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ANGIOTENSIN-II-DIRECTED GLOMERULOSA CELL FUNCTION IN FETAL ADRENAL CELLS

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Summary—These studies were undertaken to examine the role of angiotensin II (A-II) in the regulation of adrenal glomerulosa cell differentiation. We were interested particularly in the ability of A-II to support aldosterone production in fetal adrenal cells. Many *in vitro* studies on acute A-II stimulation of aldosterone synthesis in adrenocortical cells have been documented. However, it is the long-term modification of steroid-metabolizing enzyme expression that leads to the formation and release of specific adrenal steroids. Herein, we used primary cultures of fetal bovine adrenal (FBA) cells to examine the effects of A-II on aldosterone production and the expression of aldosterone synthase cytochrome *P*450 (*P*450c18). A-II treatment caused the primary cultures to maintain glomerulosa cell functions. Cells treated for 3 days with A-II increased aldosterone production by 10-fold. A-II stimulation of aldosterone production occurred rapidly (within 30 min) and in a dose-dependent manner. In addition, A-II enhanced the activity of *P*450c18, the enzyme responsible for conversion of corticosterone to aldosterone. A-II also suppressed ACTH-promoted cortisol production, while increasing ACTH-stimulated release of aldosterone. It appears that these effects of chronic treatment with A-II were mediated through an A-II type 1 (AT_1) receptor since the AT_1 receptor antagonist, Dup753, blocked aldosterone production and the increased *P*450c18 activity. Receptor binding studies suggest that FBA cells possess approx. 110,000 AT_1 binding sites/cell with $K_d = 1.8 \times 10^{-9}$ M. Via AT_1 receptors, A-II was able to stimulate both inositol phosphates and cAMP production. The stimulation of cAMP production, however, was much less than seen following ACTH treatment. These data give support to the hypothesis that A-II is involved in the differentiation of fetal adrenal cells into glomerulosa cells. This process appears to be mediated through regulation of steroid-metabolizing enzyme expression and the activation of steroid production.

INTRODUCTION

The pathways for steroidogenesis within adrenocortical cells are well established. The biogenesis of adrenocortical hormones from cholesterol is catalyzed by five forms of cytochrome *P*450 and the enzyme, 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) (reviewed in Ref. [1]). Regulation of the synthesis of the cytochrome *P*450 enzymes as well as 3β-HSD appears to occur in both cAMP-dependent and -independent fashions [2–5]. It is the relative levels of expression of these enzymes within the adrenal cortex which leads to the synthesis of zone-specific steroids, e.g. aldosterone or cortisol. The mechanisms which lead to the production of the zone-specific steroids are still unclear. Information continues to accumulate, however, which suggest that differential regulation of the steroid-metabolizing enzymes leads to formation of zone-specific steroids [6–9].

Several steroid-metabolizing enzymes have been shown to exhibit zonal differences. Two pivotal enzymes in the metabolic disposition of steroids are 17α-hydroxylase cytochrome *P*450 (*P*450c17) and 3β-HSD which act on a common substrate, pregnenolone, forming 17α-hydroxypregnenolone and progesterone, respectively. Thus, the separate regulation of 3β-HSD and *P*450c17 levels could determine the relative rates of formation of 17α-hydroxypregnenolone and progesterone and so influence the nature of the subsequent steroids secreted by these cells. We have been interested particularly in the ability of angiotensin II (A-II) to inhibit the expression of *P*450c17 following ACTH treatment [8–10]. The observation that 3β-HSD expression could be maintained suggested that A-II could block the formation of 17α-hydroxylated steroids but maintain the formation

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of 17-deoxysteroids. Such a situation is seen *in vivo* in the zona glomerulosa where *P*450c17 expression is very low. Our *in vitro* data using bovine and ovine adrenal cells are suggestive that A-II plays an important role in suppressing expression of *P*450c17 [8–10]. Suppressing *P*45017 expression is not sufficient, however, for the production of mineralocorticoids. To produce the key glomerulosa-specific steroid hormone, aldosterone, adrenal cells must also express aldosterone synthase cytochrome *P*450 (*P*450c18). In the mouse, rat and human adrenal this enzyme activity is accomplished through the product of a distinct gene, namely CYP11B2 [11–13]. Currently, however, it is not known if there are multiple CYP11B genes in the bovine adrenal.

Our previous studies on 3β-HSD and *P*450c17 expression were accomplished using adrenal fasciculata cells which do not produce aldosterone. Thus, the effects of A-II on *P*450c18 synthesis could not be determined. Indeed, there are few *in vitro* studies on the regulation of *P*450c18 expression. Several problems have slowed progress in the examination of this enzyme using *in vitro* model systems. Primary cultures of adult rat glomerulosa cells lose the ability to respond to A-II 14, whereas adult bovine glomerulosa cells exhibit rapidly decreased ability to produce aldosterone after the cells are placed in culture [15, 16]. However, *in vivo* studies using rats and mice demonstrate that *P*450c18 is localized to the zona glomerulosa of the adrenal and can be regulated by dietary salt and A-II treatment [17–19].

