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Inhibition of the expression of the gene for the angiotensin AT_1 receptor by angiotensin II in the rat adrenal gland

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Abstract

The expression of angiotensin AT_{1A} and AT_{1B} receptor mRNA after continuous angiotensin II administration was investigated in the rat adrenal gland. Angiotensin AT_1 receptor mRNA detected by Northern blot analysis decreased to $52.7 \pm 16.1\%$ of control after the administration of angiotensin II (20 μg/h) for 24 h, and to $70.8 \pm 8.0\%$ after 1 week. A low dose of angiotensin II (0.2 μg/h) also decreased angiotensin AT_1 receptor mRNA to $73.0 \pm 5.5\%$ after 1 week. Competitive reverse transcription and polymerase chain reaction (RT-PCR) experiments revealed that both angiotensin AT_{1A} and AT_{1B} receptor mRNAs decreased after administration of angiotensin II (20 or 0.2 μg/h) for 1 week. Analysis of the angiotensin AT_{1A} promoter by using luciferase–reporter system showed that angiotensin II (up to 1 μM) did not have any effects on the promoter activity ($106 \pm 5.7\%$ after 0.1 μM angiotensin II stimulation) in Y1 cells and cultured vascular smooth muscle cells, although phorbol myristate acetate (PMA) decreased the promoter activity by about 40% compared with control. These results suggest that angiotensin AT_1 receptor gene expression in the rat adrenal gland is inhibited by angiotensin II and it may not be due to suppression of promoter activity. Other mechanisms such as destabilization of angiotensin AT_1 receptor mRNA or angiotensin II-induced increased blood pressure may be involved in the inhibition.

Keywords: Angiotensin II; Angiotensin AT_1 receptor; Gene expression; Adrenal gland; Promoter

1. Introduction

The angiotensin II receptor is a central component of the renin–angiotensin system. Activation of angiotensin II receptors mediate a variety of effects such as contraction and hypertrophy of cardiac and vascular smooth muscle, catecholamine release and aldosterone secretion from adrenal glands, adrenocorticotropic hormone and prolactin secretion from the anterior pituitary gland (Peach, 1977; Ishihata and Endoh, 1993, 1995). Thus, regulation of their expression is important in cardiovascular, adrenal and central responsiveness to angiotensin II.

The existence of two types of angiotensin II receptors, AT_1 and AT_2, has been demonstrated (Timmermans et al., 1993). Although little is known about the function of angiotensin AT_2 receptors, angiotensin AT_1 receptors mediate many of those functions described above. In addition, two different types of cDNA of angiotensin AT_1 receptor, that is AT_{1A} and AT_{1B}, have been isolated in the rat (Elton et al., 1992; Langford et al., 1992; Sandberg et al., 1992). Their chromosomal location is different (Lewis et al., 1993; Szpirer et al., 1993), but they exhibit 91% nucleotide sequence homology in the coding region and have the same binding characteristics. The expression of angiotensin AT_1 receptor is regulated by various vasoactive substances, growth factors and glucocorticoids (Sato et al., 1994), and the expression of rat angiotensin AT_{1A} and AT_{1B} receptor gene is tissue-dependent. For example, vascular smooth muscle cells, kidney and heart express mainly angiotensin AT_{1A} receptor mRNA, whereas pituitary gland express primarily angiotensin AT_{1B} receptor mRNA (Kakar et al., 1992a; Kitami et al., 1992).

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Although the basal level of circulating angiotensin II does not seem to affect the level of angiotensin AT_1 receptor mRNA (Wakamiya et al., 1994), AT_1 receptors of many tissues such as kidney and vascular and bladder smooth muscle cells are reported to be downregulated by increased level of angiotensin II (Haefliger et al., 1995). By contrast, binding capacity of angiotensin AT_1 receptor was increased by angiotensin II in the rat adrenal gland (Hauger et al., 1978; Aguilera and Catt, 1978), but is decreased in bovine adrenal cells (Penhoat et al., 1988). Thus, tissue- and species-dependent differences in the regulation of angiotensin AT_1 receptor may exist. In addition, the regulation of angiotensin AT_{1A} and AT_{1B} receptors appears to be different. First, the level of angiotensin AT_{1A} receptor mRNA increased in rat infarcted ventricles without affecting angiotensin AT_{1B} mRNA (Nio et al., 1995). Also, a low sodium diet increased angiotensin AT_{1A} mRNA in the kidney but decreased levels of angiotensin AT_{1B} mRNA (Du et al., 1995). Second, dexamethasone increased the expression of angiotensin AT_{1A} but not AT_{1B} gene in rat cardiac fibroblasts and cardiomyocytes (Matsubara et al., 1994). Third, estrogen suppressed only angiotensin AT_{1B} in the rat pituitary gland (Kakar et al., 1992a). The different distribution and regulation of these two receptors suggest that two subtypes of angiotensin AT_1 receptor mRNA are regulated differently in each organ and may play an important role in pathophysiological conditions.

However, the problem of whether expression of angiotensin AT_1 receptors is under control of angiotensin II or not, is not clarified yet. Therefore, in the present study, we have investigated the effects of angiotensin II on the modulation of each subtype of angiotensin AT_1 receptor mRNA in the rat adrenal gland in vivo. In order to clarify whether angiotensin II can directly regulate the transcriptional activity of angiotensin AT_1 receptor gene, the influence of angiotensin II on the promoter activity of angiotensin AT_1 gene was examined. In addition, promoter activity was investigated both in adrenal and vascular smooth muscle cells to assess the possible existence of tissue-specific *cis*-element for angiotensin II stimulation.

2. Materials and methods

2.1. Materials

Bovine serum albumin was obtained from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal calf serum (FCS), Ham's F-10 medium, horse serum and Superscript II reverse transcriptase were from GIBCO BRL (Grand Island, NY). Angiotensin II was purchased from Peninsula Laboratories (Belmont, CA). Radiolabeled nucleotide [α-^{32}P]dCTP (3000 μCi/mmol) was obtained from New England Nuclear (Boston, MA).

