Angiotensin II early regulated genes in H295R human adrenocortical cells

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Angiotensin II (ANG II) stimulates aldosterone synthesis in the adrenal zona glomerulosa. Other aldosterone secretagogues, K+ and ACTH, with and without cycloheximide to determine whether the effect of ANG II on gene expression was caused by changes in gene transcription or translation. The RNA for the microarrays was obtained from cells contaminated with cells from the zona fasciculata, and specific mechanisms are difficult to sort out. The human adrenocortical H295R cell line was originated from a human adrenocortical carcinoma and has been established as an appropriate model to define mechanisms regulating aldosterone production (8, 42, 74). This cell has been used to study the transcriptional regulation of the aldosterone synthase (17, 21). Although much is understood about the actions of ANG II in adrenal steroidogenesis, there are several crucial steps that remain unknown. To elucidate the mechanism of intracellular signaling by ANG II stimulation in the adrenal, we have studied immediate-early regulated genes in human adrenal H295R cells using cDNA microarrays. H295R cells were stimulated with ANG II for 3 h. Gene expression was analyzed by microarray technology and validated by real-time RT-PCR. Eleven genes were found to be upregulated by ANG II. These encode the proteins for ferredoxin, Norl, Nurr1, c6orf37, CAT-1, A20, MBLL, M-Ras, RhoB, GADD45α, and a novel protein designated FLJ45273. Maximum expression levels for all genes occurred 3–6 h after ANG II stimulation. This increase was dose dependent and preceded maximal aldosterone production. Other aldosterone secretagogues, K+ and endothelin-1 (ET-1), also induced the expression of these genes with variable efficiency depending on the gene and with lower potency than ANG II. ACTH had negligible effect on gene expression except for the CAT-1 and Nurr1 genes. These ANG II-stimulated genes are involved in several cellular functions and are good candidate effectors and regulators of ANG II-mediated effects in adrenal zona glomerulosa.

adrenal; gene expression; gene regulation; aldosterone

THE RENIN-ANGIOTENSIN SYSTEM (RAS) is the most important regulator of intravascular volume and blood pressure. A key player in the RAS is the mineralocorticoid aldosterone, which acts in transporting epithelia, primarily in the kidney collecting tubule, to promote sodium retention and potassium excretion (9). Aldosterone is produced exclusively in the zona glomerulosa of the adrenal, and its secretion is regulated by angiotensin II (ANG II), potassium, and to a lesser degree by ACTH (13, 73). The regulation of aldosterone secretion occurs in two different phases. The first being an initial and rapid phase where the increased release of aldosterone reflects the increased transfer of cholesterol to the mitochondria, where it is acted upon in the inner mitochondrial membrane by the cytochrome P-450 side chain cleavage (1). This transfer is mediated by the steroidogenic acute regulator (StAR) protein (14). A later phase involves an increase in transcription and expression of the rate-limiting enzyme in the biosynthesis of aldosterone, the cytochrome P-450 aldosterone synthase or CYP11B2 gene product. This increased transcription is induced by ANG II (41). ANG II interacts with ANG II receptors in the glomerulosa cell surface and activates several intracellular signaling pathways (mainly Ca2+/PKC) that cause upregulation of several genes, including transcription factors, steroidogenic enzymes, and StAR, that ultimately lead to an increase in aldosterone production (15, 59). Although the physiology of ANG II actions has been studied for many years, there are still many unknown steps in the intracellular signaling pathway triggered by ANG II in adrenal cells leading to the increased transcription of the CYP11B2.

One of the difficulties in the studies of the mechanisms for the mediators of the transcriptional regulation of the aldosterone synthase has been the lack of a good in vitro model. Isolated adrenal cells from the zona glomerulosa are heavily contaminated with cells from the zona fasciculata, and specific mechanisms are difficult to sort out. The human adrenocortical H295R cell line was originated from a human adrenocortical carcinoma and has been established as an appropriate model to define mechanisms regulating aldosterone production (8, 42, 74). This cell has been used to study the transcriptional regulation of the aldosterone synthase (17, 21). Although much is understood about the actions of ANG II in adrenal steroidogenesis, there are several crucial steps that remain unknown. To elucidate the mechanism of intracellular signaling by ANG II stimulation in the adrenal, we have studied immediate-early regulated genes in the H295R cell using cDNA microarrays. The microarray technique was used because it efficiently screens several thousand genes, most of which have no known function and have never been studied in the adrenal gland. We report the results of our microarray survey (confirmed by real-time RT-PCR) and the characterization of the transcription regulation of the upregulated genes in H295R cells by ANG II, endothelin-1 (ET-1), K+, and ACTH, with and without cycloheximide to determine whether the effect of ANG II on gene transcription is a primary event.

