Early transcriptional effects of aldosterone in a mouse inner medullary collecting duct cell line

Michelle L. Gumz, Michael P. Popp, Charles S. Wingo, and Brian D. Cain

Department of Biochemistry and Molecular Biology and Interdisciplinary Center for Biotechnology Research, University of Florida, and Department of Veteran Affairs Medical Center, Gainesville, Florida 32610-0245

Submitted 2 October 2002; accepted in final form 21 May 2003

Gumz, Michelle L., Michael P. Popp, Charles S. Wingo, and Brian D. Cain. Early transcriptional effects of aldosterone in a mouse inner medullary collecting duct cell line. Am J Physiol Renal Physiol 285: F664–F673, 2003.—The mineralocorticoid aldosterone is a major regulator of Na\(^+\) and acid-base balance and control of blood pressure. Although the long-term effects of aldosterone have been extensively studied, the early aldosterone-responsive genes remain largely unknown. Using DNA array technology, we have characterized changes in gene expression after 1 h of exposure to aldosterone in a mouse inner medullary collecting duct cell line, mIMCD-3. Results from three independent microarray experiments revealed that the expression of many transcripts was affected by aldosterone treatment. Northern blot analysis confirmed the upregulation of four distinct transcripts identified by the microarray analysis, namely, the serum and glucocorticoid-regulated kinase (SGK), connective tissue growth factor, period homolog, and preproendothelin. Immunoblot analysis for preproendothelin demonstrated increased protein expression. Following the levels of the four transcripts over time showed that each had a unique pattern of expression, suggesting that the cellular response to aldosterone is complex. The results presented here represent a novel list of early aldosterone-responsive transcripts and provide new avenues for elucidating the mechanism of acute aldosterone action in the kidney.

kidney; SGK; period homolog; connective tissue growth factor; endothelin-1

The mineralocorticoid aldosterone is released by the cells of the adrenal zona glomerulosa in response to stimulation of the renal juxtaglomerular apparatus or changes in Na\(^+\) or K\(^+\) concentrations. Aldosterone acts directly to increase Na\(^+\) absorption in tight epithelia, including the renal collecting duct. Increased Na\(^+\) reabsorption expands blood volume, thereby leading to an increase in central venous filling pressure, cardiac output, and systemic arterial blood pressure (21). In this capacity, aldosterone plays a critical role in regulating blood pressure. To date, all cases of inherited hypertension result from abnormal regulation of aldosterone or its downstream effects (4, 31).

Known molecular targets of aldosterone action include the basolateral Na\(^+\)-K\(^+\)-ATPase and the apical epithelial sodium channel (ENaC), both of which are critical for Na\(^+\) absorption. Aldosterone has both short- and long-term effects on these ion transporters. The transcriptional effects of aldosterone on ENaC and the Na\(^+\)-K\(^+\)-ATPase occur after 4 h; these late effects of aldosterone on ion transport are well characterized (34). The more immediate transcriptional effects of aldosterone have not been fully investigated, but certain candidate genes have been identified. For example, aldosterone induces the expression of the serum and glucocorticoid-regulated kinase (SGK) as soon as 30 min after hormone treatment (19, 27). SGK has been linked to increases in both the number and the activity of ENaC (5). Expression of SGK in Xenopus laevis oocytes results in a threefold increase in the number of functional ENaCs at the cell surface (2). SGK-mediated phosphorylation of the ubiquitin ligase Nedd4 leads to inhibition of ENaC subunit degradation and therefore an increase in ENaC activity (7, 27). Aside from SGK, there is limited information on early aldosterone-responsive genes. Serial analysis of gene expression technology has recently been used to identify 34 aldosterone-induced transcripts and 29 aldosterone-repressed transcripts in a mouse kidney cortical collecting duct cell line after 4 h of aldosterone treatment (24). However, the acute transcriptional effects of aldosterone should occur in a much shorter time frame.

Studies with primary cultures derived from the inner medullary collecting duct (IMCD) have suggested that this is a target epithelium for the action of aldosterone and may be an important terminal site of Na\(^+\) absorption and acid secretion in the collecting duct (28, 29, 35). There is a dramatic increase in Na\(^+\) transport in cultured IMCD cells in response to aldosterone (12, 13). However, the factors that mediate the acute effects of aldosterone largely remain to be defined.