2.2. Surgical procedure

All experiments were performed using adult male Sprague–Dawley rats in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Angiotensin II was given in a high dose (20 μg/h) or in a low dose (0.2 μg/h) for various periods through an osmotic minipump (model 2002, Alza, Palo Alto, CA) implanted intravenously under aseptic conditions. Rats were anesthesized by intraperitoneal administration of sodium pentobarbital (40 mg/kg). To determine the effect of angiotensin II on the expression of angiotensin AT_1 receptor, rats were divided into several groups: control rats without angiotensin II infusion; rats with long-term infusion of angiotensin II (20 or 0.2 μg/h) for 1 week; rats with short-term infusion of angiotensin II (20 or 0.2 μg/h) for 24 h. Systolic blood pressure was measured by a tail-cuff method on the day of surgery, afer 3 days of infusion of angiotensin II in the long-term angiotensin II infusion group.

2.3. RNA extraction and Northern hybridization analysis

Total RNA was isolated by means of acid guanidium isothiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Total RNA (20 μg) was separated in a 1% agarose gel containing 2.2 M formaldehyde and transferred to a positively charged nylon membrane (Hybpnd N^+, Amersham, Arlington, IL). After the RNA was fixed onto the membrane by UV light, the filter was transferred to a solution of 0.5 M sodium acetate (pH 5.2) and 0.04% methylene blue for 5–10 min at room temperature to stain the RNA (Szpirer et al., 1993). Probes were labeled with [α-^{32}P]dCTP by using random primer method (Prime It II, Stratagene, La Jolla, CA). The labeled probes were separated from unincorporated nucleotides by using Sephadex G-50 spin columns (Boehringer Mannheim, Indianapolis, IN). Prehybridization was carried out in 50% formamide, 6 × standard saline citrate (SSC) (1 × SSC contains 0.15 M NaCl and 1.5 mM sodium citrate, pH 7.0), 2 × Denhardt's solution (0.1% Ficoll, 0.1% bovine serum albumin and 0.1% polyvinylpyrrolidone), 1% sodium dodecyl sulphate (SDS), 10 mM sodium phosphate buffer and 0.2 mg/ml denatured salmon sperm DNA for 4 h. Then the membrane was hybridized with a ^{32}P-labeled probe specific for AT_1 receptor in the same buffer for 16 to 24 h at 42°C. Membranes were washed successively in 2 × SSC containing 0.1% SDS at room temperature for 30 min, 0.1 × SSC containing 0.1% SDS at 65°C for 30 min twice. Membranes were exposed to Kodak XAR-5 films (Eastman Kodak, Rochester, NY). The autoradiograms were scanned by using an ES-8000C image scanner (Epson America) and quantified densitometrically. The 28S ribosomal RNA or GAPDH was used to normalize the differences in loaded and transferred RNA.

2.4. Primers for reverse transcription and polymerase chain reaction (RT-PCR)

First strand cDNAs were synthesized from total RNAs by using reverse transcriptase. The following oligonucleotide primers were used for angiotensin AT_{1A} and AT_{1B} receptor cDNAs: sense 5′-TGAGAGATTGG-TATAAAATGGCTG-3′ and 5′-AGACACACAGCCTT-TCCAGCGCCA-3′ for angiotensin AT_{1A} and AT_{1B}, respectively; antisense 5′-GTTGAACAGAACAAGT-GACCT-3′ and 5′-TTTAACAGTGGCTTTGCTCCA-3′ for angiotensin AT_{1A} and AT_{1B}, respectively (Kakar et al., 1992a,b). These dissimilar primers correspond to 5′ and 3′ non-coding regions of angiotensin AT_{1A} and AT_{1B} cDNAs, where there is little sequence homology, and there is no sequence divergence between angiotensin AT_{1A} and AT_{1B} receptors (Kakar et al., 1992a,b). The problem of contamination of RNA samples by genomic DNA was circumvented by using sense and antisense primers which correspond to the sequence of different exons.

2.5. Quantitative RT-PCR

Quantification of angiotensin AT_{1A} and AT_{1B} receptor mRNA was carried out by RT-PCR in the presence of a known concentration of deletion-mutated angiotensin AT_{1A} or AT_{1B} cRNA as an internal standard.

The angiotensin AT_{1A} and AT_{1B} receptor cDNA mutants were prepared by removing 288 bp fragment between *Msc*I–*Msc*I (New England Biolabs, Beverly, MA), self-ligation and subcloning into pBluescript II KS (+) (Stratagene). The deletion mutant cRNAs were synthesized by using T3 RNA polymerase (Promega, Madison, WI) after being linearized by *Bam*HI in angiotensin AT_{1A} cDNA or *Not*I in AT_{1B} cDNA, respectively. The mixture was digested by RNase-free DNase, and the product was extracted by phenol/chloroform and purified on a G-50 Sephadex column. Those purified RNA were used as the template for reverse transcription reaction. The expected sizes of deletion-mutated cDNAs obtained by using the primers described above were 1.6 and 1.4 kbp for angiotensin AT_{1A} and AT_{1B}, respectively.

Total RNA (0.5 μg) was dissolved in 20 μl of a reaction mixture containing 1 mM dNTPs, 100 mM dithiothreitol, 1 U RNasin (Boehringer Mannheim), 100 pM random hexamers (Boehringer Mannheim), PCR buffer (final concentrations: 50 mM KCl, 20 mM Tris–HCl, 2.5 mM $MgCl_2$, and 10 mg/ml bovine serum albumin), and 200 U Superscript II reverse transcriptase (GIBCO BRL). The deletion-mutated cRNAs were mixed simultaneously with the native RNA sample for RT-PCR to compete with the endogenous angiotensin AT_{1A} and AT_{1B} receptor mRNAs. The reaction was carried out at 42°C for 45 min, followed by inactivation of the enzyme by incubating at

