Glucocorticoid and mineralocorticoid regulation of angiotensin II type 1 receptor binding and inositol triphosphate formation in WB cells

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Abstract

Mineralocorticoids, glucocorticoids, and angiotensin II (AngII) act cooperatively to maintain body fluid homeostasis. Mineralocorticoids, such as aldosterone and deoxycorticosterone-acetate (DOCA), function synergistically with AngII in the brain to increase salt appetite and blood pressure. In addition, glucocorticoids increase AngII-induced drinking and pressor responses and may also facilitate the actions of aldosterone on salt appetite. The AngII Type 1 (AT1) receptor mediates many of the physiological and behavioral actions of AngII. This receptor is coupled to the G-protein G_q, which mediates AngII-induced inositol triphosphate (IP3) formation. The WB cell line, a liver epithelial cell line that expresses the AT1 receptor, was used to examine the cellular basis of glucocorticoid and mineralocorticoid regulation of AT1 function. In this study corticosterone and dexamethasone

treatments increased the number of AT1 receptors by activating the glucocorticoid receptor (GR). This increase in AT1 binding resulted in enhanced AngII-stimulated IP3 formation. However, only supraphysiological doses of aldosterone or DOCA increased AT1 binding, and this effect also was mediated by GR activation. Furthermore, despite evidence that mineralocorticoids and glucocorticoids function together to increase AngII-stimulated actions *in vivo*, aldosterone and dexamethasone did not act synergistically to affect AT1 binding, G_q expression, or IP3 formation. These results indicate that GR activation, and the subsequent increases in AT1 binding and in AngII-stimulated IP3 formation, may represent a cellular mechanism underlying the synergy between adrenal steroids and AngII.

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Introduction

Angiotensin II (AngII), mineralocorticoids and glucocorticoids are important in maintaining cardiovascular and body fluid homeostasis. For example, AngII causes vasoconstriction, aldosterone and vasopressin release, and sodium reabsorption. It also acts in the brain to elicit water and salt intake (reviewed by Fluharty & Sakai 1995). Although there are two subtypes of membrane-bound receptors that mediate the actions of AngII, the AngII type 1 (AT1) and the AngII type 2 receptors (Wong *et al.* 1992), the AT1 mediates most of the known physiological and behavioral actions of AngII (Fluharty & Sakai 1995). Therefore, it is the focus of this study.

Mineralocorticoid treatment increases water intake, the pressor response (Wilson *et al.* 1986), and salt appetite (Epstein 1982, Fluharty & Epstein 1983, Zhang *et al.* 1984, King *et al.* 1988) after central administration of AngII. Similarly, in conjunction with AngII, gluco-

corticoid treatment increases AngII-induced pressor responses (Krakoff *et al.* 1975, Whitworth 1987) and drinking behavior (Ganesan & Sumners 1989, Sumners *et al.* 1991). Adrenal steroids also increase AngII receptor binding in cultured vascular smooth muscle cells (Sato *et al.* 1992, 1994, Ullian *et al.* 1992), mesenteric arterioles (Schiffrin *et al.* 1984), brain (Wilson *et al.* 1986, King *et al.* 1988), and neuronal cultures (Wilson *et al.* 1986, Sumners & Fregly 1989). These effects may provide a mechanism by which adrenal steroids augment AngII-mediated actions at the cellular level (Sato *et al.* 1992, Ullian *et al.* 1992, Thornton & Nicolaidis 1994).

Intracellular receptors mediate the effects of mineralocorticoids and glucocorticoids. The mineralocorticoid receptor (MR) binds both aldosterone and corticosterone with high affinity ($K_d \sim 0.5$ nM), whereas the glucocorticoid receptor (GR) preferentially binds glucocorticoids, although at lower affinity ($K_d \sim 5$ nM) (Reul & De Kloet 1985). However, when present in

supraphysiological concentrations ($K_d \sim 20\text{--}50\text{ nM}$), mineralocorticoids can activate GR (Claire *et al.* 1989, Schmidt *et al.* 1993). AngII receptor expression is modulated by endogenous steroids and synthetic analogs of the endogenous steroids, including dexamethasone, a high-affinity glucocorticoid agonist and deoxycorticosterone acetate (DOCA), a mineralocorticoid agonist (Summers & Fregly 1989, Vallee *et al.* 1995).

The present study examines the cellular basis of adrenal steroid regulation of AT1 receptor function. It was conducted in the WB cell line, a homogeneous population of liver epithelial cells that exclusively expresses the AT1 receptor subtype. The AT1 receptor is coupled to the G-protein, Gq, which mediates AngII-induced inositol triphosphate (IP3) formation (Taylor *et al.* 1990). The WB cell line has a well characterized IP3 pathway (Bokkala & Joseph 1997) and is, therefore, an appropriate system with which to study the potential interaction of mineralocorticoids and glucocorticoids on AngII-induced IP3 formation. The initial experiments examined the steroid receptor specificity in mediating the effects of adrenal steroids on AT1 receptor binding, and subsequent experiments investigated the consequences on AngII signal transduction. Thus we examined whether these steroids might directly affect AT1 binding, Gq expression, IP3 formation, or combinations thereof, thereby amplifying the effects of AngII.

