cDNA array identification of genes regulated in rat renal medulla in response to vasopressin infusion

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Submitted 7 February 2002; accepted in final form 3 September 2002

Brooks, Heddwen L., Shana Ageloff, Tae-Hwan Kwon, William Brandt, James M. Terris, Akhil Seth, Luis Michea, Søren Nielsen, Robert Fenton, and Mark A. Knepper. cDNA array identification of genes regulated in rat renal medulla in response to vasopressin infusion. Am J Physiol Renal Physiol 284: F218–F228, 2003. First published September 11, 2002; 10.1152/ajprenal.00054.2002.—With the aim of identifying possible gene targets for direct or indirect regulation by vasopressin in the renal medulla, we have carried out cDNA array experiments in inner medullas of Brattleboro rats infused with the V2 receptor-selective vasopressin analog desamino-Cys1,D-Arg8 vasopressin (dDAVP) for 72 h. Of the 1,176 genes on the array, 137 transcripts were increased by 2-fold or more, and 10 transcripts were decreased to 0.5-fold or less. Quantitative, real-time RT-PCR measurements confirmed increases seen for six selected transcripts (Wilms’ tumor protein, β-arrestin 2, neurofibromin, casein kinase IIβ, aquaporin-3, and aquaporin-4). To correlate changes in mRNA expression with changes in protein expression, we carried out quantitative immunoblotting for 28 of the proteins whose cDNAs were on the array. For several targets including aquaporin-2, transcript abundance and protein abundance changes did not correlate. However, for most genes examined, changes in mRNA abundances were associated with concomitant protein abundance changes. Targets with demonstrated increases in both protein and mRNA abundances included neurofibromin, casein kinase IIβ, the subunit of the epithelial Na channel (β-ENaC), 11β-hydroxysteroid dehydrogenase type 2, and c-Fos. Additional cDNA arrays revealed that several transcripts that were increased in abundance after 72 h of dDAVP were also increased after 4 h, including casein kinase IIβ, β-ENaC, aquaporin-3, UT-A, and syntaxin 2. These studies have identified several transcripts whose abundances are regulated in the inner medulla in response to infusion of dDAVP and that could play roles in the regulation of salt and water excretion.

Vasopressin is a peptide hormone that controls systemic osmolality through regulation of renal water excretion. Its main site of action in the kidney is the collecting duct, where it regulates the transport of water, urea, and Na⁺ (25). In collecting duct principal cells, vasopressin binds to a Gs-coupled receptor (the V₂ receptor), which stimulates an increase in intracellular cAMP content via adenyl cyclase. Binding of vasopressin to the V₂ receptor is also associated with intracellular calcium mobilization mediated by calcium release from ryanodine-sensitive intracellular stores via the type I ryanodine receptor, which triggers calmodulin-dependent regulatory processes within the cell (6). Many of the actions of vasopressin in the collecting duct are short-term responses that do not involve activation of gene transcription, such as stimulation of aquaporin-2 trafficking to the apical plasma membrane (35) and activation of the urea transporter UT-A1 through phosphorylation (58). However, vasopressin has clear-cut long-term actions to alter the abundance of aquaporin-2 (8), aquaporin-3 (51), and the epithelial Na channel (ENaC) β- and γ-subunits (11). These long-term actions are thought to be associated with regulatory processes at a transcriptional level, involving either the transporter genes themselves or regulatory molecules that indirectly alter transporter protein abundance. In addition, in renal medulla, vasopressin may have indirect effects, owing to altered interstitial osmolality, urea concentration, or ionic strength.

Here, we have carried out cDNA array experiments with the aim of identifying possible new direct or indirect gene targets for vasopressin action in renal inner medulla. For this, we have examined levels of 1,176 transcripts after infusion of the V₂ receptor-selective vasopressin analog desamino-Cys1,D-Arg8 vasopressin (dDAVP) into Brattleboro rats, which lack endogenously circulating vasopressin.

METHODS

Brattleboro rat model. Male Brattleboro rats (180–230 g) were obtained from Harlan-Sprague Dawley (Indianapolis, IN) and maintained in a temperature- and humidity-con-
trolled room with a 12:12-h light-dark cycle (approved ACUC protocol 9-KE-5). All animals were given free access to tap water and regular pelleted rat chow during the experiments.

Under light anesthesia (isoflurane), osmotic mini-pumps (model 2001, Alza, Palo Alto, CA) were implanted subcutaneously in the rats to deliver 5 ng/h of the V2-selective vasopressin analog dDAVP (Rhone-Poulenc Rorer, Collegeville, PA). Control rats received osmotic mini-pumps loaded with isotonic saline. After dDAVP administration for time periods designated below, rats were killed and the inner medullas were isolated for RNA extraction, or cortices and inner medullas were used for protein analysis. In some experiments, serum was collected for determination of the aldosterone concentration by radioimmunoassay (Coat-A-Count, Diagnostic Products, Los Angeles, CA).

RNA isolation. Total RNA from rat inner medullas was isolated using Qiagen RNAeasy columns (74104, Qiagen, Valencia, CA) according to the manufacturer’s directions. Inner medullary tissue was initially homogenized in the manufacturer’s buffer solution, using an RNAase-free sawtooth tissue homogenizer (Omni 2000). Homogenates were then passed through the QiaShredder column (79654, Qiagen). RNA was treated with DNase while bound to the RNAeasy column. Total RNA concentration was measured by spectrophotometry and run on agarose gels to assess RNA quality.

cDNA arrays. Full documentation of cDNA array procedures and results are presented according to the Minimum Information about a microarray experiment (MIAME) guidelines (4) in the Supplemental Materials. Briefly, Clontech rat 1.2 nylon filter arrays (7854–1, Clontech Laboratories, Palo Alto, CA) were used for cDNA array analysis according to the manufacturer’s instructions. For each experiment, two filters are used, one for control RNA samples and one for experimental RNA. Twenty-five micrograms of total RNA were used for each array. For the two Brattleboro rat experiments (72- and 4-h dDAVP infusion), RNA samples were pooled from the inner medullas from 3 rats, with identical amounts added from each sample (specifically, 8 µg from each inner medulla). 32P was used for labeling in the reverse-transcription reactions, and filters were hybridized overnight at 50°C. Filters were washed at a final stringency of 0.5% SDS, 0.1× SSC at 68°C. Images were captured as TIFF images using a PhosphorImager and analyzed using the National Institutes of Health software program pSCAN (http://mscl.cit.nih.gov). Results were normalized to the overall intensity of the individual filters. To do this, the normalizing variable was total hybridization signal for the whole filter (for all 1,176 spots), allowing the relative dot density to be calculated for each individual gene.

