Angiotensin-II acute regulation of rapid response genes in human, bovine, and rat adrenocortical cells

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Abstract

Angiotensin-II (Ang-II) regulates adrenal steroid production and gene transcription through several signaling pathways. Changes in gene transcription occur within minutes after Ang-II stimulation, causing an increase in aldosterone production and subsequent increase in the overall capacity to produce aldosterone. Our goal was to compare the Ang-II regulation of early gene expression and confirm the up-regulation of selected genes using quantitative real-time RT-PCR (qPCR) across three species, such as, human, bovine, and rat. Microarray analyses were performed using samples from control and Ang-II (10 nM)-treated (1 h) cells from human adrenocortical tumor cell line H295R, and primary adrenal glomerulosa cells from bovine and rat, applied respectively to human, bovine, and rat chips. qPCR was performed to confirm up-regulation of selected genes using mRNA. The microarray comparison revealed 18% similarity among the top 50 up-regulated genes, with human/rat, 20%; human/bovine, 36%; and rat/bovine, 26% similarity. The gene list generated by this comparison included: activating transcription factor 3, B-cell translocation gene (BTG2), Nuclear receptor subfamily 4, group A, member 1 (NR4A1), NR4A2, NR4A3, early growth response 1, v-fos FBJ murine osteosarcoma viral oncogene homolog (c-FOS), FOSB, and Jun family member B (JUNB). Pretreatment of H295R cells with cycloheximide had no effect on Ang-II induction of these genes, suggesting that they are direct targets of Ang-II signaling. The Ang-II gene targets have been defined in three different adrenocortical model systems. Several of the listed genes have previously been described as being key regulators of adrenocortical function. The presence of adrenal cell common genes in such distinct cell models strengthens the hypothesis that these genes are regulators of aldosterone production.

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Introduction

The renin–angiotensin–aldosterone system is a central component of the hormonal mechanisms that regulate blood pressure. Angiotensin-II (Ang-II) is the most potent stimulus of aldosterone secretion and is known to be a growth factor for adrenal glomerulosa cells, stimulating cell proliferation, at least under *in vivo* conditions. The effect of Ang-II on aldosterone secretion is mediated through type 1 Ang-II (AT1) receptors (Spat & Hunyady 2004) and is largely due to activation of the G_q/phospholipase C (PLC)-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to production of inositol phosphates and diacylglycerol (Guillon *et al.* 1995). The AT1 receptor is a G protein-coupled receptor that associates with G_i, G_o, and G_q thereby activating PLC, phospholipase D (PLD), and phospholipase A₂ (PLA₂; Hunyady & Catt 2006). As are several other G protein-coupled receptors, Ang-II is able to activate the mitogen-activated protein (MAP) kinases pathway, namely p42/p44^{mapk} (also called extracellular signal-regulated kinase (ERK) 1/2) in bovine (Chabre

et al. 1995) and rat (Cote *et al.* 1998, McNeill *et al.* 1998). In addition, AT1 receptors can signal through G protein-independent pathways including Janus kinases/signal transducers and activators of transcription (Jak/STAT) and the epidermal growth factor (EGF) receptor (Seta *et al.* 2002). Stimulation of these pathways leads to a range of downstream effects, including activation of calcium/calmodulin-dependent kinase, PLD, PLA₂, protein kinase C, src tyrosine kinases, and MAP kinases (Hunyady & Catt 2006, Mehta & Griendling 2007). These diverse pathways influence adrenal glomerulosa cell division, acute aldosterone production, and the capacity of adrenal cells to produce aldosterone chronically (Otis *et al.* 2007). Many of the effects of Ang-II on the adrenal cells are thought to be related to Ang-II-induced changes in gene transcription (Naville *et al.* 2001, Li *et al.* 2003, Bassett *et al.* 2004a, Romero *et al.* 2007).

