Angiotensin II Stimulates Secretion of Endogenous Ouabain From Bovine Adrenocortical Cells via Angiotensin Type 2 Receptors

James Laredo, Jui R Shah, Zhuo-ren Lu, Bruce P. Hamilton, John M Hamlyn

Abstract Angiotensin II stimulates secretion of corticosteroids and ouabain-like activity from adrenocortical cells. Distinct adrenocortical angiotensin II receptor subtypes (AT1, AT2) have been described, and the present studies investigated their roles in steroid secretion. Using primary bovine adrenocortical cell cultures under serum-free conditions, angiotensin II stimulated the secretions of aldosterone, cortisol, and endogenous ouabain as verified by high-performance chromatography. The dose-response curves for stimulated steroid secretion were parallel with unitary slopes while the half-maximally effective concentrations of angiotensin II were 0.31 to 0.38 mmol/L for secretions of aldosterone and cortisol and 2.3 mmol/L for endogenous ouabain. The nonselective mammalian antagonist (Sar'-Ile') angiotensin II blocked stimulated secretion of all three steroids without affecting basal output. In the presence of the AT1 antagonist DuP753, angiotensin II-stimulated secretions of aldosterone and cortisol were blocked while secretion of endogenous ouabain was unaffected. In the presence of the AT2 antagonist PD123319, both basal and angiotensin II-stimulated secretions of aldosterone and cortisol were normal while stimulated secretion of endogenous ouabain was inhibited. The secretion of endogenous ouabain was activated maximally by the AT2 agonist CGP42112 under conditions in which aldosterone secretion was unaffected. These results demonstrate that AT2 receptors stimulate secretion of endogenous ouabain from bovine adrenocortical cells. The specificity of AT1 and AT2 receptor stimulation indicates that separate signaling mechanisms having minimal cross-talk control the adrenocortical secretions of corticosteroids and cardiac-active steroids. Adrenocortical AT2 receptors may be important in the adaption to low-salt diets and other conditions in which angiotensin II is increased.

Key Words • adrenal • cardiac glycosides • angiotensin antagonists • losartan • DuP753 • PD 123319 • CGP42112

The effects of angiotensin II (AII), the octapeptide of the renin-angiotensin system, are mediated primarily by angiotensin receptors present on the extracellular surface of target cells. The physiological actions of AII include the stimulation of aldosterone secretion from adrenocortical cells. At least two different AII receptor subtypes, termed type 1 (AT1) and type 2 (AT2), have been identified with the use of receptor antagonists. Both receptor subtypes have been cloned, and the AT subtype accounts for 80% of the angiotensin II receptors in the rat and bovine adrenal, the other 20% being the AT2 subtype.

Studies with AT1- and AT2-specific antagonists have shown that most of the known physiological effects of AII, including stimulation of aldosterone secretion in adrenocortical cells, are mediated by the AT1 receptor. The physiological role of the AT2 receptor in the adrenal gland is not known. AT2 receptors have been shown to modulate T-type calcium channels in NG108-15 neuroblastoma/ghoma cells and synthesis of prostaglandins in human astrocytes. In the rat zona glomerulosa and PC12W cells, stimulation of AT2 receptors appears to reduce guanylyl cyclase activity, and in vascular smooth muscle cells, growth in high serum media appears to reduce the ability of AT2 receptors to bind AII.

A steroidal compound having remarkable similarity to ouabain has been described in mammalian plasma, urine, and tissues including the adrenal gland. Recent studies show that the compound in the human circulation and bovine hypothalamus is an isomer of ouabain, which may be synthesized by the adrenal gland and is a putative "endogenous ouabain" (EO). We have described the secretion of EO from bovine adrenocortical cells in primary culture and shown stimulatory effects of AII. To further examine the action of AII, we studied the effects of several angiotensin receptor antagonists on the secretion of EO, aldosterone, and cortisol. The results show that AII dose-dependently stimulates secretion of EO from bovine adrenocortical cells mediated specifically by AT2 receptors.

