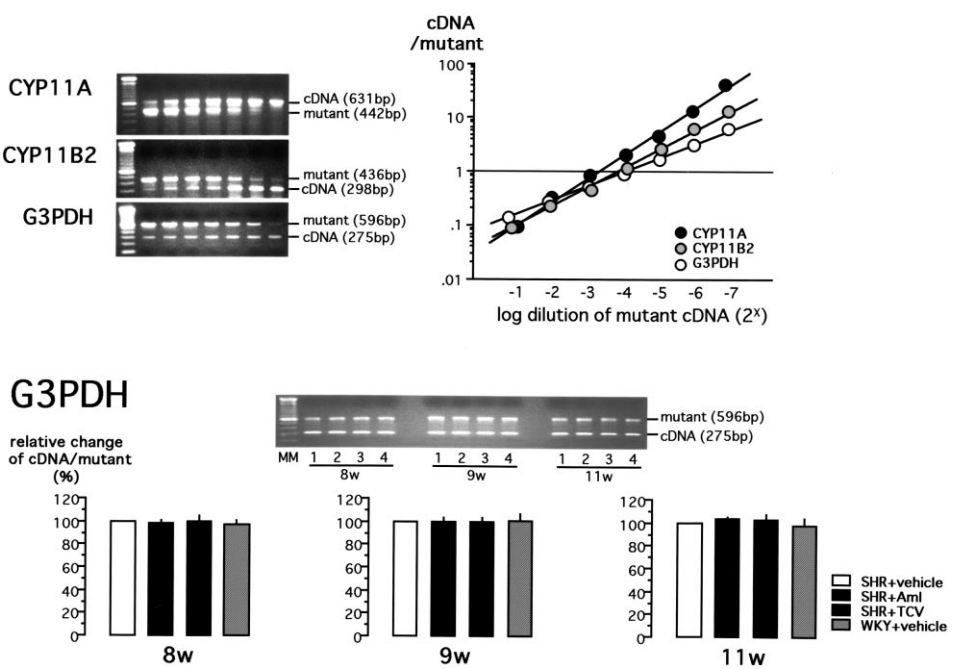

Fig. 6. Linear range for analysis of competitive RT-PCR and control study. The linear ranges for the ratios of co-amplified PCR products were determined using native cDNA obtained by RT of adrenocortical RNA from one SHR, and use of competitive mutant cDNA for CYP11A, CYP11B2 and G3PDH at serial logarithmic dilutions of 2^{-1}–2^{-7}. As a control study, quantitation of G3PDH in native cDNA sample was performed by competitive PCR mixing a constant amount (2 μl) of native cDNA (0.25 ng/μl) with 2^{-5}(3.125×10^{-3} amol/μl) of mutant G3PDH cDNA. At each week of age, the relative change in cDNA/mutant ratio was standardized to the level of cDNA/mutant in SHR + vehicle. G3PDH level did not change throughout the observation period in all groups.

