Regulation of Aldosterone Synthase Cytochrome P450 (CYP11B2) and 11 β -Hydroxylase Cytochrome P450 (CYP11B1) Expression in Rat Adrenal Zona Glomerulosa Cells by Low Sodium Diet and Angiotensin II Receptor Antagonists

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Changes in the mRNA levels for aldosterone synthase cytochrome P450 (CYP11B2 or P450_{aldo}) and 11 β hydroxylase cytochrome P450 (CYP11B1 or P450_{11 β}) in rat adrenal glands were studied in response to angiotensin II type 1 (AT1) and type 2 (AT2) receptor antagonists. CYP11B1 and CYP11B2 genes were highly homologous (88.5%) in their nucleotide sequences of the amino acid coding regions. Reverse transcription-polymerase chain reactions (RT-PCR) which are capable of discriminating between rat CYP11B1 and CYP11B2, were performed with specific primers for each P450. Upon sodium restriction (5 mmol Na⁺/kg of diet) of rats for 14 d, the amount of the CYP11B2 mRNA in the adrenal glands was increased 8.5-fold compared to that from the rats fed a normal diet (225 mmol Na⁺/kg of diet), whereas no significant change in the CYP11B1 mRNA was observed after the dietary sodium restriction. As shown by an immunoblot analysis, the adrenal capsule portions (mainly zona glomerulosa) of the rats kept on the low Na diet for 14 d expressed significantly higher levels of both CYP11B2 and CYP11B1, and contained a significantly higher amount of CYP11B2 than those from the rats fed by normal diet. The activities of the CYP11B2 enzyme were also found to be increased by about 8-fold on day 14. In concert with these alterations, the plasma aldosterone concentration (PAC) increased. However, when the specific AT1 antagonist E4177 was given to rats maintained on the low Na diet, the amount and activity of CYP11B2, as well as the PAC, were suppressed. In contrast, the increase in CYP11B2 induced by the low Na diet was not affected by the AT2-specific antagonist PD123177. These results indicate that the aldosterone synthase cytochrome P450 (CYP11B2) is an ultimate target of the regulation of aldosterone biosynthesis by an AT1 receptor antagonist.

Key words angiotensin II type 1 receptor; E4177; CYP11B2; CYP11B1; aldosterone

Aldosterone is the most potent mineralocorticoid in mammals. It is synthesized from cholesterol through a pathway which includes pregnenolone, progesterone, 11-deoxycorticosterone (DOC) and corticosterone as the metabolic intermediates. It has been shown that cytochrome P450_{11 β} in bovine adrenocortical mitochondria catalyzes the final three steps in aldosterone biosynthesis: the 11-hydroxylation of deoxycorticosterone, 18-hydroxylation of corticosterone, and 18-methyl oxidation leading to aldosterone.¹⁾ In rats and humans, however, the synthesis of aldosterone from DOC is catalyzed by two separate P450s. The 18-hydroxylation and 18-methyl oxidase activities are limited to the cells of the zona glomerulosa, whereas the 11-hydroxylation activity is present throughout the whole adrenal cortex.²⁾ The 18-hydroxylation and 18-methyl oxidase activities are catalyzed by aldosterone synthase cytochrome P450 (CYP11B2), whereas the 11-hydroxylation activity is catalyzed by 11-hydroxylase cytochrome P450 (CYP11B1).

Lauber *et al.*³⁾ demonstrated, using immunoblot analyses, that rat adrenocortical mitochondria contain two proteins which are cross-reactive to the monoclonal antibody raised against purified bovine P450_{11 β} . Their molecular masses were estimated to be 51.5 and 49.5 kDa, respectively. These two enzymes, CYP11B1 and CYP11B2, exhibited a high homology (88.5%) to each other in their nucleotide sequences of the amino acid

coding regions,⁴⁾ although their genes, CYP11B1 and CYP11B2, are expressed in different zones of the adrenal gland.^{5,6)} It is now accepted that the CYP11B2 gene is expressed primarily in the zona glomerulosa,^{5,6)} and that the CYP11B1 gene is expressed in the zona fasciculata-reticularis and zona glomerulosa of rat.⁶⁾