The current study was undertaken to develop an adrenal cell culture system which could act as a model to study the development of the glomerulosa cell function. To accomplish this goal we have used fetal bovine adrenal (FBA) cells from mid-gestational fetuses. The rational for choosing FBA cells was the relatively undifferentiated state of these cells. Our hypothesis was that FBA cells could be manipulated in culture to function as either glomerulosa or fasciculata cells depending on the treatment. Our studies demonstrate that FBA cells respond to A-II treatment with an increase in phosphoinositide hydrolysis, cAMP production and aldosterone secretion. In addition, A-II acts to increase the activity of *P*450c18. This is the first *in vitro* study that demonstrates that A-II increases *P*450c18 activity.

EXPERIMENTAL

Cell isolation and culture

Fetal bovine adrenals were obtained from fetuses of pregnant cows at a local abattoir. FBA cells were prepared by successive treatments with trypsin (0.25%) in Dulbecco's modified Eagle's (DME) and Ham's nutrient mixture F12 (Sigma, St Louis, MO) containing antibiotics as described previously. Cells were placed in monolayer culture for 24 h in the same medium containing 2% fetal bovine serum, insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenous acid (6.25 ng/ml), bovine serum albumin (1.25 mg/ml) and linoleic acid (5.35 μg/ml) added in the form of 1% ITS-plus Premix (Collaborative Research, Bedford, MA). Cells were changed on the second day of culture to this defined medium without the serum. Experiments were started as indicated in each figure legend. Media containing the various treatments were changed every 24 h until completion of the experiment. ACTH (Cortrosyn) was obtained from Organon (West Orange, NJ) and A-II (human) from Sigma (St Louis, MO). Dup753 and PD123319 were generously provided by DuPont (Wilmington, DE) and Park Davis (Ann Arbor, MI), respectively.

Protein assay

To recover cellular protein from culture dishes for subsequent protein determination, cells were solubilized in Tris–HCl (50 mM, pH 7.4), containing NaCl (150 mM), SDS (1%), EGTA (5 mM), $MgCl_2$ (0.5 mM), $MnCl_2$ (0.5 mM) and phenylmethylsulfonyl-fluoride (PMSF, 0.2 mM), and stored frozen at $-70°C$. The protein content of samples was then determined by bicinchoninic acid protein assay, using the BCA assay kit (Pierce, Rockford, IL).

Cortisol and cAMP assay

Medium cortisol contents were determined in duplicate against cortisol standards made up in defined medium using a coated tube [^{125}I]labelled cortisol immunoassay (ICN Biomedicals, Costa Mesa, CA). Aldosterone was determined using an assay kit from Diagnostic Products Corp. (Los Angeles, CA). Results were expressed as nmol/mg protein. The medium content of cAMP was determined using a specific radioimmunoassay from Advanced Magnetics (Cambridge, MA). The acetylated procedure, for increased sensitivity, was used in

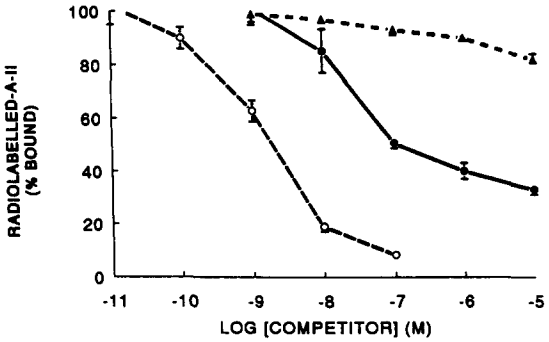

Fig. 1. Competition for the binding of [^{125}I]-labelled A-II by A-II and specific A-II receptor antagonists in FBA cells in primary culture. Data points are the mean of triplicate incubations in a representative experiment: O---O, A-II; ●—●, Dup753; ▲---▲, PD123319. In the absence of competitor (100%) the bound A-II represented 26,400 cpm/4 cm^2 culture well. Non-specific bound radiolabel was always <5%.

each assay. Results were expressed as pmol/mg protein.

[^{125}I]A-II binding displacement assay

The method of Viard *et al.* [20] was followed on cells cultured in 12 multi-well plates. [^{125}I]A-II (2000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL) and 0.05 μCi added to each well. Binding was carried out in 0.5 ml medium [DME/F12/BSA (0.5%)/Bacitracin (0.1%)/HEPES (15 mM), pH 7.4] for 1 h at 37°C. At the end of this time, wells were washed in the DME/F12 medium (4°C, 3 times) before cell lysis in 0.5 M NaOH containing deoxycholate (0.4%). Radioactivity associated with the cell lysates was then determined in a γ-counter.