Northern blotting. Northern blots were run to assess relative aquaporin-2, UT-A1, and UT-A2 mRNA abundances in total RNA samples from Brattleboro rat kidney inner medullas and cortices. Aquaporin-2 Northern blots were labeled with a digoxigenin-labeled aquaporin-2 cDNA probe as previously described (12). UT-A Northern blots were probed with 32P-labeled cDNA probes corresponding to the entire length of the UT-A1 transcript (14).

Real-time RT-PCR. Quantitative, real-time RT-PCR was used to validate selected array results as previously described (5, 40). DNase-treated (Ambion) total RNA (1 µg) from rat kidney inner medulla samples from control or dDAVP-infused rats (5 vs. 5) was reverse transcribed using oligo-dT and Superscript II reverse transcriptase (Invitrogen) following the manufacturer’s recommended protocol. RT-negative controls were performed to assess the presence of possible genomic contamination of RNA samples. PCR primers were designed to amplify targets between 80 and 150 bp in length, with minimal secondary structure. Sequences of specific primer pairs are listed in the Supplemental Materials. Real-time PCR was performed on an ABI Prism 7900HT system, using 1 µl of a 1:100 dilution of the original RT reaction product, 18 pmol (each) of gene-specific primers, and the Quantitect SYBR green PCR kit (Qiagen) according to the manufacturer’s protocol. Specificity of the amplified product was determined using melting curve analysis (5). Relative quantitation of gene expression was determined using the comparative C_T method, with validation experiments performed to determine that amplification efficiencies were equal between control and experimental groups (5) as outlined at http://docs.appliedbiosystems.com/pebiotechdocs/04303859.pdf. All experiments were repeated at least twice, on separate days, to validate results.

Antibodies. The study utilized affinity-purified rabbit polyclonal antibodies produced in our laboratory recognizing UT-A1, UT-A2, α-ENaC, β-ENaC, γ-ENaC, synaptotagmin 5, synaptophysin 2, syntaxin 3, syntaxin 4, vesicle-associated membrane protein (VAMP2), renin, aquaporin-1, aquaporin-2, aquaporin-3, and the Na-K-Cl cotransporter type 2 (NKCC2) (8, 13, 28–30, 36, 37, 51). Additional antibodies were commercially obtained: mouse monoclonal antibodies to Na-K-ATPase α1-subunit (05–369, Upstate Biotechnology, Lake Placid, NY), casein kinase IIβ (sc-12739, Santa Cruz Biotechnology, Santa Cruz, CA), calbindin D (C8666, Sigma, St. Louis, MO) and β-arrestin 2 (sc-13140, Santa Cruz Biotechnology); a sheep polyclonal antibody to 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2; AB1296, Chemicon, Temecula, CA); goat polyclonal antibodies to the Wilms’ tumor protein (WT1; sc-15422, Santa Cruz Biotechnology) and CD5 (sc-6984, Santa Cruz Biotechnology) and rabbit polyclonal antibodies to neurofibrin (sc-68, Santa Cruz Biotechnology), the endothelin B receptor (AER-002, Alomone Labs, Jerusalem, Israel), endothelial nitric oxide synthase (eNOS; 160880, Cayman Chemical, Ann Arbor, MI), neuronal (n)NOS (160870, Cayman), β-actin (A2066, Sigma), c-Fos (06–341, Upstate) and c-Jun (KAP-TF102E, StressGen Biotechnology, Victoria, BC, Canada); and a phospho-specific rabbit antibody to c-Jun phosphorylated at Ser 73 (06–659, Upstate).

Protein sample preparation, SDS-PAGE electrophoresis, and immunoblotting. Kidneys were dissected into regions and homogenized in ice-cold isolation solution (250 mM sucrose, 10 mM triethanolamine, pH 7.6, containing 1 mg/ml leupeptin, 0.1 mg/ml phenylmethylsulfonyl fluoride) using a tissue homogenizer (Omni 1000 fitted with a microsawtooth generator) at maximum speed for 15–30 s. Total protein concentrations were measured (BCA kit, Pierce, Rockford, IL), and the samples were solubilized in Laemmli sample buffer at 60°C for 15 min.

Semi-quantitative immunoblotting was carried out as previously described (22, 51) to assess the relative abundances of individual proteins in the dDAVP-treated Brattleboro rats compared with control Brattleboro rats. To confirm that protein loading of the gels was equal, preliminary 12% polyacrylamide gels were stained with Coomassie blue, as previously described (51).

Proteins were separated on 10 or 12% polyacrylamide gels by SDS-PAGE and transferred to nitrocellulose membranes electrophoretically (Bio-Rad Mini Trans-Blot Cell). Membranes were blocked for 1 h at room temperature with 5% nonfat dry milk and probed overnight at 4°C with the appro-
priate affinity-purified polyclonal antibody. Membranes were washed and exposed to one of the following horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature: donkey anti-sheep IgG (1713–035–147, diluted to 1:5,000, Jackson Laboratories), goat anti-rabbit IgG (31463, diluted to 1:5,000, Pierce), mouse anti-goat IgG (31400, diluted 1:5,000, Pierce), or rabbit anti-mouse IgG (31450, diluted to 1:5,000, Pierce). After a washing, bands were visualized using a luminol-based enhanced chemiluminescence substrate (LumiGLO, Kirkegaard and Perry Laboratories, Gaithersburg, MD). Band densities were determined by laser densitometry (Personal Densitometer SI, Molecular Dynamics, San Jose, CA).

