Abstract
Recently, we demonstrated that glucocorticoid potentiates inositol trisphosphate production evoked by angiotensin II in vascular smooth muscle cells. To clarify this mechanism, we investigated the effects of dexamethasone on the modulation of angiotensin II type 1 receptor and on postreceptor mechanisms in vascular smooth muscle cells. The number of angiotensin II type 1 receptors began to increase after 12 hours' incubation with dexamethasone. After 48 hours, the Bmax value reached 27±3 fmoL/mg protein in dexamethasone-treated cells and 15±3 fmoL/mg protein in control cells. However, binding affinity did not change. A glucocorticoid antagonist, RU 38486, completely blocked these effects of dexamethasone. Also, to elucidate the effects of dexamethasone on postreceptor mechanisms, GTP analogue-induced inositol trisphosphate production in permeabilized cells was examined. Pretreatment with 1 μmol/L dexamethasone for 48 hours did not affect these inositol trisphosphate productions. Moreover, dexamethasone had no effect on the level of Gαo protein. Furthermore, steady-state levels of angiotensin II type 1 receptor messenger RNA were increased 2.2±0.3-fold after 30 minutes' exposure to 1 μmol/L dexamethasone and 7.8±0.4-fold after 24 hours. We conclude that glucocorticoid induced expression of the angiotensin II type 1 receptor gene and resulted in an increase in the number of angiotensin II type 1 receptors through the glucocorticoid-specific receptor, without significant effect on postreceptor systems in vascular smooth muscle cells. (Hypertension. 1994;23:25-30.)

Key Words • dexamethasone • postreceptor signaling systems • RNA, messenger • receptors, angiotensin • smooth, muscle, vascular

Hypertension induced by glucocorticoid hormone is not uncommon in clinical practice. Although the exact mechanisms of glucocorticoid-induced hypertension are still uncertain, enhancement of vascular responsiveness has been considered one of the major contributing factors. Previously, we demonstrated that the pressor responses to angiotensin II (Ang II) and norepinephrine are enhanced in patients with Cushing's syndrome and experimental models with glucocorticoid-induced hypertension.

Very recently, we found that glucocorticoid potentiates inositol trisphosphate (IP3) production evoked by Ang II in cultured vascular smooth muscle cells (VSMCs), and these results showed that glucocorticoid treatment would increase sensitivity to Ang II at the cellular level. In that study, the action of glucocorticoid was, in part, through prostanoid synthesis inhibition; however, the possibility that glucocorticoid directly modulates Ang II receptor-mediated signaling pathways in VSMCs is suggested. This, together with other experimental data, makes it conceivable that glucocorticoid enhances transcription and expression of α1b-adrenergic receptors and β2-adrenergic receptors in smooth muscle cells and that glucocorticoid directly modulates the function of G proteins in several other kinds of cells.

As to Ang II receptors, the development of nonpeptide Ang II antagonists has allowed the identification of at least two different subtypes, Ang II type 1 (AT1) receptor and Ang II type 2 (AT2) receptor. Recently, Murphy et al succeeded in complementary DNA (cDNA) cloning of AT1 receptor from rat VSMCs. These significant advances have revealed that the actions of Ang II on VSMCs are mediated primarily through the AT1 receptor.

In the present study, to clarify the mechanism for glucocorticoid-enhanced Ang II receptor-mediated responses in VSMCs, the effect of glucocorticoid on AT1 receptor expression and the transcription of AT1 receptor gene in VSMCs were examined. Furthermore, the effect of glucocorticoid on postreceptor signaling systems in VSMCs was studied.

Methods

Materials
Reagents for these studies were purchased as follows: dexamethasone; the peptidease inhibitors antipain, phosphoramidon, leupeptin, pepstatin, bestatin, amastatin, and bacitracin; and bovine serum albumin (BSA) were from Sigma Chemical Co, St Louis, Mo. The nonpeptide AT1 receptor antagonist DuP 753 was a gift from Dr Smith (Du Pont de Nemours Co, Wilmington, Del), PD 123319, an AT1 receptor antagonist, was a gift from Dr Taylor (Parke-Davis Pharmaceutical Research, Warner-Lambert Co, Ann Arbor, Mich). Ang II was from the Peptide Institute, Osaka, Japan. 125I-Ang II and anti-Gαo protein were from Du Pont NEN Research Products, Boston, Mass. Fetal calf serum (FCS) was purchased from GIBCO Laboratories, Grand Island, NY. Dulbecco's modified Eagle medium (DMEM) and phosphate-buffered saline (PBS) were from Nissui Pharmaceutical Co, Ltd, Tokyo, Japan. RU 38486, a glucocorticoid antagonist, was kindly provided by Roussel Uclaf, Paris, France.