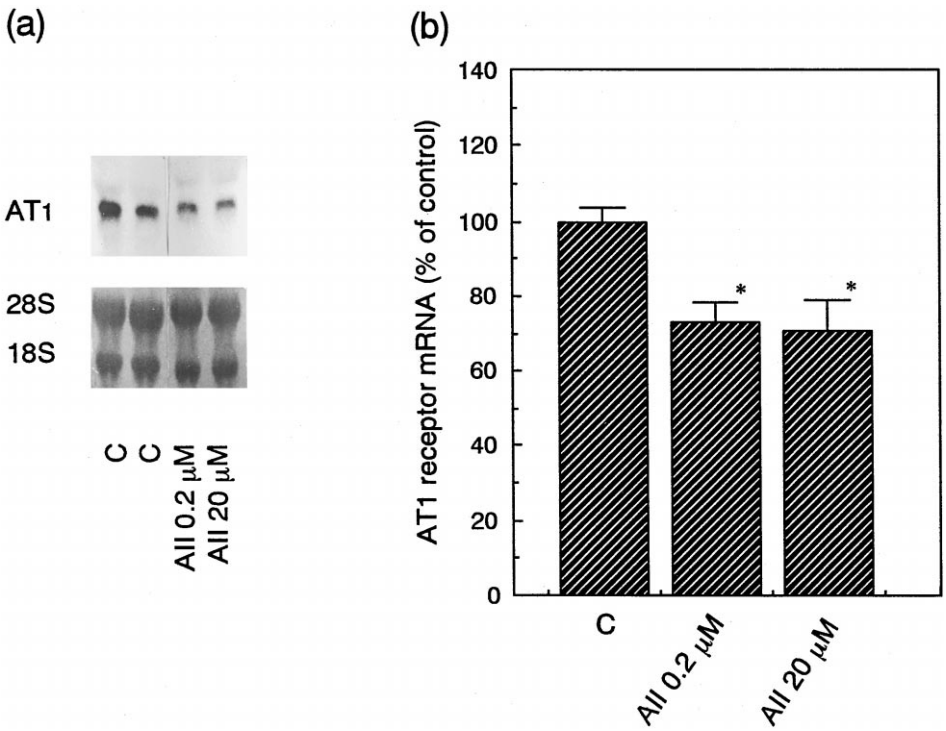

Fig. 1. Changes in angiotensin AT_1 receptor gene expression in rat adrenal glands after administration of 20 or 0.2 μg/h of angiotensin II for 1 week. (a) A typical example of Northern blotting analysis. (b) The amount of angiotensin AT_1 mRNA was normalized to 28S ribosomal RNA or GAPDH and expressed as percent of control. The data represent mean ± S.E.M. of 5–10 experiments. Asterisks indicate significant difference vs. control ($P < 0.05$).

70°C for 15 min. The samples were kept on ice. To amplify the resulting cDNA, aliquots (2 μl) of reverse transcribed samples were used for PCR. The PCR reaction mixture contained PCR buffer (GeneAmp Kit, Perkin Elmer Cetus Instruments, Norwalk, CT), 25 pmol of each primer, 1 mM dNTPs, 5 U *Taq* DNA polymerase (Perkin Elmer Cetus Instruments) and a trace amount of [α-^{32}P]dCTP (3 μCi) to label the PCR products. The reaction was performed on a thermal cycler (Perkin Elmer) under the following conditions: denaturation at 94°C for 45 s, annealing at 64°C for 1 min, and extension at 72°C for 1 min for 30 cycles. In order to avoid the amplification of non-specific products, annealing of the primers to the templates were carried out under high stringency conditions (Kakar et al., 1992a,b). After completion of the reactions, 10 μl of the 100 μl reaction mixture was electrophoresed on acrylamide gels, vacuum-dried and subjected to autoradiography using Kodak XAR-5 films. The autoradiographic signals were measured densitometrically.

To ascertain whether there were linear relationships between the native and deletion-mutated cRNA, the following approach was taken. For angiotensin AT_{1A} and AT_{1B} reactions, the amount of native total RNA containing angiotensin AT_{1A} and AT_{1B} receptor mRNAs was kept constant (0.5 μg per reaction), and that of mutated cRNA was varied (25.6, 12.8, 6.4, 3.2, 1.6 and 0.8 pg per reaction). The range of concentrations of sample RNA and deletion-mutated cRNA, and the cycles of PCR were chosen from the exponentially increasing phase of the product formation. Absolute quantities of mRNAs were determined by extrapolation of the competition curves generated in each sample for native and deletion-mutated RNAs.

2.6. Culture of rat aortic vascular smooth muscle cells and Y1 cells

Rat aortic vascular smooth muscle cells were isolated from adult male Sprague–Dawley rats purchased from Charles River Laboratories, Wilmington, MA, by the method of Gunther et al. (1980). Briefly, the thoracic aortas of 12 week old rats were dissected from the surrounding tissues. After adipose tissues were removed, aortas were digested in Dulbecco's modified Eagle medium (DMEM) containing 0.7 mg/ml collagenase (type IA, Sigma), 0.25 mg/ml elastase (type III, Sigma), 0.4 mg/ml soybean trypsin inhibitor and 1 mg/ml bovine serum albumin for 30 min at 37°C. Dissociated vascular smooth muscle cells were seeded into plastic tissue culture dishes and grown in DMEM containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO_2–95% air. The medium was changed every 3–4 days. Cultured vascular smooth muscle cells were used at 6–10 passages.

Y1 cells, which were derived from a mouse adrenocortical tumor, were obtained from American Type Culture Collection. These cells were grown in Ham's F-10 medium containing 15% horse serum, 2.5% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO_2–95% air.

2.7. Plasmid constructs

The method for construction of plasmids was described before (Uno et al., 1994). In brief, a 2.9 kb long fragment in the 5′-flanking region of the rat angiotensin AT_{1A} receptor gene from just upstream of exon 1 (−1) to a *Not*I site (−2887 upstream of the 5′-end of exon 1) was amplified by polymerase chain reaction (PCR) and *Bgl*II sites