Methods

Adrenocortical Cell Culture

Bovine adrenal glands were obtained from a local slaughterhouse and transported to the laboratory in PBS on ice. Processing of the glands to produce either mixed adrenocortical cells or zona glomerulosa–enriched cells was performed by methods described elsewhere in F12 media containing 15 mg/mL collagenase and 25 μg/mL DNAse (Worthington Enzymes). Tissue fragments were digested...
for four periods of 30 minutes on a shaker platform at 37°C. Digestion was terminated by adding the digestion mixture to ice-cold horse serum followed by centrifugation at 1000g for 10 minutes. The cell pellets were washed twice with ice-cold DMEM containing 1% BSA. The cell preparations were purified on discontinuous Percoll gradients, harvested, and resuspended in DMEM containing 10% horse serum, 2% fetal bovine serum, and penicillin 100 U/mL, streptomycin 0.1 mg/mL, and amphotericin B 0.25 μg/mL. In some experiments, the serum concentration was altered systematically as indicated. Aliquots of the cell suspension were plated out on 6-well plates (2 ml/well) to contain approximately 4 million cells per well, and the cells were grown at 37°C in 5% CO₂. Typical yields were 200 to 350 million cells from five adrenal glands. At plating, viability was >93% by trypan blue exclusion. After 24 hours, the cell culture media was discarded and replaced with 2 mL/well of fresh DMEM.

Steroid Secretion Experiments

Secretion experiments were performed on cells 42 to 48 hours after plating. At this time, the culture medium was removed, and the cells were washed twice with a buffered SM containing (in mM) NaCl 154, KCl 5, CaCl₂ 1, MgCl₂ 5, IEPES 18, and glucose 5.6; pH 7.4. The SM alone (6 mL) or containing various pharmacological agents was added to each well, and the cells were incubated at 37°C in 5% CO₂ for 2 to 4 hours as indicated. After incubation, the media were harvested and centrifuged to remove free cells. The supernatants were divided, and one portion was used for cortisol and aldosterone assays without extraction while the remainder was acidified with an equal volume of water containing 0.1% redistilled trifluoroacetic acid, passed through 0.2-μm nylon filters, and pumped onto a Beckman model 342 chromatograph with a semipreparative scale (10×250 mm). Bound materials were eluted with a gradient program of acetone as described. Fractions of 3 mL were collected, dried by vacuum centrifugation, and assayed as described above.

High-Performance Chromatography

In some cell-incubation experiments, the secretion media were harvested, acidified with an equal volume of water containing 0.1% redistilled trifluoroacetic acid, passed through 0.2-μm nylon filters, and pumped onto a Beckman model 342 chromatograph with a semipreparative scale (10×250 mm). C18 columns at a flow rate of 3 mL/min. Bound materials were eluted with a gradient program of acetone as described. Fractions of 3 mL were collected, dried by vacuum centrifugation, and assayed as described above.

Regression and Statistical Analyses

Dose-response curves for all were fitted to the indicated equation by iterative nonlinear regression using a personal computer. Evaluations of significance were performed using ANOVA and Fisher's specific test. Results are expressed as mean±SD or SEM as indicated, and values of P<0.05 were considered significant.

Materials

Losartan (DuP753) was a gift of Dr Ronald Smith, DuPont Merck, Wilmington, Del. PD was obtained as the DTFA form as a gift of Dr David Taylor, Warner-Lambert, Ann Arbor, Mich. It was repurified by HPLC to remove DTFA before use. CGP was obtained from Dr Marc de Gasparo, Geigy, Basel, Switzerland. Collagenase type I and DNAse type I were obtained from Worthington Biochemicals. Percoll was obtained from Pharmacia. Bethune bovine serum, lot No. 35N3313, and DMEM were obtained from Gibco-BRL. AII, [Sar₁, Ile₆]-AII, and all other reagents were obtained from Sigma Chemical Co.

