The role of 70-kDa heat shock protein in dDAVP-induced AQP2 trafficking in kidney collecting duct cells

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Submitted 20 August 2012; accepted in final form 7 January 2013

Park EJ, Lim JS, Jung HJ, Kim E, Han KH, Kwon TH. The role of 70-kDa heat shock protein in dDAVP-induced AQP2 trafficking in kidney collecting duct cells. Am J Physiol Renal Physiol 304: F958–F971, 2013. First published January 9, 2013; doi:10.1152/ajprenal.00469.2012.—It has been reported that several proteins [heat shock protein 70 (Hsp70 and Hsc70), annexin II, and tropomyosin 5b] interact with the Ser256 residue on the COOH terminus of aquaporin-2 (AQP2), where vasopressin-induced phosphorylation occurs for mediating AQP2 trafficking. However, it remains unknown whether these proteins, particularly Hsp70, play a role in AQP2 trafficking. Semiquantitative immunoblotting revealed that renal expression of AQP2 and Hsp70 was significantly increased in water-restricted or dDAVP-infused rats. In silico analysis of the 5′-flanking regions of AQP2, Hsp70-1, and Hsp70-2 genes revealed that transcriptional regulator binding elements associated with cAMP response were identified at both the Hsp70-1 and Hsp70-2 promoter regions, in addition to AQP2. Luciferase reporter assay demonstrated the significant increase of luminescence after dDAVP stimulation (10−8 M, 6 h) in the LLC-PK1 cells transfected with luciferase vector containing 1 kb of the 5′-flanking region of Hsp70-2 gene. Hsp70-2 protein expression was also increased in mpkCCDc14 cells treated by dDAVP in a concentration-dependent manner. Cell surface biotinylation analysis demonstrated that forskolin (10−6 M, 15 min)-induced AQP2 targeting to the apical plasma membrane was significantly attenuated in the mpkCCDc14 cells with Hsp70-2 knockdown. Moreover, forskolin-induced AQP2 phosphorylation (Ser256) was not significantly induced in mpkCCDc14 cells with Hsp70-2 knockdown. In contrast, Hsp70-2 knockdown did not affect the dDAVP-induced AQP2 abundance. In addition, siRNA-directed knockdown of Hsp70 significantly decreased cell viability. The results suggest that Hsp70 is likely to play a role in AQP2 trafficking to the apical plasma membrane, partly through affecting AQP2 phosphorylation at Ser256 and cell viability.

The role of 70-kDa heat shock protein in dDAVP-induced AQP2 trafficking in kidney collecting duct cells. Am J Physiol Renal Physiol 304: F958–F971, 2013. First published January 9, 2013; doi:10.1152/ajprenal.00469.2012.—It has been reported that several proteins [heat shock protein 70 (Hsp70 and Hsc70), annexin II, and tropomyosin 5b] interact with the Ser256 residue on the COOH terminus of aquaporin-2 (AQP2), where vasopressin-induced phosphorylation occurs for mediating AQP2 trafficking. However, it remains unknown whether these proteins, particularly Hsp70, play a role in AQP2 trafficking. Semiquantitative immunoblotting revealed that renal expression of AQP2 and Hsp70 was significantly increased in water-restricted or dDAVP-infused rats. In silico analysis of the 5′-flanking regions of AQP2, Hsp70-1, and Hsp70-2 genes revealed that transcriptional regulator binding elements associated with cAMP response were identified at both the Hsp70-1 and Hsp70-2 promoter regions, in addition to AQP2. Luciferase reporter assay demonstrated the significant increase of luminescence after dDAVP stimulation (10−8 M, 6 h) in the LLC-PK1 cells transfected with luciferase vector containing 1 kb of the 5′-flanking region of Hsp70-2 gene. Hsp70-2 protein expression was also increased in mpkCCDc14 cells treated by dDAVP in a concentration-dependent manner. Cell surface biotinylation analysis demonstrated that forskolin (10−6 M, 15 min)-induced AQP2 targeting to the apical plasma membrane was significantly attenuated in the mpkCCDc14 cells with Hsp70-2 knockdown. Moreover, forskolin-induced AQP2 phosphorylation (Ser256) was not significantly induced in the mpkCCDc14 cells with Hsp70-2 knockdown. In contrast, Hsp70-2 knockdown did not affect the dDAVP-induced AQP2 abundance. In addition, siRNA-directed knockdown of Hsp70 significantly decreased cell viability. The results suggest that Hsp70 is likely to play a role in AQP2 trafficking to the apical plasma membrane, partly through affecting AQP2 phosphorylation at Ser256 and cell viability.

Aquaporin; collecting duct; heat shock protein; phosphorylation; vasopressin

Water balance in the human body is regulated by vasopressin, which increases osmotic water permeability of the collecting duct, allowing water reabsorption through aquaporin-2 (AQP2), an apical water channel protein expressed in the principal cells of the collecting ducts (29, 34). In response to vasopressin stimulation, intracellular AQP2 is translocated to the apical plasma membrane and AQP2 protein expression is increased (2, 20, 26, 27, 29, 37). The trafficking of AQP2 from the intracellular vesicle to the apical plasma membrane is mediated by phosphorylation of serine residue (Ser256) of the COOH terminus of AQP2 through activation of the cAMP/Protein kinase A (PKA) signaling pathway (9, 27, 37). In contrast, in the disease conditions such as nephrogenic diabetes insipidus (NDI), vasopressin resistance occurs, resulting in decreased AQP2 trafficking and AQP2 protein expression despite high plasma vasopressin level, and hence decreased urinary concentration and polyuria could develop (20).