**Immunocytochemistry.** Control and dDAVP-treated Brattleboro rats were prepared as described above. The kidneys were fixed by perfusion with cold PBS (pH 7.4) for 15 s via the abdominal aorta, followed by cold 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 min. The kidneys were removed and postfixed for 1 h, followed by 3×10-min washes with 0.1 M cacodylate buffer (pH 7.4). The tissue was dehydrated in graded ethanol and left overnight in xylene. The tissue was embedded in paraffin and 2-μm sections were cut on a rotary microtome (Micron). Localization of 11β-HSD2 was carried out using indirect immunoperoxidase labeling as described (19). The primary and secondary antibodies were the same as described above for immunoblotting. For immunoperoxidase labeling, counterstaining was done using Mayer’s hematoxylin. Microscopy was carried out with a Leica DMRE light microscope.

**Comparative genomic analysis of 5’-flanking region of 11β-HSD2 gene.** The human 11β-HSD2 mRNA sequence was downloaded from GenBank via Entrez (http://www.ncbi.nlm.nih.gov/Entrez/index.html). The mRNA sequence was used in a homology search of the human genome using the genome browser available at the University of California, Santa Cruz (Jim Kent curator, http://genome.ucsc.edu/cgi-bin/hgBlat?command=start) to identify the human gene. The browser was used to locate the 5’-flanking region and a 7,000-bp length immediately upstream of the transcription start site was chosen for further analysis. Regions that were conserved between human and mouse were identified by the browser. The conserved human and mouse sequences were further analyzed by a string search using TESS (http://www.cbil.upenn.edu/tess/index.html) to compare the conserved sequences with elements of a transcription factor binding motif database (TRANSFAC version). The conserved sequences between human and mouse were entered into TESS, which returned a list of potential transcription factor binding sites. Only perfect matches with elements of the database were included, and sites not found in common between the two species were eliminated.

**RESULTS**

To generate hypotheses concerning the direct or indirect long-term action of vasopressin in the renal collecting duct, we carried out cDNA array experiments using mRNA isolated from Brattleboro rat inner medullary tissue samples after 72 h of dDAVP or vehicle treatment. Figure 1 summarizes the distribution of dot-density ratio responses for an experiment that compared RNA pooled from three dDAVP-infused Brattleboro rats with RNA pooled from three vehicle-infused rats. Of the 1,176 genes on the array, 137 transcripts were increased by 2-fold or more in response to dDAVP infusion, and 10 transcripts were decreased to 0.5-fold or less (dashed lines, Fig. 1). Full results including TIFF images of the arrays are presented in accordance with MIAME guidelines (4) in the Supplemental Materials.

**Validation of array results.** Among the 1,176 genes on the arrays, several of them were selected for further analysis because of the potential physiological significance of changes (or lack of changes) that were detected. These targets were studied further using both real-time RT-PCR to confirm the responses at an
mRNA level and semiquantitative immunoblotting to test whether the mRNA changes are associated with corresponding changes in protein abundance. The results of real-time RT-PCR determinations of mRNA responses to dDAVP are shown in Table 1. Real-time RT-PCR determinations were carried out in different total RNA samples than used for the arrays. These samples were isolated from inner medullas of separate, identically treated Brattleboro rats (5 dDAVP-treated vs. 5 vehicle-infused Brattleboro rats). As can be seen in Table 1, the genes with the largest increases in mRNA in response to dDAVP on the cDNA arrays (namely, WT1, β-arrestin 2, neurofibromin, and casein kinase IIβ) were found to be associated with significant increases in mRNA in response to dDAVP infusion by real-time RT-PCR. These are novel responses, which have potential significance regarding the mechanism of the cellular response to vasopressin in collecting duct cells (see DISCUSSION). In addition, among the three aquaporins expressed in the renal collecting duct, aquaporin-2, -3, and -4, there was reasonable agreement between cDNA array results and real-time RT-PCR results. Both aquaporin-3 and aquaporin-4 manifested substantial increases in mRNA in response to dDAVP, while, somewhat surprisingly, aquaporin-2 either did not change (cDNA array) or increased only modestly (real-time RT-PCR). Interestingly, GAPDH and β-actin, both of which are considered housekeeping genes, manifested increases in mRNA in response to dDAVP when measured by real-time RT-PCR.

The lack of an increase in aquaporin-2 mRNA abundance was surprising in view of previous reports of upregulation of aquaporin-2 protein in inner medulla in response to dDAVP (8, 51). Therefore, we decided to run Northern blots to assess aquaporin-2 mRNA abundance changes in renal inner medulla and cortex in response to long-term dDAVP infusion (Fig. 2). Consistent with findings with the cDNA arrays and real-time RT-PCR, there was no substantial change in aquaporin-2 mRNA in the inner medullas of Brattleboro rats in response to dDAVP infusion (normalized band densities: vehicle infused, 100 ± 18; dDAVP infused, 85 ± 17, not significant). In contrast, a substantial increase in aquaporin-2 mRNA was detected in RNA samples isolated from the renal cortices of the same rats (normalized band densities: vehicle infused, 100 ± 82; dDAVP infused, 1,072 ± 485, P < 0.05), consistent with previous reports based on Northern blot analysis of whole kidney samples (12).