Many of the effects of Ang-II relate to the induction of a group of ‘rapid’ response genes, which include the nerve growth factor-induced clone B (NGFIB), v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), JUN, and early growth response (EGR) families of

transcription factors. These genes respond to Ang-II treatment in a variety of tissues including smooth muscle, brain, and cardiac myocytes, as well as the adrenal gland (Sadoshima & Izumo 1993, Herbert 1996, McKay *et al.* 1998). *In vitro* and *in vivo* studies of Ang-II action in the adrenal have shown that several rapid response genes are involved in steroidogenesis as well as adrenal cell growth and differentiation. Although adrenal glomerulosa cells represent the primary adrenal cell target for Ang-II, no studies have been performed to define their Ang-II rapid response genes.

Ang-II gene targets in glomerulosa cells have only been defined in one cell culture model, the H295R adrenal carcinoma cell line (Romero *et al.* 2004). This cell line is of human origin, grows well in culture, expresses AT1 receptors, and responds to Ang-II with increased aldosterone production and aldosterone synthase (CYP11B2) expression (Gazdar *et al.* 1990, Bird *et al.* 1993, 1994, Rainey *et al.* 2004). However, these cells, like their parental tumor, also produce large amounts of cortisol and dehydroepiandrosterone (DHEA)-sulfate (Rainey *et al.* 1993). Thus, they have retained some characteristics of each of the human adrenocortical zones and do not represent a specific model for the zona glomerulosa (Rainey *et al.* 1994, 2004, Bollag *et al.* 2007). There are two additional primary glomerulosa cell culture models (bovine and rat adrenal in origin) that are widely used to study Ang-II receptors and aldosterone production (Aguilera 1992, Lu *et al.* 1996, Bollag *et al.* 2007). Because of the non-specific zonal nature of the H295R adrenocortical carcinoma cell model, the current study was undertaken to compare the Ang-II gene targets between the H295R cells and the two other common glomerulosa cell models. Thus far, such cross species comparison of Ang-II target genes has not been performed.

Materials and methods

Cell culture and treatments

NCI-H295R (H295R) human adrenocortical tumor cells were cultured in modified Eagle's medium (DME)/Ham's F12 medium (Invitrogen) supplemented with 10% cosmic calf serum (Hyclone, Logan, UT, USA), 1% penicillin/streptomycin (Invitrogen), and 0.01% gentamicin (Invitrogen). Cells were maintained in a 37 °C humidified atmosphere (5% CO₂). Cells were subcultured into six-well culture dishes (Corning Costar, Corning, NY, USA) at a density of 1 200 000 cells/well for subsequent treatments and RNA isolation. Prior to treatments, adrenal cells were maintained overnight in low-serum medium (DME/Ham's F12 medium supplemented with 0.1% cosmic calf serum medium, 1% penicillin/streptomycin, and 0.01% gentamicin).

In addition, cells were pre-incubated with or without 35 µM cycloheximide (15 min) and then stimulated with or without 10 nM Ang-II for 1 h.

The bovine adrenal glomerulosa cells were isolated from male and female near-term fetal calves and cultured overnight in Falcon Primaria dishes (Becton Dickinson Labware, Lincoln Park, NJ, USA) in a DME/Ham's F12 medium (1:1) containing 10% horse serum (vol/vol), 2% fetal bovine serum (vol/vol), 100 µM ascorbate, 1.2 µM α -tocopherol, 0.05 µM Na₂SeO₃, 50 µM butylated hydroxyanisole, 5 µM metyrapone, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. After replacement of the serum-containing medium with serum-free medium (+0.2% BSA), the cells were incubated for an additional 20–24 h before use. The cells were then rinsed with bicarbonate-buffered Krebs–Ringer solution containing 2.5 mM sodium acetate (Krebs–Ringer buffer (KRB)+) and incubated for 30 min (in 5% CO₂) in KRB+ before addition of KRB+ with or without 10 nM Ang-II. After an additional 1-h incubation, supernatants were collected and frozen for subsequent RIA of aldosterone (Diagnostic products, Los Angeles, CA, USA). Cells were rinsed once with PBS-lacking divalent cations and immediately placed at –80 °C until processing to obtain RNA.

