Gene expression profile in rat adrenal zona glomerulosa cells stimulated with aldosterone secretagogues

Damian G. Romero,1,2 Maria W. Plonczynski,1 Bronwyn L. Welsh,1,2 Celso E. Gomez-Sanchez,1,2 Ming Yi Zhou,4 and Elise P. Gomez-Sanchez1,2,3

1Division of Endocrinology, G. V. (Sonny) Montgomery Veterans Affairs Medical Center, and Departments of 2Medicine and 3Pharmacology and Toxicology, The University of Mississippi Medical Center, Jackson, Mississippi; and 4DNA Core, University of Missouri-Columbia, Columbia, Missouri

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Romero DG, Plonczynski MW, Welsh BL, Gomez-Sanchez CE, Zhou MY, Gomez-Sanchez EP. Gene expression profile in rat adrenal zona glomerulosa cells stimulated with aldosterone secretagogues. Physiol Genomics 32: 117–127, 2007. First published September 25, 2007; doi:10.1152/physiolgenomics.00145.2007.—The mineralocorticoid aldosterone, mainly produced by the adrenal gland, is essential for life, but an abnormally excessive secretion causes severe pathological effects including hypertension and target organ injury in the heart and kidney. The aim of this study was to determine the gene regulatory network triggered by aldosterone secretagogues in a nontransformed cell system. Freshly isolated rat adrenal zona glomerulosa cells were stimulated with the two main aldosterone secretagogues, angiotensin II and potassium, for 2 h and subjected to whole genome expression studies using multiple biological and bioinformatics tools. Several genes were differentially expressed by ANG II (n = 133) or potassium (n = 216). Genes belonging to the nucleic acid binding and transcription factor activity categories were significantly enriched. A subset of the most regulated genes was confirmed by real-time RT-PCR, and then their expression was analyzed in time curve studies. Differentially expressed genes were grouped according to their time response expression pattern, and their promoter regions were analyzed for common regulatory transcription factor binding sites. Finally, data mining with gene promoters, transcription factors, and literature databases was performed to generate gene interaction networks for either ANG II or potassium. This paper provides for the first time a complete study of the genes that are regulated, and the interaction between them, by aldosterone secretagogues in rat adrenal cells. Increasing our knowledge of adrenocortical and aldosterone synthase and gene regulation in nontransformed cell systems could lead us to a better approach for the discovery of candidate genes involved in pathological conditions of the adrenal cortex.

adrenal cortex; angiotensin; potassium; mineralocorticoid; genomics

ALDOSTERONE CONTROLS sodium and potassium balance and also influences acid-base homeostasis in vertebrates (42, 60, 61). Its major physiological targets are the epithelial cells, of which the most important are located in the distal nephron. It increases Na+ reabsorption as well as K+ and H+ excretion. Through changes in sodium balance, aldosterone influences fluid balance and blood pressure. Aldosterone secretion is increased during acute or chronic sodium depletion or fluid loss, erect postural position, and dietary potassium loading. In addition to its epithelial actions, aldosterone influences the function of the cardiovascular system by direct actions in the heart, vessels, and central nervous system (15, 19, 22).

The adrenal zona glomerulosa is localized in the outer cortex of the adrenal gland and secretes several steroids, of which the most important is the mineralocorticoid aldosterone. Aldosterone secretion is primarily under the control of the renin-angiotensin system (RAS) and extracellular potassium concentration (13, 34, 60, 61).

Angiotensin II (ANG II) not only regulates steroid secretion by zona glomerulosa adrenal cells, but it also regulates cell proliferation and cell size (hypertrophy) (38, 39, 43).

We have previously described several novel genes regulated by ANG II in the human adrenocortical cell line H295R (50). More recently, we reported a comprehensive analysis of the transcription regulatory proteins whose expression levels are modulated by ANG II and their role in the expression of the last steroidogenic enzymes of the mineralocorticoid and glucocorticoid biosynthetic pathways, aldosterone synthase and 11β-hydroxylase, respectively (51).

Although several high-throughput gene expression profiling studies have examined various aspects of adrenal gland physiology, none has focused on gene expression regulation by aldosterone secretagogues such as ANG II or potassium in normal adrenal zona glomerulosa cells.