The COOH terminus of AQP2 has been known to be a critical region for membrane trafficking of AQP2 (13). Particularly, phosphorylation at the Ser256 in the COOH terminus (PKA phosphorylation consensus site) is important for the AQP2 trafficking to the apical plasma membrane (3). Recently, several proteins were identified that can interact with Ser256 residue on the COOH terminus of AQP2. Heat shock protein 70 (Hsp70), heat shock cognate 70 (Hsc70), α-tropomyosin 5b, annexin II, and heat shock protein 70-5 (known as Bip) are demonstrated to interact with AQP2 at the Ser256 (13), suggesting the potential role of these proteins in the regulation of AQP2 trafficking (22, 29, 36). Interestingly, it was demonstrated that Hsp70, Hsc70, and annexin II interact more with Ser256-unphosphorylated AQP2, whereas Bip binds more to Ser256-phosphorylated AQP2 (43).

Among them, 70-kDa heat shock proteins (Hsc70 and Hsp70) are abundantly expressed in the hypertonic renal medulla (41), and Hsc70 is involved in clathrin-mediated endocytosis including AQP2 internalization (7, 22). However, the role of Hsp70, which has a considerable homology of amino acid sequence to Hsc70, is poorly understood in the AQP2 translocation. Since TonEBP/NFAT5 stimulates the transcription of Hsp70 in response to hypertonicity (41) and the interaction between AQP2 and Hsp70 is increased in response to vasopressin treatment (22), we hypothesized that Hsp70 could play a role in AQP2 trafficking.

The main purpose of the present study was to examine whether the proteins that interact with AQP2, particularly Hsp70, play a role in AQP2 trafficking. For this purpose, we examined 1) whether the expression of AQP2-binding proteins that can interact with Ser256 residue (Hsp70, annexin II, tropomyosin 5b, and Bip) are regulated in the kidneys of rats with water restriction/water loading, long-term dDAVP infusion, or hypokalemia-induced nephrogenic diabetes insipidus (NDI) by semiquantitative immunoblotting; 2) whether the transcription of Hsp70 is regulated by the dDAVP-induced cAMP/PKA pathway by both in silico analysis and luciferase reporter assay; and 3) whether siRNA-mediated knockdown of Hsp70 expression affects AQP2 phosphorylation (Ser256), AQP2 translocation to the apical plasma membrane, and AQP2 abundance by semiquantitative immunoblotting and cell surface biotinylation assay.

Materials and Methods

Experimental protocol for water restriction, water loading, 5-day dDAVP infusion, and hypokalemia-induced NDI in rats. Pathogen-free male Sprague-Dawley (SD) rats (200–250 g) were obtained from Charles River (Orient Bio, Seongnam, Korea). The animal protocols were approved by the Animal Care and Use Committee of the Kyungpook National University, Korea. For water restriction and
water loading, male SD rats were kept individually in metabolic cages for 4 days. For the first 2 days, both groups of rats received daily food rations of 15 g rat chow and 25 ml tap water. Then one group of rats received food rations of 15 g rat chow and 10 ml tap water for 2 days (water-restricted group, n = 6) and the other group received food rations of 15 g rat chow and 35 ml tap water. Then one group of rats received food rations of 15 g rat chow and 10 ml tap water for 2 days (water-restricted group, n = 6) and the other group received food rations of 15 g rat chow and 35 ml tap water. For the first 2 days, both groups of rats received daily food intake were measured.

For the experiments of hypokalemia-induced NDI, SD rats were kept in metabolic cages for 8 days (Fig. 1A). Before giving the potassium-deficient diet (TD.88239, Harlan, Madison, WI) to rats, potassium-deficient diet supplemented by 2.68 mmol/day of potassium chloride (KCl) was provided for the first 2 days with free access to water. During the following 6 days, potassium-deficient diet was supplied to SD rats to induce hypokalemia (n = 9), whereas control rats were given potassium-deficient diet supplemented by 2.68 mmol/day of KCl (n = 10).

Cell culture of mpkCCDc14 cells. mpkCCDc14 cells were cultured in a 1:1 mixture of DMEM and Ham’s F-12 medium (DMEM/F12) containing 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM L-glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM HEPES, 0.1% penicillin-streptomycin solution, 20 mM d-glucose, and 2% heat-inactivated FBS at 37°C (17). The passage numbers of mpkCCD14 cells were 29–38. Cells were cultured in semipermeable filters of Transwell system (0.4-μm pore size, Transwell Permeable Supports, catalog no. 3460, Corning).

Membrane fractionation of rat kidney and electrophoresis for semi-quantitative immunoblotting. Rats were anesthetized under enfurane inhalation and right kidneys were rapidly removed. The right kidney

### Table 1. Functional data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WL (n=6)</th>
<th>WR (n=6)</th>
<th>Vehicle-Control (n=6)</th>
<th>5 days dDAVP (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UO, ml/day</td>
<td>24.5 ± 0.6</td>
<td>4.6 ± 0.3*</td>
<td>12.8 ± 1.7</td>
<td>4.6 ± 0.7*</td>
</tr>
<tr>
<td>U-Osm, mOsm/kgH2O</td>
<td>514 ± 19</td>
<td>2,758 ± 134*</td>
<td>2,100 ± 280</td>
<td>3,500 ± 440*</td>
</tr>
<tr>
<td>p-Osm, mOsm/kgH2O</td>
<td>296 ± 0.7</td>
<td>300 ± 0.3*</td>
<td>296 ± 0.6</td>
<td>297 ± 0.8</td>
</tr>
<tr>
<td>p-Na⁺, mmol/l</td>
<td>142 ± 0.7</td>
<td>144 ± 0.6</td>
<td>135 ± 0.6</td>
<td>134 ± 0.7</td>
</tr>
<tr>
<td>p-K⁺, mmol/l</td>
<td>3.6 ± 0.1</td>
<td>3.6 ± 0.2</td>
<td>4.2 ± 0.1</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>p-Urea nitrogen, mmol/l</td>
<td>3.5 ± 0.2</td>
<td>3.7 ± 0.2</td>
<td>4.9 ± 0.3</td>
<td>8.5 ± 0.6*</td>
</tr>
<tr>
<td>p-Creat, μmol/l</td>
<td>23.6 ± 1.9</td>
<td>19.2 ± 1.5</td>
<td>28.0 ± 2.7</td>
<td>28.0 ± 1.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. WL, water loaded; WR, water restricted; UO, urine output; U-Osm, urine osmolality; p-Osm, plasma osmolality; p-Na⁺, plasma sodium; p-K⁺, plasma potassium; p-Urea nitrogen, plasma urea nitrogen; p-Creat, plasma creatinine. *P < 0.05 compared with control group (WL vs. WR or Vehicle-control vs. 5 days dDAVP).
was dissected to cortex, inner stripe outer medulla (ISOM), and inner medulla (IM), and tissues were homogenized in cold dissection buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.2), as previously described (17). Tissue homogenate was centrifuged at 4,000 g for 15 min at 4°C. The supernatant was pipetted off and the protein concentration was measured (BCA protein assay kit, Thermo Scientific, Rockford, IL). SDS-PAGE was performed on 12% polyacrylamide gels, as previously described (15, 17, 21). Primary antibodies used were anti-AQP2 (Hs77, 294, 257, 256; 1:3,000), anti-AQP2-interacting proteins (Hsp70, 70 kDa; Hsp70-2, 70 kDa; annexin II, 1.6 kDa); tropomyosin 5b (Tropo 5b; 29 kDa); and -actin (42 kDa).