The rat glomerulosa cells were obtained from adrenal glands of female Long-Evans rats weighing 200–250 g and isolated according to the method previously described in detail (Gallo-Payet & Payet 1989). All protocols were approved by the Animal Care and Ethics Committee of the Université de Sherbrooke. Isolation and cell dissociation of the zona glomerulosa was performed in MEM medium, supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. After a 20-min incubation at 37 °C with collagenase (2 mg/ml) and DNase (25 µg/ml), cells were dispersed by gentle aspiration with a sterile 10 ml pipette, filtered, and centrifuged for 10 min at 1200 g. The cell pellet was then resuspended in OPTI-MEM medium supplemented with 2% FBS (fetal bovine serum), 100 U/ml penicillin, and 100 µg/ml streptomycin. Glomerulosa cells were plated at a density of 6×10^5 cells/Petri dish and were cultured at 37 °C in a humidified atmosphere composed of 95% air/5% CO₂. Glomerulosa cells were stimulated with 10 nM Ang-II for 1 h.

Microarray analysis

RNA from H295R cells was hybridized to an Affymetrix human HG_U133+2 oligonucleotide microarray set containing 54 675 probe sets representing ~40 500 independent human genes. The arrays were scanned at high resolution in the microarray core facility at the Medical College of Georgia in Augusta, GA. RNA samples isolated from bovine and rat adrenal glomerulosa cells stimulated for 1 h with Ang-II versus basal were shipped to Codon Biosciences (Houston, TX, USA) for hybridization

with bovine array (24 128 probe sets) and Rat230_2 chip (31 095 probe sets) respectively. Results were analyzed using GeneSpring software version 7.3 (Silicon Genetics, Redwood City, CA, USA) to identify differences in expression of genes after treatment with Ang-II for 1 h compared with basal, and cycloheximide compared with cycloheximide + Ang-II. Microarray data imported into GeneSpring software were normalized using RMA (robust multichip average) followed by an additional normalization to the 50th percentile of all signal values.

RNA extraction, cDNA synthesis, and real-time RT-PCR

Total RNA was extracted from cells plates using Trizol (Invitrogen) according to the manufacturer's directions. Quantification, purity, and integrity of the RNA were checked spectroscopically using Nanodrop (Nanodrop Technologies, Wilmington, DE) and Experion (Bio-Rad Laboratories) instruments. DNase I (2 µg; Ambion Inc., Austin, TX, USA)-treated total RNA was reverse transcribed using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's recommendations. Primers for the amplification of the target sequences were designed using Primer Express 3.0 (Applied Biosystems). The sequences are shown in Supplementary Table 1, which can be viewed online at <http://jme.endocrinology-journals.org/content/vol39/issue6/>.

PCR amplifications were performed using the ABI Prism 7500 sequence detection system (Applied Biosystems) following the reaction parameters recommended by the manufacturer, using 2 µg RNA per sample. For rat and bovine samples, the 30 µl total volume consisted of SYBR Green universal PCR master mix (2×; Applied Biosystems), forward and reverse primer mix, and 5 µl cDNA. For human samples, the 20 µl total volumes consisted of Fast Reagent Master Mix (Applied Biosystems), primer/probe mix, and 5 µl cDNA. 18s was used as an endogenous control gene and negative controls contained water instead of cDNA.

In all experiments, relative gene expression was calculated by the $\Delta\Delta C_t$ method. Briefly, the resultant mRNA was normalized to a calibrator; in each case, the calibrator chosen was the basal sample. Final results were expressed as the n -fold difference in gene expression relative to 18s rRNA and calibrator as follows: $n\text{-fold} = 2^{-(\Delta C_t \text{ sample} - \Delta C_t \text{ basal})}$, where ΔC_t values of the sample and calibrator were determined by subtracting the average C_t value of the transcript under investigation from the average C_t value of the 18s rRNA gene for each sample.