Several methods have been used to measure the expression of CYP11B2 and CYP11B1 mRNA in rats. Using Northern blot analysis, several studies demonstrated that a low Na diet increased CYP11B2 mRNA in the zona glomerulosa without an effect on the expression of CYP11B1 mRNA.^{2,7,8)} The increases in the levels of CYP11B2 mRNA induced by sodium depletion were diminished by simultaneous treatment with an angiotensin converting-enzyme inhibitor, captopril, indicating that the effect of a low Na diet on CYP11B2 mRNA expression was mediated by angiotensin II.²⁾ A high Na diet was shown to decrease the expression of CYP11B2 mRNA without an effect on CYP11B1 mRNA.⁸⁾ Using the RNase protection assay, a low Na diet increased CYP11B2 mRNA but had no effect on the expression of CYP11B1 mRNA.⁵⁾

The major subtypes of angiotensin II (Ang II) receptors, the Ang II type 1 (AT1) and type 2 (AT2) receptor, have been cloned, and the degree of amino acid identity between AT1 and AT2 has been shown to be approximately 34%.^{9,10)} Recent studies have shown that the AT1 receptor

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is responsible for the activation of protein kinase C (PKC) and for the inhibition of adenyl cyclase.^{11,12)} However, the physiological functions and the signaling pathway of AT2 are not yet well defined.¹³⁾ It is well known that AT1 and AT2 receptors are mainly distributed in the zona glomerulosa and the medulla, respectively.¹⁴⁾ The AT1 and AT2 receptor-specific nonpeptide Ang II receptor antagonists, E4177 (AT1 antagonist) and PD123177 (AT2 antagonist), were recently identified by radioligand-receptor binding assay and autoradiographic analysis using rat adrenal glands.¹⁵⁾

In the present study, we examined the changes in the expression of CYP11B1 and CYP11B2 in rat adrenal glands in response to the AT1 antagonist E4177 and the AT2 antagonist PD123177.

MATERIALS AND METHODS

Drugs and Chemicals The chemical structures of E4177, 4'-[2-cyclopropyl-7-methyl-3*H*-imidazo[5,4-*b*]pyridine-3-yl]methyl-2-biphenylcarboxylic acid, and PD123177, 1-[(4-Amino-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid are shown Fig. 1. They were synthesized at Eisai Co. (Tokyo, Japan). The chemical purities of both compounds were >99% based on high-performance liquid chromatography (HPLC) analysis. All other reagents were obtained from commercial suppliers and were of analytical grade.

Rats and Diets Six groups of male Sprague-Dawley rats (SLC, Shizuoka, Japan), weighing 200–250 g were used. In each experiment, five rats per group were used throughout the study. The rats were kept under climate-controlled conditions and given rat chow and water *ad libitum*. The animals were fed one of two different diets, a low Na diet (5 mmol Na⁺ and 119 mmol K⁺/kg) or a normal diet (225 mmol Na⁺ and 119 mmol K⁺/kg), purchased from Oriental Yeast Japan (Tokyo, Japan), for 14 d, as described by Lauber *et al.*³⁾ The experimental animals were divided into six groups: normal diet group, normal diet plus E4177 oral administration group, normal diet plus PD123177 oral administration group, low Na diet group, low Na diet plus E4177 oral administration group, and low Na diet plus PD123177 oral administration group. The E4177 (5 mg/kg/d) and PD123177 (100 mg/kg/d) were administered only during the last 4 d of the 14 d period.

Preparation of Mitochondria from Adrenal Glands The

rats were killed by exsanguination from the heart under light ether anesthesia on the 14th d after the start of the feeding experiment. The adrenal glands were excised from the animals, and the capsular portion (mainly zona glomerulosa) was separated from the adrenal gland as described previously.³⁾ Mitochondria were prepared from the capsular portion and the cortex of the adrenal gland according to the conventional procedure at 4 °C. A solution containing 250 mM sucrose, 0.5 mM EDTA, 2 mM Tris buffer, pH 7.4, 0.1% bovine serum albumin (BSA), and 0.1 mM NAD was used during the isolation of the mitochondria. The mitochondria were finally resuspended in 2 mM Tris, pH 7.4 containing 250 mM sucrose and 0.5 mM EDTA. The protein concentration was determined by the method of Lowry *et al.*¹⁶⁾ using BSA (Sigma, St. Louis, MO, U.S.A.) as the standard.