Immunohistochemistry. Left kidneys were fixed by retrograde perfusion via the aorta with 3% paraformaldehyde in PBS, pH 7.4. Immunolabeling was performed on sections from vibratome (50 μm thickness) and paraffin-embedded preparation (2 μm thickness) using methods as described previously (11, 16, 21).

Computer-based in silico analysis of transcription factor binding sites. To identify putative transcriptional factor binding sites of AQP2, Hsp70-1 and Hsp70-2, the 5'-flanking regions of AQP2 (3,000 bp), Hsp70-1 (2,000 bp), and Hsp70-2 (2,000 bp) genes were analyzed using MatInspector (Genomatix Software, Ann Arbor, MI).

Genomic DNA isolation and polymerase chain reaction (PCR). Genomic DNA was isolated from mpkCCDc14 cells by DNeasy kit (Qiangen, Valencia, CA) according to manufacturer's instruction. Mouse AQP2, Hsp70-1, and Hsp70-2 promoter regions were verified from the promoter database of Cold Spring Harbor Laboratory (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home). The 1.7-kb promoter regions of AQP2 gene and the 1-kb promoter regions of Hsp70-1 and Hsp70-2 genes were amplified by KOD-plus polymerase (Toyobo, Osaka, Japan) with primers which contained endonuclease sites. The following components were added in the reaction vial: less than 50 ng of genomic DNA, 1.5 pmol of sense and antisense primers, 5 μl of 10 × KOD reaction buffer, 5 μl of 2 mM dNTP mix, 2 μl of 25 mM MgCl2, and 1 U of KOD polymerase in a total of 50 μl. Amplification was performed for 30–35 cycles with 15 s/96°C denaturation, 30 s/60°C annealing, and 2 min/68°C extension. The primer sequences were as follows: 1.7 kb of AQP2, sense 5'-GAGTCACAG-GTACCCCTTCTCAAGCAAGATTCTCT-3' and antisense 5'-GGGGGACCAGGTTGCTGTGCGGATAC-3'; 1 kb of Hsp70-1,
sense 5'-GAAGATCTTACACAAAACCTTAACATGGACAA-3' and antisense 5'-CCCAAGCTTGCGCTTCGAGCTGAGCG-3'; 1 kb of Hsp70-2, sense 5'-GGGTACCTCTACTTCTTCCTCAAAAAGTCT-3' and antisense 5'-CCCTCGAGGGGCGACTAGGGTGCTCC-3'.

Luciferase reporter assay. pGL3-basic vector (promoterless luciferase vector containing a firefly luciferase reporter gene, Promega, Madison, WI) was used to identify the promoter activity. The 1.7-kb construct (0.5 μg) and the pRL-SV40 vector (0.5 μg, Promega) per well were cotransfected using 0.5 μl Dharmafect (Thermo Scientific, Rockford, IL) and then cells were treated by vehicle or forskolin (10-5 M) for 15 min at 37°C at both the apical and the basolateral sides. Cells were washed three times with ice-cold PBS-CM (10 mM PBS containing 1 mM CaCl2 and 0.1 mM MgCl2, pH 7.5) and incubated for 45 min at 4°C in ice-cold biotinylation buffer (10 mM triethanolamine, 2 mM CaCl2, 125 mM NaCl, pH 8.9) containing 1 mg/ml sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-biotin, Thermo Scientific, Rockford, IL) on the apical side. Cells were washed once with quenching buffer (50 mM Tris-HCl in PBS-CM, pH 8.0) followed by washing twice with PBS-CM. Lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 1.15 mM PMSF, 0.4 μg/ml leupeptin, 0.1 mg/ml pepabloc, 0.1 μM okadaic acid, 1 mM Na2VO3, 25 mM NaF) was added and the lysates were sonicated 2 × 6 pulses at 20% of amplitude. The supernatant was transferred to columns which were previously loaded 200 μl Neutravidin agarose resin (Thermo Scientific, Rockford, IL) and incubated for 60 min at room temperature (RT) with end-over-end mixing. After four times washing with PBS containing protease inhibitors, 1× sample buffer containing DTT was added to the column and incubated 60 min at RT. Samples were heated at 65°C for 10 min.