The array results showed that there was an apparent increase in the mRNA abundance for UT-A in response to dDAVP infusion (dDAVP-to-vehicle ratio: 2.8). This gene codes for a major urea transporter UT-A1 in inner medullary collecting duct. This finding contrasts with the observed lack of increase in UT-A1 protein in inner medulla in response to dDAVP infusion (52). Real-time RT-PCR was impractical for UT-A1 because its sequence overlaps with other splice variants from the same gene (33). Hence, we carried out Northern blotting for UT-A isoforms, using the same samples used for real-time quantitative RT-PCR studies (Fig. 3). There was a striking increase in the mRNA level and semiquantitative immunoblotting to test whether the mRNA changes are associated with corresponding changes in protein abundance. The results of real-time RT-PCR determinations of mRNA responses to dDAVP are shown in Table 1. Real-time RT-PCR determinations were carried out in different total RNA samples than used for the arrays. These samples were isolated from inner medullas of separate, identically treated Brattleboro rats (5 dDAVP-treated vs. 5 vehicle-infused Brattleboro rats). As can be seen in Table 1, the genes with the largest increases in mRNA in response to dDAVP on the cDNA arrays (namely, WT1, β-arrestin 2, neurofibromin, and casein kinase IIβ) were found to be associated with significant increases in mRNA in response to dDAVP infusion by real-time RT-PCR. These are novel responses, which have potential significance regarding the mechanism of the cellular response to vasopressin in collecting duct cells (see DISCUSSION). In addition, among the three aquaporins expressed in the renal collecting duct, aquaporin-2, -3, and -4, there was reasonable agreement between cDNA array results and real-time RT-PCR results. Both aquaporin-3 and aquaporin-4 manifested substantial increases in mRNA in response to dDAVP, while, somewhat surprisingly, aquaporin-2 either did not change (cDNA array) or increased only modestly (real-time RT-PCR). Interestingly, GAPDH and β-actin, both of which are considered housekeeping genes, manifested increases in mRNA in response to dDAVP when measured by real-time RT-PCR.

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Table 1. Comparison of mRNA changes in inner medullas of Brattleboro rats estimated using cDNA arrays vs. real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Array (dDAVP-to-vehicle ratio)</th>
<th>Real Time RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle (n = 5)</td>
<td>dDAVP (n = 5)</td>
</tr>
<tr>
<td>WT1</td>
<td>24</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>β-Arrestin 2</td>
<td>16</td>
<td>1.00 ± 0.15</td>
</tr>
<tr>
<td>Neurofibromin</td>
<td>8.0</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>Casein kinase II</td>
<td>5.5</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>Aquaporin-4</td>
<td>2.1</td>
<td>1.00 ± 0.16</td>
</tr>
<tr>
<td>Aquaporin-3</td>
<td>1.8</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1.4</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>β-Actin</td>
<td>0.90</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>Aquaporin-2</td>
<td>0.90</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>Na-K-ATPase -α1</td>
<td>0.53</td>
<td>1.00 ± 0.10</td>
</tr>
<tr>
<td>NKCC2</td>
<td>0.49</td>
<td>1.00 ± 0.15</td>
</tr>
</tbody>
</table>

Values for the arrays are dot-density ratio for paired cDNA arrays, using pooled RNA samples from 3 desamino-Cys1, dArg8 vasopressin (dDAVP)-infused vs. 3 vehicle-infused Brattleboro rats. Values for real time RT-PCR are means ± SE for mRNA levels in total RNA samples from vehicle-infused and dDAVP-infused Brattleboro rats. WT1, Wilms’ protein; NKCC2, Na-K-Cl cotransporter 2. mRNA abundance significantly different in dDAVP-infused vs. vehicle-infused Brattleboro rats, P < 0.05.

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abundance of UT-A2 mRNA (transcript size: 2.9 kb), whereas the abundance of UT-A1 mRNA (transcript size: 3.9 kb) changed comparatively little. Consequently, real-time RT-PCR was run for UT-A2, targeting the single exon unique to this isoform. The real-time RT-PCR analysis indicated that UT-A2 mRNA abundance was increased 56-fold in inner medulla by dDAVP infusion. A check of the sequence of the cDNA probe for UT-A on the array indicated that it overlaps sequence for both UT-A1 and UT-A2 (proprietary information, Clontech). Thus, although UT-A1 is the dominant isoform in the inner medulla, an extremely large increase in UT-A2 (a splice variant expressed in thin descending limbs of Henle’s loops) was apparently chiefly responsible for the marked increase detected by cDNA array analysis.

Correlation between mRNA changes and protein changes for selected genes. To correlate changes in mRNA expression detected on the cDNA arrays with changes in protein expression, we carried out quantitative immunoblotting for 28 of the proteins corresponding to genes on the array. For this, we utilized kidneys from a different set of dDAVP-infused Brattleboro rats than those used for the arrays (Fig. 4, Table 2). As shown in Fig. 4, several transcripts exhibiting very large increases in mRNA levels in response to dDAVP infusion (based on cDNA array analysis) were also associated with significant increases in the corresponding protein abundances. This group included neurofibromin, casein kinase IIβ, and 11β-HSD2. WT1 (24-fold increase in mRNA) was not detectable in the inner medulla by immunoblotting despite positive controls showing strong labeling of heterologously expressed WT1 protein (Sean Lee, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, personal communication). Two proteins previously recognized to be upregulated by vasopressin, β-ENaC (11) and aquaporin-3 (13, 51), also showed substantial increases at the protein and mRNA levels in the present study (Fig. 4). In addition, c-Fos showed a relatively modest increase in mRNA abundance by cDNA array analysis (1.7-fold) but exhibited a large increase in protein abundance (4.9-fold). Conversely, two proteins (the α1-subunit of the Na-K-ATPase and the type 2 Na-K-2Cl cotransporter) showed corresponding decreases in mRNA and protein (Fig. 4). [Analysis of serum samples from these rats revealed a significant increase in serum concentrations of aldosterone in dDAVP-infused (1.9 ± 0.5 nM, n = 6) compared with vehicle-infused Brattleboro rats (0.5 ± 0.1 nM, n = 6), ruling out a decrease in circulating aldosterone level as a cause of the decrease in Na-K-ATPase α1-subunit protein expression.]