Material and Methods

Cell culture and steroid treatment

WB cells were maintained in Richter's minimal essential medium (Irvine Scientific, Santa Ana, CA, USA) containing 5% fetal bovine serum (Hyclone, Logan UT, USA), 20 mM HEPES, 25 mM sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin. Aldosterone, corticosterone, dexamethasone, and DOCA (Sigma, St Louis, MO, USA) and steroid antagonists RU38486 and RU28318 (kindly donated by Roussel-Uclaf, Romainville, France) were dissolved in 100% ethanol, then serially diluted in medium at the concentration described. Final ethanol concentration was 0.002%.

Membrane receptor binding

WB cells were rinsed twice with ice-cold 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, then incubated for 10 min at 4 °C in 20 mM Tris-HCl (pH 7.4), scraped with a rubber spatula, and homogenized with a Dounce homogenizer. The membrane suspension was centrifuged at 48 000 *g* for 20 min. The membrane pellet was resuspended in buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl₂, aprotinin (0.3 trypsin inhibitory units (TIU)/ml) and 1,10 phenanthroline

(0.1 mg/ml), and homogenized. Membrane preparations were added to tubes containing 0.5 nM [¹²⁵I]-AngII (2200 Ci/mmol; NEN/Dupont, Boston, MA, USA), except for the saturation isotherm experiment, in which the concentration of [¹²⁵I]-AngII was in the range 0.3–7 nM. The preparation was then incubated for 1 h at 22 °C, and binding was terminated by dilution with ice-cold 10 mM Tris-HCl (pH 7.4), 150 mM NaCl and rapid vacuum filtration through 0.3% polyethylenimine presoaked Whatman GF/B filters using a Brandell cell harvester. Dried filters were counted for 1 min in an LKB gamma-scintillation counter at 65% efficiency. Non-specific binding was determined in tubes with unlabelled Sarlle (1 µM) (Wong *et al.* 1992). Protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Triplicate values were averaged and calculated as specific fmol/mg protein. The 'vehicle-treated' level of AT1 binding in Fig. 4 differs from that of Fig. 2 because the latter value is a Bmax value calculated from a saturation binding experiment, whereas the former reflects binding carried out with a concentration of 0.5 nM [¹²⁵I]-AngII. In Figs 1, 3, and 5, where AT1 binding was expressed as '% increase above vehicle,' the vehicle baseline binding values are shown in the figure legends.

IP3 measurements

IP3 assays were conducted as described previously (Bokkala & Joseph 1997). WB cells were grown in Dulbecco's modified inositol-free medium (Life Technologies, Gaithersburg, MD, USA) and treated with 5 µCi/ml *myo*-[³H]inositol (American Radiolabelled Chemicals, St Louis, MO, USA) for 20 h. The medium was then removed and replaced with media containing AngII for 30 s. Cells were washed twice with phosphate buffered saline and lysed with 1 ml 10% trichloroacetic acid. The cell lysates were centrifuged and supernatants were extracted five times with two volumes of water-saturated diethyl ether. Protein determination of cell lysates were performed by the BCA method. The aqueous phase was neutralized with 600 mM sodium bicarbonate and 300 mM EDTA. Samples were run over AG 1-X8 columns (Biorad, Hercules, CA, USA) and inositol phosphates were sequentially eluted with increasing concentrations of ammonium formate. Radioactivity of fractions was measured with liquid scintillation counting (True-Count, IN/US systems).

Western blot analysis

Cells were rinsed twice with ice-cold 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, then incubated for 10 min at 4 °C in 20 mM Tris-HCl (pH 7.4), scraped with a rubber spatula, and homogenized with a Dounce

homogenizer. The membrane suspension was centrifuged at 48 000 *g* for 20 min. The membrane pellet was resuspended in buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl₂, aprotinin (0.3 TIU/ml) and 1,10 phenanthroline (0.1 mg/ml), and homogenized and assayed for protein concentration by the BCA method. Membrane samples were boiled for 3 min in electrophoresis sample buffer, then subjected to SDS/10% polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and nonspecific sites were blocked with 3% gelatin in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) overnight at 4 °C. The blocking solution was discarded and replaced with 5% nonfat dry milk for 30 min at 22 °C. The filters were incubated with a primary Gq_α antiserum kindly provided by D Manning (University of Pennsylvania) in 1% gelatin/TBS (1 : 1000) for 90 min with gentle shaking at 22 °C. This antiserum has equivalent specificity for Gq_{α11} and Gq_{α4}, but not for Gq_{α15} or Gq_{α16} (Lounsbury *et al.* 1993). Blots were washed with TBS 0.05%–Tween 20 (TBST) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (New England Biolabs, Boston, MA, USA) in TBS (1 : 1500) for 60 min. After TBST washes, the blots were developed by Phototope Western blotting reagents (New England Biolabs) as described by the manufacturer. Films were processed and analyzed using computer densitometry (NIH Image).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from WB cells and adult Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) anterior pituitary (Seltzer *et al.* 1992) was extracted using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Ten micrograms total RNA were reverse-transcribed using M-MuLV reverse transcriptase (Pharmacia Biotech, Piscataway, NJ, USA) and 0.2 µg random primed hexamers for 37 °C for 1 h. The PCR reaction was carried out in 50 µl volume with 5 µl 10 × PCR buffer II (Perkin Elmer, Foster City, CA, USA), 3 µl 25 mM MgCl₂, 4 µl 2.5 mM dNTPs, 1 µl cDNA template, 50 ng each MR primer, and 2.5 U AmpliTaq-DNA Polymerase (Perkin Elmer). To avoid evaporation, mixes were overlaid with 50 µl mineral oil. Amplification was performed over 30 cycles (30 s denaturing at 94 °C, 1 min annealing at 58 °C, 1 min extension at 72 °C) in a DNA thermal cycler (Perkin Elmer Cetus). The 5' and 3' MR primers correspond to bp 4875–4901 and bp 5550–5524, respectively, of the rat MR mRNA coding sequence as described elsewhere (Patel *et al.* 1989). The identity of the amplified MR RT-PCR product (695 bp) was confirmed by EcoR1 restriction enzyme cleavage. The RT-PCR products were analyzed by 2% agarose gel electrophoresis, and visualized by ethidium bromide staining under u.v. light.