MATERIALS AND METHODS

Cell culture. H295R human adrenocortical cells (a generous gift from Dr. W. Rainey) were grown in complete media containing DMEM:F12 (1:1) supplemented with 2% Ultroser G (Biosera, Ville-neuve-la-Garenne, France), ITS-Plus (Discovery Labware, Bedford, MA) and antibiotic/antimycotic mix (Invitrogen, Carlsbad, CA). Cells were grown in 175-cm2 flasks to about 95% confluence. Cell culture media was replaced with fresh complete media (40 ml) containing 0.1–100 nM ANG II (Sigma, St. Louis, MO), 100 nM ACTH (1–24) (Cortrosyn; Amphastar Pharmaceuticals, Rancho Cucamonga, CA), 10 nM ET-1 (Sigma), or 16 mM potassium chloride. The RNA for the microarrays was obtained from cells incubated for 3 h with 100 nM ANG II. Cells were incubated for
Table 1. Real-time PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (sense/antisense)</th>
<th>Amplicon Size, bp</th>
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<tbody>
<tr>
<td>MBlL</td>
<td>GCCAGAAAGTGCAGCAGGCTTAC</td>
<td>100</td>
</tr>
<tr>
<td>Nor1</td>
<td>GTGGCTTGGACTAGCTGGCAACC</td>
<td>98</td>
</tr>
<tr>
<td>Ferredoxin</td>
<td>CACCTGCTCATCTTGAAGTGG</td>
<td>110</td>
</tr>
<tr>
<td>FLJ45273</td>
<td>GCTTAAAACAGAGGGGATTTGA</td>
<td>100</td>
</tr>
<tr>
<td>M-Ras</td>
<td>GTGTCAGGCAAGAAGGATGTT</td>
<td>99</td>
</tr>
<tr>
<td>c6orf37</td>
<td>AGACGGTATATGTTCTGGGAGA</td>
<td>98</td>
</tr>
<tr>
<td>CAT-1</td>
<td>TCTAGAGGGAGAGGAGATGTA</td>
<td>101</td>
</tr>
<tr>
<td>A20</td>
<td>AGTGGCATCTCTTGGCAAGCTG</td>
<td>99</td>
</tr>
<tr>
<td>RhoB</td>
<td>GTGCTGGGTCTTTGCGGATGTC</td>
<td>100</td>
</tr>
<tr>
<td>GADD45a</td>
<td>AGCTAGCAAGATATTGGCTAGTA</td>
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<tr>
<td>Nurr1</td>
<td>AAGTCAGATATTGCTGCCTCC</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>CTCCTGGATCATGGTCTTGA</td>
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</tr>
<tr>
<td>β-Actin</td>
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<tr>
<td>γ-Actin</td>
<td>AGGACAGAGGAGAGGAGATTG</td>
<td>100</td>
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Table 2. Upregulated genes by ANG II in H295R cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>UniGene</th>
<th>Microarray Clone IMAGE No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBlL</td>
<td>Hs.372571</td>
<td>273048</td>
</tr>
<tr>
<td>Nor1</td>
<td>Hs.80561</td>
<td>777025</td>
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<td>Ferredoxin</td>
<td>Hs.744</td>
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<tr>
<td>FLJ45273</td>
<td>Hs.30646</td>
<td>446307/26084</td>
</tr>
<tr>
<td>M-Ras/R-Ras3</td>
<td>Hs.349227</td>
<td>44495</td>
</tr>
<tr>
<td>c6orf37</td>
<td>Hs.348967</td>
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<td>CAT-1</td>
<td>Hs.14846</td>
<td>491440</td>
</tr>
<tr>
<td>A20</td>
<td>Hs.211600</td>
<td>322799</td>
</tr>
<tr>
<td>RhoB</td>
<td>Hs.204354</td>
<td>5823226</td>
</tr>
<tr>
<td>GADD45a</td>
<td>Hs.80409</td>
<td>472085/491046</td>
</tr>
<tr>
<td>Nurr1</td>
<td>Hs.82120</td>
<td>38452</td>
</tr>
</tbody>
</table>

various times at 37°C, and media was removed and stored for steroid quantification.

RNA extraction and RT-PCR. Total RNA was extracted with the RNeasy Midi kit (Qiagen, Valencia, CA) and on-column DNase digested. For reverse transcription, 2 µg total RNA was incubated with T12 VN and SuperScript II (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Real-time PCR primers were designed with the Primer3 software (79) to generate ~100-bp amplicons (Table 1). Real-time PCR was performed with 1 µl RT product, 1 µl Titanium Taq DNA polymerase (Clontech, Palo Alto, CA), 1:20,000 diluted SYBR Green I (Molecular Probes, Eugene, OR), 0.2 mM dNTPs, and 0.1 µM each primer. Cycling conditions were 1 min 95°C, 50 cycles of 15 s 95°C, 15 s 60°C, and 1 min 72°C. Real-time data were obtained during the extension phase and critical threshold cycle values were obtained on the log phase of each gene amplification curve. PCR product quantification was performed by the relative quantification method (71) and standardized against housekeeping genes (GAPDH, β-actin, and γ-actin). Efficiency for each primer pair was assessed by using serial dilutions of RT product. CYP11B2 mRNA was quantified as previously reported (26) using SureStart Taq DNA polymerase (Stratagene, La Jolla, CA). Standard curves were constructed with serial dilutions (10^1 to 10^6 copies/well) of plasmid encoding the coding region of human CYP11B2.