Previous studies that have examined the early aldosterone-responsive genes used nonmammalian cell lines or considered responses several hours after exposure to the hormone (24, 30). Here, we report the use of microarray technology to analyze a mouse kidney IMCD cell line (mIMCD3) for acute aldosterone regulation after 1 h of exposure to the hormone. Numerous, previously unreported aldosterone-regulated tran-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: B. D. Cain, Dept. of Biochemistry and Molecular Biology, PO Box 100245, Gainesville, FL 32610-0245 (E-mail: bcain@biochem.med.ufl.edu).

First published May 27, 2003; 10.1152/ajprenal.00353.2002.
scripts have been identified using this method, and the
regulation of selected genes has been verified using
traditional biochemical approaches. These findings
represent a novel list of early aldosterone-regulated
transcripts.

MATERIALS AND METHODS

Tissue culture. mIMCD3 cells between passages 15 and 25
were used for all experiments. Cells were grown in a T-75
flask (Corning) in DMEM-F-12 media plus 10% FBS (Invitro-
gen) before being plated on collagen-coated Costar Trans-
well-COL inserts (Fisher) in DMEM-F-12 plus 10% FBS.
Cells were grown 1 day past confluency. Twenty-four hours
before treatment with aldosterone, the media were changed
to phenol red-free DMEM-F-12 (Invitrogen) plus 10% char-
coal/dextran-stripped FBS (Biosource). Cells grown for this
length of time on Transwell-COL inserts typically exhibit a
transepithelial resistance of 703.3 ± 25.4 Ω cm² (Xia S.-L.,
personal communication). Cells were treated for varying
lengths of time with vehicle (ethanol) or aldosterone (Sigma)
at concentrations ranging from 10⁻⁶ to 10⁻³ M. Inhibitor
studies were performed using 1 μM mifepristone (RU-486)
and/or 1 μM spironolactone (Sigma). For the microarray
experiment, cells were treated for 1 h with vehicle or 10⁻⁶ M
aldosterone.

RNA isolation. Total RNA was isolated using TRIzol re-
agent (Invitrogen) essentially according to the manufactur-
er’s instructions with the following modifications. Cells were
lysed directly on the Transwell-COL inserts for 5 min using
1 ml of TRIzol reagent/insert. The organic and aqueous
phases were separated by centrifugation at 3,200 g for 20
min. RNA was precipitated from the aqueous layer by addi-
tion of 3 ml of isopropanol. The solution was allowed to sit
at room temperature for 15 min, and then the RNA was pelleted
by centrifugation at 3,200 g for 15 min. The RNA pellet was
washed in 6 ml of 75% ethanol and resuspended in 100 μl
diethyl pyrocarbonate-treated water.

RT-PCR. Two micrograms of total RNA were used in a
reverse transcriptase (Superscript II, Invitrogen) reaction
primed with random decamers (Ambion). Of the RT reaction,
25% was used as a template in the subsequent PCR reaction.
Primers MG24 and MG25 (GCCAGATCAGCCTTCAGTT-
CAGC) were designed based on the published mouse mineralocorticoid receptor
(MR; AW190225) to amplify a 238-bp product. Primers MG81
and MG82 (GTGTCTCTCCCCACCCTCGCAGC/CTGCGC-
CCCCGTCTAGTTGCAAGGC) were designed to the publi-
ished sequence of the α-subunit of the ENaC (ENaC-α;
AF112185) to amplify a 505-bp product. Primers MG38
and MG84 (CTGGAAATCACCAAGGCCCACACG/CAGGAACT-
CTGGCCTTGCCG; CTGF KF3/4 CCCCTGTCCGAATCC-
ATGCTG) were designed based on the published mouse
mineralocorticoid receptor (MR) sequence of 11β-hydroxysteroid dehydrogenase type 2
(11βHSD2; NM_000829) to amplify a 480-bp product. PCR
was performed using Taq PCR Master Mix plus Q solution
(Qiagen) and the following cycling parameters: 94°C × 5 min
preheat; 25 cycles of 94°C × 30 s, 62.5°C (ENaC-α) or 61.8°C
(11βHSD2) or 56.6°C (MR) × 30 s, 72°C × 1 min; and 72°C ×
10-min final extension. Products were purified from a 1%
agarose gel and either cloned into the TA cloning vector
(pCR2.1, Invitrogen) or sent directly for sequence analysis.
Probes for connective tissue growth factor (CTGF), period
homolog, sgk, and endothelin were prepared in the manner
described above (Table 1). Primer sets were as follows: sgk
MG34/35 CTCCTCAACCCCTACGCGCAAC/CTTCTCCAGAG-
GTGCCTTGGCC; CTGF KF3/4 CCCCTGTCGAATCCAG-
GGCTCA/GCGGACGTTCATGCCTGATAG; period homolog
MG52/53 CACCGGTCGGGAGAGCTTCG/GGCAGTGGTCG-
GAGCTGCGG; and preproendothelin MG54/54
GTTGCGTGAATCTCCAGGGCTC/TCTACGTTCATCAACT-
CCGCAGAAC.