Enzyme Assay The assay system for 11 β -hydroxylation (CYP11B1) and aldosterone synthase activities (CYP11B2) used the addition of fresh mitochondria (0.5 mg protein) to 1 ml of a buffer containing sucrose (250 mM), isocitrate (25 μ M), deoxycorticosterone (10 μ M) and Tris-HCl (50 mM, pH 7.4). Following an incubation for 10 min at 37 °C in air, the steroid end-products were extracted with 8 ml methylene chloride after adding 300 pmol cortisol (internal standard). The organic phase was washed twice and evaporated to dryness under a stream of air at 37 °C. The steroids were redissolved in 60% (v/v, methanol/water) and separated by a reverse-phase HPLC (YMC-AM 312 ODS column, 6 $\times$ 150 mm, YMC Co., Kyoto, Japan) with a solvent system of 60% (v/v, methanol/water) at a flow rate of 1 ml/min, and were measured with an ultraviolet detector (SPD 10A, Shimadzu, Kyoto, Japan) at 240 nm.

The peak fractions corresponding to aldosterone (ALDO), 18-hydroxycorticosterone (18-hydroxy-B), corticosterone (B) and cortisol were quantitated by the integration of respective peak height.

Hormone Assay and Determination Blood collected from the heart of each rat was used for the measurements of plasma renin activity (PRA) and plasma aldosterone concentration (PAC). The blood sample was centrifuged at 3000 rpm for 15 min immediately after the collection. The PRA level was determined by a radioimmunoassay (RIA) of angiotensin I generated from the endogenous angiotensinogen during a 60-min incubation at 37 °C using a commercially available kit (Dainabot Co., Tokyo, Japan). The PAC was assayed with an aldosterone RIA kit (Dainabot Co., Tokyo, Japan).

Gel Electrophoresis and Immunoblot Analysis For immunoblotting analyses, six groups of five rats were used. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the mitochondrial lysates from the capsular portion was carried out on 8% gels according to the method of Laemmli.¹⁷⁾ Each lane was loaded with approximately 30 μ g of mitochondrial protein. The transfer of proteins from the gel onto polyvinylidene difluoride membranes was performed as described previously.¹⁸⁾ The membranes were blocked with skim milk, treated with anti-bovine cytochrome P450_{11 β} immunoglobulin G (IgG) which cross-reacted with both rat cytochrome CYP11B1 and CYP11B2,^{19,20)} and then

Fig. 1. Chemical Structure of E4177 and PD123177

treated with an anti-rabbit IgG biotin complex and a horseradish peroxidase-avidin conjugate. The protein bands were stained by reaction with diaminobenzidine. The band signals in different lanes were quantitated using a laser densitometer (CS-9000, Shimadzu, Kyoto, Japan).

Amplification of Reverse Transcribed RNA using a Polymerase Chain Reaction (RT-PCR) For RNA analyses, six groups of five rats were used. In one experiment, total RNAs were prepared from the frozen adrenal capsule tissues of each of the six groups of five rats as described earlier.²¹⁾ cDNA (RT-product) was synthesized from 5 μ g of the total RNA using a random hexadeoxynucleotide²²⁾ to prime the 2 units of M-MLV (moloney-murine leukemia virus) reverse transcriptase (Amersham, UK) reaction in 10 μ l of reaction mixture (final volume). The control reactions were identical except for the omission of reverse transcriptase in one set of reactions (to control for the amplification of genomic DNA) and the omission of RNA in another set (to control for the cDNA contamination of reagents).

Oligonucleotide primers were used to amplify 284 and 272 base pair fragments of the rat CYP11B1 and CYP11B2 from the RT products, respectively. The sequences of the CYP11B1 primers were 5' ACCTGAAAGAAGAGAA-TGCTTGG 3' (sense) and 5' CTCAGAGATGATATC-CCAGG (antisense). The CYP11B2 primers were 5' AGGTGCGTCAGAATGCTCGA 3' (sense), and 5' TCAGAGATGACATCCCAGGC 3' (antisense). Primers that amplified a 382 base pair cDNA for the rat β -actin²³⁾ were included to control reaction efficiency and variations in the concentrations of mRNA in the original RT-reaction. The β -actin transcript has been used previously as an internal control for studies of the expression of CYP11B1 and CYP11B2 mRNA in the rat adrenal gland.²⁴⁾ The sequences of the β -actin primers were 5' CATTGTAACCAACTGGGGACG 3' (sense) and 5' TGAAGCTGTAGCCACGCTC (antisense). Preliminary assays were also conducted to determine the optimal number of cycles necessary for producing PCR products within the exponential phase of amplification. Based upon these experiments, CYP11B1 and CYP11B2 cDNA were amplified for a total 23 cycles in subsequent assays. The

amplification of the β -actin cDNA was carried out with only 20 cycles.