Figure 1 Effect of corticosterone (●) and dexamethasone (■) on AT1 binding. Dose–response curve of corticosterone and dexamethasone on 0.5 nM ¹²⁵I-AngII binding. WB cells were treated with steroid at doses of 1–1000 nM for 20 h before harvest. The data are reported as percentage increase in AT1 binding above that of vehicle-treated cells. The vehicle baseline AT1 binding was 408 ± 121 fmol/mg; *n*=5, mean ± s.d. **P*<0.05.

Statistical analysis

Results are represented as mean ± standard deviation (s.d.). Statistical analysis was done via one-way ANOVA, followed by Student–Newman–Keuls' *post hoc* comparisons, with *P*<0.05 considered statistically significant.

Results

The aim of this study was to determine the cellular basis of glucocorticoid and mineralocorticoid effects on AT1 binding and intracellular signalling. WB cells were treated with corticosterone and dexamethasone at doses of 1, 10, 100, and 1000 nM for 20 h before harvest. The baseline AT1 binding in vehicle-treated cells was 408 ± 121 fmol/mg. Treatment with corticosterone increased ¹²⁵I-AngII binding in a dose-dependent manner (*P*<0.05), maximally increasing AT1 binding 102 ± 17% at the highest dose of 1 µM corticosterone (Fig. 1). Similarly, treatment with dexamethasone significantly increased ¹²⁵I-AngII binding (*P*<0.05), with a maximal increase at 165 ± 48% above control. Dexamethasone was more effective at stimulating AT1 binding than corticosterone (dexamethasone EC₅₀=0.21 ± 0.19 nM; corticosterone EC₅₀=41.7 ± 17 nM, *n*=5).

In subsequent experiments, radioligand binding techniques were used to examine whether the stimulatory

Figure 2 Representative saturation isotherm. WB cells were treated with vehicle (□), corticosterone (50 nM) (CORT, ●) or dexamethasone (5 nM) (DEX, ■) for 20 h before harvest. Saturation binding was done with concentrations of ¹²⁵I-AngII from 0.3 nM to 7 nM. Nonspecific binding was determined with unlabelled Sarile (1 μM). Triplicate values were averaged and calculated as specific fmol/mg protein. The Bmax and K_d values shown in the table represent data from three separate experiments; mean ± S.D. *P<0.05.

effects of corticosterone and dexamethasone on AT1 binding resulted from an increased number of AT1 receptors rather than a change in the affinity of the receptor for the ¹²⁵I-AngII ligand. Saturation binding showed that treatment with corticosterone and dexamethasone increased the number of AT1 receptors as reflected by a significant increase in the Bmax ($F[2,6]=68.2$, $P<0.0001$, $n=3$) without changing the K_d of the AT1 receptor (Fig. 2; graph and accompanying table). The effect of dexamethasone on Bmax was greater than the effect of corticosterone on Bmax and could result from the greater affinity of dexamethasone for the GR (McEwen *et al.* 1986), as the dose of dexamethasone used exceeded its EC₅₀, whereas the dose of corticosterone was comparable to its EC₅₀.

As both corticosterone and dexamethasone significantly increased AT1 binding (Fig. 1), and as either can interact with both the MR and GR (Reul & De Kloet 1985), we next determined which receptors mediate the increase in AT1 binding. Cells were treated with the MR antagonist, RU 28318 (1 μM), or the GR antagonist, RU 38486 (1 μM), along with corticosterone (50 nM) or dexamethasone (5 nM). These doses of steroid were used in the antagonist experiments because it was necessary to show that the stimulatory effect of dexamethasone and

Figure 3 Effects of corticosterone (Cort) and dexamethasone (Dex) on AT1 binding are via GR. WB cells were pretreated with vehicle (no antagonist), MR antagonist RU 28318 (1 μM), the GR antagonist RU 38486 (1 μM), or both, for 2 h before addition of vehicle (no steroid), dexamethasone (5 nM) or corticosterone (50 nM). Cells were harvested 20 h later and prepared for 0.5 nM ¹²⁵I-AngII binding. Binding is presented as % increase (mean ± S.D.) above that achieved with vehicle. Baseline values for vehicle was 305 ± 53 fmol/mg protein, $n=6$, *P<0.05 compared with vehicle.