Phosphoinositidase C measurement

Cells cultured in 24 well plates were incubated for 48 h in fresh culture medium supplemented with 10 μCi/ml [^{3}H]inositol (NEN DuPont, 17.9 Ci/mmol). Labelling medium was then removed, replaced with 0.5 ml DME/F12, and cells incubated for 15 min. The medium was replaced with 0.45 ml DME/F12/10 mM LiCl, and incubation continued for a further 15 min. Agonists and antagonists were then added (to the concentrations shown) to a final total volume of 0.5 ml. The treatment of cells was allowed to proceed for 30 min, after which 0.25 ml ice-cold perchloric acid (15%) was added to each well. Cells were scraped from the well using the plunger of a 1 ml disposable syringe, and the well contents transferred to a 1.5 ml microfuge tube, with a 0.5 ml water wash. Acid-insoluble material was pelleted by brief centrifugation (12,000 *g*, 3 min) and the acid supernatant neutralized, after transfer to a glass tube, by mixing thoroughly with 1.5 ml 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine (1:1 v/v). After brief centrifugation to separate the three phases formed, 0.9 ml of the neutral aqueous (upper) phase was recovered and stored at −20°C prior to analysis. To assess the labelling associated with the inositol phosphates, neutralized aqueous extracts were loaded onto 0.6 ml columns of AG1-X8 anion exchange resin, and columns washed free of unbound [^{3}H]inositol with 2 × 4 ml water. Bound [^{3}H]inositol phosphates ($InsP_1$ to $InsP_4$ inclusive) were then recovered by sequential elution with the following buffers (5 × 2 ml each): 60 mM ammonium formate/5 mM disodium tetraborate (elutes glycerophosphoinositol); 200 mM ammonium formate/0.1 M formic acid (elutes InsP); 400 mM ammonium formate/0.1 M formic acid (elutes $InsP_2$); 800 mM ammonium formate/0.1 M formic acid (elutes $InsP_3$); 1.2 M ammonium formate/0.1 M formic acid (elutes $InsP_4$). The radioactivity of the combined inositol phosphates fractions was then determined then by liquid scintillation counting.

RESULTS AND DISCUSSION

A-II receptors on FBA cells

In the first series of experiments the binding of radiolabelled A-II by FBA cells was examined. Non-radiolabelled A-II inhibited the specific binding of [^{125}I]A-II in a concentration-dependent manner with an IC_{50} of 1.1×10^{-9} M (Figs 1 and 2). The development of specific non-peptide antagonists to A-II binding has aided in the characterization of two A-II receptor subtypes (AT_1 and AT_2). Using Dup753 (an

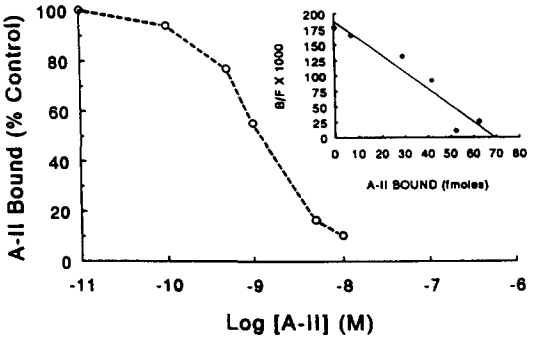

Fig. 2. Analysis of AT_1 receptor binding. Radiolabelled A-II displacement was examined in the presence of AT_2 receptor antagonist (PD123319; 10 μM). *Inset*: Scatchard analysis using the data from the displacement curve. Data points are the mean of quadruplicate wells of FBA cells.

Table 1. Effect of A-II on inositol phosphate production in FBA cells

	[^{3}H]Inositol phosphates (dpm/well)			
	$InsP_1$	$InsP_2$	$InsP_3$	Total $InsP_n$
Control	6959 ± 457	800 ± 118	292 ± 33	8051 ± 473
A-II	123,997 ± 16,721	26,870 ± 3618	6541 ± 840	157,338 ± 17,129
A-II + PD123319	108,322 ± 10,969	22,868 ± 3033	4585 ± 1192	135,775 ± 10,964
A-II + Dup753	14,621 ± 1861	1353 ± 187	502 ± 74	16,476 ± 1872

2-Day-old cultures of FBA cells were labelled for 48 h with D-myo-[^{3}H]inositol (10 μCi/ml). The medium was removed and cells incubated in radioinert medium, containing 10 mM LiCl without (control) or with A-II (10 nM) or Dup753 (10 μM) or PD123319 (10 μM) for 30 min. The results are the mean ± SD for 4 wells of cells.

antagonist to AT_1 sites) and PD123319 (an antagonist to AT_2 sites) the displacement of [^{125}I]A-II was examined using FBA cells (Fig. 1). Dup753 (0.1 to 10 μM) displaced radiolabelled A-II in a concentration-dependent manner. A maximal displacement of 75% was observed using a concentration of 10 μM Dup753, while the IC_{50} was approx. 10^{-7} M. The AT_2 antagonist (0.1–10 μM) decreased A-II binding, but only by 22% at the highest concentration. We have also examined the displacement of radiolabelled A-II in primary cultures of fetal human, fetal ovine, adult bovine and adult ovine adrenal cells. In all cell types, >70% of radiolabelled A-II was displaced by the AT_1 antagonists (data not shown). These relative levels of receptor subtypes are similar to those published using rat adrenal cells, ovine adrenal cells and a recent study of adult bovine adrenal cells [10, 20–23].

Because of the known importance of the AT_1 receptor in the regulation of adrenal cell function, we designed experiments to examine specifically AT_1 binding sites on FBA cells. Using data from displacement curves conducted in the presence of PD123319 (10 μM), we were able to specifically examine AT_1 receptor binding (Fig. 2). From these data a Scatchard analysis demonstrated the presence of a single population of high-affinity binding sites (~110,000 sites/cell) with an apparent K_d of 1.8×10^{-9} M. The number of AT_1 binding sites and the K_d are similar to those found for A-II receptors on adult bovine and ovine fasciculata cells [10, 23].