The PCR amplification was performed under the following reaction conditions: 30 μ l buffer (10 mM Tris-HCl, 2.5 mM MgCl₂, 50 mM KCl, 0.001% gelatin) containing 0.2 mM each of dATP, dCTP, dGTP and dTTP; 45 pmol of each PCR primer in water; 0.01% BSA; 0.3 μ l [α -³²P]-labeled dCTP (Amersham, UK); 11.25 U *Taq* DNA polymerase (Promega, Madison, WI); each 10 μ l RT sample was added to a 500- μ l microcentrifuge tube. Each mixture was brought to a final volume of 50 μ l with distilled water, overlaid with 30 μ l mineral oil, and amplified with a thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT). The amplification of cDNA products involved denaturation at 93 °C for 1 min, annealing at 61 °C for 1 min, and extension at 72 °C for 1.5 min. An aliquot (20 μ l) of each PCR reaction mixture was subjected to electrophoresis on a 5% polyacrylamide gel in Tris borate-EDTA buffer. The relative abundance of mRNAs for CYP11B1, CYP11B2 and β -actin in the PCR products was quantitated by scanning the gel with a BAS-2000 (Fuji, Tokyo, Japan). To validate the RT-PCR assay used, dose-response curves were established using different amounts of input RNA (0.5–16 μ g).

Statistical Analysis Data of enzyme assay and hormone assay are expressed as the mean $\pm$ S.E.M. The statistical significance was determined by Duncan's test for multiple group comparisons. *p* values of less than 0.01 were considered significant.

RESULTS

Effects of Low Na Diet and of Ang II Receptor Antagonist on the Activity of Aldosterone Synthase Cytochrome P450 (CYP11B2) and 11 β -hydroxylase Cytochrome P450 (CYP11B1) The enzymatic activities in the rats fed by normal diet and those fed by low Na diet were clearly distinct from each other. Figure 2 shows the chromatograms of the products obtained from the DOC substrate with the capsule portion (mainly zona glomerulosa) of the normal diet and low Na diet group. The main products of the normal diet group were corticosterone (B), whereas

Fig. 2. Chromatograms of Products from 11-Deoxycorticosterone (DOC) Produced by the Normal Diet and Low Na Diet Treatments

those of the low Na diet group were aldosterone (ALDO), 18-hydroxy-deoxycorticosterone (18-hydroxy-B) and B. These results confirmed that the retention times of these products were identical to those of the corresponding authentic steroids.

As seen in Table 1, the feeding of a low Na diet to rats for 14 d produced significantly higher levels of 18-hydroxy-B and ALDO (aldosterone synthase activity) than those observed in the rats fed a normal diet, by at least 6- and 8-fold, respectively.

When E4177 was administered with the low Na diet, the increase in the aldosterone synthase activity was completely suppressed to the control level. In contrast, when PD123177 was administered with the low Na diet, the increase in this activity was not altered significantly. The administration of either E4177 or PD123177 with the normal diet did not significantly alter the aldosterone synthase activity of the rats.

In parallel with these determinations, we examined the corticosterone synthesizing activity (11 β -hydroxylase activity) in these same mitochondria of the respective groups. The activity in the mitochondria from the zona glomerulosa was not affected by the dietary conditions or by E4177 or PD123177, in contrast to the aldosterone synthase activity.

PRA and PAC Table 2 shows the PRA level and PAC in the blood of the experimental animals. The PRA level was found to increase both under the low Na diet and by E4177 administration ($p < 0.01$). The normal diet plus PD123177 group showed the same PRA level as that of the normal diet group. The PAC increased significantly under the low Na diet compared with the normal diet ($p < 0.001$), and the increase was nearly suppressed to the control (normal diet) level by E4177 administration ($p < 0.001$). PD123177 administration to the low Na diet group had no effect on the PAC.