### Table 2. Immunoblotting results from long-term dDAVP infusion experiment

<table>
<thead>
<tr>
<th>Protein</th>
<th>mRNA Change on Array</th>
<th>Normalized Band Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1</td>
<td>Increased</td>
<td>ND</td>
</tr>
<tr>
<td>β-Arrestin 2</td>
<td>Increased</td>
<td>1.00 ± 0.37</td>
</tr>
<tr>
<td>ET-B receptor</td>
<td>Increased</td>
<td>1.00 ± 0.42</td>
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<td>Neurofibromin</td>
<td>Increased</td>
<td>1.00 ± 0.27</td>
</tr>
<tr>
<td>Casein kinase II</td>
<td>Increased</td>
<td>1.00 ± 0.11</td>
</tr>
<tr>
<td>CD5</td>
<td>Increased</td>
<td>1.00 ± 0.11</td>
</tr>
<tr>
<td>Syntaxin-2</td>
<td>Increased</td>
<td>1.00 ± 0.34</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>Increased</td>
<td>1.00 ± 0.10</td>
</tr>
<tr>
<td>11β-HSD2</td>
<td>Increased</td>
<td>1.00 ± 0.10</td>
</tr>
<tr>
<td>Renin</td>
<td>Increased</td>
<td>1.00 ± 0.16</td>
</tr>
<tr>
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<td>Increased</td>
<td>1.00 ± 0.16</td>
</tr>
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<td>1.00 ± 0.16</td>
</tr>
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<td>c-Jun</td>
<td>Increased</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>Calbindin 28</td>
<td>Increased</td>
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</tr>
<tr>
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<td>1.00 ± 0.19</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>Increased</td>
<td>1.00 ± 0.20</td>
</tr>
<tr>
<td>c-Fos</td>
<td>Increased</td>
<td>1.00 ± 0.12</td>
</tr>
<tr>
<td>Syntaxin-4</td>
<td>Unchanged</td>
<td>1.00 ± 0.14</td>
</tr>
<tr>
<td>Syntaxin-3</td>
<td>Unchanged</td>
<td>1.00 ± 0.26</td>
</tr>
<tr>
<td>eNOS</td>
<td>Unchanged</td>
<td>1.00 ± 0.15</td>
</tr>
<tr>
<td>nNOS</td>
<td>Unchanged</td>
<td>1.00 ± 0.16</td>
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<tr>
<td>β-Actin</td>
<td>Unchanged</td>
<td>1.00 ± 0.26</td>
</tr>
<tr>
<td>γ-ENaC</td>
<td>Unchanged</td>
<td>1.00 ± 0.23</td>
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<td>Aquaporin-2</td>
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<tr>
<td>Na-K-ATPase-α1</td>
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</tr>
<tr>
<td>NKCC2</td>
<td>Decreased</td>
<td>1.00 ± 0.18</td>
</tr>
</tbody>
</table>

Values are means ± SE. CD5, lymphocyte differentiation antigen-CD5; ENaC, epithelial sodium channel; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; UT-A1, urea transporter A isoform 1; eNOS and nNOS, endothelial and neuronal nitric oxide synthase; VAMP2, vesicle-associated membrane protein. Increased, >1.5-fold change; no change, 0.67- to 1.5-fold change; decreased, <0.67-fold change. *Significantly different for dDAVP-infused vs. vehicle-infused rats.
First, let's focus on the study of 11β-HSD2 regulation by vasopressin in greater detail. Additional Brattleboro rats were studied to localize the increase in 11β-HSD2 protein abundance in response to dDAVP (Fig. 5). As shown in Fig. 5A, immunoblot analysis of inner medullary protein samples from Brattleboro rats infused with dDAVP for 3 days again showed an increase in 11β-HSD2 protein abundance compared with vehicle-infused rats. Densitometry analysis of the immunoblots revealed that the normalized band density was significantly increased to 169 ± 20 compared with 100 ± 25 in control inner medulla, P < 0.05. Similar increases were seen in renal cortical samples (not shown; dDAVP infused, 242 ± 9 vs. vehicle-infused, 100 ± 12, P < 0.05). Figure 5B shows immunoperoxidase labeling of 11β-HSD2 in renal inner medullas of vehicle- and dDAVP-infused Brattleboro rats. Labeling conditions and exposure settings on the microscope were identical for both images. The 11β-HSD2 labeling was present in the principal cells of the collecting ducts (arrows, Fig. 5B), whereas intercalated cells were not labeled (arrowheads). dDAVP treatment markedly increased 11β-HSD2 immunostaining in principal cells (arrows, Fig. 5B-A) compared with vehicle-infused Brattleboro rats (arrow, Fig. 5B-A). Similar observations were made in two additional pairs of rats. Thus we conclude that long-term dDAVP infusion increases the abundance of 11β-HSD2 protein in the inner medullary collecting ducts of Brattleboro rats.

To assess putative binding sites for transcription factors in the 5'-flanking region of the 11β-HSD2 gene, we carried out comparative genomic analysis using the newly available genomic sequence data for humans and mice as described in METHODS. Figure 6 reports the putative transcription factor binding motifs present in the 5'-flanking regions that were conserved between the human and mouse 11β-HSD2 genes. Notably, there was no conserved cAMP responsive element binding protein (CREB) site. (A CREB site was present in the human sequence, but the corresponding mouse sequence had a 1-bp difference.) The analysis also confirms previous identification of a conserved Sp1 site in both mouse and human 11β-HSD2, positioned between...
B438 and B1088 (32). However, our analysis also identified three additional putative Sp1 sites upstream of this region, which were conserved between mice and humans. Sp1 cis-regulatory elements have been associated with cAMP-mediated transcriptional regulation (43). Finally, Fig. 6 also shows the presence of a conserved putative AP1 binding element that binds c-Fos/c-Jun heterodimers. Such sites have also been shown to be involved in cAMP-mediated regulation of transcription (50). The c-fos gene is known to be transcriptionally regulated by cAMP, although CREB-dependent and CREB-independent means (3), and an AP1 site in the 5′-flanking region of the aquaporin-2 gene has previously been demonstrated to play a central role in the upregulation of its transcription by vasopressin in LLC-PK1 cells (57).