Microarrays. Total RNA samples were labeled with Cy3 and Cy5 dyes using an indirect aminoallyl-dUTP method (12). Briefly, 10 µg total RNA was reverse transcribed with 5 µg T12 VN, 2 µl SuperScript II (200 U/µl) (Invitrogen), and 0.5 mM of each deoxynucleotide and a dUTP:aminoallyl-dUTP ratio of 1:1. The reaction was incubated at 37°C; after 1 h, an additional 2 µl was added, and the incubation continued for an additional hour. RNA was degraded by acid treatment and separated from labeled cDNA by Microcon-30 filtration (Millipore, Billerica, MA). Aminoallyl-labeled cDNA was coupled with monofunctional NHS-ester Cy3 or Cy5 (Amersham, Piscataway, NJ), quenched with hydroxylamine, cleaned by filtration, then ethanol precipitated. Control and treated labeled samples were combined, resuspended in Digi Easy HYB solution containing 0.5 mg/ml yeast tRNA (Sigma) and 0.5 mg/ml calf thymus DNA (Sigma), heated for 5 min at 65°C, and allowed to cool to room temperature. The samples were hybridized with cDNA-spotted microarrays containing 19,000 human genes (Microarray Center, University Health Network, Toronto, Canada) and incubated overnight at 37°C. Slides were washed three times in 1× SSC containing 0.1% SDS for 10 min at 50°C. Finally, slides were extensively washed in 1× SSC at room temperature and dried. Hybridizations were performed in sextuplicates with dye swap. Slides were immediately scanned using a ScanArray (Packard Biosciences, Boston, MA) and spot quantified with QuantArray (Packard Biosciences). Spot intensities on each slide were normalized using the rank invariant method followed by Loess re-gression (87). Statistical analysis of regulated genes was done using the statistical analysis of microarray data (SAM) software (88) using the one-class protocol with k-nearest neighbor method for missing data estimation and the maximum number of permutations allowed. Microarray hybridization data was submitted to Gene Expression Omnibus database (GEO, National Center for Biotechnology Information) under accession number GSE1030.

Angiotensin II caused a transient and acute induction of the primers. Further studies were performed with the one-class protocol with k-nearest neighbor method (35).

Statistical analysis. Results are expressed as means ± SE. Results were analyzed by ANOVA followed by Tukey (multiple comparisons) or Dunnett (comparisons vs. control) contrasts using the Statis-tica package version 6.1 (Statsoft, Tulsa, OK).

RESULTS

ANG II immediate-early regulated genes. H295R cells were stimulated with ANG II (100 nM) for 3 h to study immediate-early regulated genes. Genes identified as up- or downregulated using SAM software were validated by real-time RT-PCR. Table 2 shows the list of upregulated genes identified and confirmed by real-time RT-PCR. Eleven genes were acutely upregulated by ANG II treatment in H295R cells. No down-regulated genes were confirmed by real-time RT-PCR. All PCR reactions were validated by melting curve analysis and agarose gel electrophoresis. Figure 1 shows an agarose gel electrophoresis of a typical PCR reaction showing the specificity of the primers. Further studies were performed with real-time RT-PCR validated genes.

ANG II time curve. An ANG II time curve analysis of gene expression was performed on validated genes. H295R cells were treated with ANG II (100 nM) for different time periods between 3 and 48 h, and RNA levels were quantified by real-time RT-PCR. ANG II caused a transient and acute induc-
tion of mRNA levels of all genes studied (Fig. 2). In all cases maximum RNA levels were reached after 3–6 h of ANG II stimulation. ANG II also caused a time-dependent increase in aldosterone synthase mRNA levels, reaching maximum values after 6–12 h (Fig. 2), and produced a continuous aldosterone accumulation in the media (Fig. 2).