Effect of Low Na Diet and of Ang II Receptor Antagonists on the Amount of CYP11B2 in the Zona Glomerulosa Cells of the Adrenal Gland Figure 3 illustrates the results of the immunoblot analyses of CYP11B2 and CYP11B1 in the capsule portion (mainly zona glomerulosa) of the adrenal glands from each group of rats detected with anti-bovine P450_{11 β} IgG. The band of CYP11B1 with

an apparent molecular weight of 51.5 kDa was always detectable from the adrenals of the animals on day 14, irrespective of the dietary conditions and different Ang II receptor antagonists. Furthermore, the protein levels of CYP11B1 did not change by these conditions. In contrast, the band of CYP11B2 (49.5 kDa) was recognized only in the adrenal glomerulosa. The band of CYP11B2 was detectable in the adrenal glands of the rats fed by low Na diet, but the protein level decreased by 85% when E4177

Table 2. Effects of Low Na Diet, E4177 and PD123177 Administrations on the Plasma Aldosterone Concentration (PAC) and Plasma Renin Activity (PRA) of Rats

Treatments	PAC (ng/dl)	PRA (ng/ml · h)
Normal diet	21.9 $\pm$ 3.2	4.9 $\pm$ 0.7
Normal diet + E4177	19.1 $\pm$ 2.7	32.8 $\pm$ 0.6 ^{a)}
Normal diet + PD123177	22.8 $\pm$ 3.6	4.8 $\pm$ 0.4
Low Na diet	680.3 $\pm$ 49.1 ^{b)}	38.0 $\pm$ 4.9 ^{a)}
Low Na diet + E4177	47.3 $\pm$ 5.4 ^{c)}	45.4 $\pm$ 0.9 ^{a)}
Low Na diet + PD123177	545.0 $\pm$ 58.1 ^{b)}	35.4 $\pm$ 1.8 ^{a)}

PAC and PRA were determined by radioimmunoassays with the plasma isolated from the truncular blood. Values are the mean $\pm$ S.E.M. of five animals. The significance of differences was calculated by Duncan's test where a), $p < 0.01$; b), $p < 0.001$ vs. the value in the normal diet group, and c), $p < 0.001$ vs. the value in the low Na diet group.

Fig. 3. Immunoblot Analysis of CYP11B1 and CYP11B2 in the Lysates of Mitochondria from the Zona Glomerulosa of Rat Adrenal Glands

Anti-bovine cytochrome P450_{11 β} IgG was used for the immunoblot analysis of rat CYP11B1 and CYP11B2. Treatments of the animals are indicated in the figure. The amount of protein per lane was 30 μ g mitochondria protein, respectively. Arrows indicate the positions of the proteins with 51.5 kDa and 49.5 kDa molecular weights. SDS-PAGE electrophoresis and immunostaining are shown in the top panel. The relative densitometric profiles are shown in the middle and bottom panels.

Table 1. Activities of Aldosterone Synthase (ALDO, 18-Hydroxy-B) and 11 β -Hydroxylase (B) in the Adrenal Mitochondria of the Zona Glomerulosa of Rats Treated with and without Low Sodium and Ang II Receptor Antagonists

Treatments	Conversion levels to products		
	ALDO	18-Hydroxy-B	B
Normal diet	31 $\pm$ 3	44 $\pm$ 2	706 $\pm$ 29
Normal diet + E4177	29 $\pm$ 3	49 $\pm$ 2	725 $\pm$ 39
Normal diet + PD123177	29 $\pm$ 2	41 $\pm$ 3	738 $\pm$ 27
Low Na diet	252 $\pm$ 17 ^{a)}	268 $\pm$ 19 ^{a)}	702 $\pm$ 22
Low Na diet + E4177	31 $\pm$ 2 ^{b)}	42 $\pm$ 1 ^{b)}	732 $\pm$ 59
Low Na diet + PD123177	236 $\pm$ 10 ^{a)}	253 $\pm$ 15 ^{a)}	690 $\pm$ 21

Aldosterone synthase (ALDO, 18-hydroxy-B) and 11 β -hydroxylase (B) activities are expressed as picomoles per min/mg protein, respectively. Data are the means of triplicate assays. The significance of differences was calculated by Duncan's test where a), $p < 0.001$ vs. the value in the normal diet group, and b), $p < 0.001$ vs. the value in the low Na diet group.

was given to the animals. The protein level of CYP11B2 from the rats fed by low Na diet was not affected by PD123177 administration. The rats kept on a normal Na diet with or without Ang II receptor antagonists (E4177 or PD123177) did not show detectable levels of CYP11B2.