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Cell Culture

VSMCs were prepared from the thoracic aorta of 6-week-old Wistar-Kyoto rats by an enzyme method previously described and were cultured in DMEM containing 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cultures were kept at 37°C in a humid atmosphere consisting of 5% CO₂ in air, and the medium was changed every 3 or 4 days. After the cells reached confluence, the medium was changed to culture media containing various doses of dexamethasone or ethanol vehicle as a control for various periods of time before the experiments. Cells between the second and fourth passages were used for the experiments. The cells had typical smooth muscle morphology, including the presence of hills and valleys seen under a phase-contrast microscope. Cell viability was estimated by trypan blue exclusion to be approximately 90%. We used dexamethasone because of its more potent and pure glucocorticoid action than the endogenous glucocorticoid.

Radioiodinated Receptor Binding Assay

The specific binding of 125I-Ang II to cell membrane receptors was determined by use of intact VSMCs. Confluent monolayers of VSMCs grown in 24-well plates were rinsed twice with PBS, pH 7.4. Equilibrium binding studies were performed at room temperature for 60 minutes in PBS, pH 7.4, containing increasing concentrations of 125I-Ang II, 1.6% heat-inactivated BSA, and the peptide inhibitors antipain, phosphoramidon, leupeptin, pepstatin, bestatin, and amastatin, each at 1 μg/mL, and bacitracin at 100 μg/mL. Preliminary experiments demonstrated that under these conditions, equilibrium was reached. After incubation, the cells were washed three times with ice-cold PBS, pH 7.4. The attached cells were dissolved with 1.0 mL of 0.2N NaOH and transferred to three times with ice-cold PBS, pH 7.4. The attached cells were dissolved with 1.0 mL of 0.2N NaOH and transferred to polypropylene tubes. The dishes were rinsed with 1.0 mL of distilled water, and the rinses were combined with the original samples. Radioactivity in the dissolved cells was determined in an autogamma system (Aloka Co, Tokyo, Japan). A portion of the dissolved cells was used for determining the protein content. Nonspecific binding was determined in the presence of 10 μmol/L unlabeled Ang II. Receptor density (B max values) and dissociation constants (K d) for 125I-Ang II were calculated from Scatchard plots. Furthermore, to characterize Ang II receptor subtypes in VSMCs, monolayers of VSMCs were also incubated with the fixed 125I-Ang II and increasing concentrations of Ang II, AT, receptor antagonist DuP 753, and AT receptors antagonist PD 123319.

Isolation of Total Cellular RNA

The cells were washed three times with PBS and then lysed in 4 mol/L guanidine isothiocyanate. Total cellular RNA was isolated by the acid guanidinium/phenol/chloroform method. After extraction with phenol/chloroform and precipitation with ethanol, the RNA pellet was resuspended in 50 μL of TE buffer (Tris-HCl, EDTA), pH 7.4, and quantified by absorbance measurements at 260 nm. The integrity of the purified RNA was determined by visualization of the 28S and 18S ribosomal RNA bands after electrophoresis of each RNA sample through a 1% agarose gel.

Northern Blot Hybridization

For Northern blot analysis, RNA (total cellular RNA, 20 μg) was denatured with formamide and formalin at 65°C for 10 minutes and fractionated by electrophoresis through a 1% agarose gel. The RNA was transferred to a nylon filter (blotting membrane Hybond-N) was purchased from Amsbergh International, Ltd, Buckinghamshire, England). Prehybridization was conducted at 65°C for 2 hours in 5x SSPE (1x SSPE = 0.18 mol/L sodium chloride, 10 mmol/L sodium phosphate, and 1 mmol/L EDTA, pH 7.7), 5x Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and heat-denatured salmon sperm RNA (1 mg/mL). A Km I–Sac I fragment that encodes the rat AT, receptor, kindly provided by Dr. Inagami (Vanderbilt University, Nashville, Tenn), was used as a probe for hybridization. The filters were then hybridized at 65°C for at least 12 hours to the probe that was radiolabeled with [α-32P]dCTP by the random primer synthesis method (Random Primers DNA Labeling Kit, TaKaRa Shuzo Co, Ltd, Kyoto, Japan). After hybridization, the filter was washed twice in 2x SSC (sodium chloride and sodium citrate buffer) and 0.1% SDS at 65°C and successively in 1x SSC and 0.1% SDS once and in 0.5x SSC and 0.1% SDS once. The autoradiograms were scanned with a BAS 2000 (Fuji Film, Tokyo, Japan) and quantified.