**Effect of aldosterone secretagogues.** The effect of increasing concentrations of ANG II and other aldosterone secretagogues for 3 h on ANG II immediate-early regulated gene expression was studied. Aldosterone accumulation in media was measured in parallel experiments at 3 and 24 h after stimulation (Fig. 3). Although media was extracted and concentrated for aldosterone quantification, steroid accumulation was just beginning after 3-h stimulation, so clearer results are seen in parallel experiments after 24-h stimulation. K⁺ (16 mM) and ET-1 (10 nM) both increased aldosterone accumulation in the media, although to a lesser extent than ANG II. ACTH had no effect on aldosterone accumulation. Forskolin, an adenylyl cyclase activator that mimics ACTH, stimulated aldosterone production. ANG II caused a dose-dependent increase in the RNA expression levels of all genes studied (Fig. 4). K⁺ (16 mM) increased mRNA levels of only some genes and to a lower level than the maximal ANG II stimulation. ET-1 was also a low-potency inducer of gene expression, and its efficiency varied depending on the gene. ACTH had little effect on the RNA expression of these genes except for that of ferredoxin, FLJ45273, and A20. The effect of forskolin on mRNA expression varied among the genes, from almost no effect to an induction similar to maximal ANG II stimulation in Nor1, ferredoxin, CAT-1, and RhoB.

**Cycloheximide effect on ANG II-mediated stimulation.** H295R cells were incubated with ANG II (100 nM) in the presence or absence of cycloheximide (10 μg/ml) for 3 h. Cycloheximide is a protein synthesis inhibitor and is used to discriminate between primary and secondary event-regulated genes. As expected, cycloheximide treatment completely blocked the ANG II-mediated aldosterone accumulation in the culture media (Fig. 5). MBLL and ferredoxin are primary event genes, as the ANG II-mediated stimulation of their mRNA is not blocked by cycloheximide. ANG II-mediated stimulation of FLJ45273 was completely blocked by cycloheximide, indicating that increased expression of this gene is a secondary event requiring protein synthesis induced by ANG II for its induction. The evaluation of M-Ras, c6orf37, and CAT-1 generated mixed results. Cycloheximide treatment caused a superinduction of Nor1, A20, RhoB, Nurr1, and GADD45α; their expression levels were higher with ANG II + cycloheximide than with ANG II treatment alone.

**DISCUSSION**

We identified several immediate-early regulated genes whose transcription is significantly altered by 3-h treatment with ANG II in H295R human adrenocortical cells. The genes identified are diverse, belonging to different functional families or having unknown functions. Some are newly discovered. The combination of microarray and real-time RT-PCR techniques allows us to be confident that the genes described in this work are truly ANG II regulated. These genes are only a subset of ANG II-regulated genes. There are known ANG II-regulated genes that were not detected and/or validated in the microarrays and real-time RT-PCR, respectively. There are probably also several unknown ANG II-regulated genes that were not detected due to several causes, such as absence in the microarray, lack of sensitivity, alternative splicing, etc. For these reasons, in the present work ANG II-unregulated genes are not reported, as we do not know whether they are in fact not regulated (true negatives) or we lack the ability to detect the regulation (false negatives). Special care was taken in the microarray design to account not only for hybridization replicates but also for biological replicates. Dye-swap compensation was also used in the microarray hybridizations to avoid dye-specific effects. We used a rank invariant method followed by Loess regression normalization for microarray data analysis to avoid intensity-dependent effects. SAM software was used to select regulated genes, as it avoids the assumption of normal distribution for each gene, a condition difficult to achieve due to the small number of replicates used in microarray experiments. SAM software also uses the false discovery rate to indicate the significance of each regulated gene instead of the P value, allowing a better interpretation of the results and use of resources. A brief description of the genes found to be upregulated follows.

Adrenodoxin (ferredoxin) and adrenodoxin reductase form an electron transfer system that transfers electrons from NADPH to cytochrome P-450 enzymes (36, 64). These include cytochrome P450sc (side chain cleavage of cholesterol to form pregnenolone), CYP11B1 (11β-hydroxylase, the last step in cortisol synthesis), and CYP11B2 (aldosterone synthase). ACTH increases adrenodoxin mRNA levels severalfold in bovine adrenal zona fasciculata-reticularis cells (66). Adrenodoxin mRNA is also regulated in Y-1 mouse adrenocortical cells by cAMP (3, 62) and the calcium ionophore A23187 (95). Forskolin upregulates adrenodoxin mRNA in H295 cells (95). Adrenodoxin mRNA expression in our H295R cells is rapidly and transiently induced by ANG II. The induction pattern is similar to the one observed in Y-1 cells transfected with a reporter gene, although a much slower response was observed when the endogenous level of adrenodoxin RNA was measured (62). ACTH minimally alters mRNA levels in H295R cells, consonant with reports that these cells exhibit a very low ACTH response (70, 75). When H295R cells were transfected with plasmids expressing the ACTH receptor, no ACTH response was observed, suggesting that the lack of biological response to ACTH is due to an unknown alteration in the cascade leading to the activation of PKA (70). Cycloheximide did not block the ANG II increase in adrenodoxin mRNA level.
Fig. 2. Angiotensin II (ANG II) time curve in gene expression RNA levels. H295R cells were treated with ANG II (100 nM) for different time periods, and RNA levels were quantified by real-time RT-PCR. Aldosterone was measured in cell culture supernatants by ELISA. *P < 0.05 and **P < 0.01, control vs. ANG II.
ANGIOTENSIN II EARLY REGULATED GENES IN ADRENAL CELLS