We used freshly isolated rat adrenal cells, a well-characterized model for studying adrenal gland cell physiology and metabolism in a nontransformed cell system (29, 47), and high-throughput screening with high-density oligonucleotide microarrays, a promising tool for the discovery of regulated genes, to identify genes differentially expressed by ANG II or potassium. We analyzed gene expression patterns in time response curve studies to cluster co-regulated genes. Finally, we applied several bioinformatics methods to further characterize genes that were differentially expressed with either treatment into functional classes and to find the transcription regulatory networks involved in their regulation.

MATERIALS AND METHODS

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee of the G. V. Montgomery Veterans Affairs Medical Center. All animal protocols were performed in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Male Sprague-Dawley rats (3 mo old) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained on standard rat chow (Teklad, Harlan, catalog no. 8640; containing 0.40% sodium and 1.00% potassium) and tap water in an environment with a 12:12-h light-dark cycle. Animals were anesthetized with isoflurane, and...
adrenal glands were removed, excised of fat, and placed in ice-cold saline. Adrenal cells were isolated within an hour of tissue harvest.

**Cell preparation and incubations.** Rat adrenals were separated into capsules and cores, and adrenal glomerulosa cells were obtained from the adrenal capsules as previously described (23, 46) by a combination of enzymatic and mechanical dispersion. In short, capsules were incubated in DMEM-F12 (1:1) media without phenol red containing 0.2% collagenase CLS-1 (Worthington Biochemical, Lakewood, NJ), 0.5% fatty acid-free bovine serum albumin, and 0.02% DNase I (Sigma Chemical, St. Louis, MO). Capsules were incubated at 37°C with agitation for 15 min and decanted for 1 min, and the collagenase solution was discarded and replaced with a fresh one. Capsules were mechanically dispersed, incubated for an additional 15 min, and mechanically dispersed again. Dispersed cells were cleared of debris by decantation and filtration through gauze. Cells were extensively washed by centrifugation and resuspension in DMEM (1:1), followed by 1 ml of RT product at a cell density of 50,000 cells/ml. Incubations were performed at 37°C in a 5% CO₂ incubator for different time periods, as described below. Cells were treated with either 100 nM ANG II (American Peptide, Sunnyvale, CA) or 16 mM potassium chloride (Sigma Chemical) (final concentrations); no corrections for changes in osmolality were performed to the media in either case. At the end of the incubations, cells were resuspended and centrifuged at 10,000 g for 10 s. Cell culture supernatants were saved for aldosterone determination by ELISA as previously reported (18), and cell pellets were saved for RNA extraction. As is standard in rat adrenal zona glomerulosa cell preparations, a small degree of contamination with adrenal zona fasciculata cells (reported to be no more than 2–9% by different authors; Refs. 4, 26, 62) is unavoidable.

**RNA extraction.** Total RNA was extracted with the RNeasy Midi kit (Qiagen, Valencia, CA), on-column deoxyribonuclease digested, and quantified by UV absorbance using the Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

**Microarray analysis.** Total RNA for microarray analysis was extracted and DNase digested as previously described (50). RNA was prepared in 6-ml plates or in 0.5 ml of media in 24-well plates at a cell density of 50,000 cells/ml. Incubations were performed at 37°C in a 5% CO₂ incubator for different time periods, as described below. Cells were treated with either 100 nM ANG II (American Peptide, Sunnyvale, CA) or 16 mM potassium chloride (Sigma Chemical) (final concentrations); no corrections for changes in osmolality were performed to the media in either case. At the end of the incubations, cells were resuspended and centrifuged at 10,000 g for 10 s. Cell culture supernatants were saved for aldosterone determination by ELISA as previously reported (18), and cell pellets were saved for RNA extraction. As is standard in rat adrenal zona glomerulosa cell preparations, a small degree of contamination with adrenal zona fasciculata cells (reported to be no more than 2–9% by different authors; Refs. 4, 26, 62) is unavoidable.

**Real-time PCR.** Five micrograms of total RNA were reverse transcribed with 0.5 μg of T_{7} RNA polymerase, and Superscript III (Invitrogen, Carlsbad, CA) following the manufacturer’s suggested protocol. Primer pairs were designed with Primer3 software (55) to generate 80- to 120-bp amplicons based on microarray target sequences (NETAFFX, Affymetrix) (Table 1). GAPDH primers were previously described (52). Real-time PCR was performed with 1 μl of RT product, 1 μl of Titanium Taq DNA polymerase (Clontech, Mountain View, CA), a 1:20,000 dilution of SYBR Green I (Molecular Probes, Carlsbad, CA), 0.2 mM dNTPs and 0.1 μM each primer. Cycling conditions were 1 min at 95°C, 50 cycles of 15 s at 95°C, 15 s at 60°C, and 1 min at 72°C. Real-time data were obtained during the extension phase and threshold cycle values were obtained at the log phase of each gene amplification. PCR product quantification was performed by the relative quantification method (45) and standardized against GAPDH. Efficiency for each primer pair was assessed by using serial dilutions of RT product. Results are expressed as arbitrary units and normalized against GAPDH mRNA expression.