corticosterone on AT1 binding could be significantly decreased with a GR antagonist. Using lower (submaximal) doses may have not illustrated this result. The MR antagonist RU28318, GR antagonist RU 38486, or both antagonists together, had no significant effect on AT1 binding compared with vehicle treatment (Fig. 3). Corticosterone increased AT1 binding by 107 ± 9% over vehicle treatment ($P<0.05$ compared with vehicle, $n=6$). This stimulatory effect was not significantly changed by treatment with the MR antagonist, RU 28318 (119 ± 14%; $P<0.05$). However, treatment with RU 38486 significantly decreased AT1 binding to 41 ± 10%, which was not significantly different from the effect of GR antagonist RU 38486 or vehicle alone. In addition, treatment with both RU 28318 and RU 38486 did not further affect AT1 binding versus the RU 38486 alone. This indicates that the stimulatory effect of corticosterone on AT1 binding is mediated by the GR exclusively. Similarly, cells treated with dexamethasone (5 nM) alone showed a 128 ± 21% increase in AngII binding over vehicle treatment ($P<0.05$). This increase in AT1 binding was not significantly affected (84 ± 33%) by the presence of RU 28318. However, treatment with RU 38486

Figure 4 Effects of aldosterone (□) and DOCA (■) on AT1 binding. Dose-response curve of aldosterone and DOCA on 0.5 nM 125 I-AngII binding. WB cells were treated with steroid at doses of 1–1000 nM for 20 h before harvest. The data are presented as AT1 binding in fmol/mg protein and represent the mean $\pm$ S.D. of three experiments, * P <0.05 compared with vehicle.

prevented the increase in AT1 binding ($3 \pm 10\%$), whereas the effect of treatment with both RU 28318 and RU 38486 was not different than that with RU 38486 alone ($17 \pm 23\%$ increase over vehicle). Thus, as with corticosterone, the stimulatory effect of dexamethasone on AT1 binding is mediated by the GR and not by the MR.

We next examined the role of mineralocorticoids in this system and whether they act cooperatively with glucocorticoids to modulate the effects on AT1 binding or function. WB cells were treated with increasing doses of two mineralocorticoids, aldosterone and DOCA at doses of 1, 10, 100, and 1000 nM for 20 h before harvest. DOCA possesses mostly mineralocorticoid activity, with some glucocorticoid activity, and may have a longer half-life than aldosterone (Vallee *et al.* 1995). Cells treated with aldosterone showed no increase in AT1 binding at the lower doses, but at the supraphysiological doses of 100 nM and 1 μ M, they increased AT1 binding by 90% and 152%, respectively (Fig. 4). Similarly, DOCA treatment increased AT1 binding, but only at the highest doses of 100 nM and 1 μ M, with which binding was increased by 40% and 87%, respectively. Treatment with higher doses of aldosterone or DOCA (10 μ M) resulted in AT1 binding equivalent to that of 1 μ M doses (data not shown). Also, at the 100 nM and 1 μ M doses, aldosterone treatment elicited greater AT1 binding than DOCA, although the increased potency (aldosterone $EC_{50}=89 \pm 14$ nM; DOCA $EC_{50}=221 \pm 73$, $n=3$) did not reach the level of statistical significance ($P=0.15$).

Figure 5 Effects of aldosterone (ALDO) and DOCA on AT1 binding are via GR. WB cells were pretreated with vehicle (no antagonist), MR antagonist RU 28318 (10 μ M), the GR antagonist RU 38486 (10 μ M), or both, for 2 h before addition of vehicle (no steroid), aldosterone (1 μ M) or DOCA (1 μ M). Cells were harvested 20 h later and prepared for 0.5 nM 125 I-AngII binding. Binding is presented as % increase (mean $\pm$ S.D.) over vehicle. Baseline values for vehicle was 141 ± 15 fmol/mg protein, $n=3$, * P <0.05 compared with vehicle.

Although physiological doses of aldosterone and DOCA are believed to mediate their effect via the MR, both of these steroids can bind to the GR at pharmacological doses (Claire *et al.* 1989, Schmidt *et al.* 1993). Because high doses of aldosterone and DOCA were necessary to increase AT1 binding significantly, the next experiment determined if this effect was mediated by MR or GR. Cells were treated with the MR antagonist RU 28318 (10 μ M) and the GR antagonist RU 38486 (10 μ M), along with 1 μ M aldosterone or DOCA – doses that stimulated AT1 binding (Fig. 4). In this experiment, a 10 μ M antagonist dose was used, rather than 1 μ M antagonist (Fig. 3), because higher doses of steroid agonist were used. The MR antagonist RU 28318 (10 μ M) or the GR antagonist RU 38486 (10 μ M), given alone or together, did not significantly increase AT1 binding compared with vehicle (Fig. 5). Treatment with 1 μ M aldosterone increased AT1 binding over vehicle by $231 \pm 67\%$ (P <0.05 compared with vehicle, $n=3$). Similarly, administration of 1 μ M DOCA increased AT1 binding over vehicle by $176 \pm 57\%$ (P <0.05 compared with vehicle). As shown in Fig. 5, pretreatment of cells with RU 28318 along with 1 μ M aldosterone or 1 μ M DOCA did not significantly affect AT1 binding compared with steroid