Results

Before investigating the effects of AII receptor antagonists, we investigated the effect of the serum content of the DMEM on the basal and AII-stimulated secretion of aldosterone and EO from zona glomerulosa cells. Fig 1 shows that growth of the cells in DMEM containing serum ranging from 0.3% to 12% had no significant effect on the basal secretion of aldosterone from washed cells incubated under serum-free conditions. However, the cells grown in DMEM containing 0.3% and 12% serum showed a threelfold and fivefold increase in aldosterone secretion in response to AII, respectively. In contrast, the amount of EO secreted under both basal and AII conditions was highest in cells grown in DMEM containing 0.3% serum and lowest in cells grown in DMEM containing 12% serum. However, the ratio of the AII-stimulated to basal secretions was greatest (~threefold) in the cells grown in 12% serum, and we chose that growth condition for the subsequent experiments. The EO content of the native sera used for growing the cells in these experiments was 0.58 nmol/l.

Fig 2 shows the effect of AII (10 and 100 nmol/L) on the secretion of EO, aldosterone, and cortisol from mixed adrenocortical cells. In each case, the effects of AII were dose dependent and relative to basal output, 10 nmol/L AII stimulated the secretion of aldosterone, cortisol, and EO by 2.4-, 4.2-, and 2.7-fold, respectively. Follow-up experiments shown in Fig 3 examined the dose-response relationships for the AII-stimulated component in more de-
FIG 1 Effect of serum concentration in the growth medium on basal and All-stimulated steroid secretion into a serum-free medium. Zona glomerulosa cells were grown for 48 hours in media containing the indicated serum levels. They were washed, incubated for 4 hours in serum-free medium in the presence or absence of 10 nmol/L All, and assayed as described under "Methods." In each case, the amount secreted in the presence of All was greater than basal (P < 0.05).

tail. In each case, the dose-response curves were parallel, sigmoidal in nature, showed Hill coefficients between 0.9 and unity, and exhibited maximal responses between $10^{-10}$ and $10^{-8}$ mol/L. All. The concentrations of All that half-maximally stimulated aldosterone and cortisol secretions, derived from iterative nonlinear regression analyses, were 0.383 and 0.314 nmol/L, respectively. For EO secretion, the EC50 for All was 2.28 nmol/L, and maximal secretion occurred at concentrations >10 nmol/L. The concentrations of All required to stimulate aldosterone and EO secretion 10% above baseline were ~42 and ~250 pmol/L, respectively. In subsequent experiments using All receptor antagonists, a concentration of All (10 nmol/L) was selected. This dose increased the secretion of all three steroids to values >85% of maximal while permitting reasonable concentrations of All receptor ligands to be used.

The results of HPLC of the serum-free secretion medium following a 4-hour incubation with mixed cells in the presence and absence of 10 nmol/L All are shown in Fig 4. Immunoassay of the fractions with the appropriate techniques showed three primary peaks of immunoreactivity at fractions 20, 47, and 49 corresponding to EO, aldosterone, and cortisol, respectively. In each case, the peaks were baseline resolved and the retention times of the secreted materials were indistinguishable from tracer amounts of pure standards run under the same conditions immediately following the sample runs. In response to All, the amount of immunoreactivity in each peak was increased >5-fold. In addition to the major peak of EO secretion.
in fraction 20, a small peak of immunoreactivity was found in fraction 18 that, based on retention characteristics and immunoreactivity, appears to be the aglycon of EO (AGEO).