**Gene ontology analysis.** Gene ontologies of regulated genes were annotated using the Database for Annotation, Visualization and Integrated Discovery (DAVID 2007) (10). Gene ontology-overrepresented categories were selected with the Functional Annotation Chart with a count threshold = 2 and Expression Analysis Systematic Explorer (EASE) threshold = 0.1 under level 2 annotation (27). Functional Annotation Clustering was performed using all available annotations (up to the deepest level) in DAVID 2007. Clusters with a group enrichment score, the geometric mean (in –log scale) of a member’s P values in a corresponding annotation cluster, >1 are reported.

**Transcription factor binding site analysis.** Transcription factor binding sites (TFBS) were screened with MATCH software (17) using software (GCOS version 1.2; Affymetrix). Data were normalized by the invariant set method, smoothed by running the median, and modeled using the model-based expression with mismatch probe (PM/MM difference) for background subtraction using dChip software (version Jan. 4, 2007) (31). Differentially expressed genes were selected by ANOVA with "P < 0.005 using absolute correlation. Genes were filtered if they were called present in at least two-thirds of arrays and were considered differentially expressed for each treatment if they were regulated >1.5-fold with a P value < 0.05. Microarray hybridization data were submitted to the Gene Expression Omnibus database (National Center for Biotechnology Information; NCBI) under accession number GSE8421.

### Table 1. Real-time PCR primers

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<td>TCCTCAAGGAGAGCTTTGATA</td>
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<td>CTTCATGCTTCGACCTCTT</td>
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<tr>
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Fig. 1. Gene ontology classification of differentially expressed genes by ANG II or potassium in rat adrenal glomerulosa cells. Genes were classified according to their biological process (A) or molecular function (B). The various categories of functions are listed/indicated. Functional categories significantly enriched are denoted with an asterisk.

Table 2. ANG II and potassium differentially expressed genes (>4-fold)

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Affymetrix Probe</th>
<th>Microarray, Fold Change</th>
<th>Real-Time RT-PCR, Fold Change</th>
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<td>Potassium</td>
</tr>
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<td>0.54±0.07</td>
</tr>
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<td>0.70±0.06</td>
</tr>
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<td>Spr1a</td>
<td>1371248_at</td>
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<td>2.31±0.12</td>
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<td>Dcamkl1</td>
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<td>1.82±0.30</td>
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<td>0.62±0.07</td>
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<td>3.45±0.11</td>
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<td></td>
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<tr>
<td>Ypel4</td>
<td>1389715_at</td>
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<td>6.22±0.81</td>
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<td>1387410_at</td>
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<td>5.09±0.31</td>
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<tr>
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<td>4.36±0.30</td>
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<td>1.42±0.04</td>
<td>4.06±0.29</td>
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<td>1380028_at</td>
<td>1.02±0.02</td>
<td>0.22±0.01</td>
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</table>

Values are means ± SE. Boldface values are significant genes, P < 0.005.
high-quality vertebrate matrices from the Transfac database (37) and the option to minimize the sum of false-positive and -negative error rates.

Overrepresented TFBS were obtained by use of Transcription Factor Matrix Explorer software with the default parameters (9). Overrepresented TFBS were considered significant if they were present in all the cluster sequences and had a P value < 0.0005.

Promoter and gene interaction analysis. Genes and their promoters were analyzed for interactions at the literature and promoter level using Bibliosphere PathwayEdition software version 6.26 (Genomatix) as previously described (51). Literature data mining was performed with all regulated transcription regulatory genes based on the >15 million NCBI PubMed collections of abstracts. Co-citation was set at the maximum stringency, showing genes connected by literature mining only if they are co-cited at least three times and in the same sentence of the abstract with the order “gene... word function... gene.”