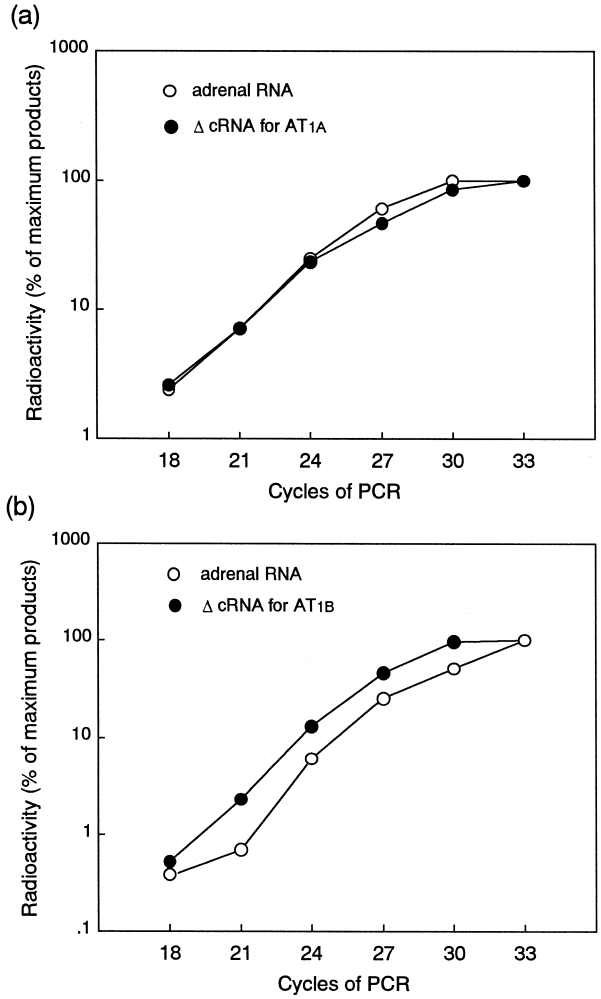

Fig. 2. RT-PCR of angiotensin AT_{1A} receptor mRNA in rat adrenal glands. (A) Cycle dependency of PCR products for angiotensin AT_{1A}. Total RNA from rat adrenal glands (0.5 μg) and deletion-mutated cRNA (Δ cRNA: 25 pg) for AT_{1A} as internal standards were amplified for different numbers of PCR cycles after reverse transcription. (B) Cycle dependency of PCR products for angiotensin AT_{1B}. Total RNA from rat adrenal glands (0.5 μg) and Δ cRNA (25 pg) for angiotensin AT_{1B} were amplified for different numbers of PCR cycles after reverse transcription. A trace amount of [α-^{32}P]dCTP (3 μCi) was included in each reaction mixture to measure the amount of PCR products. The amount of PCR products is expressed as a percentage of the maximal amount produced after 33 of PCR cycles. The same results were obtained in three different experiments.

were added to both ends. This fragment was inserted into the *Bgl*II site of a pGL-2 basic vector (Promega), and named pATLu(−2887/−1). This luciferase–reporter plasmid contained 2887 bp upstream from exon 1 of the rat AT_{1A} gene (Uno et al., 1994).

2.8. Transient transfections and luciferase assay

Vascular smooth muscle cells were seeded at a density of 5×10^5 cells per 60 mm dish and grown in DMEM containing 10% fetal calf serum for 24 h. Y1 cells were seeded at a density of 1×10^6 cells per 60 mm dish and grown in Ham's F-10 medium containing 15% horse serum and 2.5% fetal calf serum for 24 h. Transient transfections were performed by using the DEAE-dextran method as recommended by the manufacturer (Promega). For luciferase assays, cells were cotransfected with 6 μg of plasmid DNA and 2 μg of a pSV-β-galactosidase vector (Promega) to normalize transfection efficiencies. After transfection, cells were grown in DMEM containing 10% fetal calf serum for 24 h. Then, the medium was changed to DMEM without serum and cells were incubated for another 24 h. After that, cells were stimulated by angiotensin II for 18 h. After stimulation, cells were washed with phosphate-buffered saline and harvested with lysis buffer (25 mM Tris–HCl, pH 7.8; 2 mM dithiothreitol; 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; 10% glycerol and 1% Triton X-100) for the luciferase and β-galactosidase assays. Cells were scraped with a rubber policeman, transferred to microcentrifuge tubes, and spun at 12 000 rpm for 5 min. The supernatant was transferred to new tubes and used for luciferase assay. A glass tube containing 20 μl of supernatant was placed in a luminometer (Optocomp I, MGM Instruments, MA) and 100 μl of 470 μM luciferin was added automatically. The integrated peak luminescence was measured over a 45 s window after a 5 s delay. For β-galactosidase assay, 100 μl of supernatants was mixed with the same volume of 2× buffer (200 mM Na_2HPO_4, 90 mM β-mercaptoethanol and 8 mg/ml *O*-nitrophenol-b-D-galactopyranoside) and β-galactosidase activity was determined by absorbance at 405 nm in a spectrophotometer after incubation for 3 h. Transfections were carried out in quadriplicate.

2.9. Analysis of data

Amplification products obtained after PCR were electrophoretically separated on 5% polyacrylamide gels. Autoradiography images were analyzed densitometrically by the using NIH Image 1.58 program. The ratio of intensity of the bands between the endogenous mRNA and their respective deletion-mutated cRNA was calculated for each reaction to make a competition curve. Results are expressed as means ± S.E.M. The data were analyzed statistically by one-way analysis of variance (ANOVA). *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Changes of blood pressure and heart rate

Mean arterial pressure was increased after infusion of angiotensin II (0.2 μg/h) for 3 days from 116 ± 4 mmHg to 140 ± 8 mmHg ($n = 13$), and declined to near the basal level (117 ± 6 mmHg, $n = 10$) after 1 week. Infusion of angiotensin II (20 μg/h) increased mean arterial pressure from 115 ± 6 mmHg to 158 ± 4 mmHg ($n = 15$) after 3 days, and the increase continued until 1 week (167 ± 8 mmHg, $n = 10$).

3.2. Changes in adrenal AT_1 receptor mRNAs

Angiotensin II was infused into rats intravenously for 1 week or 24 h with a dose of 20 μg/h or 0.2 μg/h. Then,

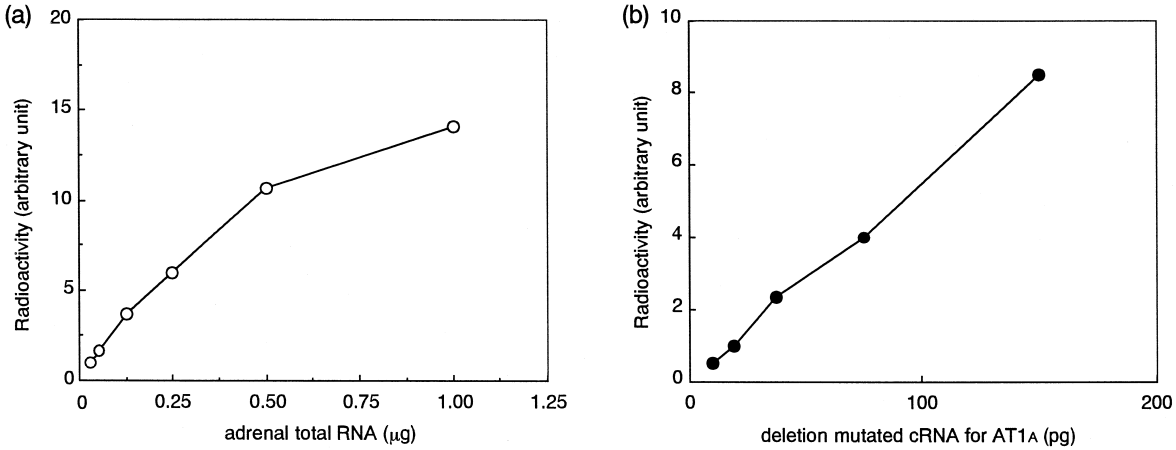

Fig. 3. Concentration-dependent amplification of (a) angiotensin AT_{1A} and (b) deletion-mutated cRNA (ΔcRNA) for angiotensin AT_{1A} receptor. Serial dilutions of adrenal total RNAs (2, 1, 0.5, 0.25, 0.125, 0.0625 μg) or ΔcRNA (150, 75, 37, 5, 18.8, 9.4 pg) were amplified for 30 cycles. [α-^{32}P]dCTP (3 μCi) was included in each reaction mixture. This is the representative of three different experiments.