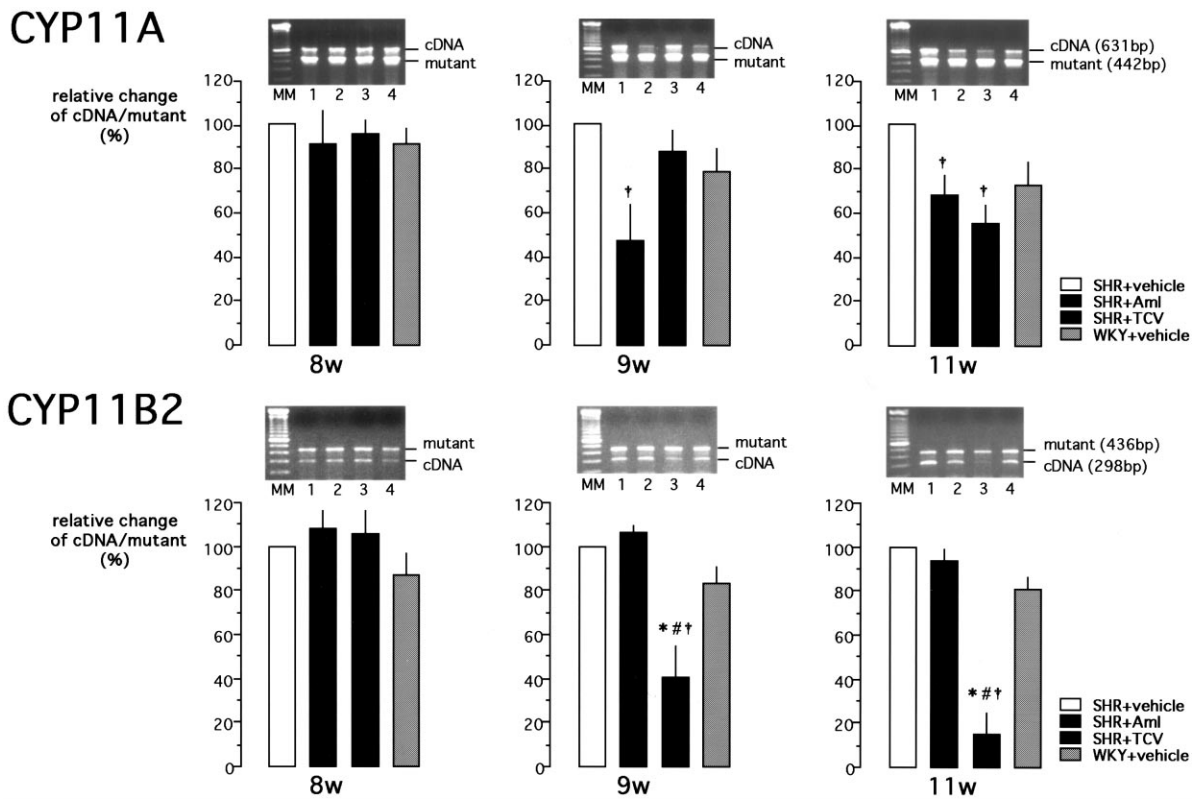

Fig. 7. Quantitation of adrenocortical mRNA expression by competitive RT-PCR. For quantitation of native cDNA samples, competitive RT-PCR was performed by mixing a constant amount (2 μl) of native cDNA (0.25 ng/μl) with the following dilutions of mutant cDNAs: $2^{-3}(1.25 \times 10^{-3}$ amol/μl) for CYP11A and $2^{-4}(6.25 \times 10^{-4}$ amol/μl) for CYP11B2. Densitometric quantitation of PCR products demonstrated that the levels of CYP11A and CYP11B2 mRNAs at the age of 8 weeks were not significantly different among the four groups; however, at the age of 9 weeks, CYP11A level was significantly reduced by amlodipine, and then at the age of 11 weeks, the CYP11A level was significantly lowered both by the amlodipine and TCV-116 treatments. CYP11B2 level was significantly reduced during the age of 9–11 weeks only by TCV-116 treatment. Data are mean ± S.E.M. ($n = 5$); *, $P < 0.01$ vs. WKY + vehicle; #, $P < 0.01$ vs. SHR + Aml; and †, $P < 0.01$ vs. SHR + vehicle.

4. Discussion

The present study demonstrates that in vivo Aldo synthesis is differentially modified by Ca^{2+}a and AT_1a despite their equivalent antihypertensive effect, and comparable serum and urinary electrolyte levels. The marked difference between these two drugs reflects their inhibitory effects on enzyme mRNAs involved in Aldo synthesis in vivo.

First, although p-Aldo levels in SHR are lower than in WKY rats in the prehypertensive phase, in the established hypertensive phase, p-Aldo levels in SHR are higher than in WKY rats. These results are in agreement with those of Moll et al. [25]; Komanicky et al. [26] also reported changes in adrenal steroidogenesis in SHR during the course of development of hypertension and that, in the prehypertensive stage of SHR, the synthesis of adrenocortical Aldo was noticeably potentiated. Our results showed that TCV-116 treatment significantly reduced p-Aldo level compared with amlodipine. To confirm the level of Aldo secretion in the adrenal cortex, we further evaluated the daily u-Aldo excretion, PRA and p-Aldo/PRA ratio. The daily u-Aldo excretion in SHR was significantly higher than in WKY rats throughout the observation period. Urinary 19-noraldosterone, another mineralocorticoid synthesized with Aldo in the zona glomerulosa, has also been reported to be increased at the prehypertensive stage in SHR [27]. The p-Aldo/PRA ratio, a sensitive indicator of responsiveness of adrenocortical Aldo secretion [28], was not different between SHR and WKY rats throughout the observation period.