Semiquantitative immunoblotting of phosphorylated AQP2 (Ser256) and AQP2 in mpkCCDc14 cells with Hsp70-2 knockdown. For examining the changes of forskolin-induced pAQP2 (Ser256) expression in

Fig. 3. Semiquantitative immunoblotting of AQP2 and AQP2-interacting proteins in the inner medulla from vehicle-treated control rats (Con) and rats with dDAVP-infusion for 5 days (dDAVP-5D). A: immunoblots of AQP2 (deglycosylated 29 kDa and glycosylated 35–50 kDa), Hsp70, and Hsp70-2 expression, dDAVP (10−11–10−8 M) was treated at the basolateral side of the polarized cells for either 24 h or 48 h. Cells were lysed by RIPA buffer, and changes of protein expression were analyzed by semiquantitative immunoblotting analysis.

Cell surface biotinylation assay. mpkCCDc14 cells were seeded (5 × 106 cells/well) on semipermeable filters of Transwell system and cultured for 3 days. Hsp70-2 siRNA was transfected for 48 h with Dharmafect (Thermo Scientific, Rockford, IL) and then cells were treated by vehicle or forskolin (10−5 M) for 15 min at 37°C at both the apical and the basolateral sides. Cells were washed three times with ice-cold PBS-CM (10 mM PBS containing 1 mM CaCl2 and 0.1 mM MgCl2, pH 7.5) and incubated for 45 min at 4°C in ice-cold biotinylation buffer (10 mM triethanolamine, 2 mM CaCl2, 125 mM NaCl, pH 8.9) containing 1 mg/ml sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-biotin, Thermo Scientific, Rockford, IL) on the apical side. Cells were washed once with quenching buffer (50 mM Tris-HCl in PBS-CM, pH 8.0) followed by washing twice with PBS-CM. Lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 1.15 mM PMSF, 0.4 μg/ml leupeptin, 0.1 mg/ml pepabloc, 0.1 μM okadaic acid, 1 mM Na2VO3, 25 mM NaF) was added and the lysates were sonicated 2 × 6 pulses at 20% of amplitude. The supernatant was transferred to columns which were previously loaded 200 μl Neutravidin agarose resin (Thermo Scientific, Rockford, IL) and incubated for 60 min at room temperature (RT) with end-over-end mixing. After four times washing with PBS containing protease inhibitors, 1× sample buffer containing DTT was added to the column and incubated 60 min at RT. Samples were heated at 65°C for 10 min.

Semiquantitative immunoblotting of phosphorylated AQP2 (Ser256) and AQP2 in mpkCCDc14 cells with Hsp70-2 knockdown. For examining the changes of forskolin-induced pAQP2 (Ser256) expression in
the mpkCCDc14 cells with Hsp70-2 knockdown, cells were seeded at approximately 80–90% confluence on semipermeable filters of the Transwell system and cultured for 7 days to induce cell polarity. Hsp70-2 siRNA (50 nM) was transfected for three times during the last 3 days with Dharmafect (Thermo Scientific, Rockford, IL). Cells were incubated with 0.5 mM IBMX for 30 min at the apical and basolateral sides, and then treated with vehicle or forskolin (10−5 M) for 15 min at 37°C at both the apical and basolateral sides. Cells were lysed with RIPA buffer (10 mM Tris-HCl, 0.15 M NaCl, 1% NP-40, 1% Na-deoxycholate, 0.5% SDS, 0.02% sodium azide, 1 mM EDTA, pH 7.4) containing proteinase and phosphatase inhibitors (0.4 µg/ml leupeptin, 0.1 mg/ml pefabloc, 1 mM Na3VO4, 25 mM NaF, and 0.1 µM okadaic acid). For immunoblotting of phosphorylated AQP2 (pAQP2, Scy206), PVDF (polyvinylidene fluoride) membrane (0.45 µm pore size, Millipore) was used. Primary antibodies used were anti-pAQP2 (K0307AP), and the blots were visualized with horseradish peroxidase-conjugated secondary antibodies (DAKO, P448).

For examining whether Hsp70-2 knockdown affects the dDAVP-induced upregulation of total AQP2 expression in the mpkCCDc14 cells, a mixture of Hsp70-2 siRNA and Dharmafect was treated to mpkCCDc14 cells on semipermeable filters of the Transwell system. Cells were cultured for 7 days to induce cell polarity, and at day 8, cells were incubated in serum-free and hormone-deprived medium for 24 h before dDAVP treatment. dDAVP (10−9 M) was treated at the basolateral side of the polarized cells for 48 h. Protein expression of AQP2 and Hsp70-2 was analyzed by semiquantitative immunoblot analysis.

Cell viability assay in mpkCCDc14 cells with Hsp70 knockdown. Changes of cell viability in mpkCCDc14 cells in response to Hsp70-siRNA or Hsp-70-2-siRNA-mediated knockdown were analyzed by MTT assay. The cells (70% confluence) were transfected by 50 nM of siRNAs using Dharmafect (Thermo Scientific, Rockford, IL) in 12-well plates. After 24 h or 48 h, 20 µl of MTT solution (5 mg/ml) was added, and the plates were incubated at 37°C for 2 h. To solubilize the MTT formazan crystals, DMSO was added to the cells and incubated with gentle shaking at RT for 2 h. The absorbance of the formazan-solubilized DMSO was measured at the wavelength of 595 nm using a microplate reader (Tecan Sunrise, Grödig, Austria). The cell viability was calculated by the following equation: cell viability = [(absorbance of Hsp70- or Hsp-70-2-siRNA-treated well) – (absorbance of DMSO)]/[(absorbance of control-siRNA-treated well) – (absorbance of DMSO)] × 100. Protein knockdown in response to siRNA treatment was confirmed by immunoblot analysis.

Statistical analysis. Quantitative data are presented as means ± SE. Comparisons were made by unpaired t-test (between two groups) or one-way ANOVA followed by Tukey’s multiple comparisons post hoc test (more than two groups). Multiple comparisons tests were only applied when a significant difference was determined in the ANOVA (P < 0.05). P values < 0.05 were considered statistically significant.