A-II coupling to second messengers in FBA cells

Because acute effects of A-II on FBA cells have not been reported previously, we evaluated changes in phosphoinositol production and cAMP production following A-II treatment. A-II (10 nM) enhanced by 10-fold the production of total inositol phosphates after 30 min treatment (Table 1). This was associated with a significant ($P < 0.01$) accumulation of each of the three inositol phosphates measured. As expected in the presence of Li^+, the labelling increase in $InsP_1$ (10-fold) was higher than that of $InsP_2$ (3-fold) which, in turn, was higher than $InsP_3$ (2-fold). In direct comparisons the FBA cells appeared more responsive to A-II than similarly cultured adult bovine adrenal cells (data not shown). The A-II stimulation was abolished by Dup753 (10 μM) but unaffected by PD123319 (10 μM), suggesting a role for AT_1 receptors in the activation of phosphoinositidase C activation. The involvement of AT_1 receptors in phosphoinositidase C activation is

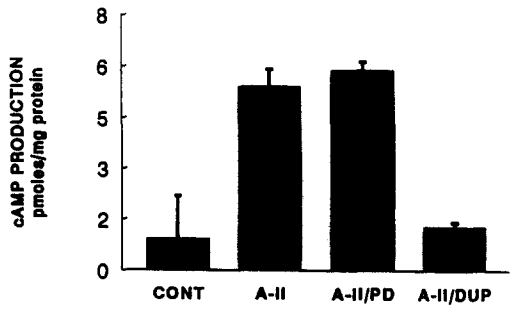

Fig. 3. Effects of A-II and A-II antagonists on cAMP production by FBA cells in primary culture. FBA cells on day 4 of culture were rinsed and treated as indicated for 3 h. Data points are the mean of quadruplicate incubations in a representative experiment. Concentrations were for A-II (10 nM), Dup753 (10 μM) and PD123319 (10 μM).

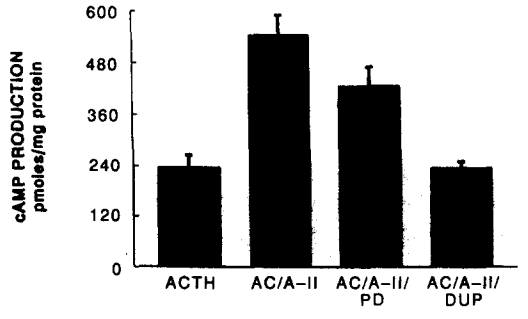

Fig. 4. Effects of A-II and A-II antagonists on cAMP production by FBA cells stimulated by ACTH. FBA cells on day 4 of culture were rinsed and treated as indicated for 3 h. Data points are the mean of quadruplicate incubations in a representative experiment. Concentrations were for A-II (10 nM), Dup753 (10 μM), PD123319 (10 μM) and ACTH (10 nM).

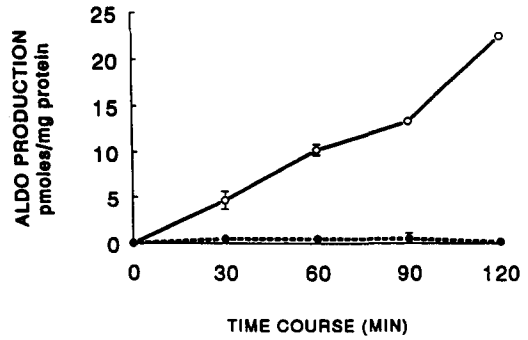

Fig. 5. Acute effects of A-II on FBA cell production of aldosterone. FBA cells on day 4 of culture were rinsed and treated for the indicated time with A-II (100 nM). Data points are the mean of quadruplicate incubations in a representative experiment.

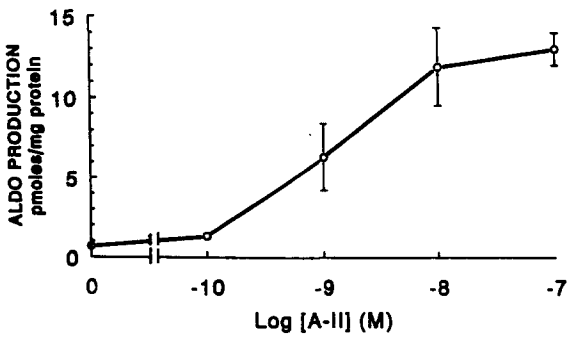

Fig. 7. Concentration dependence for A-II stimulation of aldosterone production in FBA cells. FBA cells on day 4 of culture were rinsed and treated with A-II for 24 h. Data points are the mean of quadruplicate incubation in a representative experiment.

similar to that reported for dispersed adult rat glomerulosa cells [22, 23], adult ovine fasciculata cells in culture [10] and adult bovine adrenal cells [23].

Several reports using a variety of tissue types have demonstrated that A-II can stimulate cAMP production [24–29] Therefore we examined the effects of A-II on FBA cell production of cAMP (Fig. 3). A-II (10 nM) stimulated a 3- to 5-fold increase in the accumulation of cAMP in the incubation medium. This stimulation was blocked by Dup753 (10 μM) but not PD123319 (10 μM). Thus, AT_1 receptor activation appears to increase cAMP production in FBA cells. The stimulation by A-II was much less than that observed by ACTH (10 nM) as can be seen by the difference in the ordinate parameters of Figs 3 and 4. Interestingly, A-II was also able to double the amount of cAMP produced in response to ACTH (Fig. 4). The ability of A-II to enhance ACTH stimulation of cAMP production was also mediated by AT_1 receptors, as demonstrated by the Dup753 inhibition of A-II action.