Evaluation of the changes in u-Aldo level and p-Aldo/PRA ratio revealed that TCV-116 potently suppressed adrenocortical Aldo secretion after 1 week of treatment, while the reduction in Aldo by amlodipine occurred after 4 weeks of treatment. Wada et al. [29] reported that treatment with TCV-116 for 2 weeks dose-dependently reduced systemic blood pressure and p-Aldo level in SHR without exerting any effect on plasma corticosterone levels. The potent Aldo-suppressive effect of oral TCV-116 was confirmed in the plasma, urine and p-Aldo/PRA ratio. On the other hand, the effect of amlodipine on Aldo secretion be-

came evident 4 weeks after the commencement of treatment manifested by changes in u-Aldo and p-Aldo/PRA ratio levels.

Several in vitro studies have established the importance of Ca^{2+} influx through Ca^{2+}-channels on Aldo synthesis in response to stimuli of Ang II and K^+ [15,16,19,30,31]. In addition, in bovine adrenal glomerulosa cells containing both L- and T-type Ca^{2+}-channels, T-type Ca^{2+}-channel activity is thought to be closely correlated with Aldo biosynthesis [32–34]. Although the in vitro inhibitory effect of Ca^{2+}a on the synthesis of adrenocortical Aldo is well documented [33,35,36], the in vivo effects on Aldo secretion have been inconsistent and controversial [37–42]. Recently, Fiad et al. [28] reported that 8-day nifedipine treatment markedly lowered the p-Aldo/PRA ratio both in normotensive and hypertensive patients suggesting that the previously reported in vitro inhibition of Aldo biosynthesis in adrenal cells is reproducible even in vivo.

Second, using immunohistochemistry, we estimated the change of Aldo-synthesizing cells localized in the zona glomerulosa of the adrenal cortex. Treatment with either compound did not alter Aldo staining compared with control sections, although the Aldo-immunoreactivity was localized at the external layer of the adrenal cortex, the zona glomerulosa. Examination of HE stained sections showed no marked differences in the structure of the adrenal zona glomerulosa among SHR, WKY and both treated groups; however, the relative thickness of the glomerular layer was significantly increased by both antihypertensive agents compared with WKY rats. Frink et al. [43] morphometrically demonstrated increased volume of the zona glomerulosa following reduction of arterial blood pressure by various antihypertensive drugs such as hydralazine, reserpine and hydrochlorothiazide, and that such change reflected increased density of lipid droplets. They postulated that these morphological changes might be associated with a decrease in Aldo production in the zona glomerulosa [43].

Finally, the most important finding in the present study was the in vivo effect of amlodipine and TCV-116 on adrenocortical enzyme mRNAs involved in Aldo synthesis. The level of CYP11A mRNA coding for P450scc was markedly suppressed within 2 weeks of commencement of amlodipine treatment. Furthermore, TCV-116 also reduced CYP11A mRNA levels, although this effect did not appear until 4 weeks after commencement of treatment. Our results also showed that the level of CYP11B2 mRNA coding P450aldo was markedly suppressed within 2 weeks of commencement of TCV-116 treatment and continued throughout therapy. Cholesterol side-chain cleavage to pregnenolone by P450scc is classically defined as the rate-limiting step in the production of adrenal steroids [21]. It is also recognized that mobilization of cholesterol from cellular stores to the mitochondrial outer membrane and the subsequent transfer of this cholesterol to P450scc in the inner mitochondrial membrane are the rate-limiting steps in steroid production [44]. Tremblay et al. [45] showed that both low sodium and high potassium intake significantly increased P450scc and P450c11β (the latter mediates 11β-hydroxylation from 11-deoxycorticosterone to corticosterone) at both mRNA and protein levels, in the zona glomerulosa of rat adrenal cortex in vivo. They suggested that high levels of P450scc and P450c11β in the zona glomerulosa are closely associated with Aldo synthesis in response to Aldo-positive regulators. Although we did not investigate the mechanism of amlodipine-induced suppression of CYP11A, it is possible that amlodipine suppressed the upstream steroidogenesis pathway, and resulted in a slow and moderate reduction of u-Aldo and p-Aldo/PRA levels. Furthermore, the relative increase in serum cholesterol level in amlodipine treated SHR might be associated with such inhibition of P450scc.