alone. However, pretreatment of the cells with RU 38486 significantly decreased aldosterone- or DOCA-induced AT1 binding ($P<0.05$). In addition, treatment with both RU 28318 and RU 38486 did not further affect AT1 binding compared with the inhibitory effect of RU 38486 alone. Thus, the stimulatory effects of high-dose aldosterone and DOCA on AT1 binding are mediated by the GR exclusively.

The subsequent experiments were aimed at determining if aldosterone could act cooperatively with dexamethasone to modulate AT1 binding or AT1-mediated actions. Cells were treated with a dose of aldosterone (1 nM) that primarily interacts with MR only (Reul & De Kloet 1985), along with two doses of dexamethasone (1 nM and 5 nM). The 1 nM dose represents a submaximal dose of dexamethasone that would allow detection of a potential synergy with aldosterone on AT1 binding. Treatment with aldosterone alone did not increase AT1 binding (aldosterone 1 nM 182 ± 24 fmol/mg compared with vehicle 188 ± 3 fmol/mg; $n=3$), similar to the results shown in Fig. 4. Also, the 76% increase in AT1 binding after 1 nM dexamethasone treatment ($P<0.05$ compared with vehicle) was not further increased by combined treatment with aldosterone (dexamethasone 1 nM 330 ± 24 fmol/mg; aldosterone (1 nM)+dexamethasone (1 nM) 315 ± 27 fmol/mg). Similarly, the 176% increase in AT1 binding after 5 nM dexamethasone treatment ($P<0.05$ compared with vehicle) was not further increased by combined treatment with aldosterone (dexamethasone (5 nM) 520 ± 37 fmol/mg; aldosterone+dexamethasone 474 ± 4 fmol/mg), indicating that no cooperativity was occurring between the two steroids at the level of AT1 binding.

AngII binding to the AT1 receptor leads to the formation of IP3. We examined if the increase in AT1 receptor seen with dexamethasone leads to an increase in AngII-induced IP3 formation. Cells were loaded with *myo*-[3 H]inositol and treated with dexamethasone (5 nM) or vehicle for 20 h, followed by stimulation with AngII. As seen in Fig. 6, there was a main treatment effect, with dexamethasone increasing AngII-stimulated IP3 formation ($F[1,72]=4.39$, $P=0.039$, $n=10$). However, there was no significant difference in EC_{50} values between vehicle and dexamethasone treatment (vehicle $EC_{50}=37 \pm 8$ nM; dexamethasone $EC_{50}=92 \pm 38$ nM). Cells also were tested for 125 I-AngII binding. Dexamethasone increased AT1 binding, which was correlated with an increase in IP3 formation that accompanied the increase in AT1 binding ($r=0.73$, $P<0.05$).

Although physiological doses of aldosterone did not result in increased AT1 binding (Fig. 4), we examined if aldosterone affected AngII-induced IP3 formation, possibly suggesting a role for aldosterone in AT1 signal transduction. Thus, cells were loaded with *myo*-[3 H]inositol and treated with aldosterone (1, 10, 100, 1000 nM) or vehicle for 20 h, followed by stimulation with AngII.

Figure 6 Effect of dexamethasone on AngII-stimulated IP3 formation: dexamethasone treatment leads to an increase in AngII-stimulated IP3 formation. WB cells were loaded with 5 μ Ci/ml *myo*-[3 H]inositol and were treated with dexamethasone (5 nM) (■) or vehicle (□) for 20 h. After treatment, cells were stimulated with increasing doses of AngII (0, 1, 10, 100, 1000 nM) for 30 s and assayed for IP3 formation. Values are represented as % increase in IP3 formation (c.p.m./mg) above basal (no AngII stimulation) of the same treatment. Baseline value for unstimulated vehicle treatment was 135 ± 37 c.p.m./mg and was comparable to unstimulated dexamethasone treatment (135 ± 9 c.p.m./mg), demonstrating that there was no difference in basal IP3 levels. There was a main treatment effect of dexamethasone ($n=10$, $P=0.039$).