RNA extraction and amplification. Total RNA from tissue response curve incubations was extracted with the RNeasy Micro kit (Qiagen), on-column deoxyribonuclease digested and quantified with the RiboGreen RNA Quantitation kit (Invitrogen).

To overcome the limited amount of total RNA obtained from rat adrenal cell incubations, we performed whole transcriptome amplification using the Transplex Whole Transcriptome Amplification (WTA) kit (Rubicon Genomics, Ann Arbor, MI), which consists of two steps, reverse transcription and library amplification. For reverse transcription, 50 ng of total RNA were supplemented with 2.5 μl of library synthesis buffer and 2.5 μl of library stabilization buffer, heated at 70°C for 5 min, and immediately cooled in ice. One microliter of library synthesis enzyme was added, and samples were incubated at 24°C for 15 min, followed by 2 h at 42°C. The reaction was terminated by incubation at 95°C for 5 min. For library amplification, reverse transcription reactions were split into four tubes and PCR amplified using Titanium Taq DNA polymerase (Clontech, Palo Alto, CA). Samples were incubated at 95°C for 3 min, followed by cycles of 94°C for 20 s and 65°C for 5 min. Reaction progress was monitored in real time using SYBR Green I (Invitrogen) on iCycler IQ (Bio-Rad, Hercules, CA). Reactions were stopped two to three cycles after the plateau phase was reached (16–19 cycles). PCR products were pooled and purified with QIAquick PCR Purification kit (Qiagen).

Statistics. All results are expressed as means ± SE. Multiple experimental groups under one treatment were analyzed by one-way ANOVA, followed by Dunnett’s contrasts against the control group. Multiple experimental groups under two treatments (or a 1-treatment time series) were analyzed by two-way ANOVA, followed by Bonferroni contrasts. Correlations were analyzed by the Pearson correlation coefficient. Statistical calculations were performed with Graphpad Prism package version 4.03 (Graphpad Software, San Diego, CA). All experiments were repeated at least three times. Differences were considered significant if P < 0.05.

RESULTS

Global analysis of regulated genes by aldosterone secretagogues. To study zona glomerulosa adrenal cell gene expression regulation under aldosterone stimulatory conditions, we treated freshly isolated rat adrenal cells with the two main in vitro aldosterone secretagogues, ANG II and potassium, for 2 h. Total RNA from three independent experiments was isolated, labeled, and hybridized to whole genome high-density oligonucleotide microarrays (Affymetrix Rat Genome 230 2.0). Aldosterone secretion by adrenal cells in cell culture supernatants was measured to assess treatment efficacy. Both ANG II (27.3 ± 5.0 ng aldosterone/ml, P < 0.05) and potassium (32.6 ± 2.9 ng aldosterone/ml, P < 0.05) significantly increased aldosterone secretion over basal conditions (8.4 ± 0.9 ng aldosterone/ml).

Probe level intensities were analyzed by ANOVA, and genes differentially expressed >1.5-fold for each treatment were selected. ANG II and potassium treatment caused differential expression of 133 and 216 genes, respectively. Differentially expressed genes for each treatment were classified according to their gene ontology categories for “biological process” and “molecular function.” Categories representing <=2% of the genes were grouped in “other” for easier visualization (Fig. 1).

Although potassium regulated a significantly higher number of genes, differentially expressed genes by both ANG II and potassium present a similar pattern when classified according to the biological process in which they are involved or the molecular function that they represent.

We then determined whether any of these categories were significantly enriched, i.e., high ratio of regulated genes to total number of genes for any particular category, with either treatment. Several categories at the biological process or molecular function classification were significantly enriched by ANG II or potassium (Fisher exact P value ≤0.05). These categories are indicated in Fig. 1.

To study differentially expressed genes with a wider biological point of view, we performed “Functional Annotation Clustering” using all available annotations in the DAVID database. Gene clusters significantly enriched are reported in Supplemental Table S1 (supplemental data are available at the online version of this article) with their enrichment scores and genes. We found that ANG II and potassium generate three and nine enriched gene clusters, respectively.