Amplification of Reverse Transcribed RNAs for CYP11B1 and CYP11B2 using the RT-PCR To test the validity of the RT-PCR assay as a tool for the semi-quantitative assessment of CYP11B1, CYP11B2 and β -actin mRNA, dose-response curves were established using different amounts of mRNA extracted from low Na diet-treated rat adrenals. The results showed that for the CYP11B1, CYP11B2 and β -actin mRNA RT-PCR assays,

Fig. 4. Dose-Response Curves for CYP11B1, CYP11B2 and β -Actin mRNA RT-PCR Assays

Different quantities (0.5–16 μ g) of RNA extracted from the adrenal capsular portions of low Na diet-treated rats adrenals were used as input RNA. CYP11B1 and CYP11B2 cDNA were amplified for a total of 23 cycles. β -actin cDNA was amplified for a total of 20 cycles.

Fig. 5. Gel Electrophoresis of PCR Products of the RNA from Adrenal Zona Glomerulosa

Total RNA preparation, RT reaction and PCR condition were performed as described in Materials and Methods. Twenty μ l samples of each 50 μ l PCR reaction mixture fractionated by electrophoresis on a 5% polyacrylamide gel in Tris borate-EDTA buffer were prepared. Specific primers for CYP11B1 and CYP11B2 genes were used to amplify the 284 and 272 bp fragments of these genes, respectively. The PCR product of β -actin mRNA (382 bp) is also shown as the standard.

there was a steady increase in the response as the doses were increased from 0.5 to 16 μ g input RNA. The preliminary assays demonstrated that the range of mRNA amounts produced PCR products that were linear with respect to the initial CYP11B1, CYP11B2 and β -actin concentrations ($r^2=0.99$ for all PCR products; Fig. 4).

Thus, we used six groups of rats among which the aldosterone synthesis was altered by a sodium restricted diet and by Ang II receptor antagonists, and the RNA preparations from the capsule portion (mainly zona glomerulosa) of the adrenal glands were analyzed by RT-PCR assay (Fig. 5). There was no modulation of the levels of β -actin mRNA with sodium restriction or Ang II receptor antagonist treatment. The administration of Ang II receptor antagonists (E4177 or PD123177) to the rats kept on a normal diet did not change the level of CYP11B1 or CYP11B2 mRNA in the adrenal cortex zona glomerulosa (Table 3). In the rats that received the low Na diet, the level of CYP11B1 mRNA was not different from the control (normal diet) level, whereas the amount of CYP11B2 mRNA was increased by 8.5-fold. When E4177 was given to the rats maintained on the low Na diet, the level of CYP11B2 mRNA decreased markedly, almost to the control level. The level of CYP11B1 mRNA was not affected by E4177 administration. The PD123177 administration did not affect the levels of CYP11B1 and CYP11B2 mRNAs of the low Na diet animals.

DISCUSSION

In the present study, we examined the effects of AT1-specific and AT2-specific antagonists, *i.e.*, E4177 and PD123177, on the expression of aldosterone synthase cytochrome P450 (CYP11B2) and 11 β -hydroxylase cytochrome P450 (CYP11B1) in the rat adrenal glands. E4177 is a potent and selective AT1-specific antagonist whose specific binding to the AT1 receptor on the plasma membrane of the adrenal zona glomerulosa cells has been confirmed by light- and electron-microscopic observations.¹⁵⁾ PD123177 inhibits the AT2 receptor which is localized in the adrenal medulla.^{14,15)} It is known that CYP11B2 is specifically expressed in the zona glomerulosa cells, where AT1 receptors are mainly distributed.^{5,6)} The zonation of CYP11B1 has recently been questioned. CYP11B1 is expressed in the zona fasciculata and zona reticularis cells as judged by immunostaining with specific

Table 3. Relative Abundance of CYP11B1 and CYP11B2 mRNAs Estimated by RT-PCR of the Adrenal Total RNAs of the Treated Rats

Treatments	CYP11B1	CYP11B2
Normal diet	1.0	1.0
Normal diet + E4177	1.0	0.9
Normal diet + PD123177	1.0	1.0
Low Na diet	1.0	8.5
Low Na diet + E4177	1.0	1.5
Low Na diet + PD123177	0.9	8.0

The bands of 284 bp (CYP11B1) and 272 bp (CYP11B2) DNA fragments on the gels shown in Fig. 5 were analyzed and quantitated with a BAS-2000. The relative intensities of the bands are shown in the table. The data are the means of two independent experiments after correction using data obtained with β -actin. After correction, the control values (normal diet group) were set arbitrarily at 1.0.

antibody.^{25,26)} According to an *in situ* hybridization study, CYP11B1 transcripts were found in all three zones of the rat adrenal cortex.⁶⁾ This finding suggests a post-translational dissociation and/or that the adrenal capsular cells were contaminated with subcapsular cells.