we examined whether angiotensin II altered the expression of angiotensin AT_1 receptor mRNA in the adrenal gland. In the first series of experiments, angiotensin AT_1 receptor mRNA was determined by blotting of total RNA extracts followed by hybridization with a labeled cDNA probe specific for angiotensin AT_1 receptor (Fig. 1a).

The representative autoradiogram in Fig. 1 indicates that angiotensin AT_1 receptor mRNA decreased significantly after infusion of high dose of angiotensin II (20 μg/h) for both 24 h and 1 week. In rats treated by angiotensin II (20 μg/h) for 24 h, the level of angiotensin AT_1 mRNA was decreased from control ($104.0 \pm 3.4\%$, $n = 10$) to $52.7 \pm 16.1\%$ ($n = 5$). Also, angiotensin AT_1 mRNA was decreased to $70.8 \pm 8.0\%$ ($n = 10$) in rats treated by angiotensin II for 1 week. However, there was no difference in the expression of angiotensin AT_1 gene

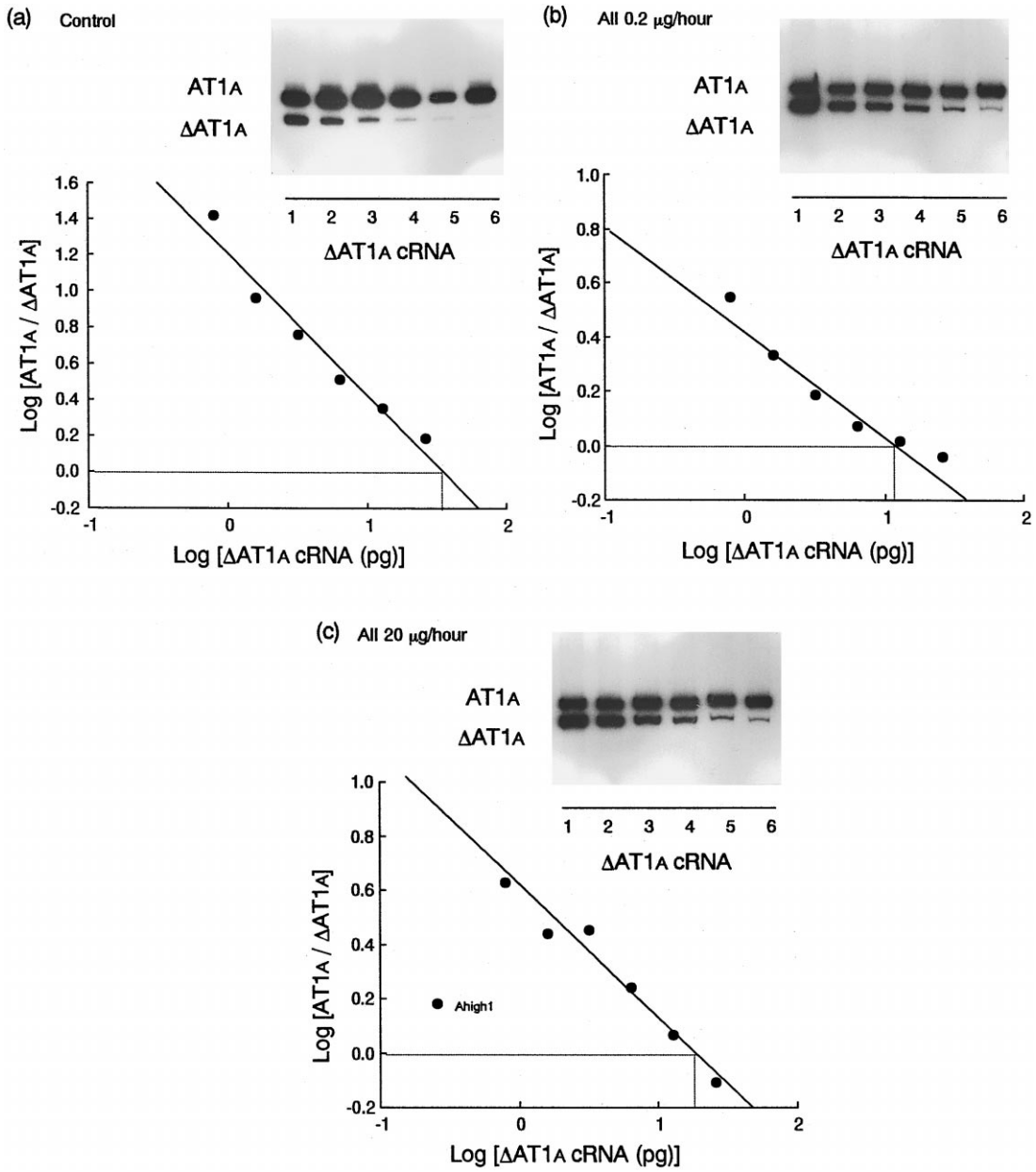

Fig. 4. RT-PCR analysis of adrenal angiotensin AT_{1A} receptor mRNA levels. Rats were treated with angiotensin II (20 or 0.2 μg/h) or saline by osmotic minipumps for 1 week, and then the adrenal glands were isolated. A 0.5 μg sample of total RNA from rat adrenal glands and various amounts of deletion mutant (Δ) cRNA were reverse transcribed and amplified for 30 cycles. RT-PCR products were electrophoresed on 5% polyacrylamide gels. The autoradiographic signals were analyzed densitometrically and the ratio of angiotensin AT_{1A} to ΔAT_{1A} receptor mRNAs was calculated. The amounts of angiotensin AT_{1A} receptor mRNA were calculated by extrapolating each curve in which the ratio of products was plotted against the amount of ΔcRNA. (a) control, (b) low dose of angiotensin II (0.2 μg/h) treatment for 1 week, (c) high dose angiotensin II (20 μg/h) treatment for 1 week. This is representative of at least three experiments. ΔAT_{1A}: deletion-mutated AT_{1A}.

between groups treated for 24 h and that for 1 week. In rats treated with a low dose of angiotensin II (0.2 μg/h), angiotensin AT_1 mRNA was also decreased both after 24 h (75.8 ± 4.0, $n = 3$) and after 1 week (73.0 ± 5.5, $n = 10$)(Fig. 1b).