With regard to the significance of Ca^{2+} in the regulation of Aldo synthesis, several in vitro studies have suggested that Ca^{2+}-influx through Ca^{2+}-channels is commonly associated with increased CYP11B2 gene expression via stimulation of Ang II and K^+ [46–48]; however, amlodipine treatment did not significantly affect CYP11B2 mRNA level in the present in vivo experiment. Taken together, it appears that the mechanisms that regulate adrenocortical Aldo synthesis described in in vitro studies are at least in part different from the in vivo regulation of Aldo synthesis noted in our study in SHR. We found that amlodipine, a Ca^{2+}a, suppresses the early step of Aldo synthesis and that TCV-116, an AT_1a, potently suppresses the last step of Aldo synthesis with no difference in terms of systemic blood pressure and electrolytes. These results suggest that in SHR a key-regulator of CYP11A is the influx of extracellular Ca^{2+} and that the action of Ang II through AT_1-receptors regulates CYP11B2 predominantly through a direct mechanism, e.g. transcription factors that have been recognized in vitro [49,50], rather than Ca^{2+}-influx which is considered to be a common effector in in vitro Aldo synthesis.

In conclusion, we have demonstrated in the present study the in vivo effects of chronic blockade of Ca^{2+}-channels and AT_1-receptors on Aldo synthesis in the adrenal cortex of SHR. These findings indicate that each antihypertensive agent, amlodipine and TCV-116, directly modulates adrenocortical Aldo synthesis in SHR through different pathways in the chronic stage under the same physiological and biochemical conditions. This modification comprises the suppression of both the first step (CYP11A predominantly responsive to Ca^{2+}a) and the last step (CYP11B2 in response to AT_1a), of the process of adrenocortical Aldo synthesis. The reduction of Aldo synthesis caused by either Ca^{2+}

a or AT_1a is at least in part involved in the antihypertensive action of amlodipine and TCV-116 and their protective role against hypertension-related organ damage. In view of the advantage of reduced action of Aldo, AT_1a:TCV-116 is considered to be a beneficial direct inhibitor of Aldo synthesis in vivo. The present results suggest that inhibition of action of Ang II, which is an upstream stimulator of Aldo synthesis, by AT_1a might be effective in suppressing the production and secretion of Aldo compared with the inhibition of Ca^{2+}-influx, which is a downstream common mediator of Aldo synthesis following Ang II and K^+, by Ca^{2+}a. To resolve the discrepancy between the in vitro and in vivo studies on the regulation of Aldo synthesis, further analysis is necessary.

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References

[1] J.H. Laragh, J.E. Sealey, The renin–angiotensin–aldosterone system in hypertensive disorders: a key role to two forms of arteriolar constriction and a possible clue to risk of vascular injury (heart attack and stroke) and prognosis, in: J.H. Laragh, B.M. Brenner (Eds.), Hypertension, Pathophysiology, Diagnosis, and Management, Raven Press, New York, 1990, pp. 1329–1348.

[2] F. Otsuka, T. Yamauchi, H. Kataoka, Y. Mimura, T. Ogura, H. Makino, Effects of chronic inhibition of ACE and AT_1 receptors on glomerular injury in Dahl salt-sensitive rats, Am. J. Physiol. 274 (1998) R1797–R1806.

[3] E.R. Lumbers, Invited review. Angiotensin and aldosterone, Regul. Pept. 80 (1999) 91–100.

[4] J. Muller, Aldosterone: the minority hormone of the adrenal cortex, Steroids 60 (1995) 2–9.