RESULTS
Decreased urine output and increased urine osmolality in water-restricted rats or dDAVP-treated rats. Rats subjected to water restriction (10 ml of water intake/day for 2 days, n = 6) demonstrated significantly decreased urine output (4.6 ± 0.3 vs. 24.5 ± 0.6 ml/day, P < 0.05) and increased urine osmolality...
(2,758 ± 134 vs. 514 ± 19 mOsm/kgH2O, \( P < 0.05 \)) compared with the water-loaded rats (35 ml of water intake/day for 2 days, \( n = 6 \), Table 1). Moreover, rats were treated by dDAVP via osmotic minipumps (dDAVP 40 ng/h sc for 5 days). Urine output was significantly decreased in dDAVP-infused rats (4.6 ± 0.7 ml/day, \( n = 6 \), \( P < 0.05 \)) compared with vehicle-treated control rats (12.8 ± 1.7 ml/day, \( n = 6 \)) at day 5. Consistent with this, urine osmolality was markedly increased in dDAVP-infused rats (3,500 ± 440 mOsm/kgH2O, \( P < 0.05 \)) compared with the control rats (2,100 ± 280 mOsm/kgH2O). The functional data are summarized in Table 1.

**Increased urine output and decreased urine osmolality in hypokalemic rats (HypoK).** Rats receiving potassium-deficient diet for 6 days demonstrated significantly decreased plasma potassium level (3.6 ± 0.1 vs. 2.5 ± 0.2 mmol/l, \( P < 0.05 \), Table 2). Urine output was gradually increased in hypokalemic rats after 3 days on potassium-deficient diet (Fig. 1B). At day 6, urine output in control rats on potassium-supplemented diet was 7.8 ± 0.8 ml/day, whereas it was significantly increased in hypokalemic rats (17.3 ± 2.5 ml/day, \( P < 0.05 \)). Consistent with this, urine osmolality was markedly decreased in hypokalemic rats (611 ± 70 mOsm/kgH2O, \( P < 0.05 \)) compared with control rats (1,800 ± 320 mOsm/kgH2O, Table 2). Plasma sodium and osmolality levels were unchanged (Table 2).

**Altered expression of renal AQP2 and AQP2-binding proteins after water restriction or dDAVP infusion.** Water-restricted rats demonstrated significantly increased AQP2 expression in the IM (184 ± 16% of the level in water-loaded rats; \( P < 0.05 \), Fig. 2). Moreover, expression of Hsp70 (134 ± 8%, \( P < 0.05 \)) and Hsp70-2 (146 ± 9%, \( P < 0.05 \)) in the IM was also markedly increased (Fig. 2), indicating that Hsp70 is regulated by the changes of body water balance. In contrast, AQP2-binding proteins (annexin II, BiP, tropomyosin 5b) were unchanged (Fig. 2).

In dDAVP-infused group, AQP2 protein expression in the IM was significantly increased (153 ± 9% of the vehicle-
treated control level, \( P < 0.05 \), Fig. 3). Moreover, expression of Hsp70 (129 ± 5%, \( P < 0.05 \)) and Hsp70-2 (173 ± 12%, \( P < 0.05 \)) in the IM were markedly increased (Fig. 3), indicating that Hsp70 is also regulated by long-term vasopressin infusion. In contrast to the model of water restriction (vs. water loading), long-term dDAVP infusion increased protein expression of Bip and decreased tropomyosin 5b (Fig. 3). This finding suggested that exogenous administration of a high dose of dDAVP exerted other effects, compared with the results seen in a model of water restriction/loading.

Immunoperoxidase microscopy demonstrated that AQP2 labeling was observed at the apical plasma membrane and cytoplasm of the collecting duct cells in vehicle-treated controls (Fig. 4A), whereas AQP2 labeling was more prominent at the apical plasma membrane in dDAVP-infused rats (Fig. 4B). Hsp70 and Hsp70-2 labeling was associated with collecting duct cells and thin limb structures in the IM (Con, Fig. 4, C and E), and the labeling intensity was markedly increased after dDAVP administration (dDAVP 5 days, Fig. 4, D and F).

Altered expression of renal AQP2 and AQP2-binding proteins in hypokalemia-induced NDI. In the kidney IM, AQP2 protein expression in hypokalemic rats was significantly decreased (60 ± 7% of control level, \( P < 0.05 \), Fig. 5), consistent with the observed polyuria and decreased urine osmolality (Table 2). Hypokalemia was also associated with significantly decreased Hsp70 (44 ± 4%, \( P < 0.05 \)) and annexin II (69 ± 3%, \( P < 0.05 \)) expression, whereas expression of BiP and tropomyosin 5b was unchanged (Fig. 5). The data of changes in protein expression levels in the kidney cortex, outer medulla, and inner medulla are summarized in Table 3.

Immunohistochemistry of kidney sections from rats with hypokalemia-induced NDI confirmed decreased AQP2 labeling intensity in the collecting duct principal cells of the IM, ISOM, and cortex (Fig. 6), as demonstrated previously (23). In addition, dilatation of collecting duct was observed in the IM, distinct from that of control (Fig. 6, F vs. E). Immunohistochemistry demonstrated that Hsp70 (Fig. 7, A and B) and annexin II (not shown) were mostly expressed in the IM, whereas the immunolabeling intensity was weaker in the ISOM and cortex (not shown). In the IM, the labeling intensity of Hsp70 and annexin II was decreased in hypokalemia-induced NDI (Fig. 7, C–F), consistent with semiquantitative immunoblotting. Hsp70 labeling was mainly cytosolic in the collecting...
duct cells, and annexin II labeling was mostly seen at the plasma membranes (Fig. 7, C–F).