Measurement of Inositol 1,4,5-Trisphosphate Production

Production of IP₃ in VSMCs was measured with a radioimmunoassay kit (TRK1000, Amersham), as previously described. To examine the effects of dexamethasone on postreceptor signaling systems, the accumulation of IP₃ was determined in permeabilized VSMCs by use of the nonhydrolyzable GTP analogue guanosine-5’-O-(3’-thiotriphosphate) (GTPγS) in dexamethasone-treated (1 μmol/L for 48 hours) or nontreated cells. VSMCs were exposed to 200 U/mL a-toxin (Life Technologies, Inc, Gaithersburg, Md), a transmembrane pore-making exoprotein produced by Staphylococcus aureus, for 20 minutes at 37°C in buffer containing (mmol/L) glutamate potassium salt, 150; EGTA, 5; magnesium chloride, 1; and 1,4-piperazine diethanesulfonic acid (PIPES), 10; pH 7.2, adjusted with KOH. The cells were then rinsed with the above medium and incubated with 100 μmol/L GTPγS for 10 minutes. IP₃ production was measured with a competitive binding assay that used a bovine-derived adrenal binding protein specific for IP₃.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting

The cells were washed three times with ice-cold PBS and added sample buffer (60 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.1% bromophenol blue) and boiled for 5 minutes before resolution by SDS–polyacrylamide gel electrophoresis on gels containing 12% acrylamide. The protein was loaded in 150-μg aliquots to achieve optimal blots. Low molecular weight protein standards (Bio-Rad Laboratories, Hercules) were run in parallel with the samples under study. After electrophoresis, proteins were transferred to nitrocellulose membrane (TEF Co, Nagano, Japan), which was then blocked overnight in blocking solution (5% BSA in buffer comprising 150 mmol/L NaCl in 20 mmol/L Tris-HCl, pH 7.5). After washing, the blot was incubated for 2 hours with the first antiserum (anti-Gqα) at a 1:1000 dilution in 0.1% SDS at 65°C and successively in 1x SSC and 0.1% SDS once. The autoradiograms were scanned with a BAS 2000 (Fuji Film, Tokyo, Japan) and quantified.

Protein Determination

The protein levels in VSMCs were determined by the method of Pierce (Pierce Chemical Co, Rockford, Ill) using BSA as a standard.

Statistical Analysis

The results are expressed as mean±SEM. Statistical significance was assessed by Student’s t test. Values of P<.05 were considered to be significant.

Results

Effects of Dexamethasone on Angiotensin II Type 1 Receptor

Fig 1 shows the competition curves with Ang II, DuP 753, and PD 123319 in 1 μmol/L dexamethasone-
was through the glucocorticoid receptor, VSMCs were incubated with 1 μmol/L dexamethasone for 48 hours. Although RU 38486 alone had no effect on the number of AT$_1$ receptors, it completely blocked the effect of 1 μmol/L dexamethasone (Fig 5). These results suggested that the effect of dexamethasone on AT$_1$ receptor was through the glucocorticoid-specific receptor.

Next, to investigate the effect of dexamethasone on AT$_1$ receptor, VSMCs were incubated with 1 μmol/L dexamethasone for various periods before the cells were harvested. AT$_1$ receptor number, measured with the ligand $^{125}$I-Ang II, was unchanged during the first several hours of exposure to dexamethasone. The receptor number was found to be increased after 12 hours' exposure to dexamethasone and progressively increased to 185.6±9.8% of control at 48 hours (Fig 2). Fig 3 shows the typical binding curves (A) and Scatchard plot (B) for $^{125}$I-Ang II binding in dexamethasone-treated and control cells at 48 hours. The $B_{max}$ value was 27±3 fmol/mg protein in dexamethasone-treated cells and 15±3 fmol/mg protein in control cells. Conversely, the $K_d$ of $^{125}$I-Ang II did not change significantly (dexamethasone-treated cells, 1.2±0.3 nmol/L; control cells, 1.1±0.2 nmol/L).