AP1-mediated transcriptional regulation typically occurs via two mechanisms: 1) regulation of c-Fos protein abundance and 2) phosphorylation of the c-Jun protein. Addressing these two mechanisms, Fig. 7 shows immunoblots of inner medullary homogenates from Brattleboro rats. dDAVP infusion indeed increased c-Fos abundance to 489 ± 108% (P < 0.05) of vehicle-infused controls (Fig. 7A). Application of a phosphorylation-specific (Ser73) c-Jun antibody using the same protein samples (Fig. 7B) revealed an increase in phospho-c-Jun abundance relative to vehicle-infused Brattleboro rats (normalized band densities: 722 ± 225 vs. 100 ± 28% in control rats, P < 0.05).

Response to 4-h dDAVP infusion. Figure 8 summarizes the overall pattern of mRNA abundance changes in response to 4-h treatment with dDAVP compared with 72-h treatment, as determined by cDNA microarray analysis. Predictably, the range of response ratios is not as great after 4-h compared with 72-h dDAVP infusion. Furthermore, in a large number of cases, responding genes at 72 h were different from responding genes at 4 h. Among the 18 transcripts that were increased in Tables 1 or 2, only 5 of them were also increased (dDAVP:vehicle > 1.5) after 4 h of dDAVP infusion (casein kinase IIβ, syntaxin-2, β-ENaC, UT-A1, and aquaporin-3), while 9 were unchanged after 4-h dDAVP treatment (neurofibromin, CD5, 11β-HSD2, renin, synaptotagmin 5, c-Jun, calbindin 28, α-ENaC, and c-Fos) and 4 were decreased (dDAVP:vehicle < 1.5) after 4-h treatment (WT1, ET-B receptor, β-arrestin 2, and aquaporin-4). Full results of the 4-h dDAVP/Brattleboro rat array experiments, including TIFF images of the arrays, are presented in accordance with MIAME guidelines (4) in the Supplemental Materials.

DISCUSSION

Previous studies have established that vasopressin regulates gene expression in the kidney, at least with regard to the genes that code for aquaporin-2 and -3 (12, 51), the three ENaC subunits (11, 34), the urea transporter UT-A2 (55), and the Na-K-2Cl cotransporter NKCC2 (22). In this paper, we used cDNA arrays to broaden these observations, identifying several additional genes for which expression is altered in the renal inner medulla in response to administration of the V2 receptor-selective vasopressin analog dDAVP. The studies were done in the Brattleboro rat, which lacks circulating vasopressin due to a mutation in the vasopressin-neurophysin gene (46) and thus provides a vasopressin-free host in which to test responses to dDAVP infusion. Because these studies were done in vivo, responses to dDAVP infusion in this study could be either direct, i.e., responses to increased phosphor-

![Fig. 7. Effect of long-term dDAVP infusion on c-Fos, c-Jun, and phosphorylated (phospho-)Jun protein abundance in Brattleboro rat inner medullas. Immunoblots were performed using inner medullary samples from vehicle- and dDAVP-infused Brattleboro rats. Blots were loaded with 15 μg of total protein/lane and probed with polyclonal antibodies, anti-c-Fos, anti-c-Jun, and anti-phospho-Jun (Ser73). Band density for c-Fos and phospho-Jun were significantly increased by dDAVP infusion (P < 0.05).](image-url)
ylation of transcription factors by vasopressin-activated kinases (protein kinase A or calmodulin-dependent kinases) in collecting duct cells, or indirect, i.e., due to a more complex response that is triggered by increased V2-receptor occupation but is not an immediate consequence of vasopressin receptor-mediated signaling. The latter would include several categories of responses including 1) activation of additional signaling cascades in collecting duct cells such as the MAP kinase pathway; 2) induction of hierarchical transcription factors downstream of transcription factors immediately activated by vasopressin-induced signaling; 3) secondary changes in circulating hormone levels; or 4) responses to an altered inner medullary interstitial environment. This paper does not attempt to discriminate these different types of responses. In the following, we discuss, first, the regulatory targets identified and then address some general issues raised by the results.

**β-Arrestin 2.** β-Arrestin 2 is a member of a family of proteins involved in G protein-coupled receptor desensitization (39). These proteins bind to the phosphorylated COOH termini of G protein-coupled receptors and mediate receptor desensitization in part by inducing receptor endocytosis. In the present studies, identification of β-arrestin 2 upregulation in response to dDAVP infusion points to a potential role for the induction of this protein in the vasopressin-escape phenomenon (12), which has been found to be due to V2 receptor downregulation (10, 53) and is crucial in limiting the degree of extracellular fluid dilution in the syndrome of inappropriate antidiuresis. Recently, the β-arrestins have also been found to play a role as ligand-activated scaffold proteins for two MAP kinase pathways, the ERK cascade (7, 27) and the c-Jun NH2-terminal kinase 3 (JNK3) cascade (31). It is therefore conceivable that induction of β-arrestin 2 by vasopressin is involved in the observed activation of MAP kinase cascades in the renal inner medulla during antidiuresis (56) and may provide part of the explanation for the dDAVP-induced increase in c-Jun phosphorylation demonstrated in the present study (Fig. 7).

**Neurofibromin.** The renal inner medullary expression of neurofibromin was markedly increased at both the mRNA (Table 1) and protein (Table 2) levels in response to long-term dDAVP infusion. Neurofibromin was originally identified as the protein product of the disease gene responsible for the autosomal dominant genetic disease neurofibromatosis type 1 (18). It is a member of the GTPase-activating protein (GAP) family and has been implicated as a key factor in limiting of the growth-promoting action of the small GTP-binding protein Ras. Recently, however, an additional function has been identified for the protein, direct activation of CNS-specific adenyl cyclase isoforms (17, 54). It is unknown whether neurofibromin interacts in a similar manner with renal isoforms of adenyl cyclase. If it does, upregulation of neurofibromin expression in the kidney could contribute to long-term regulation of water, urea, and sodium ion transport in the renal tubule.