In the next experiment, we used a quantitative RT-PCR method to detect the change in the mRNA for angiotensin AT_{1A} and AT_{1B} receptors individually. Because it is crucial for the precise quantitation to choose an optimal phase of PCR, PCR cycles and amounts of total RNA and

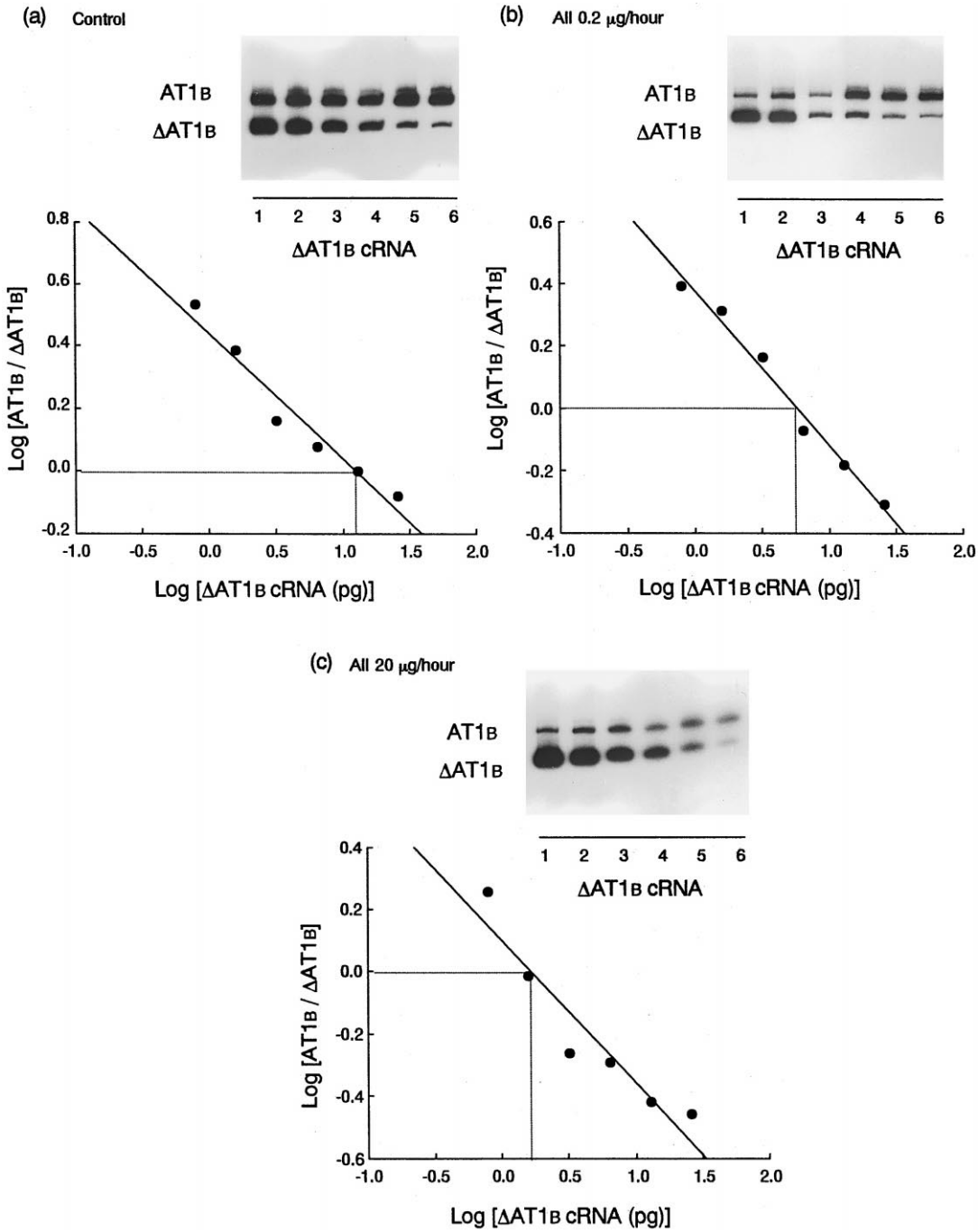

Fig. 5. RT-PCR analysis of adrenal angiotensin AT_{1B} receptor mRNA levels. Rats were treated with angiotensin II (20 or 0.2 μg/h) or saline by means of osmotic minipumps for 1 week, and then the adrenal glands were isolated. A 0.5 μg sample of total RNA from rat adrenal glands and various amounts of deletion mutant (Δ) cRNA were reverse transcribed and amplified for 30 cycles. RT-PCR products were electrophoresed on 5% polyacrylamide gels. The autoradiographic signals were analyzed densitometrically and the ratio of angiotensin AT_{1B} to ΔAT_{1B} receptor mRNAs was calculated. The amounts of angiotensin AT_{1B} receptor mRNA were calculated by extrapolation from curves in which the ratio of products was plotted against the amount of ΔcRNA. (a) control, (b) low dose of angiotensin II (0.2 μg/h) treatment for 1 week, (c) high dose angiotensin II (20 μg/h) treatment for 1 week. This is representative of at least three experiments.