Statistical analysis

For qPCR analysis, all values were expressed as mean $\pm$ S.E.M. Statistical calculations were performed with

Ms Office Excel 2003 (Microsoft Corporation). Statistical differences between qPCR samples were determined by Student's t -test. $P < 0.05$ was required for statistically significance.

Results

Ang-II target genes in human H295R cells

The H295R cell line is the only human adrenal cell model that responds to Ang-II and has been used to study 'acute' and chronic production of aldosterone (Bassett *et al.* 2000). Adrenal H295R cells were treated for 1 h with Ang-II (10 nM) followed by isolation of RNA and microarray analysis. Of the 54 675 probe sets on the Affymetrix array, 28 718 were considered detectable on both the basal and Ang-II samples and these probe sets were used for data analysis. A fivefold increase took place in 16 transcripts, whereas no gene was down-regulated more than fivefold (Fig. 1, Panel A). The genes with the highest fold change were further studied using qPCR from three independent experiments (Fig. 1, Panel B). These replicate experiments confirmed increased expression for each of these genes identified by microarray analysis. Analyses of RNA from Ang-II-treated H295R cells from three different experiments compared with basal showed four transcription factors among the top five up-regulated genes. In order of increased expression determined by microarray analysis, the identified genes are: nuclear receptor subfamily 4, group A, member 2 (NR4A2), FOS, FOSB, NR4A3, and EGR4.

To determine whether genes were direct or indirect targets of Ang-II treatment, the H295R cells were treated with cycloheximide to block new protein synthesis. Array analysis was compared between cells treated with cycloheximide versus those exposed to Ang-II plus cycloheximide. Genes were determined to be detectable on at least one of the arrays and these genes were used for data analysis. More than fivefold increase was found in 80% of the genes in Fig. 1 (Panel A) which also increased by more than fivefold in the presence of cycloheximide. Of the top 20 genes, 12 genes shown to increase following Ang-II treatment in the absence of cycloheximide were also increased in its presence. These data suggest that a great number of genes that change expression following 1 h of Ang-II treatment are direct targets of Ang-II signaling pathways and not regulated by the proteins whose expression changes acutely after Ang-II stimulation.

Ang-II target genes in primary cultures of bovine glomerulosa cells

Because of the adrenal size and their availability at many abattoirs, bovine adrenals have been used by many

Figure 1 Microarray analysis of human H295R cells after treatment for 1 h with 10 nM Ang-II versus basal (panel A). B shows confirmation of gene up-regulation of the top five genes, which act as transcription factors, with the exception of an unknown sequence (EST). The following abbreviations are conventionally used by the GeneSpring Software: FOS, v-fos FBJ murine osteosarcoma viral oncogene homolog; FOSB, FBJ murine osteosarcoma viral oncogene homolog B. NR4A2, Nuclear receptor subfamily 4, group A, member 2; NR4A3, Nuclear receptor subfamily 4, group A, member 3; EST, expressed sequence tag (unknown sequence). qPCR data represent the mean $\pm$ S.E.M. for three independent experiments. All gene increases were statistically significant from basal levels ($P < 0.05$).

groups to isolate Ang-II-responsive glomerulosa cells (Lu *et al.* 1996, Smith *et al.* 1998, Cherradi *et al.* 2003, Bollag *et al.* 2007). Current arrays for bovine only partially cover the transcriptome with 24 128 probe sets. Of these, 13 568 were detectable in both basal and Ang-II-treated cells. Among these, seven were increased by more than fivefold upon Ang-II treatment while none decreased by fivefold (Fig. 2, panel A). The primary bovine glomerulosa cell microarray data also showed four transcription factors among the top five up-regulated genes: NR4A3, EST (Genbank: CB431046), NR4A2, FOS, and NR4A1. The qPCR analysis of each gene was performed in three independent bovine glomerulosa experiments (Fig. 2, Panel B). The qPCR showed positive correlation between the fold increase determined in independent experiments and confirmed the results obtained in the microarray analysis.