**Putative transcriptional factor binding sites of AQP2, Hsp70-1, and Hsp70-2 genes.** To identify the putative binding sites for transcriptional factors in AQP2, Hsp70-1, and Hsp70-2 genes, in silico analysis of 5′-flanking regions of AQP2 (3,000 bp), Hsp70-1 (2,000 bp), and Hsp70-2 (2,000 bp) genes was performed using the Genomatix Software Suite (Genomatix Software, Ann Arbor, MI). In the 3,000 bp of the 5′-flanking region of AQP2 gene, 725 transcriptional regulator binding elements (TRBEs) were found, respectively. Among them, cAMP-responsive elements were selected. Fifteen TRBEs (AP1, AP-1F, AP-2, SP-1, CREB, NFAT, HAND, P53, SMAD, ETSF, THAP, CEBP, CAAT, KLFS, and GATA) were identified at the AQP2 promoter region (Fig. 8A). Moreover, six TRBEs (CEBP, SP1, AP1R, THAP, CREB, and AP2) in the Hsp70-1 promoter region and 10 TRBEs (HAND, SP1, KLFS, NFKB, CAAT, AP1R, AP2, CREB, CEBP, and NFAT) in the Hsp70-2 promoter region were identified (Fig. 8A). This suggests that Hsp70 could be regulated by vasopressin-induced cAMP stimulation.

**Luciferase reporter assay for AQP2 and Hsp70 in response to dDAVP stimulation.** To further examine whether transcription of Hsp70-1 and Hsp70-2 are regulated by cAMP pathway induced by vasopressin, LLC-PK1 cells transfected by constructs of 1.7 kb of the 5′-flanking region of AQP2 gene, and 1 kb of the 5′-flanking region of Hsp70-1 gene or Hsp70-2 gene, respectively, were stimulated by dDAVP (10⁻⁸ M) for either 3 h or 6 h. When 1.7 kb of the AQP2 promoter region was transfected to LLC-PK1 cells, the level of transcription was unchanged in response to 3 h dDAVP treatment, but it was significantly increased after 6-h dDAVP treatment (160 ± 13% of vehicle-treated control group, *P* < 0.05, Fig. 8B). Moreover, LLC-PK1 cells transfected by 1 kb of the 5′-flanking region of Hsp70-2 construct revealed significantly increased luminescence at 6-h dDAVP stimulation (135 ± 12% of control level, *P* < 0.05, Fig. 8D). In contrast, cells transfected by luciferase vector containing 1 kb of the 5′-flanking region of Hsp70-1 gene did not demonstrate any changes despite the stimulation with 10⁻⁸ M dDAVP for 6 h (Fig. 8C). In addition, there were no significant changes in the transcription in the cells transfected either by Hsp70-1 or Hsp70-2 promoter constructs when they were treated by 10⁻⁸ M dDAVP for 3 h (Fig. 8, C and D).

**dDAVP concentration-response in expression of AQP2 and Hsp70 in mpkCCDc14 cells.** To examine the response of Hsp70 protein expression to dDAVP stimulation in vitro, Hsp70 expression in mpkCCDc14 cells was examined in response to various concentrations of dDAVP. Serially diluted dDAVP (10⁻¹¹–10⁻⁸ M) was added to the basolateral side of mpkCCDc14 cells cultured on semipermeable filters for either 24 h or 48 h. AQP2 expression was increased in the cells treated by the concentrations from 10⁻¹⁰ to 10⁻⁸ M of dDAVP for either 24 h or 48 h, compared with vehicle-treated cells (Fig. 9, A–C). Although it was not concentration dependent, expression of total Hsp70 was not changed in response to dDAVP (Fig. 9, A, B, and D). In contrast, expression of Hsp70-2 was increased in the range of dDAVP concentrations from 10⁻¹⁰ to 10⁻⁸ M for 24 h in a concentration-dependent manner (Fig. 9, A, B, and E). The results indicated that Hsp70-2 expression was increased in response to dDAVP stimulation, consistent with the luciferase reporter assay (Fig. 8).

**Cell surface biotinylation assay for the changes of AQP2 expression in the apical plasma membrane of mpkCCDc14 cells with siRNA-directed knockdown of Hsp70-2.** To identify the role of Hsp70-2 in AQP2 trafficking to the apical plasma membrane of the kidney collecting duct cells, cell surface biotinylation was performed in the mpkCCDc14 cells with siRNA-directed knockdown of Hsp70-2 (74 ± 4% compared with control siRNA-treated cells, *P* < 0.05, Fig. 10, A and B). In the control siRNA-transfected cells, short-term forskolin treatment (10⁻⁵ M, 15 min) induced a significant increase of AQP2 expression in the apical plasma membrane (AQP2 expression of the biotinylated apical plasma membrane to total...
AQP2 expression: 280 ± 21% of vehicle-treated control, \( P < 0.05 \), Fig. 10, C and D). In contrast, in the cells with Hsp70-2 knockdown, forskolin treatment (10^{-5} M, 15 min) did not increase AQP2 expression at the apical plasma membrane [90 ± 25% of vehicle-treated cells with Hsp70-2 knockdown, not significant (NS), Fig. 10, C and D].

Moreover, to demonstrate whether Hsp70-2 exerts an effect on the phosphorylation of AQP2 (Ser^{256}), semiquantitative immunoblotting was done in the mpkCCDc14 cells in a condition of Hsp70-2 specific knockdown (68 ± 4% compared with control siRNA-treated cells, \( P < 0.05 \), Fig. 11, A and B). In the control siRNA-transfected mpkCCDc14 cells, the ratio of phosphorylated AQP2 at Ser^{256} residue to total AQP2 expression was markedly increased when stimulated by 10^{-5} M forskolin for 15 min (180 ± 34% of vehicle-treated cells, \( P < 0.05 \), Fig. 11, A and C). In contrast, forskolin treatment did not induce AQP2 phosphorylation (Ser^{256}) in the mpkCCDc14 cells with siRNA-directed knockdown of Hsp70-2 (80 ± 6% of vehicle-treated cells, NS, Fig. 11, A and C).

dDAVP-induced AQP2 protein expression in mpkCCDc14 cells with Hsp70-2 knockdown. We examined whether Hsp70-2 knockdown could affect dDAVP-induced upregulation of AQP2 expression. Hsp70-2 expression in mpkCCDc14 cells was decreased by treatment of Hsp70-2 siRNA (50 nM): 64 ± 5% of the control siRNA-treated cells (\( P < 0.05 \), Fig. 12A). In control siRNA-treated cells, AQP2 expression was significantly increased in response to dDAVP treatment (10^{-9} M) for 48 h (138 ± 10%, \( P < 0.05 \), Fig. 12, A and B). In Hsp70-2 siRNA-treated cells, AQP2 expression was also increased by dDAVP treatment (143 ± 13%, \( P < 0.05 \), Fig. 12, A and B). The results indicate that dDAVP-induced up-regulation of AQP2 was not affected by siRNA-mediated Hsp70-2 knockdown.