Despite a wide range of doses of aldosterone (Table 1), the increase in IP3 formation did not reach statistical significance ($F[4,50]=1.74$, $P=0.15$, $n=3$). However, it was possible that aldosterone or dexamethasone could synergistically exert post-receptor effects on mediators of AngII signal response, such as IP3 formation. Therefore, cells were loaded with *myo*-[3 H]inositol and treated with vehicle, dexamethasone (5 nM), aldosterone (1 nM), or both, followed by AngII-stimulation. Aldosterone treatment alone had no effect on IP3 formation compared with control (Fig. 7). Dexamethasone had a significant stimulatory effect on IP3 formation ($P<0.05$), as was seen previously (Fig. 6). In addition, dexamethasone+aldosterone stimulated IP3 formation above vehicle ($P<0.05$), but no differently than dexamethasone alone. Also, there was no significant difference in EC_{50} values between the groups (vehicle, 11.6 ± 3 nM; aldosterone, 13.5 ± 2.4 ; dexamethasone, 19.25 ± 5.2 ; aldosterone+dexamethasone, 18.7 ± 4.1 ; $n=3$). In parallel, WB cells also were tested for 125 I-AngII binding. Consistent with the results in which dexamethasone and aldosterone+dexamethasone, but not aldosterone alone, increased AT1

Table 1 Effect of aldosterone on AngII-stimulated IP3 formation: aldosterone treatment does not increase AngII-stimulated IP3 formation. WB cells were loaded with 5 μ Ci/ml myo-[3 H]inositol and were treated with aldosterone (1 nM–1 μ M) or vehicle for 20 h. After treatment, cells were stimulated with increasing doses of AngII (0, 1, 10, 100, 1000 nM) for 30 s and assayed for IP3 formation. Values are represented as IP3 formation (c.p.m./mg) above basal (no AngII stimulation) of the same treatment (mean $\pm$ s.d. of three experiments). Baseline values for unstimulated vehicle treatment were comparable to those of the aldosterone treatment groups.

AngII	Aldosterone				
	Vehicle	1 nM	10 nM	100 nM	1 μ M
1 nM	8 $\pm$ 29	100 $\pm$ 145	138 $\pm$ 36	199 $\pm$ 66	77 $\pm$ 47
10 nM	214 $\pm$ 22	450 $\pm$ 198	467 $\pm$ 59	429 $\pm$ 22	499 $\pm$ 9
100 nM	871 $\pm$ 27	906 $\pm$ 302	1097 $\pm$ 173	1105 $\pm$ 51	1023 $\pm$ 68
1 μ M	789 $\pm$ 93	831 $\pm$ 112	1037 $\pm$ 92	965 $\pm$ 31	857 $\pm$ 16

Figure 7 Effect of aldosterone+dexamethasone on AngII-stimulated IP3 formation. Aldosterone treatment does not increase the stimulatory effects of dexamethasone on AngII-stimulated IP3 formation. WB cells were loaded with 5 μ Ci/ml myo-[3 H]inositol and were treated with vehicle (V, $\square$), aldosterone (1 nM) (A, $\triangle$), dexamethasone (5 nM) (D, $\blacksquare$), or aldosterone+dexamethasone (A+D, $\blacklozenge$) for 20 h. After treatment, cells were stimulated with AngII (0, 1, 10, 100, 1000 nM) for 30 s and assayed for IP3 formation. Values are represented as % increase in IP3 formation (c.p.m./mg) over basal (no AngII stimulation) of the same treatment. Baseline values for unstimulated vehicle treatment were comparable to those of A, D, and A+D treatment groups. Dexamethasone and dexamethasone+aldosterone had a significant stimulatory effect on IP3 formation compared with vehicle ($P<0.05$), but no different than dexamethasone alone (mean $\pm$ s.d. of three experiments).

binding, there was also an increase in IP3 formation that accompanied the increase in AT1 binding ($r=0.87$, $P<0.05$). These data indicate that the stimulatory effect of dexamethasone on IP3 formation was not further affected by aldosterone, indicating the lack of synergy between the two hormones at the level of IP3 formation.

The AT1 receptor is coupled to the G-protein, Gq, which mediates AngII-induced IP3 formation (Taylor *et al.* 1990, Langlois *et al.* 1994). To determine the effect of adrenal steroids on factors distal to the receptor, Western blot analysis was used to determine the effects of adrenal steroids on Gq expression. Cells were treated with vehicle, aldosterone (1 nM), dexamethasone (5 nM) or both, and prepared for immunodetection using antisera with equal specificity to Gq $_{\alpha 11}$ and Gq $_{\alpha q}$ (Lounsbury *et al.* 1993). As shown in Fig. 8, a 42 kDa band was detected, but there was no significant difference between any of the treatment groups.

Given the absence of an MR effect, RT-PCR was used to demonstrate the presence of MR in WB cells. Rat anterior pituitary tissue was used as a positive control (Reul *et al.* 1990, 1994, Rosenfeld *et al.* 1990, Turner 1990, Rothuizen *et al.* 1993, Schobitz *et al.* 1994, Smith *et al.* 1994, McCormick *et al.* 1998). The MR (695 bp band) was expressed in WB cells and in the anterior pituitary (Fig. 9).

Discussion

The AT1 receptor mediates many physiological and behavioral actions of AngII (Bottari *et al.* 1993). Both mineralocorticoids and glucocorticoids regulate components of the renin-angiotensin system, including the AT1 receptor. The aim of this study was to examine the direct effects of adrenal steroids on AT1 binding and post-receptor signalling mechanisms in WB cells. First, corticosterone and dexamethasone increased the number

Densitometry of Gq Immunoblot

Treatment group	Optical density
Vehicle	159 ± 13
ALDO (1 nM)	160 ± 17
DEX (5 nM)	161 ± 8
ALDO + DEX	155 ± 17

Figure 8 Immunoblot of Gq. WB cells were treated with vehicle (Veh), aldosterone (ALDO) (1 nM), dexamethasone (DEX) (5 nM), or ALDO+DEX for 20 h before harvest. Samples were separated by 10% SDS-PAGE, immunoblotted, and detected with a rabbit anti-Gq antibody. As shown, a 42 kDa band was detected. Molecular mass markers (mw) are shown. The table represents specific optical density measurements from four separate experiments (mean ± S.D.).