**Casein kinase IIβ.** Casein kinase IIβ expression was strongly increased in the renal inner medulla in response to dDAVP infusion at both mRNA and protein levels (Tables 1 and 2). A detectable increase in casein kinase IIβ mRNA after a 4-h dDAVP infusion suggests rapid, direct induction. Casein kinase IIβ is a regulatory subunit of the protein kinase CK2, a ubiquitous serine/threonine kinase, which is composed of two regulatory β-subunits and two catalytic α-subunits. Many protein substrates for CK2 have been identified, including growth factor receptors, transcription factors, cytoskeletal proteins, cell cycle regulatory proteins, and vesicle trafficking proteins. The latter includes syntaxin-4, which is believed to play a role in vasopressin-dependent aquaporin-2 trafficking to the plasma membrane (41). Recent findings point to a role for CK2 in the cellular response to various forms of cellular stress (1), raising the possibility that its induction by dDAVP may play a role in protecting inner medullary cells against osmotic stress.

**11β-HSD2.** 11β-HSD2 was upregulated in response to long-term dDAVP infusion at both mRNA and protein levels (Figs. 4 and 5). This enzyme is believed to play a central role in the regulation of ion transport in the renal collecting duct through its ability to break down glucocorticoids (cortisol in humans; corticosterone in rodents) to inactive forms (16). In the absence of 11β-HSD2 in collecting duct cells, glucocorticoids at circulating concentrations would be expected to bind to and fully activate the mineralocorticoid receptor (MR), impairing the ability of regulated changes in circulating aldosterone levels to alter gene expression. The conventional view of the role of 11β-HSD2 is that it is constitutively expressed at such high levels that only the mineralocorticoid aldosterone can reach the MR. However, previous studies identified 11β-HSD2 as a regulatory target for the short-term actions of vasopressin by a nontranscriptional mechanism (2), suggesting that regulation of 11β-HSD2 activity may play a physiological role, perhaps by controlling glucocorticoid access to the MR and glucocorticoid receptor in the collecting duct. The present finding of upregulation of 11β-HSD2 gene expression in response to long-term vasopressin treatment extends this view and could have implications for the regulation of transporter proteins in the collecting duct that are recognized targets for glucocorticoid or mineralocorticoid regulation, including the UT-A1 urea transporter (38), the Na-K-ATPase (15), and ENaC (48).

The interpretation of the effects of dDAVP infusion on corticosteroid-regulated transporters is complicated by the fact that dDAVP infusion increased the circulating level of aldosterone in this study by almost fourfold. This response might be expected to oppose the effect of increased 11β-HSD2 levels. Indeed, in the present study α-ENaC, whose abundance is strongly upregulated by aldosterone (30), was substantially increased at both the mRNA and protein levels in response to dDAVP infusion (Fig. 4, Table 2). The increase in plasma aldosterone concentration may be due in part to the ability of dDAVP to bind and activate the V1b receptor in corticotroph cells of the anterior pituitary (44), thereby increasing ACTH secretion, which is
considered a minor factor in the regulation of adrenal aldosterone secretion.

WT1. Among all cDNAs on the array, WT1 manifested the largest increase in dot density in response to long-term infusion of dDAVP (Table 1). A large increase in mRNA abundance was confirmed by real-time quantitative RT-PCR. WT1 is a zinc-finger transcription factor expressed chiefly in kidney, gonads, uterus, and spleen, which functions as a tumor suppressor (26). In addition, WT1 appears to be involved in posttranscriptional processing of mRNA (45). Mutations in WT1 are associated with a high incidence of Wilms’ tumor, a renal neoplasm arising from renal tissue of metanephric origin. In the mature kidney, WT1 is generally believed to be expressed only in the glomerular podocyte (45), although the possibility of its expression in the renal inner medulla of the adult kidney has not been investigated in detail. Available antibodies to WT1 could not convincingly demonstrate WT1 protein in the rat inner medulla (Table 2), although absolute expression levels of a functional transcription factor could conceivably be quite low. Consequently, further studies will be needed to localize WT1 expression in the renal inner medulla and to determine its role in vasopressin-mediated transcriptional regulation.

c-Fos and c-Jun. c-Fos and c-Jun are immediate early genes, whose products together constitute the transcription factor AP1. On cDNA arrays, dot densities for both c-Jun and c-Fos mRNA were observed to increase with long-term dDAVP infusion (Fig. 4). Immunoblotting demonstrated that c-Fos protein abundance was markedly increased in response to dDAVP administration, consistent with the findings of Yasui et al. (57) in LLC-PK1 cells. However, there was no demonstrable increase in the abundance of c-Jun protein. Nevertheless, there was a marked increase in the abundance of phosphorylated c-Jun, possibly resulting from activation of MAP kinases in response to dDAVP. Thus, although direct studies of transcriptional regulation are beyond the scope of this study, we postulate a critical role of the AP1 binding motif in mediating the long-term responses to vasopressin in the renal inner medulla. One gene that may be transcriptionally upregulated by AP1 may be 11β-HSD2, which has a conserved AP1 binding site in its 5'-flanking region and is markedly upregulated by vasopressin (see above).

Synaptotagmin. The synaptotagmins are postulated to play calcium-sensing roles in the regulation of exocytosis (49). In the present study, we found that synaptotagmin 5 (20) (also termed synaptotagmin IX) was upregulated in the inner medulla in response to long-term dDAVP infusion at both mRNA and protein levels (Fig. 4). Synaptotagmin 5 has recently been demonstrated to be a binding partner for the α-subunit of the protein serine/threonine kinase CK2 (9), another dDAVP-responsive protein (see above).