To determine whether the stimulatory effect of AII on EO secretion was mediated by a direct action of AII binding to angiotensin receptors, the effect of a well-characterized peptide angiotensin receptor antagonist, SI-AII, was evaluated. SI-AII inhibits AII binding to both the AT1 and AT2 receptor subtypes and blocks AII-stimulated aldosterone secretion in mammals.6,26,27 As shown in Fig 5, SI-AII (0.5 μmol/L) had no effect on the basal secretions of aldosterone, cortisol, and EO (P > 0.05 versus control for all three steroids) from mixed cells. In the presence of 10 nmol/L AII, the secretions of each of the three adrenal steroids measured were significantly increased (P < 0.001 versus control for all) while SI-AII completely blocked the stimulation by AII. These results show that the AII-stimulated secretion of each steroid measured is blocked by a nonselective antagonist of mammalian angiotensin receptors.

Experiments with specific AT1 and AT2 receptor antagonists were performed to determine whether a specific angiotensin receptor subtype mediates the stimulatory effect of AII on EO secretion. DuP753 (losartan), a nonpeptide AT1 receptor antagonist, has been shown to selectively inhibit AII binding to AT1 receptors and block the stimulated secretion of aldosterone and cortisol.6,26,27 As shown in Fig 6, DuP753 (20 μmol/L) had no effect on the basal secretions of any of the steroids measured (P > 0.05 versus control for all) from mixed cells. As expected, DuP753 blocked the AII-stimulated secretions of aldosterone and cortisol (DUP+AII versus control, P > 0.05 for both steroids), whereas this antagonist had no effect on stimulated secretion of EO (DUP+AII versus control, P < 0.001). These results suggested that AII-stimulated secretion of EO was not mediated by AT1 receptors. As shown in Fig 7, the AT2 antagonist PD (2 μmol/L) had no effect on the basal secretions of the three steroids measured (PD versus control, P > 0.05 for all) from mixed cells. However, PD blocked the AII-stimulated increase in EO secretion (PD+AII versus control, P < 0.05) without affecting the stimulated secretions of aldosterone and cortisol (PD+AII versus control, P < 0.001 for both). Further evidence for the involvement of AT2 receptors is shown in Fig 8. In this experiment using zona glomerulosa cells, neither PD (2 μmol/L) nor the AT2 agonist CGP (1

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**Fig 5** Effect of SI-AII on AII stimulation of aldosterone, cortisol, and EO secretions from mixed adrenocortical cells. Cells were incubated for 2 hours with secretion media alone (C) and in the presence of 0.5 μmol/L SI-AII (SI), 10 nmol/L AII (AII), or both ligands (SI+AII). Data are mean ± SEM, n=4 separate cultures, *P < 0.001 vs control.

**Fig 6** Effect of DuP753 on secretions of aldosterone, cortisol, and EO secretion from mixed adrenocortical cells. Cells were incubated for 2 hours with secretion media alone (C) or in media containing 20 μmol/L DuP753 (DUP), 10 nmol/L AII (AII), or both (DUP+AII). Data are mean ± SEM, n=4 separate cultures, *P < 0.001 vs control.

**Fig 7** Effect of PD on AII stimulation of aldosterone, cortisol, and EO secretions from mixed adrenocortical cells. Cells were incubated for 2 hours with secretion media alone (C) and in medium containing either 2 μmol/L PD, 10 nmol/L AII (AII), or medium containing both ligands (PD+AII). Data are mean ± SEM, n=4 separate cultures, *P < 0.001 vs control.
The suppressive effects of culture in a high-serum medium on AI-stimulated EO secretion parallel the anticipated consequences of reduced binding of AI by AT\textsubscript{2} receptors in vascular smooth muscle cells described by others.\textsuperscript{12} However, the low basal secretion of EO from cells cultured in high-serum media suggests that other mechanisms independent of AT\textsubscript{2} receptors are affected under these conditions. Kinetic studies have shown that the secretion of EO by adrenocortical cells slows as the concentration of EO rises in the external medium.\textsuperscript{21} This phenomenon was attributed to local negative feedback inhibition by EO or a cosecreted factor and provided an early indication that the control mechanisms for EO secretion differed from those involved in aldosterone and cortisol secretions. The concentration of EO in the mixed sera we employed for cell culture in these experiments was 0.38 nmol/L. Thus, in the growth media containing 0.3% or 12% serum, the nominal concentrations of EO were 0.00174 and 0.0696 nmol/L, respectively, a difference of 40-fold. The latter concentrations of EO are in the lower range where some degree of direct feedback inhibition would be expected based on a previous study.\textsuperscript{21} However, direct feedback inhibition is an unlikely explanation for the low basal secretion of EO from cells grown in high-serum medium since all secretion experiments used washed cells incubated in the absence of serum. In these experiments, direct feedback inhibition by serum-derived EO is an unlikely explanation for the low basal secretion. Moreover, the observations that growth in high-serum media suppresses basal EO but not aldosterone secretion and that the former effect is seen in washed cells indicate that a fundamental and persistent change has occurred specifically in the secretory/biosynthetic pathway for EO. The nature of this effect is unknown but presumably involves growth factors.