Nurr1 and Nor1 belong to the NGFI-B orphan receptor superfamily (34). The NGFI-B nuclear orphan receptor superfamily includes three members NR4A1 (comprising Nur77, NGFI-B, N10, TR3, NAK1, TIS1), NR4A2 (comprising Nurr1, NOT, RNR1, HZF-3, TINUR, TR3β), and NR4A3 (comprising Nor1, MINOR, TEC, CHN). Nur77 is highly expressed throughout the adrenal cortex, preferentially in the zona fasciculata (5, 91). ACTH rapidly induces Nur77 mRNA levels in Y-1 and mouse adrenocortical cells (20, 91). The same pattern of acute transient Nur77 mRNA level induction has been observed in bovine zona fasciculata adrenal cells with ACTH, ANG II, and KCl (25). Nur77 binds to the 21-hydroxylase (OHase) promoter, and its coexpression increases the 21-OHase gene (P450c21) promoter activity in Y-1 cells (91). However, Nur77 knockout mice do not show any alteration in ACTH, corticosterone, or adrenal P450c21 expression levels (19). Nor did Nur77 knockout mice differ from their wild-type counterparts in several paradigms for hypothalamic-pituitary-adrenal axis regulation (19). The same lack of effect due to absence of Nur77 expression was observed when thymic or peripheral T cell deaths were evaluated (56). These data suggest that more than one of the members of this gene family share the same biological activity.

Nurr1 is also expressed both in zona glomerulosa and zona fasciculata of the adrenal cortex (5, 20). In vivo ACTH treatment or capsaicin-induced stress induced Nur77 and Nurr1 mRNA throughout the whole adrenal cortex (20, 45). Nurr1 mRNA levels peaked 30 min after capsaicin-induced stress and remain elevated in the zona glomerulosa for more than 2 h, whereas Nur77 expression returned to basal levels within this time across the adrenal cortex (45). Nor1 is expressed in the rat and bovine adrenal (29), and ACTH and ANG II transiently and acutely upregulate Nor1 mRNA levels in bovine zona fasciculata cells (29). Like Nur77, Nor1 binds the P450c21 promoter and increases expression of a reporter gene under the control of the P450c21.

Recently Bassett et al. (5) reported that both Nur77 and Nurr1 enhance aldosterone synthease gene expression in H295R cells. Neither Nur77 nor Nurr1 modulated 11β-hydroxylase gene expression. This specific effect is probably due to the Ad5 and NBRE-1 sites localized only in the aldosterone synthase promoter. Both Nur77 and Nurr1 mRNA and protein levels were increased after 6-h treatment with ANG II and return to basal levels after 24-h treatment in H295R cells. Potassium- and ANG II-mediated stimulation of Nurr1 gene expression was blocked by CaM kinase inhibition. Both potassium and ANG II agonists converge at increasing intracellular calcium concentration, calmodulin, and CaM kinase to modulate adrenal aldosterone production. Nurr1 gene expression is regulated by CaM kinase, being probably a common pathway for ANG II- and potassium-mediated aldosterone production in adrenal cells.

In summary, both Nurr1 and Nor1 expression are increased by ANG II in H295R cells. Both genes peak at 3–6 h post-ANG II treatment and return to basal levels 12 h after stimulation. ANG II induction of Nurr1 expression is higher than for Nor1 (95- vs. 24-fold induction), and Nurr1 is also more responsive than Nor1 to ET-1, K⁺, and forskolin induction. Both nuclear receptors are superinduced by cycloheximide plus ANG II treatment.

C6orf37 is a newly described gene that maps to the human chromosome 6q14 (54). The cDNA sequence contains an open reading frame of 1,314 bp that encodes a 437-amino acid protein with a predicted molecular mass of 49.2 kDa. Northern blot analysis indicates that this gene is primarily expressed in the ovary, as well as in the heart, placenta, liver, colon, small intestine, leukocytes, prostate, spleen, and retina. C6orf37 gene