The ability of A-II to stimulate cAMP production in adrenocortical cells is controversial. Indeed, there are numerous reports where A-II was not observed to elevate cAMP production [30–39]. We also see variations from one cell type to another. Whereas FBA and adult bovine adrenal cells respond to A-II with an increase in cAMP release [29], fetal and adult ovine adrenal cells in primary culture do not (unpublished observation). The reported effects of A-II on ACTH-stimulated cAMP production also vary. In rat glomerulosa cells A-II inhibits cAMP production in response to ACTH [23, 38]. In both ovine and bovine adrenal fasciculata cells, however, A-II enhances ACTH-stimulated cAMP production apparently through a protein-kinase-C-dependent mechanism [20, 35, 39]. The reasons for the differences between species are currently not known, but may relate in part to the presence or absence of G_i coupling to the A-II receptor [35].

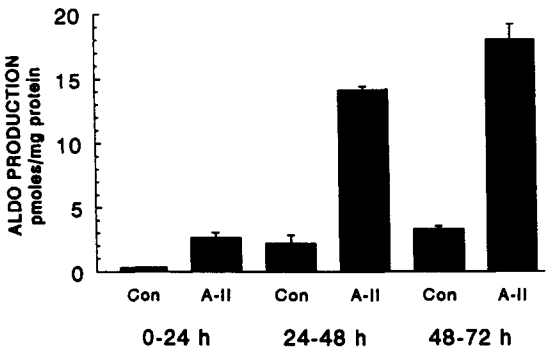

Fig. 6. Aldosterone production by FBA cells over a 3-day period of A-II treatment. FBA cells on day 4 of culture were rinsed and treated with A-II (100 nM) for 3 days. Medium and A-II were renewed every 24 h. Data points are the mean of quadruplicate incubations in a representative experiment.

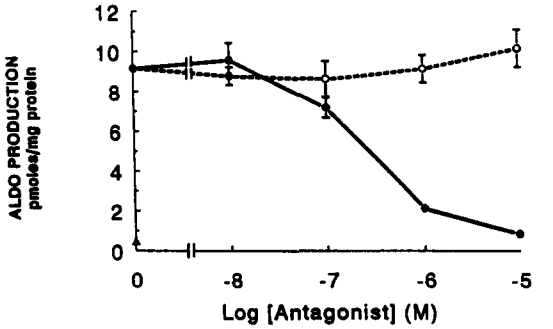

Fig. 8. Effects of A-II and A-II antagonists on the production of aldosterone by FBA cells. FBA cells on day 4 of culture were rinsed and treated with A-II (10 nM) and the antagonists as indicated for 24 h. Data points are the mean of quadruplicate incubations in a representative experiment: O---O, PD123319; ●—●, Dup753.

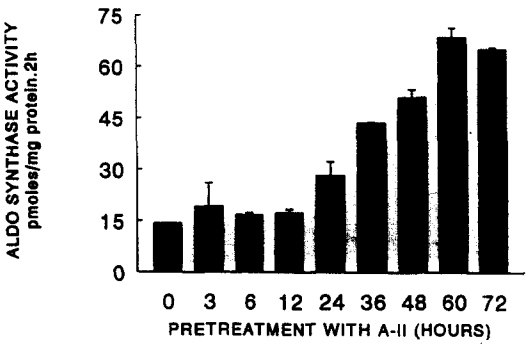

Fig. 9. Time-dependent effects of A-II on the activity of *P*450aldo. FBA cells on day 4 of culture were treated with A-II (100 nM) for the times indicated. Data points are the mean ± SD of quadruplicate incubations in a representative experiment.

A-II regulation of aldosterone production by FBA cells

Acute stimulation of FBA cells with A-II caused a linear increase in aldosterone production during the 2 h period examined (Fig. 5). Aldosterone production was also maintained during 3 days of treatment with A-II (Fig. 6). The stimulatory effects of A-II were concentration dependent (Fig. 7) with significant stimulation observed using physiologic concentrations of A-II (1 nM). In order to determine which A-II receptor was responsible for aldosterone production we examined the effects of AT_1 and AT_2 receptor antagonists. The AT_1 antagonist Dup753 (0.01–10 μM) inhibited A-II (10 nM) stimulation of aldosterone production in a concentration-dependent manner (Fig. 8). In contrast, the AT_2 antagonist PD123319 (0.01–10 μM) did not effect aldosterone production (Fig. 8).