[5] C.G. Brilla, G. Zhou, L.S. Matsubara, K.T. Weber, Collagen metabolism in cultured adult rat cardiac fibrosis: response to angiotensin II and aldosterone, J. Mol. Cell. Cardiol. 28 (1995) 809–820.

[6] D. Pitt, ACE inhibitor co-therapy in patients with heart failure: rationale for the randomized aldactone evaluation study (RALES), Eur. Heart J. 16 (Suppl. N) (1995) 107–110.

[7] R. Rocha, P.N. Chander, K. Khanna, A. Zuckerman, C.T. Stier, Jr, Mineralocorticoid blockade reduces vascular injury in stroke-prone hypertensive rats, Hypertension 31 (1998) 451–458.

[8] R. Rocha, P.N. Chander, A. Zuckerman, C.T. Stier, Jr, Role of aldosterone in renal vascular injury in stroke-prone hypertensive rats, Hypertension 33 (1999) 232–237.

[9] E.L. Schiffrin, D.J. Franks, J. Gutkowska, Effect of aldosterone on vascular angiotensin II receptors in the rat, Can. J. Physiol. Pharmacol. 63 (1985) 1522–1527.

[10] M.E. Ullian, J.R. Schelling, S.T. Limas, Aldosterone enhances angiotensin II receptor binding and inositol phosphate response, Hypertension 20 (1992) 67–73.

[11] V. Robert, C. Heymes, J.-S. Silvestre, A. Sabri, B. Swynghedauw, C. Delcayre, Angiotensin AT_1 receptor subtype as a cardiac target of aldosterone. Role in aldosterone-salt-induced fibrosis, Hypertension 33 (1999) 981–986.

[12] A.T. Chiu, W.F. Herblin, D.E. McCall, R.J. Ardecky, D.J. Carini, J.V. Duncia, L.J. Pease, P.C. Wong, R.R. Wexler, A.L. Johnson, P.B.M.W.M. Timmermans, Identification of angiotensin II receptor subtypes, Biochem. Biophys. Res. Commun. 165 (1989) 196–203.

[13] A. Tanabe, M. Naruse, K. Arai, K. Naruse, T. Yoshimoto, T. Seki, T. Imaki, M. Kobayashi, H. Miyazaki, H. Demura, Angiotensin II stimulates both aldosterone secretion and DNA synthesis via type 1 not type 2 receptors in bovine adrenocortical cells, J. Endocrinol. Invest. 21 (1998) 668–672.

[14] B. Gigante, S. Rubattu, R. Russo, A. Porcellini, I. Enea, P. De Paolis, C. Savoia, A. Natale, O. Piras, M. Volpe, Opposite feedback control of renin and aldosterone biosynthesis in the adrenal cortex by angiotensin II AT1-subtype receptors, Hypertension 30 (1997) 563–568.

[15] J.H. Pratt, J.K. Rothrock, H. Dominguez, Evidence that angiotensin-II and potassium collaborate to increase cytosolic calcium and stimulate the secretion of aldosterone, Endocrinology 125 (1989) 2463–2469.

[16] M.M. Burnay, M.D. Vallotton, A.M. Capponi, M.F. Rossier, Angiotensin II potentiates adrenocorticotrophic hormone-induced cAMP formation in bovine adrenal glomerulosa cells through a capacitative calcium influx, Biochem. J. 330 (1998) 21–27.

[17] E.I. Johnson, J.G. McDougall, J.P. Coghlan, D.A. Denton, B.A. Scoggins, R.D. Wright, Role of Ca^{2+} in response of aldosterone to stimulation by angiotensin II in vivo, Am. J. Physiol. 254 (1988) E566–E571.

[18] S.J. Quinn, M.C. Cornwall, G.H. Williams, Electrical properties of isolated rat adrenal glomerulosa and fasciculata cells, Endocrinology 120 (1987) 903–914.

[19] E.L. Schiffrin, M. Lis, J. Gutkowska, J. Genest, Role of Ca^{2+} in response of adrenal glomerulosa cells to angiotensin II, ACTH, K^+, and ouabain, Am. J. Physiol. 241 (1981) E42–E46.