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Fig. 3. Aldosterone accumulation by different agents. H295R cells were treated with ANG II (0.1–100 nM), endothelin-1 (ET-1, 10 nM), K⁺ (16 mM), or forskolin (10 μM) for 3 or 24 h. Aldosterone was measured in culture supernatants by ELISA. *P < 0.01, vs. control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3 hr Treatment</th>
<th>24 hr Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.05 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>ANG II 1 nM</td>
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<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>ANG II 10 nM</td>
<td>0.60 ± 0.06</td>
<td>1.0 ± 0.10</td>
</tr>
<tr>
<td>ANG II 100 nM</td>
<td>1.0 ± 0.20</td>
<td>2.0 ± 0.40</td>
</tr>
<tr>
<td>ET-1 1 nM</td>
<td>0.20 ± 0.02</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>K⁺ 16 mM</td>
<td>0.40 ± 0.04</td>
<td>0.60 ± 0.06</td>
</tr>
<tr>
<td>Forskolin 10 μM</td>
<td>0.80 ± 0.10</td>
<td>1.2 ± 0.20</td>
</tr>
</tbody>
</table>

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homologs are present in *Rattus norvegicus*, *Mus musculus*, *Drosophila melanogaster*, and *Caenorhabditis elegans*. Protein sequence analysis predicts that c6orf37 is a soluble, globular cytoplasmic protein containing several putative phosphorylation sites highly conserved across different species. Its physiological function is still unknown, and no conserved sequence pattern resembling any other protein domain has been observed. C6orf37 mRNA is upregulated reaching its maximum level after 3-h ANG II stimulation and decreasing to basal levels 24 h after hormone treatment.

GADD45α belongs to a gene family which also includes GADD45β and GADD45γ (86). GADD45α was initially identified as a rapidly induced gene by agents that cause DNA damage (ionizing UV light, N-acetoxy-2-acetylaminofluorene,
and H$_2$O$_2$) (31). The GADD45 family of proteins are rapidly induced by genotoxic agents as well as by terminal differentiation and apoptotic cytokines (43, 57). Their pivotal roles in negative growth control are dependent or independent of p53 depending on the cell and the GADD45 protein. They are implicated in cell cycle arrest, DNA repair, apoptosis, cell survival, and innate immunity. GADD45/H9251 overexpression in primary human fibroblasts arrested the cells at the G$_2$/M boundary of the cell cycle in a p53-dependent process. But in other systems, GADD45/H9251 overexpression promotes cell survival. This indicates that the stress stimulus encountered, the cell type, its physiological state, its genetic makeup, and interaction with other proteins modulate the function of GADD45 proteins and determine whether the outcome will be cell cycle arrest, DNA repair and cell survival, or apoptotic cell death. Interacting partners of GADD45/H9251 include PCNA, cdc2, and homo- and hetero-interactions among themselves. GADD45α-null mice exhibit a phenotype similar to that of p53-deficient mice, including genomic instability, increased radiation carcinogenesis, and a low frequency of exencephalia (44). GADD45α is induced in H295R cells after 3-h stimulation by ANG II. GADD45α mRNA levels return to near basal levels 12 h poststimulation. Cycloheximide superinduces ANG II-induction of GADD45α mRNA levels, indicating that it is a primary response gene.

M-Ras (R-Ras3) is a novel member of the Ras subfamily of GTP-binding proteins that has a unique expression pattern highly restricted to the mammalian central nervous system (51, 61). Ras proteins act as molecular switches, alternating from an inactive GDP-bound state to an active GTP-bound state. Inactive GDP-bound ras proteins are activated by interaction with members of a large structurally diverse class of proteins termed guanine-nucleotide exchange factors, which catalyze the release of GDP (24, 72). M-Ras has moderate transforming activity, promotes weak extracellular signal regulated kinase (ERK) activation required for its transforming ability, and can interact weakly with a number of other Ras effectors. M-Ras promotes cell adhesion through the activation of specific integrins on the cell surface. Overexpression of M-Ras in fibroblasts induces the formation of peripheral microspikes, actin stress fibers disappearance, and numerous actin foci formation (61). These data suggest a role in actin cytoskeleton reorganization for M-Ras. M-Ras induction in H295R cells by ANG II reaches a maximum at 3–6 h. ET-1 induces M-Ras at a value of 30% the maximal obtained with ANG II.

RhoB is a low-molecular-weight GTP binding protein that belongs to the Rho GTPase subfamily. The Rho subfamily belongs to the Ras superfamily of small GTPases and is composed of three members: RhoA, RhoB, and RhoC. Rho GTPases interact with several proteins including GEFs, Rho GDP dissociation inhibitor, and GTPase activating proteins (GAPs) that positively and negatively modulate its activity (37, 49, 80, 81). Rho protein targets include rho-associated kinase/ROCK, the myosin-binding subunit (MBS) of myosin phosphatase, protein kinase N (PKN), rhotekin, citron, and p140 Dia, among many others (49, 80).