Regulation of aldosterone synthase

A key enzyme in the production of aldosterone is *P*450c18. In human, mouse and rat there appear to be separate enzymes for the conversion of 11-deoxycorticosterone to corticosterone and corticosterone to aldosterone, but this has not been established for the bovine adrenal. Therefore, we used the conversion of exogenously added corticosterone to aldosterone to estimate the activity of *P*450c18. Treatment with A-II (100 nM) increased *P*450c18 activity in a time-dependent manner (Fig. 9). *P*450c18 appeared maximal following 60 h of A-II treatment. In order to determine which A-II receptor subtype was responsible for increasing *P*450c18, we examined the effects of PD123319 and Dup753 on A-II action. Dup753 (10 μM) inhibited completely the ability of A-II (10 nM) to increase *P*450c18 (Table 2). Thus, AT_1 receptors appear to mediate A-II effects on *P*450c18 activity. These experiments demonstrate that, *in vitro*, A-II is able to increase both aldosterone secretion and *P*450c18 expression. We next examined the effects of ACTH on *P*450c18 activity. ACTH (10 nM) treatment for 3 days increased *P*450c18 by 10-fold which was similar to that seen by A-II (Table 2). Treatment with a combination of A-II and ACTH caused an additive increase in *P*450c18 which was double the effect of either hormone alone. Again, AT_1 receptors appear responsible for increasing *P*450c18, since Dup753 blocked the additive effects of A-II. While the effects of A-II on *P*450c18 have been demonstrated *in vivo*, problems with cell culture models have made the verification *in vitro* difficult. The reason for the positive findings seen using FBA cells is not clear. Similar findings are also seen, however, when we use fetal ovine cells in culture (data not shown). The success of these models may relate to the relatively undifferentiated state of the fetal ovine and bovine adrenal cells.

Interactions between ACTH and A-II

We recently demonstrated that A-II was a potent inhibitor of cortisol production in fetal ovine adrenal cells [9]. Therefore, we examined the effect of A-II on ACTH stimulation of cortisol production. ACTH caused a 20-fold increase of cortisol production in FBA cells (Fig. 10). A-II inhibited ACTH-stimulated cortisol release in a concentration-dependent manner. A-II inhibited cortisol production by 50% in this experiment, but the amount of inhibition was seen to vary from 40 to 80%

Table 2. Effects of A-II, ACTH and A-II receptor antagonists on *P*450c18 activity

Pretreatment	*P*450c18 activity (pmol/2 h/mg cell protein)
Control	1.7 ± 0.8
A-II	24.1 ± 0.7
A-II + PD123319	21.7 ± 0.1
A-II + Dup753	1.3 ± 0.3
ACTH	23.2 ± 0.9
ACTH + A-II	52.7 ± 0.3
ACTH + A-II + PD123319	45.1 ± 1.6
ACTH + A-II + Dup753	18.9 ± 0.9

FBA cells which had been in culture for 2 days were treated as indicated for 3 days. Media containing agonists and antagonists were changed daily. At the end of treatment cells were rinsed 3 times, then incubated in medium containing corticosterone (2 μM) for 2 h. The aldosterone content of the medium was quantified in the incubation medium. The concentrations of the pre-incubation factors were: A-II (10 nM); PD123319 (10 μM); Dup753 (10 μM); ACTH (10 nM). Values represent the mean ± SD for 4 separate dishes of cells.

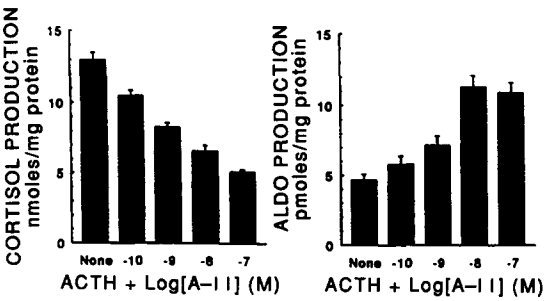

Fig. 10. Effects of A-II on ACTH-stimulated cortisol production. FBA cells on day 4 of culture were treated for 48 h with ACTH (10 nM) with the indicated concentration of A-II. The medium contents of cortisol (left) and aldosterone (right) were then determined. Data are the mean ± SD for 4 separate wells of cells.

depending on the FBA cell preparation. In the same experiment aldosterone release was examined (Fig. 10). ACTH, like A-II alone, stimulated aldosterone production. Interestingly, when A-II was included with ACTH, both aldosterone production and *P*450c18 activity were greater than that cause by either hormone alone. These data demonstrate that A-II is able to modify the profile of steroids secreted in response to ACTH towards those characteristically secreted by the zona glomerulosa cell. A paradox in these observations is the ability of A-II to enhance cAMP production in response to ACTH, but to inhibit cortisol production. The decrease in cortisol production is probably due in part to an inhibition of *P*450c17 expression, as we have shown using fetal ovine adrenal cells [9]. We hypothesize that the inhibition of cortisol production results from A-II activation of the C kinase pathway. Protein kinase C activation has previously been shown to inhibit expression of *P*450c17 [6–8].

CONCLUSION

FBA cells in primary culture respond to A-II treatment by increasing aldosterone production and *P*450c18 activity. A-II stimulation is mediated through an AT_1 receptor. Receptor activation results in increased inositol phosphate production and cAMP formation. However, cortisol production occurring in response to ACTH is partially inhibited. These data are suggestive of a central role for A-II in controlling steroidogenesis and the profile of steroids secreted by bovine adrenal cells. These *in vitro* results also suggest that A-II could be involved in maintaining adrenal glomerulosa cell differentiation and secretion of aldosterone.