As shown in Fig 4, VSMCs treated with different concentrations of dexamethasone exhibited a dose-dependent increase in AT$_1$ receptor number. The increase in AT$_1$ receptor density became apparent at as low as 10 nmol/L dexamethasone and became maximal at 1 μmol/L dexamethasone.

To demonstrate whether the effect of dexamethasone was through the glucocorticoid receptor, VSMCs were incubated with 10 μmol/L RU 38486, a glucocorticoid receptor-specific antagonist, with or without 1 μmol/L dexamethasone for 48 hours. Although RU 38486 alone had no effect on the number of AT$_1$ receptors, it completely blocked the effect of 1 μmol/L dexamethasone (Fig 5). These results suggested that the effect of dexamethasone on AT$_1$ receptor was through the glucocorticoid-specific receptor.

Effect of Dexamethasone on Angiotensin II Type 1 Receptor Messenger RNA Level

The steady-state AT$_1$ receptor mRNA level was measured by Northern blot analysis. Fig 6A shows Northern blot analysis of total RNA from rat VSMCs. A single band of 2.3 kb was observed in both dexamethasone-treated and control cells. The size of this mRNA was consistent with the previous report. After treatment with 1 μmol/L dexamethasone or ethanol vehicle for 48 hours, the cells showed that the characteristics of the Ang II receptor were similar to those in the control cells. However, there was no significant difference of IP$_3$ production between dexamethasone-treated cells and control cells. It should be noted that in the experiments conducted in the absence of α-toxin treatment, there was no rise in IP$_3$ levels in response to GTPyS in both groups (data not shown).

Effect of Dexamethasone on G$_s$ Protein α-Subunit Protein Level

To examine the effect of dexamethasone on Ang II-mediated postreceptor signaling systems in VSMCs, we first directly activated G proteins by GTPyS in permeabilized cells. GTPyS has been shown to stimulate IP$_3$ accumulation and potentiate contraction of permeabilized smooth muscle.19,21 The basal intracellular IP$_3$ content was 4.5±0.5 pmol/10$^6$ cells, consistent with the previous study. After treatment with 1 μmol/L dexamethasone or ethanol vehicle for 48 hours, the cells were stimulated with GTPyS (100 μmol/L for 20 minutes). As shown in Fig 8, increased accumulation of IP$_3$ occurred in both dexamethasone-treated and control cells. However, there was no significant difference of IP$_3$ production between dexamethasone-treated cells and control cells. It should be noted that in the experiments conducted in the absence of α-toxin treatment, there was no rise in IP$_3$ levels in response to GTPyS in both groups (data not shown).
Effect of Dexamethasone on Cell Numbers and Cell Protein

Dexamethasone had no significant effects on cell numbers and cell protein in VSMCs incubated for 48 hours compared with control.

Discussion

In the present study, we investigated the effects of glucocorticoid on AT1 receptor, its gene expression, and postreceptor signaling systems in VSMCs. Dexamethasone induced the gene expression of AT1 receptor and increased AT1 receptor number through the glucocorticoid-specific receptor, without significant effect on post-receptor systems in VSMCs. These findings could potentially explain how glucocorticoid induces vascular hypersensitivity to Ang II and are quite important in elucidating the mechanism of increased vascular sensitivity that is found in patients with Cushing's syndrome.

The radioligand binding study using the specific receptor antagonists DuP 753 and PD 123319 disclosed that the actions of Ang II on VSMCs were mediated primarily through the AT1 receptor. Moreover, treatment with dexamethasone did not alter the characteristics of Ang II receptor subtypes. From the results of the time course study, the number of AT1 receptors was increased after 12 hours' exposure to dexamethasone and maintained its levels at 48 hours. This relatively long duration might account for protein synthesis, and this behavior is consistent with the general mode of action of steroid hormones. Furthermore, dexamethasone exhibited a dose-related increase in AT1 receptor numbers.