Affymetrix GeneChip. The murine genome array U74Av2
was purchased from Affymetrix through the Interdisci-
plinary Center for Biotechnology Research at the University of
Florida. Using total RNA from aldosterone- or vehicle-
treated cells, first- and second-strand syntheses were per-
formed using Invitrogen reagents according to the protocol
provided in the Affymetrix GeneChip Expression Analysis
Manual. In vitro transcription reactions were then carried
out using the BioArray High Yield RNA Transcript Labeling
Kit (ENZO). Biotin-labeled CTP and UTP were included in
the reaction cocktail. Fragmentation of the eRNA, hybridiza-
tion, staining, and scanning of the microarray were per-
formed according to the GeneChip Expression Analysis Man-
ual provided by Affymetrix. Three independent microarray
experiments were performed.

Affymetrix GeneChip Expression Analysis. Analysis of in-
tensity data was performed using Microarray Suite Version 4
(MAS4; Affymetrix). Global scaling was applied to all arrays
such that the mean intensity of each array was equivalent. In
global scaling, the raw signal value of each probe cell is
multiplied by a scaling factor. The scaling factor is deter-
mined by first calculating the mean intensity of each array that is
equivalent to the mean raw signal value, minus
background, of probe cells, excluding the highest and lowest
2% of values. The mean intensity is multiplied by the scaling
factor to equal the target intensity. The target value of all
chips was 2,500. The scaled signal from each probe cell was used
to generate a quantitative hybridization signal for each
gene with MAS4. MAS4 was also used to perform comparison
expression analyses to examine the intensity data between
two different arrays. Initially, a comparison expression anal-
ysis was performed for two samples, untreated and treated,
where the untreated cells served as the baseline. MAS4
algorithms, which use both qualitative and quantitative
metrics, produced a list of differentially expressed genes.

Table 1. Probe generation and comparison of fold-induction values

<table>
<thead>
<tr>
<th>Transcript</th>
<th>GenBank</th>
<th>Microarray Fold-Change</th>
<th>Northern Fold-Change</th>
<th>Real-Time PCR Fold-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>sgk</td>
<td>AF205855</td>
<td>3.1 ± 0.12</td>
<td>5.1 ± 0.7*</td>
<td>5.2 ± 0.7‡</td>
</tr>
<tr>
<td>CTGF</td>
<td>M70642</td>
<td>2.1 ± 0.10</td>
<td>3.2 ± 0.5†</td>
<td>1.0 ND</td>
</tr>
<tr>
<td>Period homolog</td>
<td>AF022992</td>
<td>4.1 ± 0.20</td>
<td>7.9 ± 3*</td>
<td>7.2 ± 1.5‡</td>
</tr>
<tr>
<td>Preproendothelin</td>
<td>U35233</td>
<td>2.5 ± 0.23</td>
<td>6.2 ± 1.7†</td>
<td>3.0 ± 0.3§</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3 except for connective tissue growth factor (CTGF; n = 2) and serum and glucocorticoid-regulated kinase (sgk; n = 6), calculated using Affymetrix Suite, version 4 (microarray fold-change) and densitometry values from Northern blot analysis. Of human sgk-1, sgk-2, and sgk-3, a BLAST search indicated that mouse sgk is most similar to human sgk-1. ND, not done. * P < 0.001, † P < 0.1, ‡ P < 0.01.
The list was further filtered to include only genes whose expression was altered at least twofold. The experiment was repeated two additional times in independent RNA samples isolated from untreated and treated cells. The scaled signal values from all six hybridization experiments have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). The accession numbers are GSM6603, GSM6604, GSM6605, GSM6606, GSM6607, and GSM6623. The data series is represented by the accession number GSE4343.

Northern blot analysis. Northern blot analysis was done according to the method of Davis et al. (6). The generation of 32P-labeled probes was performed using the Redi Prime II random labeling kit (Amersham) and 20 ng of template DNA. Blots were washed at 65°C three times for 15 min in wash solution (20 mM Na2HPO4, 1% SDS, pH 7.2). After the washes, blots were exposed to Kodak Biomax MS film for an appropriate length of time. A probe for the mRNA of GAPDH was used to control for loading.