Ang-II target genes in primary cultures of rat glomerulosa cells

The rat glomerulosa cell has long been used as a model system for acute Ang-II action (Aguilera 1992, Hajnoczky *et al.* 1992). Rat adrenal glomerulosa cells were treated for 1 h with Ang-II (10 nM) followed by isolation of RNA and microarray analysis. Of the 31 095 probe sets on the Affymetrix array, 15 103 were considered detectable on both the Ang-II-treated and basal samples and these probe sets were used for data analysis. Seven transcripts were increased, and none decreased, by fivefold (Fig. 3, panel A). The five genes with the highest fold change were further studied using qPCR from three independent experiments, which confirmed the stimulatory effects of Ang II

(Fig. 3, panel B). Four of the top five Ang-II induced genes were classified as transcription factors (EGR3, NR4A1, NR4A3, and FOSB) and the fifth was BTG2, a protein involved in cell cycle regulation.

Species comparisons of Ang-II target genes

A list of the top 50 Ang-II-responsive genes with known functions was generated for the bovine, human, and rat model systems (Supplementary Table 1 which can be viewed online at <http://jme.endocrinology-journals.org/content/vol39/issue6/>). To determine similarity between the three species, genes were placed in a Venn diagram (Fig. 4). Nine genes or 18% of the total genes were found to be common across the three species. The nine common genes were: NR4A1, NR4A2, NR4A3, FOS, FOSB, JUNB, EGR1, BTG2, and activating transcription factor 3 (ATF3; Table 1). These transcripts encode either transcription factors, nuclear receptors or, in the case of BTG2, a protein that indirectly regulates cell cycle. The predominance and similarity of transcription factor upregulation support the hypothesis that activation of Ang-II receptors rapidly alters gene expression leading to both acute and chronic alterations in the glomerulosa cell phenotype.

Discussion

For the first time, we report a comparative study of the acute target genes for Ang-II in the most commonly used adrenal cell model systems: the H295R adrenocortical carcinoma cell line, primary bovine glomerulosa cells, and primary rat glomerulosa cells. Previous genomic studies have used the H295R cell model to determine genetic regulation by Ang-II (Romero *et al.* 2004). It represents the

Figure 2 Microarray analysis of bovine glomerulosa cells after 1-h stimulation with 10 nM Ang-II versus basal (panel A). Panel B shows the confirmation of gene up-regulation with qPCR of the selected top five genes, which act as transcription factors, with the exception of an unknown sequence (EST). Abbreviations are as in the legend to Fig. 1. qPCR data represent the mean $\pm$ s.e.m. for three independent experiments. All gene increases were statistically significant from basal levels ($P < 0.05$).

only human adrenal model that responds to Ang-II. However, the H295R exhibits differences from normal glomerulosa cells that support the need for comparison studies with other glomerulosa models. For example, these cells have retained the ability to produce cortisol and DHEA, expressed high levels of the enzyme 17 α -hydroxylase, and lost their response to adrenocorticotrophin (ACTH; Mountjoy *et al.* 1994, Bird *et al.* 1998). Thus, while H295R cells are highly responsive to Ang-II, these cells represent a carcinoma cell line that displays attributes consistent with each of the zones of the adult adrenal as well as some characteristics associated with fetal adrenal cells (Logie *et al.* 1999). It is for these reasons that

we compared the Ang-II target genes found in H295R cells with those observed in two primary cell cultures models arising from bovine and rat zona glomerulosa.