The stimulation of aldosterone biosynthesis in rats by the low Na diet has been well documented.^{27–29)} It was found that this diet induced the increase of a protein with a 49.5 kDa molecular weight in the rat adrenal zona glomerulosa mitochondria, which was detected by an immunoblot analysis with an anti-bovine P450_{11 β} IgG.³⁾ The 49.5 kDa protein was purified and named CYP11B2 since it catalyzed aldosterone synthesis in addition to 11 β hydroxylation.¹⁹⁾ The increase in aldosterone biosynthetic activity induced by sodium restriction was accompanied by a simultaneous and commensurate increase in the zona glomerulosa mitochondrial CYP11B2 protein level.²⁰⁾ A zona glomerulosa-specific increase in CYP11B2 mRNA level induced by sodium deficiency has been confirmed by slot blot and Northern blot hybridization analyses with specific oligonucleotide probes.^{2,7)} According to these findings, sodium restriction influences aldosterone biosynthesis, at least partly, at a pretranslational level of the expression of CYP11B2. We found that the low Na diet elevated the enzyme activity and amount of CYP11B2 in the adrenal zona glomerulosa mitochondria of the treated rats, which was accompanied by an increase in the PAC, and that the AT1-specific antagonist E4177 completely suppressed the increases in CYP11B2 and PAC. However, the AT2-specific antagonist PD123177 administered to the low Na diet animals did not lower the elevated levels of PAC or the enzyme activity or amount of CYP11B2. In previous studies, when enalapril malate or captopril, an angiotensin converting-enzyme inhibitor, was administered to the low Na rats, the increases in the amount and activity of the CYP11B2 enzyme, as well as in PAC, were suppressed only partially.^{2,20)} These results indicate that the level of CYP11B2 mRNA in the adrenal zona glomerulosa cells was elevated by the low Na diet, and the elevation was completely suppressed by the AT1-specific antagonist E4177, which strongly suggested that the synthesis of CYP11B2 is regulated at the transcriptional level by the AT1 receptor. In contrast, the level of CYP11B1 mRNA in the adrenal zona glomerulosa was not affected by the Na restriction in the diet, nor by the administration of AT1 and AT2 antagonists.

We did not detect significant effects of the dietary sodium restriction or the Ang II receptor antagonists on the expression of CYP11B1 in the adrenal cortex, which indicated that the renin-angiotensin system has no relation to the expression of CYP11B1. The CYP11B1 protein had been previously detected in rat zona glomerulosa cells by Western blot analysis.^{19,20)} Furthermore, the presence of CYP11B1 mRNA in the zona glomerulosa cells was clearly shown, although its level was more abundant in the zona fasciculata-reticularis than in the zona glomerulosa.^{30,31)} CYP11B1 mRNA in the rat zona glomerulosa and fasciculata cells also increased under corticotropin (ACTH) treatment. However, it is well known that CYP11B2 is expressed only in the zona glomerulosa cells, and that its level is not affected by ACTH treatment.^{30,31)}

It is highly likely that the expressions of these two steroidogenic P450s, CYP11B1 and CYP11B2, reflect differences in the promoter elements of their genes which regulate the expression of the genes through specific receptors for ACTH and Ang II on the adrenal cortex cells.

The AT1 receptor is linked to several signaling mechanisms which could lead to an Ang II-induced increase in intracellular calcium¹⁰⁾ or the activation of PKC.¹⁵⁾ The expression of the CYP11B2 gene is probably regulated by several signal transduction factors, including PKC, which is activated by this AT1 receptor-mediated mechanism. Although further studies of the regulation of CYP11B2 gene expression are needed to test this hypothesis, the results of our present study indicate that the regulation of aldosterone-synthesizing enzymes mediated by the AT1 receptor is a key mechanism for the adaptation of rats to a dietary sodium restriction.

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