Real-time RT-PCR. Reactions were performed using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) according to the manufacturer's instructions. Cycling parameters were as follows: 48°C × 30 min, 95°C × 10-min pre soak; 45 cycles of 95°C × 15 s, 60°C × 1 min. Primers and TaqMan probes were generated via Assays-by-Design (Applied Biosystems). The sequences of the primers and probes were as follows: sgk forward CGCCCAAGTCCCTCCTACACAA, sgk reverse TTGCCGTTGAGGTTGGGA, sgk probe 6FAM TCAACCTGGTCGCTC MGBNFQ; preproendothelin forward TGCACCTGGAGCATCATCGT, preproendothelin reverse CCTCAGCTTACATGATCGA, preproendothelin probe 6FAM AACACTCCGAGCGC MG-BFNQ; period homolog forward CCAGGTCTGTTGATTA-AATTGATCA, period homolog reverse GGCTTTTGGAGG-TCTGGATAAA, period homolog probe 6FAMTCAGAGA-CAGGGCTCT MGMFQ. As a negative control, TaqMan Rodent GAPDH Control Reagents were used to perform real-time RT-PCR for GAPDH. Reactions were carried out in a DNA Engine Opticon 2 Continuous Fluorescence Detector, and data were analyzed using Opticon Monitor 2 software (MJ Research).

Western blot analysis. mIMCD3 cells were treated as described above with aldosterone or vehicle for 6 h. Cells were trypsinized and collected in PBS. After 5 min of centrifugation, cell pellets were suspended in 1 ml cell lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% Nonidet P-40). One hundred fifty micrograms of total cellular protein were run on an 18% Tris-glycine SDS-PAGE gel. Proteins were electrophoretically blotted onto nitrocellulose in transfer buffer [25 mM Tris-base, 192 mM glycine, 20% (vol/vol) methanol, pH 8.3]. Blocking was performed in 5% nonfat milk (Bio-Rad) in TBS plus 0.1% Tween at room temperature for 1 h. To detect endothelin, either a rabbit polyclonal antibody (AB2380, Chemicon) or a mouse monoclonal antibody was used (E0771, Sigma), both at a dilution of 1:1,000. The primary antibody incubation was done in 5% nonfat milk in TBS plus 0.1% Tween overnight at 4°C. Secondary antibody incubation was performed using a 1:5,000 dilution of horseradish peroxidase-linked donkey anti-rabbit or sheep anti-mouse immunoglobulin for 1 h at room temperature in 5% nonfat milk in TBS plus 0.1% Tween. After the secondary antibody incubation, blots were washed three times for 15 min in TBS plus 0.1% Tween (Sigma). Chemiluminescence was used to detect antibody binding via the enhanced chemiluminescence system (Amersham).

Statistical analysis. Values are means ± SE. A two-way ANOVA was performed on the real-time PCR data to compare fluorescence intensity at each time point with control. The Dunnett test for error protection was used with a confidence interval of 95%. A one-way ANOVA was performed on the fold-change data for the inhibitor study to compare each condition with control. The least significant difference test for error protection was used with a confidence interval of 90%. A one-way ANOVA with the Dunnett test for error protection and a confidence interval of 95% was used to evaluate the Western blot analysis fold-change data.

RESULTS

To demonstrate that the mIMCD3 cell line had retained properties reflecting collecting duct cells, the presence of the ENaC-α was investigated as a representative collecting duct-specific transcript (19). RT-PCR was performed to amplify a 505-bp fragment of ENaC-α (Fig. 1). The resulting product was gel purified and sequenced; the product was identical to the published ENaC-α sequence. The presence of this transcript indicated that mIMCD3 cells were indeed representative of the collecting duct.

Next, the aldosterone responsiveness of mIMCD3 cells was assessed. RT-PCR was performed to amplify 480- and 238-bp fragments of 11βHSD2 and the MR, respectively (Fig. 1). 11βHSD2 and the MR represent transcripts specific to aldosterone-responsive cells. The resulting products were gel purified, and their sequences were determined. Both sequences were identical to the published sequence of the transcript in question. Another marker of aldosterone responsiveness is induction of a known aldosterone-responsive transcript, sgk. As a preliminary indication of whether aldosterone regulates sgk in this cell line, cells were treated for 1 h with 10−6 M aldosterone or vehicle (ethanol), total RNA was isolated, and Northern blot analysis was performed (Fig. 2). Densitometry analysis of the results revealed an approximate fivefold increase in sgk expression over that in control cells (Table 1), a value comparable to levels observed in other studies (19, 27). In summary, mIMCD3 cells were found to be representative of the collecting duct and also to be aldosterone responsive. These cells were therefore se-