There have been two studies published (one acute and one chronic) that examined Ang-II gene targets in the H295R adrenal cell model (Wang *et al.* 2000, Romero *et al.* 2004). Ang-II rapid-response target genes have previously been defined in the H295R human adrenocortical carcinoma cell model using microarray analysis following a 3-h treatment period with Ang-II. Our results follow the results of this longer exposure to Ang-II (below). A comparison of the 50 transcripts from each of the Ang-II-responsive cell models demonstrated an 18%

Figure 3 Microarray analysis of rat glomerulosa cells after 1-h stimulation with 10 nM Ang-II versus basal (panel A). Graph 3B shows the confirmation of gene up-regulation with qPCR of the top five genes, all classified as transcription factors. The abbreviations used are as in the legend for Fig. 1 with the following two additional abbreviations: BTG2 and BTG family, number 2; EGR3, early growth response 3. qPCR data represent the mean $\pm$ s.e.m. for four independent experiments. All gene increases were statistically significant from basal levels ($P < 0.05$).

Figure 4 Venn diagram demonstrating the absolute number of common genes among human, bovine, and rat. The list of genes used to make the diagram was based on the top 50 genes (excluding ESTs) that were up-regulated upon *in vitro* treatment with 10 nM Ang-II for 1 h in cells from each of the three adrenal glomerulosa models.

similarity across the three species. Herein, we focused on the genes found to be common between the three model systems. The common genes arranged in Table 1 include three members of the NGFI-B family; JUNB and FOS (members of the AP-1 complex), EGR-1, BTG2 and ATF3. The core group of transcription factors that were elevated in each of our three model systems were in the list of Ang-II target genes described by Romero *et al.* (2007) in H295R (3 h Ang-II). Such a common pattern of induction across three species suggests that Ang-II induction of these transcription factors may be necessary for the acute

and chronic regulation of glomerulosa cell aldosterone production.

Considerable effort has been expended to define the role of the NR4A family of nuclear hormone receptors in the regulation of adrenal cell steroid production. These transcription factors are members of the nuclear receptor family but have no identified ligand. They regulate transcription through changes in their expression level and phosphorylation (Hsu *et al.* 2004, Maxwell & Muscat 2006). Previous studies using rat, bovine, human, and mouse adrenal models have identified members of the NR4A family of nuclear hormone receptors as adrenocortical rapid response genes that increase after Ang-II or ACTH treatment (Enyeart *et al.* 1996, Fernandez *et al.* 2000, Kelly *et al.* 2004). These nuclear receptors have also been shown to increase adrenal-specific gene expression in several whole animal studies suggesting that our *in vitro* observations may have physiologic significance (Pirih *et al.* 2005, Pei *et al.* 2006). Additional studies have focused on defining the functional role of NR4A1 (previously called Nurr77, or NGFI-B, NR4A2 (previously called Nurr1) and NR4A3 (previously called Nor1) in adrenal steroidogenesis (Crawford *et al.* 1995, Fernandez *et al.* 2000, Lu *et al.* 2004). In the H295R cells, NR4A1 and NR4A2 appear to increase selectively the expression of enzymes involved in aldosterone and cortisol biosynthesis. NR4A2 and NR4A1 have selective effects on CYP11B2 and HSD3B2 transcription (Bassett *et al.* 2004b); however, no effect was observed on the transcription of CYP11B1 or CYP17. A recent study confirmed a stimulatory effect

Table 1 Common angiotensin-II (Ang-II) target genes across the human, bovine, and rat adrenal models systems

			Human mean fold increase over basal	Bovine mean fold increase over basal	Rat mean fold increase over basal
Gene function					
Name					
Gene name					
Activating transcription factor 3	Transcription factor	ATF3	2·63	2·5	2·98
BTG family, member 2	Modulator of cell cycle	BTG2	6·64	7·12	5·65
Early growth response 1	Transcription factor	EGR1	9·30	2·94	3·77
FBJ murine osteosarcoma viral oncogene homolog B	Transcription factor	FOSB	15·5	3·5	20·63
Jun B proto-oncogene	Transcription factor	JUNB	4·88	3·20	2·41
Nuclear receptor subfamily 4, group A, member 1	Nuclear receptor	NR4A1	5·4	3·02	16·08
Nuclear receptor subfamily 4, group A, member 2	Nuclear receptor	NR4A2	30·91	10·65	4·53
Nuclear receptor subfamily 4, group A, member 3	Nuclear receptor	NR4A3	12·10	27·49	10·80
v-fos FBJ murine osteosarcoma viral oncogene homolog	Transcription factor	FOS	25·45	8·17	4·66