deletion-mutated cRNA were chosen so that PCR products could increase exponentially. Each cDNA mixture was subjected to PCR between 18 and 33 cycles of amplification as described in Section 2. The radioactivity corresponding to angiotensin AT_1 mRNA or deletion-mutated angiotensin AT_1 receptor was plotted as a function of the number of PCR cycles. Under our PCR conditions, PCR products increased exponentially up to 30 cycles (Fig. 2) and amounts up to 1 μg total RNA per reaction tube (Fig. 3); however, at 33 cycles, the curve reached a plateau phase. Therefore, we used 30 cycles in quantitative RT-PCR. As shown in the cycle–PCR products relation curves, the slopes obtained from adrenal RNA and deletion-mutated cRNA (ΔcRNA) were not different, indicating that amplification efficiencies were almost the same. Figs. 4 and 5 show the results of RT-PCR analysis of angiotensin AT_1 receptor mRNAs. Angiotensin AT_{1A} or AT_{1B} mRNA was calculated by extrapolating each curve generated by plotting the ratio of products for adrenal angiotensin AT_{1A} and deletion-mutated AT_{1A} ($\log[AT_{1A}/\Delta AT_{1A}]$), or adrenal AT_{1B} and deletion-mutated AT_{1B} ($\log[AT_{1B}/\Delta AT_{1B}]$) against the amount of deletion-mutated cRNA (ΔcRNA). Angiotensin AT_{1A} receptor mRNA was decreased from control level to $78.0 \pm 18.6\%$ after 20 μg/h of angiotensin II infusion for 1 week, and to $46.7 \pm 4.0\%$ after 0.2 μg/h of angiotensin II infusion (Fig. 4). Angiotensin AT_{1B} receptor mRNA decreased to $43.7 \pm 13.1\%$ of control after infusion of angiotensin II (20 μg/h) for 1 week and to $25.6 \pm 12.5\%$ after infusion of angiotensin II (0.2 μg/h) (Fig. 5).

3.3. The promoter activity of angiotensin AT_{1A} receptor gene

Promoter activity was examined by a luciferase assay. In brief, a 2.9 kb promoter region of rat angiotensin AT_{1A} receptor was inserted upstream of the promoterless pGL-2-basic luciferase gene. The fusion construct, pATLu ($-2887/-1$), was transfected transiently into Y1 cells or rat aortic vascular smooth muscle cells. The luciferase activity of cells transfected with pATLu ($-2887/-1$) is shown in Fig. 6. In Y1 cells, angiotensin II (up to 1.0 μM) did not have any effects on the luciferase activity. Promoter activity was not significantly different from control level even after stimulation with 1 μM of angiotensin II ($106 \pm 5.7\%$, $n = 5$), whereas phorbol myristate acetate (PMA, 0.1 μM), which is known to downregulate the angiotensin AT_1 receptor in a variety of tissues, decreased the luciferase activity to $60.8 \pm 5.0\%$ ($n = 7$) of control in Y1 cells (Fig. 6). By contrast, aldosterone (10 μM) increased luciferase activity to $131.8 \pm 13.7\%$ ($n = 6$) in Y1 cells. In vascular smooth muscle cells, angiotensin II did not influence luciferase activity (102 ± 3.1 ($n = 6$), whereas PMA inhibited luciferase activity to $64.2 \pm 6.0\%$ ($n = 5$). In both Y1 cells and vascular smooth muscle cells, PMA inhibited the activity of angiotensin AT_{1A} promoter but angiotensin II did not.

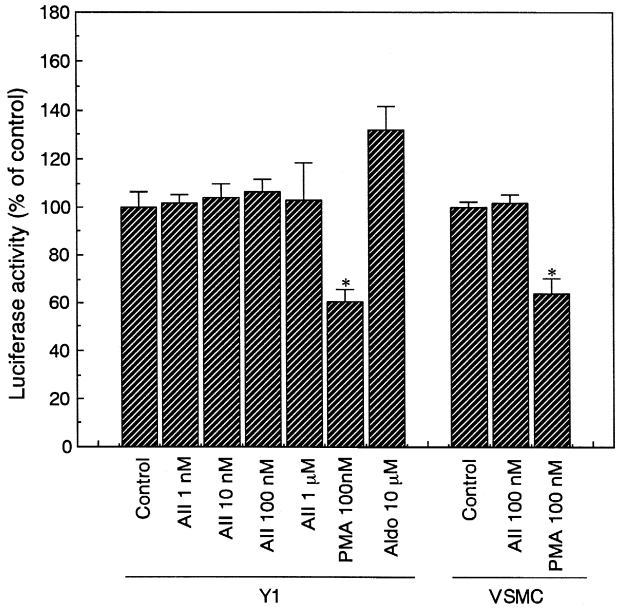

Fig. 6. Effects of angiotensin II, phorbol ester and aldosterone on the angiotensin AT_{1A} promoter activity in Y1 cells or vascular smooth muscle cells transfected with luciferase–reporter plasmid containing 5′-flanking region of the rat angiotensin AT_{1A} receptor gene, pATLu ($-2887/-1$). Transfection was carried out as shown in Section 2. After serum depletion for 24 h, cells were stimulated and incubated for another 18 h. Luciferase activity in the stimulated cells is expressed as a percentage of unstimulated control cells. Values represent means ± S.E.M. of 5–6 experiments. Asterisks indicate significant difference vs. control ($P < 0.05$).

4. Discussion

We examined the regulation of mRNAs for angiotensin AT_1 receptor subtypes (AT_{1A} and AT_{1B}) in the adrenal glands of rats that have been given a continuous infusion of angiotensin II, and the effects of angiotensin II on the promoter activity of angiotensin AT_1 receptor in Y1 cells and aortic vascular smooth muscle cells. We found the following: (a) angiotensin AT_1 receptor mRNAs were downregulated by angiotensin II. Competitive RT-PCR with deletion-mutated cRNAs for angiotensin AT_1 receptor showed that not only angiotensin AT_{1A} but also AT_{1B} receptor mRNAs were decreased after administration of angiotensin II; (b) angiotensin II had neither stimulating nor inhibiting effects on the angiotensin AT_{1A} promoter activity in Y1 cells, although PMA inhibited the angiotensin AT_{1A} promoter activity by about 40%; (c) the promoter activity of angiotensin AT_{1A} receptor was not significantly influenced by angiotensin II in both Y1 cells and vascular smooth muscle cells, indicating that there are no angiotensin II-responding tissue-specific *cis*-elements within the 5′-flanking region examined in this study. In

addition, subtype-dependent regulation of angiotensin AT_1 receptor mRNA has been reported previously, but in our case of angiotensin II stimulation, there was no remarkable difference in the regulation of the two subtypes.