Rho was initially characterized as a regulator of actin cytoskeletal organization (78). Rho proteins are involved in a variety of functions involved in cytoskeleton regulation including smooth muscle contraction, membrane ruffling, cell motil-
ity, cell migration, neurite retraction and cell rounding, cadherin-mediated cell-cell adhesion, microvillus-like structures formation, cytokinesis, and cell transformation (49, 77, 80, 83). Microinjection studies showed that Rho stimulates and regulates the formation of actin stress fibers and focal adhesions in fibroblasts and other adherent cell types (37, 49).

The Rho/Rho-associated kinase signaling pathway is involved in ANG II-induced processes in several tissues, including premyofibril formation in cardiac myocytes (2), hypertrophy of vascular smooth muscle cells (94), and Pyk2 tyrosine phosphorylation in intestinal epithelial cells (92). In the present experiments, ANG II induced RhoB mRNA in H295R cells after 3-h treatment. Cycloheximide alone increased RhoB mRNA levels, and caused superinduction when added with ANG II. Forskolin caused a 6.5-fold increase in RhoB mRNA after 3 h incubation.

CAT-1 (SLC7A1) is a member of the system y+ cationic amino acid transporter (CAT) (22, 60, 69). Four members of the human CAT family (CAT-1, CAT-2, CAT-3, and CAT-4) have transport properties resembling that of system y+ (13). These transporters exhibit high affinity for cationic amino acids (L-arginine, L-lysine, and L-ornithine) that is sodium independent. Neutral amino acids are also transported, but with a much lower affinity and in the presence of sodium. Murine CAT-1 was described as a membrane receptor for ecotropic murine leukemia viruses (ecoR) that when expressed in an heterologous system induces cationic amino acid transport (50, 90). CAT-1 is widely expressed except in the liver. As it has been reported in iNOS- and eNOS-expressing cells, CAT-1 may be implicated in nitric oxide production by regulating arginine availability for NOS. ANG II induces the transient upregulation of CAT-1 mRNA followed by cationic amino acid transport in rat vascular smooth muscle cells (58). This ANG II-induced increase in CAT-1 mRNA is similar both in time and magnitude to that observed in our H295R cells. Nevertheless, CAT-1 mRNA may not reflect a complete picture of its regulation, since CAT activity is regulated at the transcriptional, translational, and activity level, and mRNA levels do not always reflect CAT activity (16). Although the ANG II-mediated increase in CAT-1 expression in H295R cells was less than twofold, this regulation was observed in several experiments, and a relatively low level of CAT-1 mRNA regulation has been observed in other systems. In fact, CAT-1 mRNA levels not only return to basal levels after 12 h ANG II treatment but decreases by 50% compared with time 0 levels after 24–48 h ANG II treatment. This biphasic behavior is different from all other ANG II-regulated genes described.

MBLL is a recently described protein that belongs to a gene family MBL comprising MBNL, MBLL, and MBXL. All of these proteins share homology with the Drosophila gene muscleblind (6, 28). Muscleblind proteins play essential roles in the terminal differentiation of embryonic pharyngeal, visceral, and somatic muscles, as well as ommatidial photoreceptors in Drosophila (6). MBNL was isolated by its role in binding (CUG), repeat expansions of the DM1 protein kinase (DMPK) gene in fibroblasts from DM1 myotonic dystrophy patients (27, 63). MBLL and MBXL also bind to (CUG)n repeats in cells from DM1 patients, as do all three proteins in cells from DM2 patients (28). All members of the MBL gene family present a wide variety of splice variants (28, 63). The functions of the MBL proteins are as yet unknown. They contain four CCCH zinc fingers in two pairs with the cysteines spaced CX-CX2-CX2-H-CX2-CX3-H and are localized to the nucleus. MBLL is widely distributed throughout the body with the highest levels in heart and skeletal muscle (28, 63). Of all the genes studied, the response of MBLL in our H295R cells to K+ stimulation was the greatest compared with all other genes. MBLL is a primary gene, as coinucbation with cycloheximide did not modify its induction compared with that of ANG II alone. MBLL was the only gene regulated by potassium, being upregulated 3.5-fold after 3 h of incubation.