A list of the most responsive transcripts to 1 h Ang-II (10 nM) treatment (with known annotation) was compiled for H295R, bovine glomerulosa, and rat glomerulosa cells. Comparison of the top 50 transcripts provided the genes common to each model system. The complete list of the 50 transcripts for each model is available as Supplementary Tables 1, 2, and 3, which can be viewed online at <http://jme.endocrinology-journals.org/content/vol39/issue6/>. Also listed are the gene functions and fold increase compared with basal expression levels.

of NR4A1, NR4A2, and NR4A3 on CYP11B2 transcription, using transfection of H295R cells with CYP11B2 reporter constructs (Romero *et al.* 2007). Others have demonstrated that NR4A1 and NR4A3 regulate transcription of CYP21, which is also needed for aldosterone/cortisol biosynthesis (Wilson *et al.* 1993, Fernandez *et al.* 2000). Studies in mouse adrenocortical Y1 cells suggest that regulation of NR4A1 by ACTH occurs via both increased expression and phosphorylation of NR4A1 (Li & Lau 1997).

Several studies reported a rapid induction of the activating protein 1 (AP-1) factors (FOS and JUN family members) by both Ang-II and ACTH (Viard *et al.* 1992, Lehoux *et al.* 1998, Naville *et al.* 2001). AP-1 complexes function as homo- or heterodimers formed by members of the c-FOS and JUN families. Interaction between members of the two families to form heterodimers leads to a much higher affinity for AP-1 sites. Different AP-1 complexes consisting of different FOS and JUN members display distinct transcriptional regulatory activities in different cell systems (Enyeart *et al.* 1996). In H295R cells, following co-transfection of c-FOS and FOSB with CYP11B1 or CYP11B2, transcriptional activity of both steroidogenic enzymes was enhanced (Romero *et al.* 2007). However, a comparison of the fold increases of these two steroidogenic enzymes in the same study has shown greater effects on CYP11B1. This results agrees with studies performed in the rat in which FOS increases rat CYP11B1 transcriptional activity (Mukai *et al.* 1995), and with the presence of a putative AP-1 site in human CYP11B1 (Rainey 1999).

The EGR family of zinc finger proteins is coordinately stimulated in resting cells upon a growth stimulus. EGR4, which is present in the top five up-regulated genes in H295R cells, is known to be an auto-regulatory protein that causes transcription repression (Zipfel *et al.* 1997). In rat glomerulosa cells, the EGR3 was the gene that demonstrated the greatest 1-h stimulation by Ang-II. EGR3 was previously shown to be increased in the adrenal cortex of rats following stress induced by capsaicin infusion (Honkaniemi *et al.* 2000). This increase was thought to result from increased ACTH levels and was not affected by denervation of the adrenal medulla. Another EGR family member, EGR1, was up-regulated in human, bovine, and rat arrays, although it was not among the top five genes. Because of the known role of EGR1 and EGR3 in activating cell division, it is likely that these factors may be responsible for the known action of Ang II on adrenal glomerulosa cell division.

The ATF1, has been previously described by Gu and colleagues as a part of the pathway activating aldosterone synthase expression following Ang-II treatment of H295R cells (Gu *et al.* 2003). Also, interaction of ATF1 and ATF2 with the cAMP-responsive element (CRE) was shown within the promoters of CYP11B1 and CYP11B2 in H295R cells (Bassett *et al.* 2004c). No previous studies

were found to connect ATF3 with Ang-II or ATF3 with the adrenal. However, ATF3 is known to bind CRE within other target genes and therefore could play a role in CYP11B1 and CYP11B2 transcription (Liang *et al.* 1996).