The effects of glucocorticoid on the regulation of Ang II receptor have been demonstrated in in vivo and in vitro studies. Douglas et al demonstrated that dexamethasone infusion produced downregulation of glomerular Ang II receptor obtained from sodium-loaded rats. Those results contradict our present findings. Since glomerular mesangium and vascular smooth muscle share many properties, similar actions of dexamethasone on Ang II receptor are expected. Although the exact reasons for the difference between these studies and our present findings remain to be determined, several possible explanations can be suggested. The dose of dexamethasone infused in sodium-loaded rats was lower than in our in vitro study. In our experiment, low doses of dexamethasone did not induce any significant changes in the number of AT1 receptor. Second, other factors, such as low levels of circulating Ang II, may modulate the effects of glucocorticoid in sodium-loaded rats. Furthermore, it is also possible that glucocorticoid has tissue- and cell-specific regulation. Indeed, Chappell et al showed that glucocorticoid downregulated both AT1 and AT2 receptors in pancreatic acinar cells.

Several recent studies have investigated the effects of glucocorticoid on the mRNA of seven transmembrane-

Fig 9 shows the results of Western blot analysis of the proteins in 1 μmol/L dexamethasone-treated cells and control cells for various periods of time. No differences were seen in Gsa protein level between dexamethasone-treated cells and control cells.

**Effect of Dexamethasone on Cell Numbers and Cell Protein**

Dexamethasone had no significant effects on cell numbers and cell protein in VSMCs incubated for 48 hours compared with control.
type receptors. Sakaue and Hoffman have clearly demonstrated that glucocorticoid regulated expression of α1B-receptors by increasing the rate of transcription of this gene. The pattern of change in expression of AT1 receptor mRNA by dexamethasone was similar to that of the α1B-adrenergic receptor mRNA. Moreover, there was a good correlation between the increase in the expression of AT1 receptor and the change in the abundance of the receptor's mRNA, as observed in α1B-receptors.

Compared with these similarities, the reported pattern of change in β2-adrenergic receptor gene is different. β2-Adrenergic receptor mRNA increased very rapidly, within 1 to 2 hours, and returned slowly, by 10 hours, in the continued presence of glucocorticoid. These different regulatory mechanisms among AT1, α1B-, and β2-adrenergic receptor mRNA may be important, because these receptors are closely involved in cardiovascular regulation in glucocorticoid-induced hypertension. From our present study, it still remains unclear whether dexamethasone elevated transcription rate or increased the stability of AT1 receptor gene product. Our data only presented the increased accumulation of AT1 receptor mRNA. For most steroid-regulated genes, gene expression is effected primarily at the transcription rate. Glucocorticoid has been shown, however, to have effects on mRNA stability such as insulin mRNA in insulinoma cells and interleukin-1β mRNA in monocytic cell lines. Further studies will be needed.
We previously reported marked elevation of Ang II--induced IP₃ production in response to dexamethasone exposure in VSMCs. These changes (threefold) were significantly greater than could be explained by modest increases in receptor density (twofold) and led us to test the hypothesis that augmented signal transduction might be partially explained by changes in postreceptor systems. Recent advances in the investigation of G protein have demonstrated that the Ang II-linked G protein concerned in IP₃ production in VSMCs is Gq. First, therefore, α-toxin-permeabilized VSMCs were used. In these cells, GTPyS induced an increase in IP₃ production without Ang II, and 48 hours' exposure to dexamethasone did not affect the IP₃ production induced by GTPyS. It is therefore less likely that dexamethasone exerts its action on IP₃ production through postreceptor signaling systems. However, GTPyS might have the capability of stimulating all kinds of G proteins other than Gq in VSMCs. Then, we investigated the effect of dexamethasone on Gq protein level, and no significant changes in Gq protein level were found. The classification "G protein" is now used to refer to a family of G proteins. In Gq protein, the interaction and regulation with other G proteins, such as Gₛ, G₁₁, and so on, were not fully understood. Therefore, it is clear that before we conclude that there is no modulatory role of dexamethasone in postreceptor signaling systems, further precise study would be needed.

In conclusion, we have first demonstrated that glucocorticoid induced the expression of the AT₁ receptor gene and resulted in an increase in the number of AT₁ receptors through the glucocorticoid-specific receptor in VSMCs. Moreover, we also demonstrated that glucocorticoid did not exert significant action on postreceptor signaling systems. Taken together with the results of our recent study, these results indicate that glucocorticoid elevates AT₁ receptor numbers and also enhances receptor-mediated response, that is, Ang II--stimulated IP₃ production. This evidence makes clear the mechanisms of vascular hypersensitivity to Ang II in glucocorticoid-induced hypertension.

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