Fig. 1. Characterization of mouse inner medullary collecting duct (mIMCD3) cells. Primers were designed according to published sequences to amplify fragments of epithelial Na+ channel (ENaC)-α, 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), and mineralocorticoid receptor (MR) from mIMCD3 cells. RT-PCR products were separated on a 1% agarose gel and visualized with ethidium bromide. +/−, Presence or absence of RT and template. In the absence of an RNA template, an equal volume of H2O was used.
Northern blot analysis was performed using cDNA probes for samples in which 28S and 18S ribosomal RNA bands are indicated. An ethidium bromide-stained 1% agarose gel containing the RNA is shown in the top panel. The bottom panel shows the loading control throughout this study, were observed. Of the four transcripts tested, period homolog underwent the greatest induction according to the microarray data, the Northern blot data, and the real-time PCR data. The period homolog transcript was induced more than sevenfold in mIMCD3 cells exposed to aldosterone for 1 h, as measured by Northern blot analysis and real-time PCR.

To examine the effects of a range of aldosterone concentrations, mIMCD3 cells were treated with increasing amounts of the hormone for 1 h (Fig. 3). The most dramatic increase in expression for all transcripts tested was observed after treatment with $10^{-6}$ M aldosterone. Although the concentration of the hormone in these in vitro experiments was greater than the physiological concentration of aldosterone, the actual effective intracellular concentration was probably much lower. Recent studies have indicated that steroid hormones may not diffuse across cell membranes as freely as was once thought (23).

The next experiments examined the levels of expression of sgk, CTGF, period homolog, and preproendothelin as a function of time. Northern blot analysis results revealed that all four messages show a clear increase in expression after 1 h (Fig. 4). sgk mRNA decreased over the next 12 h and then showed a sharp increase beyond 24 h. A similar pattern was observed for CTGF.

**Table 2. Transcripts downregulated by aldosterone**

<table>
<thead>
<tr>
<th>GenBank</th>
<th>Fold-Change</th>
<th>Transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV332798</td>
<td>-2.5 ± 1.2</td>
<td>EST</td>
</tr>
<tr>
<td>AI843571</td>
<td>-2.1 ± 0.8</td>
<td>Similar to hypothetical protein MGC12966*</td>
</tr>
<tr>
<td>AF026771</td>
<td>-2 ± 0.7</td>
<td>Importin-a Q1 mRNA</td>
</tr>
<tr>
<td>AV277198</td>
<td>-1.9 ± 0.7</td>
<td>Weakly similar to T28931 hypothetical protein C52B9.3*</td>
</tr>
<tr>
<td>U48321</td>
<td>-1.9 ± 0.6</td>
<td>Zinc finger protein 60</td>
</tr>
<tr>
<td>X53177</td>
<td>-1.8 ± 0.3</td>
<td>$\alpha_6$-Integrin (Cd49d)</td>
</tr>
<tr>
<td>W91600</td>
<td>-1.7 ± 0.5</td>
<td>EST</td>
</tr>
</tbody>
</table>

Values are means ± SE calculated using Affymetrix Suite, version 4; $n = 2$. EST, expressed sequence tags. MGC, multinucleated giant cell.

*EST designations were amended using NetAffx.
message. In contrast, period homolog expression slightly decreased at 6 h and then remained relatively low for the duration of the experiment. Preproendothelin mRNA showed a biphasic response similar to sgk and CTGF, but with different timing. The recovery of preproendothelin mRNA was evident at 12 h and continued to increase. To control for a feeding effect, cells were treated with vehicle alone over these same time points. Northern blot analysis of these samples did not show any significant changes in expression of any of the four transcripts tested (data not shown).

To further validate, in a quantitative manner, the time-course Northern blot analysis results, real-time PCR was performed using the same RNA samples as a template (Fig. 5). Expression patterns similar to those seen in the Northern blot analysis were observed for all transcripts tested. sgk showed a sharp fivefold increase at 1 h, which decreased to threefold at 6 h. The level of sgk transcript continued to climb from 12 to 48 h, hitting a peak of approximately sevenfold at the final time point. Period homolog peaked at 1 h with an increase of greater than sevenfold. Period homolog levels then dropped and stayed between three- and fourfold for the remainder of the experiment. Preproendothelin showed a gradual increase from threefold at 1 h to a peak of greater than sixfold at 12 h. Preproendothelin levels remained high for the duration of the experiment. GAPDH showed no significant changes in expression over time.