When ouabain is present in high concentrations in the extracellular medium of certain adrenocortical clonal cell lines, it is accumulated in an endosomal compartment and subsequently secreted with a half-time of 40 to 50 hours. The uptake and secretion of ouabain from the external phase has led some to question the origin of the secreted EO.\textsuperscript{31} The present results in Fig 1 obtained with primary zona glomerulosa cell cultures show that the secretion of EO and by inference the content of this steroid in the cells are affected minimally by prior growth in media where the external EO concentrations vary 40-fold in the subnanomolar range. This result and previous studies in rats showing that the adrenal content of EO is independent of changes in circulating ouabain within the physiological range\textsuperscript{32} suggest again that the secreted EO is not the result of prior sequestration from the serum. This point is emphasized by the following calculations. In Fig 1, each well of cells grown in medium containing 0.3% serum was exposed over the 48 hours in culture to a total of \(\approx 6\) fmol of EO. When washed and incubated, the cells in each of those wells secreted on average \(\approx 330\) fmol of EO into the
secretion medium in 4 hours. Therefore, the endosomal pathway involved in the sequestration and secretion of extracellular ouabain is neither quantitatively nor kinetically viable as a means to explain the secreted EO measured under our conditions.

In response to AII, the secretions of EO, aldosterone, and cortisol were consistently elevated. The stimulatory effect of AII was blocked by SI-AII (Fig 5), compatible with the hypothesis that AII receptors mediate steroid secretion. Whereas DuP753 had no effect on AII-stimulated secretion of EO, the corresponding secretions of aldosterone and cortisol were blocked (Fig 6). On the other hand, the stimulatory effect of AII on EO secretion was completely blocked by the AT2 receptor antagonist PD at concentrations known to inhibit the binding of AII to the AT2 receptor without affecting the AT1 receptor.6,26-28 The selectivity of this antagonism was demonstrated by the lack of effect of this compound on stimulated aldosterone and cortisol secretions (Fig 7). In addition, the AT2 agonist CGP stimulated the secretion of EO but had no effect on basal or AI1-stimulated aldosterone secretion. Other studies have shown that the AT2 receptor ligands PD and CGP selectively inhibit AII binding to AT2 receptors at concentrations that do not affect AII-stimulated secretions of aldosterone and cortisol.6,26-28 Therefore, when taken together, the present results demonstrate that AI1 stimulates secretion of EO by activating the AT2 receptor subtype. AT1 and AT2 receptors have distinct signaling pathways, the latter subtype stimulating an orthovanadate-sensitive tyrosine kinase activity.5 The role of phosphorylation in EO secretion remains to be demonstrated. However, preliminary experiments suggest that orthovanadate blocks AI1-stimulated secretion of EO from zona glomerulosa-enriched primary cell cultures, a result that is consistent with the participation of phosphatase activity (J R.S. and J.M H., 1996, unpublished data).