[20] S.J. Quinn, G.H. Williams, Regulation of aldosterone secretion, Annu. Rev. Physiol. 50 (1988) 409–426.

[21] E.R. Simpson, C.R. Jefcoate, C. Brownie, G.S. Boyd, The effect of ether anaesthesia stress on cholesterol-side-chain cleavage and cytochrome P450 in rat-adrenal mitochondria, Eur. J. Biochem. 28 (1972) 442–450.

[22] R.B. Oonk, J.S. Krasnow, W.G. Beattie, J.S. Richards, Cyclic AMP-dependent and -independent regulation of cholesterol side chain cleavage cytochrome P-450 (P-450scc) in rat ovarian granulosa cells and corpora lutea. cDNA and deduced amino acid sequence of rat P-450scc, J. Biol. Chem. 264 (1989) 21934–21942.

[23] M. Zhou, C.E. Gomez-Sanchez, Cloning and expression of a rat cytochrome P-450 11β-hydroxylase/aldosterone synthase (CYP11B2) cDNA variant, Biochem. Biophys. Res. Commun. 194 (1993) 112–117.

[24] F. Otsuka, T. Ogura, T. Yamauchi, H. Kataoka, M. Kishida, N. Miyatake, Y. Mimura, J. Kageyama, H. Makino, Long-term administration of adrenocorticotropin modulates the expression of IGF-I and TGF-β1 mRNA in the rat adrenal cortex, Growth Horm. IGF Res. 9 (1999) 41–51.

[25] D. Moll, S.L. Dale, J.C. Melby, Adrenal steroidogenesis in the spontaneously hypertensive rats (SHR), Endocrinology 96 (1975) 416–420.

[26] P. Komanicky, D.L. Reiss, S.L. Dale, J.C. Melby, Role of adrenal steroidogenesis in etiology of hypertension in the spontaneously hypertensive rat, Endocrinology 111 (1982) 219–224.

[27] Y. Takeda, I. Miyamori, T. Yoneda, K. Hurukawa, S. Inaba, Y. Ito, R. Takeda, Urinary excretion of 19-noraldosterone in the

spontaneously hypertensive rat and stroke-prone spontaneously hypertensive rat, Clin. Exp. Pharmacol. Physiol. 22 (Suppl. 1) (1995) S20–S22.

[28] T.M. Fiad, S.K. Cunningham, F.J. Hayes, T.J. McKenna, Effects of nifedipine treatment on the renin–angiotensin–aldosterone axis, J. Clin. Endocrinol. Metab. 82 (1997) 457–460.

[29] T. Wada, Y. Inada, T. Sanada, M. Ojima, Y. Shibouta, M. Noda, K. Nishikawa, Effect of angiotensin II receptor antagonist, CV-11974, and its prodrug, TCV-116, on production of aldosterone, Eur. J. Pharmacol. 253 (1994) 27–34.

[30] W.P. Hausdorff, K.J. Catt, Activation of dihydropyridine-sensitive calcium channels and biphasic cytosolic calcium responses by angiotensin II in rat adrenal glomerulosa cells, Endocrinology 123 (1988) 2818–2826.

[31] V. Pezzi, C.D. Clyne, S. Ando, J.M. Mathis, W.E. Rainey, Ca^{2+}-regulated expression of aldosterone synthase is mediate by calmodulin and calmodulin-dependent protein kinases, Endocrinology 138 (1997) 835–838.

[32] X.-L. Chen, A.B. Douglas, R.J. Fern, P.Q. Barrett, A role for T-type Ca^{2+} channel in the synergistic control of aldosterone production by ANG II and K^{+}, Am. J. Physiol. 276 (1999) F674–F683.

[33] M.F. Rossier, C.P. Python, A.M. Capponi, W. Schlegel, C.Y. Kwan, M.B. Vallotton, Blocking T-type calcium channel with tetrandrine inhibits steroidgenesis in bovine adrenal glomerulosa cells, Endocrinology 132 (1993) 1035–1043.

[34] M.F. Rossier, M.M. Burnay, M.B. Vallotton, A.M. Capponi, Distinct functions of T- and L-type calcium channels during activation of bovine adrenal glomerulosa cells, Endocrinology 137 (1996) 4817–4826.