The effects of aldosterone can be mediated through the MR as well as the glucocorticoid receptor (GR). The effects of GR and MR often overlap, and the two receptors can heterodimerize to drive transcription of some genes (3, 8). It has previously been shown in primary cultures of IMCD cells that either MR or GR can activate electrogenic Na\(^+\) transport (12). Specific inhibitors of both receptors were used alone or in combination to determine the contribution of each to the aldosterone-mediated changes in gene expression (Fig. 6). Densitometry analysis of repeated Northern blot data demonstrated similar patterns of expression for all messages tested. MR and GR inhibitors, used alone or

### Table 3. Transcripts upregulated by aldosterone

<table>
<thead>
<tr>
<th>GenBank</th>
<th>Fold-Change</th>
<th>Transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF0229992</td>
<td>4.1 ± 0.3</td>
<td>Period homolog (<em>Drosophila</em>)</td>
</tr>
<tr>
<td>AF019385</td>
<td>3.4 ± 0.7</td>
<td>Heparan sulfate glucosaminyl 3-O-sulfotransferase</td>
</tr>
<tr>
<td>AW046181</td>
<td>3.1 ± 0.2</td>
<td>sgk(^*)</td>
</tr>
<tr>
<td>AF032459</td>
<td>3.0 ± 1.3</td>
<td>BimEL</td>
</tr>
<tr>
<td>AF058799</td>
<td>2.8 ± 1.6</td>
<td>14-3-3 Protein-γ</td>
</tr>
<tr>
<td>U39233</td>
<td>2.6 ± 0.8</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>AI839289</td>
<td>2.6 ± 0.8</td>
<td>SMT3 (supressor of mir 2, 3) homolog 2, pseudogene 4</td>
</tr>
<tr>
<td>D50032</td>
<td>2.5 ± 1.3</td>
<td>TGN38B</td>
</tr>
<tr>
<td>X05546</td>
<td>2.4 ± 0.8</td>
<td>DBA2 RNA fragment for gag-related peptide</td>
</tr>
<tr>
<td>U07982</td>
<td>2.3 ± 0.2</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>AF077660</td>
<td>2.3 ± 0.8</td>
<td>Homeodomain-interacting protein kinase 3</td>
</tr>
<tr>
<td>AW046627</td>
<td>2.3 ± 0.5</td>
<td>Serine threonine kinase pim 3, clone MGC:27707(^*)</td>
</tr>
<tr>
<td>AA434661</td>
<td>2.2 ± 1.0</td>
<td>EST</td>
</tr>
<tr>
<td>D13458</td>
<td>2.2 ± 0.3</td>
<td>Prostaglandin E receptor EP, subtype</td>
</tr>
<tr>
<td>U36340</td>
<td>2.2 ± 1.0</td>
<td>CACCC-box binding protein BKLF</td>
</tr>
<tr>
<td>AI845584</td>
<td>2.2 ± 0.1</td>
<td>Dual-specificity phosphatase 6(^*)</td>
</tr>
<tr>
<td>U50413</td>
<td>2.2 ± 0.8</td>
<td>Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85α)</td>
</tr>
<tr>
<td>AF072127</td>
<td>2.2 ± 0.8</td>
<td>Claudin-1</td>
</tr>
<tr>
<td>U50384</td>
<td>2.2 ± 0.5</td>
<td>Retinoic acid-responsive (NNS-4AG)</td>
</tr>
<tr>
<td>AF077658</td>
<td>2.1 ± 0.3</td>
<td>Homeodomain-interacting protein kinase 1</td>
</tr>
<tr>
<td>M70642</td>
<td>2.1 ± 0.1</td>
<td>CTGF</td>
</tr>
<tr>
<td>X61800</td>
<td>2.1 ± 0.4</td>
<td>CCAAT/enhancer binding protein (C/EBP), Δ</td>
</tr>
<tr>
<td>AA517845</td>
<td>2.1 ± 0.3</td>
<td>EST</td>
</tr>
<tr>
<td>AF036893</td>
<td>2.0 ± 0.5</td>
<td>Circadian clock protein (Per2)</td>
</tr>
<tr>
<td>Y12713</td>
<td>2.0 ± 0.1</td>
<td>Endogenous retroviral sequence MuERV-L gag, pol, and dUTPase</td>
</tr>
<tr>
<td>X66084</td>
<td>2.0 ± 0.9</td>
<td>CD44 antigen</td>
</tr>
<tr>
<td>AV380793</td>
<td>2.0 ± 0.9</td>
<td>EST</td>
</tr>
<tr>
<td>AA759910</td>
<td>1.9 ± 0.8</td>
<td>EST</td>
</tr>
<tr>
<td>AW121876</td>
<td>1.9 ± 0.7</td>
<td>Similar to hypothetical protein FLJ20505(^*)</td>
</tr>
<tr>
<td>AV351224</td>
<td>1.9 ± 0.4</td>
<td>Nuclear protein 220(^*)</td>
</tr>
<tr>
<td>AI507266</td>
<td>1.9 ± 0.4</td>
<td>Lympoid nuclear protein related to AF4-like(^*)</td>
</tr>
<tr>
<td>M29009</td>
<td>1.9 ± 0.4</td>
<td>Complement factor H-related protein</td>
</tr>
<tr>
<td>AF037437</td>
<td>1.8 ± 0.6</td>
<td>Proaposin</td>
</tr>
<tr>
<td>AW047616</td>
<td>1.8 ± 0.5</td>
<td>HLA-B-associated transcript 3(^*)</td>
</tr>
<tr>
<td>AA420397</td>
<td>1.8 ± 0.6</td>
<td>EST</td>
</tr>
<tr>
<td>AU017202</td>
<td>1.8 ± 0.5</td>
<td>Endogenous retrovirus truncated gag protein</td>
</tr>
<tr>
<td>M26005</td>
<td>1.8 ± 0.3</td>
<td>EST</td>
</tr>
<tr>
<td>AA797843</td>
<td>1.7 ± 0.5</td>
<td>EST</td>
</tr>
<tr>
<td>AI841387</td>
<td>1.7 ± 0.6</td>
<td>Clone MGC:7674 IMAGE:3496398(^*)</td>
</tr>
</tbody>
</table>