Figure 9 Ethidium bromide-stained agarose gel showing MR PCR product, 695 bp, after RT-PCR amplification of cDNA from WB cells and from rat anterior pituitary gland. Lane 1, 1 kb DNA ladder molecular weight marker; lane 2, Pituitary MR; lane 3, WB cell MR; lane 4, Pituitary negative control (no reverse transcriptase); Lane 5, WB cell negative control (no reverse transcriptase).

of AT1 receptors by activating the GR, but not the MR. Functionally, this led to an increase in AngII-induced IP3 formation, which can result in increases in intracellular calcium and distal signalling cascades (Berridge 1993). Also analyzed was the effect of aldosterone, the endogenous mineralocorticoid, and DOCA, a synthetic mineralocorticoid used *in vitro* and *in vivo* studies of AngII receptor

regulation (Sumners & Fregly 1989, Vallee *et al.* 1995). Neither aldosterone nor DOCA increased AT1 binding at physiological levels, but both stimulated AT1 binding at the highest doses. However, using specific MR and GR antagonists, it was apparent that aldosterone and DOCA stimulated AT1 binding by only activating the GR, and not the MR. Furthermore, although high-dose mineralocorticoids increased AT1 binding, AngII-induced IP3 formation was not significantly affected. This may result from insufficient coupling of the AT1 receptor to its post-receptor signalling factors. An MR-mediated effect was not detected despite MR expression in these cells, as detected by RT-PCR (Fig. 9). This technique closely correlates with the presence of functional nuclear steroid hormone receptor protein (Castagnetta & Caruba 1995, Bodine *et al.* 1997, Malayer & Woods 1998, Robinson *et al.* 1998), such as the MR in the anterior pituitary (Reul *et al.* 1990, 1994, Rosenfeld *et al.* 1990, Turner 1990, Rothuizen *et al.* 1993, Schobitz *et al.* 1994, Smith *et al.* 1994, McCormick *et al.* 1998).

Adrenal steroids regulate multiple components of the renin-angiotensin system, including angiotensinogen (Deschepper & Flaxman 1990, Bunnemann *et al.* 1993, Rifitina *et al.* 1995, Ryan *et al.* 1997) and angiotensin-converting enzyme (Mendelsohn *et al.* 1982), both of which could generate more AngII ligand. In addition, the physiological and behavioral actions of AngII can be modulated by mineralocorticoids (Epstein 1982, Fluharty & Epstein 1983, Zhang *et al.* 1984, Wilson *et al.* 1986, King *et al.* 1988) and glucocorticoids (Krakoff *et al.* 1975, Whitworth 1987, Ganesan & Sumners 1989, Sumners *et al.* 1991) at the level of the AT1 receptor (Schiffrin *et al.* 1984, Wilson *et al.* 1986, Sumners & Fregly 1989, Ullian *et al.* 1992). Furthermore, glucocorticoids enhance mineralocorticoid-induced actions (Ma *et al.* 1993, Zhang *et al.* 1993). To examine the cellular basis of this synergy, cells were treated concomitantly with 5nM dexamethasone, a concentration that augmented AT1 binding and IP3 formation, together with 1nM aldosterone, a concentration that primarily activates MR (Reul & De Kloet 1985). However, this combination did not further enhance the effect of dexamethasone alone on binding. It was possible that aldosterone and dexamethasone acted synergistically at post-receptor mechanisms, such that a direct effect on AT1 signalling mechanisms could further amplify AngII stimulation. However, aldosterone (1 nM) did not further augment the effect of dexamethasone alone on AngII-induced IP3 formation. We also examined the expression of Gq, which mediates AngII-induced IP3 formation from the AT1 receptor (Taylor *et al.* 1990, Fluharty & Sakai 1995). The results confirmed that dexamethasone did not increase Gq expression (Sato *et al.* 1994), and neither did the other steroid treatments. Thus, no synergy between aldosterone and dexamethasone was seen at the level of AT1 binding, Gq expression, or IP3 formation. However, we cannot exclude the possibility

that aldosterone and dexamethasone act together to regulate signalling factors distal to Gq or IP3 formation or other signalling molecules, such as cyclic AMP, which decreases after AngII stimulation (reviewed by Fluharty & Sakai 1995).