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REFERENCES

1. Waterman M. R. and Simpson E. R.: Regulation of steroid hydroxylase gene expression is multifactorial in nature. *Recent Prog. Horm. Res.* **45** (1989) 533–566.
2. Kramer R. E., Rainey W. E, Funkenstein B., Dee A., Simpson E. R. and Waterman M. R.: Induction of synthesis of mitochondrial steroidogenic enzymes of bovine adrenocortical cells by analogs of cyclic AMP. *J. Biol. Chem.* **259** (1984) 707–713.
3. Zuber M. X., Simpson E. R. and Waterman M. R.: Expression of bovine 17α-hydroxylase cytochrome *P*-450 cDNA in non-steroidogenic (COS1) cells. *Science* **234** (1986) 1258–1261.
4. Naville D., Rainey W. E., Milewich L. and Mason J. I.: Regulation of 3β-hydroxysteroid dehydrogenase/$\Delta^{5\to4}$-isomerase expression by adrenocorticotropin in bovine adrenocortical cells. *Endocrinology* **128** (1991) 139–145.
5. John M. E., Simpson E. R., Carr B. R., Magness R. R., Rosenfeld C. R., Waterman M. R. and Mason J. I.: Ontogeny of steroid hydroxylases: evidence for cAMP-independent gene expression. *Molec. Cell Endocr.* **121** (1987) 263–268.
6. McAllister J. M. and Hornsby P. J.: Dual regulation of 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, and dehydroepiandrosterone sulfotransferase by adenosine 3′,5′-monophosphate and activators of protein kinase C in cultured human adrenocortical cells. *Endocrinology* **122** (1988) 2012–2018.
7. Naseeruddin S. A. and Hornsby P. J.: Regulation of 11β- and 17α-hydroxylases in cultured bovine adrenocortical cells: cyclic adenosine 3′,5′-monophosphate, insulin-like growth factor-I, and activators of protein kinase C. *Endocrinology* **127** (1990) 1673–1681.
8. Rainey W. E., Naville D. and Mason J. I.: Regulation of 3β-hydroxysteroid dehydrogenase in adrenocortical cells: effects of angiotensin-II and transforming growth factor beta. *Endocrine Res.* **17** (1991) 281–296.
9. Rainey W. E., Oka K., Magness R. R. and Mason J. I.: Ovine fetal adrenal synthesis of cortisol: regulation by adrenocorticotropin, angiotensin II and transforming growth factor-β. *Endocrinology* **129** (1991) 1784–1790.
10. Bird I. M., Mason J. I. and Rainey W. E.: Angiotensin II acts via the type I receptor to inhibit 17α-hydroxylase cytochrome *P*450 expression in ovine adrenocortical cells. *Endocrinology* **130** (1992) 3113–3121.
11. Shizuta Y., Kawamoto T., Mitsuuchi Y., Toda K., Miyahara K., Ichikawa Y., Imura H. and Ulick S.: Molecular genetic studies on the biosynthesis of aldosterone in humans. *J. Steroid Biochem. Molec. Biol.* **43** (1992) 981–987.
12. White P. C., Pascoe L., Curnow K. M., Tannin G. and Rösler A.: Molecular biology of 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase enzymes. *J. Steroid Biochem. Molec. Biol.* **43** (1992) 827–835.
13. LeHoux J.-G. and Tremblay A.: *In vivo* regulation of gene expression of enzymes controlling aldosterone synthesis in rat adrenal. *J. Steroid Biochem Molec. Biol.* **43** (1992) 837–846.
14. Hornsby P. J., O'Hare M. J. and Neville A. M.: Functional and morphological observations on rat adrenal zona glomerulosa cells in monolayer culture. *Endocrinology* **95** (1974) 1240–1251.