Gene level analysis of regulated genes by aldosterone secretagogues. Supplemental Tables S2 and S3 show the genes that were differentially expressed with either ANG II or potassium. We selected the genes regulated more than fourfold to confirm by real-time PCR. Table 2 lists these genes with their fold increase as detected by microarray and real-time PCR. A highly statistical correlation was observed (Pearson r = 0.882,
GENE EXPRESSION IN RAT ADRENAL ZONA GLOMERULOSA CELLS

1. 1377226_at
   ![Graph 1]

2. Serpinb2
   ![Graph 2]

3. Ypel4
   ![Graph 3]

4. Bop1
   ![Graph 4]

5. Aldosterone
   ![Graph 5]
To further study aldosterone secretagogue-mediated gene expression in rat adrenal cells. Supplemental Table S4 lists the genes differentially expressed by ANG II and potassium.

Time curve analysis of regulated genes by aldosterone secretagogues. To further study aldosterone secretagogue-mediated gene expression regulation in rat adrenal cells, we performed time course studies. Freshly isolated rat adrenal cells were incubated for increasing time periods (0.5–4 h) in the presence of ANG II (100 nM) or potassium (16 mM). As a control of the efficacy of the treatments and responsiveness of the cells, we quantified aldosterone in cell culture supernatants. ANG II and potassium time dependently increased aldosterone secretion by rat adrenal cells (Fig. 3, bottom right). We then measured gene expression levels of the most upregulated (>4-fold) genes identified in each treatment. To overcome the limited amount of total RNA obtained from rat adrenal cell incubations, we performed whole transcriptome amplification using the Transplex WTA kit (Rubicon Genomics). Transplex WTA is a validated nonbiased amplification technique that allows nanogram amounts of RNA to be amplified ~100- to 1,000-fold for quantitative PCR and microarray analysis (44, 65). Figure 3 shows the gene expression time response curves for the most upregulated genes by ANG II and potassium.

Promoter analysis of regulated genes by aldosterone secretagogues. To analyze the promoters of ANG II- and potassium-regulated genes in rat adrenal cells, we classified the most upregulated genes according to the temporal expression pattern observed with both ANG II and potassium. There were five different groups containing two to four genes each. Assuming that genes that present a similar expression pattern are controlled by the same transcription factors, we obtained the promoter region of these genes and performed an analysis of the TFBS observed in their promoters. Supplemental Table S5 lists the TFBS found in each subset of regulated genes. One limitation of this analysis is the presence of many false-positive hits. To overcome this limitation, we analyzed the TFBS overrepresented in each subset of regulated genes. Table 3 lists overrepresented TFBS along with their matrices and overrepresented in each subset of regulated genes. One limitation of this analysis is the presence of many false-positive hits. To overcome this limitation, we analyzed the TFBS overrepresented in each subset of regulated genes. Table 3 lists the TFBS found in each subset of regulated genes. One limitation of this analysis is the presence of many false-positive hits. To overcome this limitation, we analyzed the TFBS overrepresented in each subset of regulated genes. Table 3 lists overrepresented TFBS along with their matrices and P values for each subset of regulated genes.

Literature mining of regulated genes by aldosterone secretagogues. Genes and their promoters were analyzed for interactions at the literature and promoter level using Bibliosphere Pathway Edition software (59). Figures 4 and 5 show the gene-gene interaction networks of differentially expressed genes and the transcription factors known to modulate them, based on previously reported gene interactions in the literature. In the network of ANG II-regulated genes, Cdkn1a is a central node that also modulates other subnodes, including Runx1, Ptgs2, Creb1, Fos, and Nkfb1. In the network of potassium-regulated genes, Nr4a1 is a central node that also modulates other subnodes, including Junb, Fos, Nkfb1, Creb1, Egr1, and Ptgs2.

Table 3. Overrepresented TFBS in clusters of co-regulated genes

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Cluster nos. refer to gene clusters of similar time course expression pattern as nos. matching the ones indicated in Fig. 3. Matrix refers to the vertebrate Transfac matrix table giving the nucleotide distribution matrix for the binding sites of the specified transcription factors. TFBS, transcription factor binding sites.
Fig. 4. ANG II differentially expressed genes and their network of transcription factors. Regulated genes are highlighted in an orange (upregulated)-to-blue (downregulated) color scale. Transcription factors known to regulate or interact with differentially expressed genes are enclosed in white boxes. If a gene that codes for a transcription factor is connected to a gene that is known to contain a binding site for this transcription factor in its promoter, the connecting line is colored green over one-half of its length near the gene containing the binding site. Arrows symbolize interaction between genes. Truncated lines symbolize inhibition. The length of the connection lines reflects the no. of abstracts in which the genes are co-cited; shorter lines mean more co-citations.
Fig. 5. Potassium differentially expressed genes and their network of transcription factors. Regulated genes are highlighted in an orange (upregulated)-to-blue (downregulated) color scale. Transcription factors known to regulate or interact with differentially expressed genes are enclosed in white boxes. If a gene that codes for a transcription factor is connected to a gene that is known to contain a binding site for this transcription factor in its promoter, the connecting line is colored green over one-half of its length near the gene containing the binding site. Arrows symbolize interaction between genes. Truncated lines symbolize inhibition. The length of the connection lines reflects the no. of abstracts in which the genes are co-cited; shorter lines mean more co-citations.
GENE EXPRESSION IN RAT ADRENAL ZONA GLOMERULOSA CELLS