Cell viability of mpkCCDc14 cells in response to Hsp70 knockdown. Hsp70 has been known to be associated with cellular survival mechanisms against cellular stress (6), and suppression of Hsp70 expression has shown decrease of cell viability under cellular stress (33). Cell viability of mpkCCDc14 cells after siRNA-directed knockdown of Hsp70-1 or Hsp70-2 was examined by MTT assay. Treatment of siRNA against Hsp70-1 or Hsp70-2 revealed time-dependent reduction of Hsp70 (24 h, 31 ± 1%; and 48 h, 5 ± 1% of Hsp70 expression compared with control siRNA-treated cells, \( P < 0.05 \), Fig. 13, A–C) and Hsp70-2 (24 h, 74 ± 8%; and 48 h, 40 ± 6% of Hsp70-2 expression compared with control siRNA-treated cells, \( P < 0.05 \), Fig. 13, A, B, and D), respectively. Viability of mpkCCDc14 cells treated by 50 nM Hsp70-1 siRNA was decreased at 24 h and 48 h, respectively (89 ± 1% and 74 ± 0.3% compared with

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**Fig. 8.** In silico analysis of the 5'-flanking region of AQP2, Hsp70-1, and Hsp70-2 and luciferase reporter assay of AQP2, Hsp70-1, and Hsp70-2 genes in response to dDAVP stimulation. A: in silico analysis of 5'-flanking region of AQP2 (3,000 bp), Hsp70-1 (2,000 bp), and Hsp70-2 (2,000 bp) genes. In each gene, transcriptional regulator binding elements that are associated with cAMP response were detected. B–D: Luciferase reporter assays for AQP2 (B), Hsp70-1 (C), and Hsp70-2 (D). The significant increase of luminescence was detected after 6 h stimulation of dDAVP (10^{-8} M) in the LLC-PK1 cells transfected with either 1.7 kb of the 5'-flanking region of AQP2 gene (B) or 1 kb of the 5'-flanking region of Hsp70-2 gene (D). V, vehicle treatment; D, dDAVP treatment. \( n \), no. of cell lysate preparations from 2 independent experiments of AQP2, Hsp70-1, and Hsp70-2. \* \( P < 0.05 \) compared with control group.
discrepancy between the changes of AQP2 and Hsp70 protein expression in mpkCCDc14 cells treated by the concentrations from $10^{-11}$ to $10^{-8}$ M of dDAVP for 24 h or 48 h compared with vehicle-treated cells, although it was not dependent on the concentration of dDAVP. Expression of total Hsp70 was not changed in response to dDAVP, whereas Hsp70-2 expression was increased in the range of dDAVP concentration from $10^{-10}$ to $10^{-8}$ M for 24 h in a concentration-dependent manner.

**DISCUSSION**

In this study, we demonstrated that water restriction or long-term dDAVP infusion in normal rats increased renal abundance of AQP2 and Hsp70, along with increased urine concentration. In contrast, renal abundance of AQP2 and Hsp70 was significantly decreased in rats with hypokalemia-induced NDI. In silico analysis of 5′-flanking regions of AQP2, Hsp70-1, and Hsp70-2 genes demonstrated that TRBEs associated with cAMP response were identified in both the Hsp70-1 and Hsp70-2 promoter regions, in addition to AQP2. Consistent with this, luciferase reporter assay demonstrated an increase of Hsp70-2 transcription after 6-h stimulation of dDAVP, in addition to AQP2. Moreover, Hsp70-2 protein expression was increased in mpkCCDc14 cells treated by dDAVP in a concentration-dependent manner for 24 h. Cell surface biotinylation analysis demonstrated that forskolin-induced AQP2 targeting to the apical plasma membrane was significantly decreased in the mpkCCDc14 cells with Hsp70-2 knockdown. In addition, Hsp70-2 knockdown did not affect the dDAVP-induced AQP2 abundance.

Heat shock proteins belong to a class of functionally related proteins which play a role in the folding and unfolding of other proteins. Hsp70 acts as a molecular chaperone and is involved in several processes, e.g., protein folding, reassembly of misfolded proteins and aggregated amyloid, and trafficking of proteins (12, 24, 30). Activated heat shock factor by the nonnative proteins that accumulate in response to a variety of stress including heat binds to heat shock element and stimu-
lates transcription of Hsp70 and other chaperones. Consistent with this, previous studies demonstrated the protective effects of Hsp70 against cell injury (12, 24, 35). For instance, expression of the major heat-inducible protein Hsp70 protects cells from heat-induced apoptosis (10), the harmful effects of a high urea concentration in renal medulla (40), and ischemic injury in the kidney (39). In the present study, MTT assay demonstrated that cell viability of mpkCCDc14 cells was affected by siRNA-directed knockdown of Hsp70-1 or Hsp70-2. This could be one of the potential factors to decrease the trafficking of AQP2-containing vesicles, particularly in the presence of a nonphysiological stimulation like forskolin.