Values are means ± SE calculated using Affymetrix Suite, version 4; n = 2 except n = 3 for AF0229992, AW046181, and U35233 (averaged results from 3 hybridization experiments). Transcript of CTGF was previously named fibrolast-inducible-secreted protein. TGN, trans-Golgi network; BKLF, basic Kruppel-like factor; HLA, human leukocyte antigen. \(^*\)EST designations were amended using NetAffx.
in combination, dropped expression to levels that were not significantly different from control. Use of GR- or MR-specific inhibitors suggested, then, that both receptors contribute to the aldosterone-mediated effects on gene expression in mIMCD3 cells. This is not a surprising result, given that MR and GR are known to heterodimerize with each other.

Induction of preproendothelin. To correlate protein expression with the changes in mRNA levels, a cell lysate was prepared from aldosterone- or vehicle-treated cells after 6 h of hormone exposure. Western blot analysis was conducted using a monoclonal antibody raised against endothelin-1. A representative blot is pictured in Fig. 7A. In addition, an anti-endothelin-1 polyclonal antibody was used (data not shown). Both antibodies recognized the same band at ~23 kDa, which is the expected size of unprocessed preproendothelin. As determined by densitometry, preproendothelin protein levels were increased more than fivefold in aldosterone-treated cells compared with control cells in four independent experiments (Fig. 7B). Equal loading of samples was visualized on a duplicate gel stained with Coomassie brilliant blue (data not shown).

DISCUSSION

The late effects of aldosterone on ion channels and transporters such as ENaC and the Na⁺-K⁺-ATPase have been well characterized, but the genes that are immediately affected by aldosterone are not as well known. We have used oligonucleotide array technology to examine the effects of aldosterone on gene expression after 1 h in a mouse IMCD cell line. Not surprisingly, the results of three hybridization experiments suggested that the expression of numerous genes changed in aldosterone-treated cells compared with control cells. Northern blot analysis directly confirmed the upregulation of sgk, CTGF, period homolog, and preproendothelin transcripts. Time course studies showed a biphasic response of sgk and preproendothelin mRNAs to aldosterone treatment, indicating that aldosterone signaling is complex in mIMCD3 cells, with a long-term component in addition to the acute response. The use of MR- and GR-specific inhibitors indicated that both receptors contribute to the aldosterone-mediated regulation of the transcripts tested. Western blot analysis demonstrated that the increases in preproendothelin mRNA levels also resulted in increased levels of protein expression.