DISCUSSION

In this study, we report for the first time a high-throughput screening of differentially expressed genes modulated by ANG II or potassium in freshly isolated rat adrenal zona glomerulosa cells using high-density oligonucleotide microarrays. We performed a series of bioinformatics analyses to better characterize the sets of genes regulated by both aldosterone secretagogues.

Differentially expressed genes were classified according to the biological process in which they are involved or the molecular function that they perform. Several biological process categories were significantly enriched with both ANG II and potassium treatment, including the categories of positive regulation of biological process, regulation of cellular process, and regulation of physiological process. While some biological process categories were significantly enriched with either ANG II or potassium treatment, several of their members were also regulated with the other treatment, although the category enrichment did not reach statistical significance. When differentially expressed genes were classified according to their molecular function, two categories (nucleic acid binding and transcription factor activity) were significantly enriched by either treatment, indicating that these genes are highly regulated by aldosterone secretagogues in adrenal cells. This data suggest that a complex transcription regulatory network is triggered by ANG II and potassium to regulate several biological, cellular, and physiological processes in rat adrenal cells.

We validated our microarray results independently with real-time RT-PCR. We found an excellent correlation on gene expression levels between microarray and real-time RT-PCR data, validating the use of microarray gene expression data for high-throughput screening of differentially expressed genes under our assay conditions. It is important to stress that primer pairs used for real-time RT-PCR were designed using the reported target sequences used for microarray probe design rather than using database accession numbers to avoid any confounding difference due to annotation problems.

For the set of most differentially expressed genes, we studied the transcription factors that may mediate the effects observed in gene expression. A limitation of present transcription factor analysis is the generation of lists with numerous false positives. One way to overcome this limitation is to study sets of co-regulated genes under the assumption that genes whose expression regulation follows a similar pattern over time will be modulated by the same set of transcription factors. To classify the differentially expressed genes in sets of co-regulated genes, we performed time course studies of the most regulated genes with either ANG II or potassium. Time course studies allowed us to classify the set of the 19 most regulated genes with either ANG II or potassium into five subsets according to their temporal gene expression pattern with ANG II and potassium. We then obtained their promoter regions to study the TFBS overrepresented in each cluster of co-regulated genes. Since each cluster has a small number of genes, we used very stringent conditions to select overrepresented TFBS.

To analyze the interaction between the differentially expressed genes and the transcription factors that modulate their expression, we constructed gene regulatory networks using our gene expression data in addition to known gene product interactions and the presence of TFBS on the promoter regions already reported in the literature. We observed that there are strong similarities between main gene nodes of the gene regulatory networks triggered by ANG II and potassium stimulation of adrenal cells. Although the networks look slightly different because of the observed regulation of different gene sets with both aldosterone secretagogues, several gene nodes (Creb1, Fos, Nfkb1, and Ptg2) were present in both networks. Cdkn1a and Nr4a1, which are located in central positions in the ANG II- and potassium-triggered networks, respectively, are also upregulated by the other aldosterone secretagogue, although their relative positions in the two networks of regulated genes are different. RunX1, which may interact with Cdkn1a, was exclusively regulated by ANG II.