A recent study demonstrated that 70-kDa heat shock proteins (Hsc70 and Hsp70) interact with AQP2 (22). Lu et al. (22) identified the Hsc70/Hsp70 as AQP2-interacting proteins by yeast two-hybrid screening of a human kidney cDNA library. Interaction was also confirmed by mass spectrometry, pull-down, and coimmunoprecipitation studies (22), showing that vasopressin stimulation in cells enhances the interaction of Hsc70 with AQP2 and induces an increased colocalization of hsc70 and AQP2 in vivo. Functional inhibition of Hsc70 activity, which is a clathrin-decoating ATPase, induces membrane accumulation of AQP2, demonstrating that Hsc70 is involved in the clathrin-mediated endocytosis (22). Another study revealed that cytosolic chaperone Hsc70 promotes the trafficking of intracellular retained melanocortin-4 receptor mutants to the cell surface (25), suggesting that Hsc70 plays a role in the trafficking and endocytosis of proteins including AQP2. In contrast, immunolabeling intensity of Hsp70 at the apical plasma membrane of the collecting duct cells was not increased after vasopressin stimulation, as demonstrated in the present study and others in a previous study (22). Thus,
AQP2 trafficking and expression and impaired urine concentration in hypertonic conditions are well documented, and altered expression of AQP2 has been observed in a variety of body water balance disorders (18). In brief, vasopressin acts by binding to the V2 receptor, which activates the adenyl cyclase through the GTP binding protein Go. The adenyl cyclase induces the production of cAMP from ATP that binds to the regulatory subunit of protein kinase A (PKA) and induces the phosphorylation of various proteins including AQP2, which is important for the short-term regulation of AQP2, i.e., AQP2 trafficking. Also cAMP mediates the long-term adaptation of AQP2, by increasing the levels of the catalytic subunit of PKA in the nuclei that phosphorylates the transcription factors cyclic AMP responsive element binding protein (CREB) and c-Jun/c-Fos, resulting in AQP2 synthesis (42). Several previous studies support that cAMP plays a critical role in AQP2 regulation: First, a mouse strain with NDI (polyuric DI +/+ severe mice) exhibiting low cAMP levels due to increased cAMP-phosphodiesterase activity demonstrates a significant downregulation of AQP2 (8, 14); second, lithium treatment in rats results in decreased adenylyl cyclase activity and cAMP levels in the medullary collecting duct cells along with decreased AQP2 expression (4, 19, 28, 31); third, phosphodiesterase inhibitor treatment in rats with hypercalcemia inhibits the downregulation of AQP2 and impaired urine concentration (38); and fourth, urine concentration was impaired in mice lacking adenylyl cyclase type IV (32).

The present study supports the view that cAMP plays a role in the regulation of Hsp70 expression, as in the regulation of AQP2 expression. From the computational analysis, Hsp70-1 and Hsp70-2 have several binding sites for potential transcription factors that could be regulated by cAMP. It could imply that expression of Hsp70-1 and Hsp-2 could be mediated by cAMP/PKA signaling pathway, e.g., vasopressin-induced stimulation. This is compatible with the results showing that the response of Hsp70-2 promoter region was substantially augmented by dDAVP stimulation and Hsp70-2 protein expression in mpkCCDc14 cells was increased by dDAVP in a concentration-dependent manner. From these results, Hsp70 is, at least in part, regulated by vasopressin under the control of a common signaling pathway for AQP2 regulation.

A recent study further demonstrated the interactions between AQP2 and Hsc70, Hsp70-1, Hsp-70-2, and annexin II (43). Interestingly, these proteins were found to bind more to the nonphosphorylated form of AQP2 (at Ser256) than to the phosphorylated AQP2 (Ser256), whereas another heat shock protein, Hsp70-5 (BiP/grp78), bound to phosphorylated AQP2 more (41). These results suggest that different interaction of Hsp70 family proteins to the COOH-terminal tail of AQP2 could affect the phosphorylation of AQP2 at Ser256 and regulate AQP2 trafficking. Importantly, cell surface biotinylation revealed that AQP2 expression at the apical plasma membrane was not increased in response to forskolin stimulation in the cells with Hsp70-2 knockdown. Furthermore, during the Hsp70-2 specific knockdown condition, AQP2 phosphorylation at Ser256 residue was not significantly induced by forskolin stimulation. These results suggest that Hsp70-2 plays a role in AQP2 trafficking, partly through the regulation of AQP2 phosphorylation at Ser256 residue.

In summary, we demonstrated that renal abundance of AQP2 and Hsp70, the AQP2-interacting protein, was regulated by vasopressin, as observed in the conditions of water loading/
restriction and dDAVP infusion in rats. In silico analysis demonstrated that a number of TRBEs associated with cAMP response were identified at both the Hsp70-1 and Hsp70-2 promoter regions, in addition to AQP2. This was further demonstrated by luciferase reporter assay, suggesting that Hsp70 could be regulated by a cAMP-induced signaling pathway, and, accordingly, Hsp70-2 protein expression was increased in mpkCCDc14 cells treated by dDAVP in a concentration-dependent manner. Cell surface biotinylation analysis demonstrated that forskolin-induced AQP2 targeting to the apical plasma membrane was significantly decreased in the cells with Hsp70-2 knockdown. Moreover, AQP2 phosphorylation at Ser256 residue was significantly decreased in the cells with Hsp70-2 knockdown in response to forskolin stimulation. This could suggest that Hsp70 is likely to play a role in AQP2 trafficking to the plasma membrane, by affecting phosphorylation of AQP2 at Ser256. In addition, siRNA-directed knockdown of Hsp70 significantly decreased cell viability of mpkCCDc14 cells. Since Hsp70 plays a role against cellular stress, the observed decreased AQP2 trafficking in the cells where Hsp70 expression was decreased was also partly due to the decreased cell viability.

GRANTS
This study was supported by a National Research Foundation Grant (2010-0008225 and 2010-0019393) funded by the Ministry of Education, Science and Technology (MEST), Korea, and the Korea Healthcare Technology R&D Project, Ministry of Health and Welfare, Korea (A111345).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
REFERENCES