Several interesting genes were increased in each of the three *in vitro* models, but not to a level placing them in the top 50 genes (Supplementary Tables 2–3, which can be viewed online at <http://jme.endocrinology-journals.org/content/vol39/issue6/>). For example, regulator of G-protein signaling 2 (RGS2) was in the top 50 up-regulated genes in rat and bovine. In the human, RGS2 has previously been described as a regulator of AT1-receptor signaling in adrenocortical cells (Romero *et al.* 2006). Over-expression of the RGS2 gene decreases the Ang-II-induced synthesis of aldosterone. MAP phosphatase-1 (MKP-1), also called dual specificity phosphatase 1, was up-regulated in all the three adrenal models and was one of the top ten Ang-II-responsive genes for human and bovine cells. In a recent study using bovine adrenal glomerulosa cells, Ang-II was also shown to induce expression of MPK-1. Interestingly, MPK-1 over-expression was shown to cause inactivation of ERK1/2, leading to a decrease in mineralocorticoid production (Casal *et al.* 2007). In contrast, Ang-II activation of ERK1/2 has been shown to play an important role in the activation of rat adrenal glomerulosa cell aldosterone production (Otis & Gallo-Payet 2006). Taken together, these results suggest that the synthesis of MKP-1 works as an intracellular negative feedback mechanism to dampen Ang-II action. nuclear factor, interleukin 3 (NFIL3 regulated) is also included in the list of up-regulated genes in the three species. NFIL3 has been previously identified as a transcription factor with defined roles in cell survival (Cowell 2002). With regard to the adrenal, a previous study identified NFIL3 as one of the genes responsive to Ang-II treatment of H295R cells (Romero *et al.* 2007). Furthermore, studies suggested that NFIL3 regulates CYP11B2 transcription (10). Interestingly, few mRNAs encoding steroidogenic enzymes changed in the 1-h treatment period with Ang-II. CYP21A1 was increased by approximately threefold in rat glomerulosa cells, and steroidogenic regulatory protein (StAR) mRNA was one of the top 10 genes increased in bovine adrenal cells. The functional role of this protein in the acute transport of cholesterol inside the mitochondria suggests that the bovine glomerulosa system may be more acutely coupled than either the rat or human adrenal cell model.

Although Ang-II plays critical roles in numerous physiological processes, there have been few studies directed at defining its target genes using microarray analysis. Vascular smooth muscle cell Ang-II targets were examined using filter arrays containing ~5000 genes (Braam *et al.* 2003, Campos *et al.* 2003). In addition, Ang-II

target genes were studied in human renal proximal tubular cells using targeted filter arrays that contained 287 genes (Braam *et al.* 2003). While it is difficult to directly compare these studies with our study, it is interesting that none of the transcripts observed in the vascular smooth muscle or proximal tubular cells were observed in the top 50 genes observed to change in the adrenal cell models. However, future studies using a similar microarray format would be useful to better determine if there are common gene targets.

In summary, the effects of Ang-II on aldosterone biosynthesis can be divided into acute and chronic phases. Acutely (minutes to hours after a stimulus), aldosterone production is controlled by the movement of cholesterol into the mitochondria, which is mediated by post translational modification of and increased expression of StAR protein (Christenson & Strauss 2001). Chronically (hours to days), aldosterone production is regulated at the level of steroid-metabolizing enzymes including aldosterone synthase (CYP11B2; Bassett *et al.* 2004a). Considerable evidence suggests that the acute and chronic phases of aldosterone production rely on the transcription of target genes and their translation to proteins. Herein, the acute target genes for Ang-II were defined in the three most commonly used adrenal cell model systems: the H295R adrenocortical carcinoma cell line, primary bovine glomerulosa cells, and primary rat glomerulosa cells. All three models responded to Ang-II stimulation with expression of a variety of transcription factors that we hypothesize are responsible for converting the transient ligand/receptor signal into lasting changes in the phenotype of the cell and its capacity to produce steroids.

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Disclosures

The authors have nothing to disclose.

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