Two spots on the microarray representing different cDNA fragments map to a novel gene encoding a hypothetical protein (FLJ45273). FLJ45273 localizes to the human chromosome 2. It has an open reading frame that encodes for a 511 amino acid polypeptide with a predicted molecular mass of 57.4 kDa and a pl of 5.5. Prediction of its subcellular localization indicates that this protein may have a mitochondrial or cytosolic localization (PSORT II, TargetP V1.0, Predotar, MitoProt II 1.0a4). This putative protein does not have charge runs, hydrophobic or transmembrane domains, repetitive structures, or multiplets (SAPS). Nor were myristoylation (NMT) or GPI-anchor (big-PI) sequences detected. Phosphorylation analysis indicates the presence of several potential high-score sites at serine, threonine, and tyrosine residues (NetPhos 2.0). FLJ45273 contains a RING zinc finger motif and a LON protease domain. The RING finger is a specialized type of Zn finger of 40–60 residues that binds two atoms of zinc and is probably involved in mediating protein-protein interactions (10, 32, 82). The RING domain is a protein interaction domain that has been implicated in a range of diverse biological processes. E3 ubiquitin-protein ligase activity is intrinsic to the RING domain of c-Cbl and is likely to be a general function of this domain; various RING fingers exhibit binding to E2 ubiquitin-conjugating enzymes (4, 33, 47). ATP-dependent Lon (La) protease is a highly conserved enzyme that is present in archaeabacteria and eubacteria, as well as in mitochondria of lower and higher eukaryotes (76, 85). Mitochondrial Lon mediates the degradation of misfolded, unassembled, or oxidatively damaged polypeptides, preserving mitochondrial function (11). Lon proteases bind DNA and are involved in controlling gene expression by either modulating transcription factor levels or mRNA transcript stability. FLJ45273 has not been studied, so its protease and DNA binding activities have not been defined. FLJ45273 mRNA was transiently upregulated by ANG II and by ET-1 in H295R cells. FLJ45273 mRNA seems to require the synthesis of other proteins for its ANG II-mediated induction, since cycloheximide completely blocks its ANG II-mediated upregulation.

A20 was first identified as a gene that was rapidly induced by tumor necrosis factor-α (TNF) in human umbilical vein cells (HUVECs) (23). A20 mRNA levels are increased within 15 min of TNF treatment and are near maximal by 1 h (23). The human A20 gene encodes a 790-amino acid protein (67). The COOH-terminal half of A20 contains seven Cysz/Cysz2 zinc fingers that are identical in the murine and human A20, except for a single substitution. Six of the zinc finger motifs are Cys-X2-Cys-X11-Cys-X2-Cys; one is Cys-X2-Cys-X11-Cys-X2-Cys. Because of this unusual spacing of cysteines, A20 is considered a new type of zinc finger protein. Costimulation with cycloheximide results in the stabilization of the A20 mRNA (23), consistent with the fact that the 3’-untranslated
region of A20 mRNA contains four copies of the canonical sequence ATTTA, which is known to confer instability on a number of short-lived transcripts (93).

The first observed biological function of A20 was that overexpression confers resistance to TNF-induced apoptosis in a number of cell lines (68). But this anti-apoptotic role is not universal; it varies with the cell line and the apoptosis inducer (7). As A20 is not a universal apoptosis inhibitor, its role is probably very early in one or more of the pathways before they converge into the common NF-κB complex (7).

A20 functions as a potent inhibitor of NF-κB-dependent gene expression and negatively regulates its own NF-κB-dependent expression (53). Overexpression of A20 blocks the activation of NF-κB by TNF, IL-1, LPS, and phorbol esters in different cell types (18, 30, 40, 46). Consistent with these results, overexpression of A20 is able to prevent TNF-induced production of NF-κB-dependent proteins such as E-selectin, I-κB, IL-6, and IL-8 (30, 40).

A20 interacts with several proteins involved in NF-κB signaling pathway including TRAF1, TRAF2, 14-3-3, ABIN2, TXBP151, LMP1, IκK/γ/NEMO, as well as itself (38–40, 52, 84, 89, 96). However, the molecular mechanism by which A20 regulates NF-κB and apoptosis is still unknown. A20 knockout mice develop severe inflammation and cachexia, are hypersensitive to LPS and TNF, and die prematurely (55). A20-deficient cells fail to terminate TNF-induced NF-κB responses. These cells are also more susceptible to TNF-mediated apoptosis. This observation suggests that A20 is critical for limiting inflammation by terminating TNF-induced NF-κB responses in vivo.

ANG II treatment of H295R cells transiently induced A20 mRNA after 3–6 h; cycloheximide caused superinduction of this gene, as has been observed in other systems.

In summary, microarray technology and real-time RT-PCR were used to identify several genes whose expression is acutely increased in the human adrenal carcinoma H295R cells by ANG II. This induction was verified and characterized by further study of mRNA transcription patterns in the H295R cells with several other aldosterone secretagogues, ET-1, ACTH, and K⁺, in addition to ANG II. The crucial role of ferredoxin, or adrenodoxin, in multiple steps of adrenal steroid synthesis by cytochrome P-450 enzymes is clear. Nur1 has been implicated in aldosterone synthesis suppression. All the other genes studied have unknown function in adrenal cells. The chronic stimulation of the zona glomerulosa results in the increase in the number of glomerulosa cells, presumably by a combination of cell proliferation and decrease in apoptosis (65). The genes described in the present paper are good candidates for both steroid synthesis and cell proliferation regulingation of adrenal cells.

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GRANTS

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