The observation that adrenocortical cells have two classes of functional AII-dependent affinities (Fig 3) also supports the notion that different receptors for AII are involved. Based on the pharmacological dissection presented, it appears that the stimulated secretions of aldosterone and cortisol in response to AII exhibit an apparent EC50 value of ~0.3 nmol/L and are mediated exclusively by AT1 receptors. These results are consistent with the results from earlier studies.26-32 However, the present results showed that the effective dose of AII required (~2.3 nmol/L) for half-maximal secretion of EO was eightfold higher than that needed for aldosterone and cortisol secretions.

Studies that evaluated the binding of labeled AII to bovine, human, and rat adrenocortical membranes have suggested a single class of high-affinity sites with dissociation constants ranging broadly from 0.2 to 4.0 nmol/L.28,32-34 It seems likely that the Scatchard analyses used in some studies may not reliably discriminate between classes of receptors whose affinities differ by less than one order of magnitude, especially where the lower affinity component represents >20% of the sites. Others have reported biphasic AII binding to angiotensin receptors from adrenal cortex and other tissues.1,5,35 However, three lines of evidence suggest that the low-affinity sites identified from these binding studies may not reflect AT2 receptors. First, the difference in affinity between the high- and low-affinity binding sites previously described tends to be large, typically 15 to 2 log units. Second, the dissociation constants for the low-affinity site typically appear to lie outside of the reported range for effects of AII (eg, rat heart Kd ~50 nmol/L). Third, the low-affinity sites are not always present in cellular membranes from different tissues where AT2 receptors are now known to be present.39 Thus, the low-affinity sites for AII may represent desensitized AT1 receptors or membrane-associated peptides.6,30

The EC50 for stimulated secretion of EO (~2.3 nmol/L) is ~35-fold and >10-fold higher than the level in mixed venous plasma from cattle observed under sodium-replete (~0.65 pmol/L) or acutely depleted (~230 pmol/L) conditions, respectively.31 The calculated concentrations of AII required to stimulate aldosterone and EO secretions by 10% were ~42 and ~250 pmol/L. Therefore, according to this analysis, the normal plasma levels of AII in cattle would be sufficient to stimulate aldosterone secretion by 10% to 12% and, during acute sodium depletion, the plasma levels of AII would be sufficient to provide a similar stimulus to EO secretion. Furthermore, high plasma levels of AII may also be an important stimulus to EO secretion in patients with renin-secreting tumors and in congestive heart failure.38

Renin granules have been described in the zona glomerulosa, and the results of several studies imply that the intra-adrenal concentrations of AII are higher than arterial blood levels by a significant margin.39-41 While our results do not exclude AII as a tonic stimulus to EO secretion in the sodium-replete state, they suggest that the physiological role of adrenocortical AT2 receptors may be increasingly important during prolonged adaptation to low-sodium diets, at least in free-ranging cattle. Recent studies in other tissues and species seem to reinforce the notion that AT2 receptors are functionally significant under low-sodium conditions.42 In addition, AII stimulates aldosterone secretion and binds with greater affinity than AII to AT2 receptors in several species, and other components of the renin-angiotensin system not explored here may be relevant to the control of EO secretion.32,43

The bulk of human evolution is believed to have occurred under the stress of a sodium-poor environment.44 Under such conditions, elevated circulating levels of EO, like ouabain, may amplify the pressor effects of aldosterone,45 and we have suggested that this effect may have played a critical role in the maintenance of heightened vascular tone and normal blood pressure critical to survival.46 Under sodium-replete conditions, amplification of the effects of aldosterone by EO may be of pathological significance in view of the large portion of patients with essential hypertension and Conn's syndrome in whom markedly elevated circulating levels of one or both steroids have been described.47

In summary, AII stimulates secretion of endogenous ouabain from adrenocortical cell cultures. These effects appear to be mediated specifically via functional AT2 receptors and provide additional evidence that novel cellular mechanisms within the adrenal gland mediate the synthesis and secretion of polyhydroxylated steroids similar to ouabain.

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