The regulation of angiotensin AT_1 receptors by angiotensin II depends on tissue and species investigated. Several previous reports showed that angiotensin AT_1 receptors increased in the adrenal gland of the rat. Our results are in agreement with the inhibitory effect of angiotensin II on angiotensin AT_1 in human adrenocortical H295 cells (Naville et al., 1993) and in the adrenal glands administered a higher dose of angiotensin II (Aguilera et al., 1980; Pernollet et al., 1977).

A number of factors including aldosterone (Schiffrin et al., 1985), glucocorticoids (Sato et al., 1994), estrogen (Douglas, 1987), potassium (Linas et al., 1990) and catecholamines (Myers and Sumners, 1989) are suggested to modulate the expression of angiotensin AT_1 receptor protein and mRNA. One possible condition regulating the expression of angiotensin AT_1 mRNAs is increased plasma renin activity induced, for example, by sodium restriction, high plasma K^+ and renovascular hypertension. However, the influence of plasma renin activity on angiotensin AT_1 mRNA are inconsistent: sodium restriction and high K^+ have been reported to increase adrenal angiotensin AT_1 receptors, but renovascular hypertension did not change or decrease the level of angiotensin AT_{1A} and AT_{1B} mRNA, respectively (Lehoux et al., 1994; Llorens et al., 1994). The other factor may be aldosterone. Chronic angiotensin II infusion increases plasma aldosterone and induces systemic hypertension. Aldosterone may induce transcription factors through mineralocorticoid receptors, which is followed by an increase in angiotensin II receptors in vascular smooth muscle, adrenal gland and in neuronal cultures (Schiffrin et al., 1985; Ullian et al., 1992), but not in uterine smooth muscles (Douglas and Brown, 1982). This inconsistency suggests that there is tissue-specific synthesis of nuclear transcription factors. In this study, although we did not examine the effects of aldosterone antagonists on the angiotensin II-induced angiotensin AT_1 receptor downregulation, it is not probable that the reduced angiotensin AT_1 gene expression was exerted through aldosterone because aldosterone would have had a stimulating effect on angiotensin AT_1 expression via transcriptional activation in our promoter–luciferase assay.

It is important to consider the effects of hypertension on the expression of angiotensin II receptors. Downregulation of adrenal angiotensin AT_1 receptor gene expression has been described in three models of experimental hypertension. In the two-kidney, one-clip hypertension model, renal angiotensin AT_{1A} receptor as well as adrenal AT_{1B} receptor mRNA (but not AT_{1A}) decreased by 50% (Haefliger et al., 1995; Llorens et al., 1994). In salt-loaded hypertensive rats and in deoxycorticosterone and salt-hypertensive rats, adrenal angiotensin AT_1 receptor mRNA decreased by 66% and by 33%, respectively (Elijovich et al., 1997). These models include every category of hypertension with different levels of plasma renin activity, suggesting that high blood pressure status is directly or indirectly related to angiotensin AT_1 receptor downregulation irrespective of the level of plasma renin activity. This downregulation of adrenal angiotensin AT_1 mRNA may be in compensation for the hypertension. The adrenal glands of spontaneously hypertensive rat expressed more angiotensin AT_1 receptor mRNA than that of Wistar–Kyoto rats, suggesting that increased angiotensin AT_1 mRNA without physiological control may be a pathogenic factor in genetic hypertension (Song et al., 1994). If the angiotensin AT_1 receptor responds to high blood pressure directly, 'blood pressure-response elements' may exist in the promoter region of the angiotensin AT_1 gene. However, such *cis*-elements have not yet been identified. In cultured bovine adrenal cells and human adrenocortical H295 cells on which the effects of hypertension are negligible, angiotensin II decreased angiotensin AT_1 receptor binding and angiotensin AT_1 receptor mRNA (Naville et al., 1993; Ouali et al., 1997). Therefore, hypertension is important for the regulation of angiotensin AT_1 expression, but in addition, angiotensin II-induced factors independent of hypertension may also be able to downregulate angiotensin AT_1 gene expression.

Because several factors, such as transcriptional regulation and degradation of mRNA through angiotensin II-induced proteins may determine angiotensin AT_1 mRNA, the effects of angiotensin II will depend on the synthesis of transcription and/or degradation factors (Bird et al., 1994). The first possible mechanism for downregulation of angiotensin AT_1 mRNAs by angiotensin II is the inhibition of the transcription of angiotensin AT_1 genes, because the 5′-flanking region of angiotensin AT_{1A} receptor gene is reported to have several positive and negative regulatory sequences (Murasawa et al., 1993). The second possible mechanism is the accelerated degradation of angiotensin AT_1 receptor mRNA as there are several reports suggesting that angiotensin II alters the stability of its mRNA (Klett et al., 1994, 1993; Lassegue et al., 1995). In order to clarify the molecular basis for the regulation of angiotensin AT_1 gene expression, we examined the effects of angiotensin II on the promoter activity. Our results showed no angiotensin II-induced regulation of promoter activity. However, because promoter–luciferase reporter assay is a method for identifying promoter activity of the region examined, and does not give any information on the rate of mRNA degradation, destabilization of angiotensin AT_1 receptor mRNA cannot be ruled out. Alternatively, angiotensin II-induced hypertension per se may affect angiotensin AT_1 mRNA transcription.

In this study, we used Northern blotting analysis to detect angiotensin AT_1 mRNA of both subtypes, and quantitative RT-PCR to evaluate the mRNA for each angiotensin AT_1-subtype. For measurement of the amount of target mRNA, quantitative RT-PCR was a valuable tech-

nique, especially because the RNA samples from adrenal glands were limited in amount and angiotensin AT_1 subtype mRNAs were difficult to measure separately by Northern blotting analysis due to their high homology. There are several reports suggesting that angiotensin AT_1 mRNA expression is not simply correlated with angiotensin AT_1 receptor density (Kitami et al., 1992; Llorens et al., 1994). For further study, combination of another method such as Western blotting analysis will be needed to estimate the amount of protein. We could not do this becuase the adrenal glands were not big enough to do so many experiments.

5. Conclusion

mRNA expression for both angiotensin AT_{1A} and AT_{1B} receptor was decreased by angiotensin II in the rat adrenal gland. Although the 5′-flanking region of the angiotensin AT_{1B} receptor was not examined, the decrease in angiotensin AT_{1A} mRNA was not due to angiotensin II-induced suppression of the promoter activity. Although further studies are required to understand the exact mechanism, another regulational mechanism, such as destabilization of angiotensin AT_1 mRNAs and/or hypertension per se, may be responsible for this downregulation.

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