The genes located in the central nodes of ANG II- and potassium-triggered networks have been found previously to be expressed in the adrenal gland and play important roles in adrenal gland physiology and pathophysiology, validating our whole transcriptome approach using bioinformatics tools. NR4A1 (also known as Nur77, NGFI-B, N10, TR3, NAK1, TIS1) belongs to the nuclear orphan receptor superfamily that also includes NR4A2 and NR4A3. NR4A1 is highly expressed throughout the adrenal cortex in humans and rodents (3, 25, 28, 33, 69). NR4A1 levels are increased at the mRNA and protein levels by ANG II, potassium, and ACTH in multiple adrenocortical cell experimental models (8, 14, 25, 69). NR4A1 upregulates the expression of multiple steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase, 21-hydroxylase, and aldosterone synthase (2, 3, 36, 69). NR4A1 expression has been reported to be significantly increased in aldosterone-producing adenomas (1, 33). Cdkn1a (also known as p21, Cip1, Waf1, Sdi1, Cap20, p21Cip1) has been implicated in cell cycle regulation, through inhibition of the cyclin-dependent protein kinase and DNA replication, but it is also associated with transcriptional regulation, apoptosis, and senescence (6, 7, 70). Cdkn1a is expressed in the human and rodent adrenal gland (11, 12). Cdkn1a expression is highly upregulated by adrenocortical cell dispersion and culture in vitro (72) and adrenal gland ischemia/reperfusion, sepsis, and acute pancreatitis in vivo (11, 12).

Osmolality regulates adrenal gland cell physiology, including cell volume, surface area, and steroidsogenesis (24, 35, 57, 58). Osmolality inversely modulates ANG II- or potassium-mediated aldosterone secretion in perfused adrenal glands and in isolated adrenal cells (32, 35, 57, 58, 64). Osmolality effect is more pronounced at suboptimal stimulatory concentrations of ANG II or potassium, while it has no significant effects under basal conditions or maximal stimulatory doses of the mentioned aldosterone secretagogues (32, 56, 57, 67). Since our experimental setup was not specifically designed to test the effect of osmolality, we did not control for it, although, undoubtedly, addition of potassium caused an increase in media osmolality. Increased osmolality decreases aldosterone secretion, although its effect is minimal at the potassium concentrations used in our study (32), and our results indicate a stimulatory effect on aldosterone secretion similar to a maximal ANG II stimulatory dose. Although potassium treatment probably has an effect on cell volume, and some of the effects on gene expression may reflect a physiological response to this, it also has a strong aldosterone stimulatory effect.

Aldosterone exerts its effect not only in epithelial target tissues, where its main function is regulation of sodium/
potassium and water balance, but also in nonepithelial tissues such as the brain and the heart. In experimental animal models, aldosterone acts both peripherally and centrally to increase blood pressure (16, 20, 21). Furthermore, aldosterone administration in the presence of an elevated salt intake causes severe organ injury in the heart and kidney (5, 48, 49). In humans, high aldosterone levels have been associated with severe target organ injury in the heart and the kidney of hypertensive patients with primary aldosteronism (53, 54, 63). In addition, excess aldosterone in nonhypertensive patients has been reported to correlate with an increased risk to suffer an increase in blood pressure or develop hypertension (41, 66).

ANG II and potassium have rapid and long-lasting aldosterone secretagogue effects (30, 34, 40, 61, 71). Rapid aldosterone secretagogue effects are mainly mediated by a rapid increase in cholesterol transport to the inner mitochondrial membrane, mediated by the steriodogenic acute regulatory (STAR) protein. Long-lasting aldosterone secretagogue effects are mainly mediated by regulation of steriodogenic enzyme expression, as well as STAR protein. We think that the genes that we found differentially expressed by ANG II and potassium in rat adrenal cells are probably involved in the regulatory network that modulates long-lasting aldosterone secretion but not in the rapid secretagogue effect, since most genes are modulated during or after the raise in aldosterone secretion. Long-lasting aldosterone secretion regulation is critically important under physiological (i.e., sodium deprivation) and pathophysiological (i.e., primary aldosteronism) conditions.

In summary, we have reported the genes that are differentially expressed by the aldosterone secretagogues ANG II and potassium in freshly isolated rat adrenal glomerulosa cells. Using multiple bioinformatics analysis tools, we described the gene interaction networks and the transcription factors triggered by both steroid secretion stimuli. We have shown that, despite the fact that ANG II and potassium differentially regulate different subsets of genes, both aldosterone secretagogues modulate similar gene regulatory networks. A better understanding of the gene interaction networks and the transcription factors modulated by the aldosterone secretagogues triggered under physiological conditions would allow us to further refine the search of candidate genes and consequent therapies addressing the mitigation of deleterious effects of excess aldosterone-mediated target organ injury and hypertension. We expect that the present report will promote research by other groups to further elucidate the role of reported genes in adrenocortical cell function.

GRANTS

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