15. Crivello J. F. and Gill G. N.: Induction of cultured bovine adrenocortical zona glomerulosa cell 17-hydroxylase activity by ACTH. *Molec. Cell. Endocr.* **30** (1983) 97–107.
16. Crivello J. F., Hornsby P. J. and Gill G. N.: Metyrapone and antioxidants are required to maintain aldosterone synthesis by cultured bovine adrenocortical zona glomerulosa cells. *Endocrinology* **111** (1982) 469–479.
17. Tremblay A., Parker K. L. and Lehoux J.-G.: Dietary potassium supplementation and sodium restriction stimulate aldosterone synthase but not 11β-hydroxylase *P*-450 messenger ribonucleic acid accumulation in rat adrenals and require angiotensin II production. *Endocrinology* **130** (1992) 3152–3158.
18. Domalik L. J., Chaplin D. D., Kirkman M. S., Wu R. C., Liu W., Howard T. A., Seldin M. F. and Parker K. L.: Different isozymes of mouse 11β-hydroxylase produce mineralocorticoids and glucocorticoids. *Molec. Endocr.* **5** (1991) 1853–1861.
19. Aguilera G., Menard R. H. and Catt K. J.: Regulatory actions of angiotensin II on receptor and steroidogenic enzymes in adrenal glomerulosa cells. *Endocrinology* **107** (1980) 55–60.
20. Viard I., Rainey W. E., Capponi A. M., Begeot M. and Saez J. M.: Ovine adrenal fasciculata cells contain angiotensin-II receptors coupled to intracellular effectors but are resistant to the steroidogenic effects of this hormone. *Endocrinology* **127** (1990) 2071–2078.
21. Chiu A. T., McCall D. E., Aldrich P. E. and Timmermans P. B. M. W. M.: [^{3}H]DUP 753, a highly potent and specific radioligand for the angiotensin II-1 receptor subtype. *Biochem. Biophys. Res. Commun.* **172** (1990) 119–202.
22. Chiu A. T., McCall D. E., Price W. A., Wong P. C., Carini D. J., Duncia J. V., Wexler R. R., Yoo S. E., Johnson A. L. and Timmermans P. B. M. W. M.: Nonpeptide angiotensin II receptor antagonists. VII. Cellular and biochemical pharmacology of DUP 753, an orally active antihypertensive agent. *J. Pharmac. Exp. Ther.* **252** (1990) 711–718.
23. Balla T., Baukal A. J., Eng S. and Catt K. J.: Angiotensin II receptor subtypes and biological responses in the adrenal cortex and medulla. *Molec. Pharmac.* **40** (1991) 401–406.
24. Peytremann A., Nicholson W. E., Brown R. D., Liddle G. W. and Hardman J. G.: Comparative effects of angiotensin and ACTH on cyclic AMP and steroidogenesis in isolated bovine adrenal cells. *J. Clin. Invest.* **52** (1973) 835–842.
25. Bing R. F. and Schulster D.: Adenosine 3′,5′-cyclic monophosphate production and steroidogenesis by isolated rat adrenal glomerulosa cells. Effects of angiotensin II and [Sar, Ala8]angiotensin II. *Biochem. J.* **176** (1978) 39–45.
26. Boarder M. R., Plevin R. and Marriott D. B.: Angiotensin II potentiates prostaglandin stimulation of cyclic AMP levels in intact bovine adrenal medulla cells but not adenylate cyclase in permeabilized cells. *J. Biol. Chem.* **263** (1988) 15,319–15,324.
27. Welsh C., Dubyak G. and Douglas J. G.: Relationship between phospholipase C activation and prostaglandin E_2 and cyclic adenosine monophosphate production in rabbit tubular epithelial cells: effects of angiotensin, bradykinin, and arginine vasopressin. *J. Clin. Invest.* **81** (1988) 710–719.
28. Missale C., Memo M., Sigala S., Carruba M. O. and Spano PF.: Angiotensin II differentially affects cyclic AMP formation in intact adrenal glomerulosa cells and in purified membrane preparations. *Regul. Peptides* **24** (1989) 167–178.
29. Rainey W. E., Byrd E. W., Sinnokrot R. H. and Carr B. R.: Angiotensin-II activation of cAMP and corticosterone production in bovine adrenocortical cells: effects of nonpeptide angiotensin-II antagonists. *Molec. Cell. Endocr.* **81** (1991) 33–41.
30. Douglas J., Saltman S., Williams C., Bartley P., Kondo T. and Catt K.: An examination of possible mechanisms of angiotensin II-stimulated steroidogenesis. *Endocrine Res. Commun.* **5** (1978) 173–188.
31. Shima S., Kawashiima Y. and Hirai M.: Studies on cyclic nucleotides in the adrenal gland. VIII. Effects of angiotensin on adenosine 3′,5′-monophosphate and steroidogenesis in the adrenal cortex. *Endocrinology* **103** (1978) 1361–1367.
32. Fakunding J. L., Chow R. and Catt K. J.: The role of calcium in the stimulation of aldosterone production by adrenocorticotropin, angiotensin II, and potassium in isolated glomerulosa cells. *Endocrinology* **105** (1979) 327–333.
33. Fujita K., Aguilera G. and Catt K. J.: The role of cyclic AMP in aldosterone production by isolated zona glomerulosa cells. *J. Biol. Chem.* **254** (1979) 8567–8574.
34. Begeot M., Langlois D., Vilgrain I. and Saez J. M.: Angiotensin II (A-II) steroidogenic refractoriness in Y-1 cells in the presence of A-II receptors negatively coupled to adenylate cyclase. *Endocrine Res.* **13** (1987) 301–316.
35. Begeot M., Langlois D., Penhoat A. and Saez J. M.: Variations in guanine-binding proteins (Gs, Gi) in cultured bovine adrenal cells. Consequences on the effects of phorbol ester and angiotensin II on adrenocorticotropin-induced and cholera-toxin-induced cAMP production. *Eur. J. Biochem.* **174** (1988) 317–321.
36. Williams B. C., Lightly E. R., Ross A. R., Bird I. M. and Walker S. W.: Characterization of the steroidogenic responsiveness and ultrastructure of purified zona fasciculata/reticularis cells from bovine adrenal cortex before and after primary culture. *J. Endocr.* **121** (1989) 317–324.
37. Bell J. B. G., Tait J. F., Tait S. A. S., Barnes G. D. and Brown B. L.: Lack of effect of angiotensin on levels of cyclic AMP in isolated adrenal zona glomerulosa cells from the rat. *J. Endocr.* **91** (1981) 145–154.
38. Woodcock E. A. and Johnston C. I.: Inhibition of adenylate cycle in rat adrenal glomerulosa cells by angiotensin II. *Endocrinology* **115** (1984) 337–341.
39. Brami B., Vilgrain I. and Chambaz E. M.: Sensitization of adrenocortical cell adenylate cyclase activity to ACTH by angiotensin II and activators of protein kinase C. *Molec. Cell Endocr.* **50** (1987) 131–137.