The human genes for sgk (36), CTGF (26), period homolog (33) and preproendothelin (14) have been reported. Acute transcriptional regulation suggested that each gene should be directly under the control of a hormone receptor, so the human gene promoters were examined for sterol-response elements (SREs) using the TRANSFAC program (10). Apparent SRE sequences were found in all four promoters. These sites
matched the consensus sequence ATCACCCCAC (16), with a threshold value of at least 80%. Whether these SREs mediate the acute effects of aldosterone in IMCD3 cells remains to be seen.

These findings represent a novel list of aldosterone-regulated transcripts. The physiological significance of these studies must be viewed at two levels. The first level is composed of the immediate mediators of aldo-
Steroid action. Endothelin and CTGF have emerged from the present study as two candidates that may underlie the mechanism of the known physiological actions of aldosterone; these actions are the enhancement of renal net acid excretion with the attendant metabolic alkalosis and the effects of aldosterone on cardiac and renal fibrosis.

The second level is only made possible with the use of oligonucleotide array technology, which enables us to examine the global, genomic effects of aldosterone. Although caution must be exercised because these effects reflect changes at the mRNA level, several of the transcripts listed in Tables 2 and 3 have well-known functions and, in many cases, are part of well-established signaling pathways. Three independent methods, i.e., microarray analysis, Northern blot analysis, and real-time PCR, were used to validate the aldosterone responsiveness of sgk, period homolog, and preproendothelin. Induction of CTGF was verified by two of these methods. The consistent aldosterone-mediated response of these transcripts lends validity to the remaining transcripts on the lists. The aldosterone-induced and -repressed transcripts contain several kinases, transcription factors, and signaling proteins. It is becoming apparent that these molecules must play a vital role in mediating aldosterone action by regulating existing transporters or causing changes in the expression of transporters.

To our knowledge, these results represent the first investigation into aldosterone regulation as early as 1 h after treatment using microarray technology. Previous studies (24) examined the effect of aldosterone at 4 h, at which time the most immediate effects of aldosterone have likely already occurred. There are far-reaching implications of these genes being regulated by a mineralocorticoid. Recently, a role for sgk in Na⁺ absorption was proposed. When coexpressed with ENaC, sgk is able to stimulate Na⁺ current (22). In addition, sgk activity has been shown to lead to translocation of ENaC subunits to the apical membrane (18). CTGF functions in several renal diseases (38). Increased expression of CTGF was observed in diabetic nephropathy and glomerulonephritis (15). Period homolog regulates circadian rhythms, and at least one other circadian rhythm gene, Per 2, also appeared in the list of upregulated transcripts. Expression of period homolog in the kidney has been observed previously, and a circadian pattern of expression in the suprachiasmatic nucleus was also seen (32).

Preproendothelin undergoes significant posttranslational processing with enzymatic hydrolysis by two enzymes before the production of the active form, a 21-amino acid peptide (1). Endothelin-1 is a peptide hormone secreted by vascular endothelial cells and is the most potent vasoconstrictor known. In the mature animal, endothelin-1 has other effects aside from vasoconstriction. Most notably, endothelin-1 has been shown to affect both Na⁺ transport (9) and H⁺ secretion (37) in the collecting duct. In addition, evidence supports the role of endothelin-1 in the pathogenesis of renal interstitial fibrosis, K⁺ depletion, and diabetic nephropathy (11). Aldosterone is a known stimulator of H⁺ secretion (20). The finding that preproendothelin is one of the early genes stimulated by aldosterone suggests an important role for this hormone as a mediator of aldosterone action. Given aldosterone's known effect on cardiac fibrosis (25) and our observation that aldosterone also stimulates CTGF, a more coordinated action of aldosterone may emerge from these studies. Further investigation into the relationship among aldosterone, CTGF, and endothelin-1 must be made to elucidate the mechanisms governing each of their roles in cardiovascular disease.

The expression of several additional transcripts was affected by aldosterone as described by the microarray data, and these effects remain to be confirmed and studied. The identity of these genes indicates that the aldosterone-signaling pathways are more complex than previously thought. Subsequent investigation into the functions of these early-response genes, together with the results presented here, will provide greater insight into the signaling pathways initiated by aldosterone and will further clarify our knowledge of the critical role aldosterone plays in regulating ion homeostasis and cardiovascular disease.

We thank Dr. Rena Bahjat for assistance with statistical analysis programs.
DISCLOSURES

This work was supported by Public Health Service Grant RO1-54721 (to B. D. Cain), Department of Veteran Affairs Merit Review Award 0001 (to C. S. Wingo), and National Institute of Diabetes and Digestive and Kidney Diseases Training Grant DK-07518 (to M. L. Gumz.).

REFERENCES