[35] N. Blanchouin-Emeric, M. Zenatti, G. Defaye, B. Aupetit, Verapamil directly inhibits aldosterone synthesis by adrenal mitochondria in vitro, J. Steroid Biochem. 30 (1988) 453–456.

[36] J.L. Fakunding, K.J. Catt, Dependence of aldosterone stimulation in adrenal glomerulosa cells on calcium uptake: effects of lanthanum and verapamil, Endocrinology 107 (1980) 1345–1353.

[37] E.I. Johnson, J.G. McDougall, J.P. Coghlan, D.A. Denton, K.J. Hardy, B.A. Scoggins, R.D. Wright, Ca antagonists do not alter aldosterone secretion during established Na depletion, Am. J. Physiol. 248 (1985) E676–E680.

[38] L.M. Resnick, J.P. Nicholson, J.H. Laragh, Calcium, the renin–aldosterone system, and the hypotensive response to nifedipine, Hypertension 10 (1987) 254–258.

[39] M.W. Roy, G.P. Guthrie, Jr, F.P. Holladay, T.A. Kotchen, Effects of verapamil on renin and aldosterone in the dog and rat, Am. J. Physiol. 245 (1983) E410–E416.

[40] L. Terzoli, G. Leonetti, R. Pedretti, R. Bragato, L. Sampieri, M. Fruscio, L. Boselli, A. Zanchetti, Nifedipine does not blunt the aldosterone and cardiovascular response to angiotensin II and potassium infusion in hypertensive patients, J. Cardiovasc. Pharmacol. 11 (1988) 317–320.

[41] I. Zofkova, The effect of long-term verapamil treatment on the secretion of cortisol and aldosterone in subjects with normal and high blood pressure, Exp. Clin. Endocrinol. 85 (1985) 217–222.

[42] P.R. Conlin, E.W. Seely, N.K. Hollenberg, G.H. Williams, Dissociation of vascular and adrenal responsiveness to angiotensin II following calcium channel blockade, Endocr. Res. 24 (1998) 127–139.

[43] R. Frink, P.P. Krupp, D.T. Root, D.M. Warshaw, Effects of blood pressure reduction on the ultrastructure of the adrenal cortex of the spontaneously hypertensive rat, Exp. Cell Biol. 48 (1980) 245–254.

[44] C.R. Jefcoate, B.C. McNamara, L. Artemenko, T. Yamazaki, Regulation of cholesterol movement to mitochondrial cytochrome P450scc in steroid hormone synthesis, J. Steroid Biochem. Mol. Biol. 43 (1992) 751–767.

[45] A. Tremblay, M.R. Waterman, K.L. Parker, J.-G. Lehoux, Regulation of rat adrenal messenger RNA and protein levels for cytochrome P-450s and adrenodoxin by dietary sodium depletion or potassium intake, J. Biol. Chem. 266 (1991) 2245–2251.

[46] C.D. Clyne, P.C. White, W.E. Rainey, Calcium regulates human CYP11B2 transcription, Endocr. Res. 22 (1996) 485–492.

[47] K. Denner, W.E. Rainey, V. Pezzi, I.M. Bird, R. Bernhardt, J.M. Mathis, Differential regulation of 11β-hydroxylase and aldosterone synthase in human adrenocortical H295R cells, Mol. Cell. Endocrinol. 121 (1996) 87–91.

[48] P. Sarazin, A. Lefebvre, J.-G. LeHoux, The role of calcium channels in hamster CYP11B2 gene expression, Endocr. Res. 24 (1998) 633–636.

[49] C.D. Clyne, Y. Zhang, L. Slutsker, J.M. Mathis, P.C. White, W.E. Rainey, Angiotensin II and potassium regulate human CYP11B2 transcription through common *cis*-elements, Mol. Endocrinol. 11 (1997) 638–649.

[50] J.G. LeHoux, A. Lefebvre, Transcriptional activity of the hamster CYP11B2 promoter NCI-H295 cells stimulated by angiotensin II, potassium, forskolin and bisindolylmaleimide, J. Mol. Endocrinol. 20 (1998) 183–191.