Aquaporin-2 and -3. Aquaporin-3, a basolateral water channel in collecting duct principal cells, was found to be upregulated at both the mRNA (Table 1) and protein (Table 2) levels, consistent with prior results (12, 13, 51). The inner medullary protein abundance of aquaporin-2, the apical water channel in collecting duct principal cells, was also increased (Table 2), consistent with previous findings (8, 51). In the context of these previous findings, we were surprised to find that there was little or no increase in aquaporin-2 mRNA abundance in the inner medulla in response to dDAVP infusion as demonstrated on cDNA arrays (Fig. 4), by Northern blotting (Fig. 2), and by real-time RT-PCR (Table 1). This result suggests that posttranscriptional mechanisms may be involved in the regulation of aquaporin-2 protein abundance in the renal inner medulla. In contrast, aquaporin-2 mRNA abundance was strongly increased in the cortex in response to dDAVP infusion (Fig. 2), consistent with previous findings (10).

Urea transporters. The UT-A gene codes for several urea transporter proteins expressed in inner medulla that arise from alternative splicing (33). UT-A1, the predominant form in the inner medullary collecting duct (37, 47), has been shown to be downregulated in the inner medulla in response to dDAVP infusion (52). Hence, we were somewhat surprised to find that the mRNA dot density for UT-A was increased 2.8-fold on the cDNA array (Table 1), whereas immunoblotting confirmed a lack of increase in UT-A1 protein in the inner medulla in response to dDAVP infusion (Fig. 4). Further analysis by real-time quantitative RT-PCR revealed a 56-fold increase in the abundance of a second splicing variant, UT-A2, which is expressed chiefly in the thin descending limbs of Henle in the outer medulla (37, 47) but is relatively nonabundant in the inner medulla (55). Interrogation of the commercial supplier of the array revealed that the sequence of the UT-A cDNA on the array overlaps both UT-A1 and UT-A2. Hence, it appears that a very large change in a relatively nonabundant splicing variant gave a result on cDNA array analysis that was not representative of changes in the most abundant splice variant.

General observations and conclusions. cDNA array analysis has revealed several genes that are upregulated in the inner medulla of the Brattleboro rat in response to dDAVP infusion. Further studies will be needed to investigate further the role of these genes and their protein products in the overall response to vasopressin. Clearly, the data presented here provide only an initial glimpse of the response to vasopressin, with a detailed view only of the long-term response, which may consist of both direct and indirect effects of vasopressin on gene expression. A general comparison of the response to vasopressin at a 4-h vs. a 72-h time point (Fig. 8) reveals a much different pattern of mRNA abundance changes at the pre-steady-state time point. Detailed time course studies will be required to work out the sequence of events involved in the vasopressin response and to determine which genes are upregulated in direct response to vasopressin-mediated signaling vs. secondary responses, which might be related to vasopressin-mediated changes in local osmolality, local calcium ion concentrations, luminal pH, luminal flow rate, and other factors altered by vasopressin.

An important component of the present study was a detailed comparison of mRNA abundance and protein abundance responses to dDAVP infusion, with an assessment of 28 different protein products by quantita-
tive immunoblotting (Table 2, Fig. 4). For this element of the study, we used a large number of polyclonal antibodies developed in this laboratory for targeted proteomic studies (24) as well as a selection of antibodies from commercial sources for which the specificities were clearly documented. Although for many of the genes examined in this manner there was a clear correlation between changes in mRNA and those in protein, it is important to emphasize that several genes exhibited mRNA responses that were qualitatively different from protein responses. Some genes showed increases in protein with no change in mRNA levels, whereas some showed changes in mRNA abundance without coordinate changes in protein abundance. Similar observations have been made in studies using large-scale proteomics and gene expression arrays (21). Clearly, there are physiologically important mechanisms by which levels of specific proteins can change without changes in mRNA levels, including translational regulation and regulation of protein half-life (23). Hence, although cDNA arrays provide an important means of generating new hypotheses about physiological regulation at a molecular level, a complete evaluation of such mechanisms requires protein measurements.

A previous study reported genes whose transcript abundances were upregulated or downregulated in response to exposure of cultured mpkCCDc14 to 10^{-8} M arginine vasopressin for 4 h as determined by SAGE (42). There was no overlap between the responding genes reported in that study to those up- or downregulated in the present study at either the 4- or 24-h time point (see list of responding genes in Supplemental Materials). However, it is well recognized that there are many differences in gene expression and regulation between the cortical collecting duct and the inner medullary collecting duct. One difference was illustrated in the present study: aquaporin-2 mRNA levels are increased by vasopressin in the renal cortex but not the renal medulla (Fig. 2).

The strength of the cDNA array approach is that a large number of transcripts can be assessed simultaneously. However, costs limit the number of observations per transcript to relatively low numbers, yielding severe statistical limitations with regard to probabilities of both false positives and false negatives. Furthermore, even if large numbers of array experiments can be completed, ambiguities can remain, as evidenced by the finding that the “urea transporter” spot on the array actually recognizes two different splicing variants of the UT-A gene with much different physiological roles (see above). Complete evaluation of complex physiological responses therefore requires application of other methodologies, such as Northern blotting and real-time RT-PCR, for quantitative assessment of abundance changes in specific transcripts. As noted above, assessment of protein abundance changes can, in principle, substitute for confirmation at an mRNA level if the question being addressed pertains to the physiological function of proteins.

The authors thank Dr. Sean Lee (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) for advice regarding WT1 antibodies.

This study was funded by the Intramural Budget of the National Heart, Lung, and Blood Institute (National Institutes of Health, project no. Z01-HL-01282-KE, to M. A. Knepper). Studies at Aarhus University were supported by the Danish Medical Research Council, the Karen Elise Jensen Foundation, the Commission of the European Union (EU-TMR Program and K.A. 3.1.2 Program) and Dongguk University. The Water and Salt Research Center, Aarhus University, is supported by The Danish National Research Foundation (Danmarks Grundforskningsfond). A. Seth was supported by the Biomedical Engineering Student Internship Program of the Whitaker Foundation.

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