At physiological doses ($K_d \sim 0.5$ nM), mineralocorticoids activate the MR and increase AT1 binding (Sumners & Fregly 1989, DeNicola *et al.* 1993, Shelat *et al.* 1998). However, by using specific MR and GR antagonists, it has been shown that pharmacological doses of mineralocorticoids ($K_d \sim 20$ – 50 nM) bind the GR and mimic glucocorticoid activation (Reul & De Kloet 1985, Claire *et al.* 1989, Schmidt *et al.* 1993). Similar cases of GR activation by supraphysiological concentrations of mineralocorticoids have been reported, including sodium transport in A6 kidney cells, which express both the MR and GR (Claire *et al.* 1989, Schmidt *et al.* 1993), inhibition of glucose transport in primary neuronal and glial cell cultures (Horner *et al.* 1990), modulation of microglial morphology (Tanaka *et al.* 1997), and regulation of endothelin and AngII binding in vascular smooth muscle cells (Provencher *et al.* 1995). In the latter study, the increase in AngII binding in vascular smooth muscle cells after 100 nM aldosterone treatment was only partially blocked by the GR antagonist, RU 38486 (Provencher *et al.* 1995). Although this study did not look at the effect of MR antagonists and GR antagonists, alone and together, on suppressing the aldosterone effect on AngII binding, it would have been interesting to see if the combined treatment with the GR and MR antagonist together completely suppressed the aldosterone effect on AngII binding, thereby showing that the aldosterone effect was mediated partly by MR and partly by GR. In contrast, the present study showed that the stimulatory effect of supraphysiological doses of both aldosterone and DOCA was completely blocked by the GR antagonist, RU 38486 (Fig. 5). Furthermore, treatment with both the MR antagonist RU28318 and the GR antagonist RU 38486 together did not further affect AT1 binding compared with the inhibitory effect of RU 38486 alone (Fig. 5). Thus the stimulatory effects of high-dose aldosterone and DOCA on AT1 binding were mediated by the GR exclusively. Another study showed that a 1 μ M dose of aldosterone increased AngII receptor binding and AngII-induced IP3 response in cultured vascular smooth muscle cells (Ullian *et al.* 1992). This effect may also have reflected GR activation because, in these same cells, dexamethasone increased AngII receptor binding (Sato *et al.* 1994) and AngII-induced IP3 formation (Sato *et al.* 1992) by acting at the GR. In the present study, dexamethasone, corticosterone, and high-dose aldosterone and DOCA increased AT1 binding by acting at the GR.

There are several possible reasons why activation of MR did not increase AT1 binding in this study. This result occurred despite the presence of MR mRNA detected by RT-PCR (see above). First, at low concentrations (1 and 10 nM), aldosterone and DOCA may have been

metabolized to inactive compounds. Secondly, there may have been insufficient MR protein, because of translational or post-translational effects. Consequently, the levels of GR may have far exceeded those of MR, as is true in many tissues (McEwen *et al.* 1986), and, therefore, activation of the MR may not have been detected. In this case, because of the lower affinity of aldosterone for GR (Claire *et al.* 1989, Schmidt *et al.* 1993), only high mineralocorticoid concentrations could activate GR and increase AT1 binding. Future studies will also examine the effects of mineralocorticoid and glucocorticoid on the relative levels of MR and GR after steroid treatment. Lastly, it is possible that the necessary repertoire of cell-specific transcriptional factors was not present for MR-mediated actions on AT1 binding.

Several lines of evidence suggest that steroids act genomically to modulate AT1 receptors. First, the promoter region of the AT1A receptor, the predominant AT1 receptor subtype in liver cells, contains an active glucocorticoid response element (Pearce & Yamamoto 1993, Guo *et al.* 1995). Secondly, in the WB cells (Shelat, unpublished data) and in other systems (Provencher *et al.* 1995, Wintersgill *et al.* 1995), the time-course of glucocorticoid action is consistent with steroids acting genomically (Tsai & O'Malley 1994). Thirdly, the steroid effects can be abolished with inhibitors of protein synthesis (Ullian *et al.* 1992). Fourthly, the stimulatory effects of corticosterone, dexamethasone and high-dose aldosterone and DOCA can be suppressed by blocking the GR, a known nuclear transcription factor (Carson-Jurica *et al.* 1990). Lastly, adrenal steroid effects on AT1 binding are accompanied by parallel changes in AT1 RNA (Sato *et al.* 1994, Aguilera *et al.* 1995, Wintersgill *et al.* 1995). Thus the changes in AT1 binding seen in this study probably reflect a genomic action of steroids in these cells.

The present study was conducted in WB cells – a homogeneous liver epithelial cell population that exclusively expresses the AT1 receptor subtype. Like other cell types that express the AT1 receptor, they can respond to glucocorticoids (because of the ubiquitous expression of GR (McEwen *et al.* 1986)), and they respond to AngII stimulation by increasing IP3 formation (Bokkala & Joseph 1997). However, the WB cells may not precisely represent the cellular environment in all cell types, as another study showed that glucocorticoid treatment in a human hepatoma cell line decreased AT1 receptor expression (Wintersgill *et al.* 1995). This disparity suggests that cell-specific factors are necessary for steroid receptor activation, which may account for the failure of physiological doses of mineralocorticoids to modulate AT1 binding or IP3 formation. This study shows that activation of the GR can amplify AngII-mediated actions by increasing the expression of AT1 receptors and IP3 formation. These effects may provide the cellular basis of how glucocorticoids enhance AngII-mediated physiological and behavioral actions.

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