Changes of renal AQP2, ENaC, and NHE3 in experimentally induced heart failure: response to angiotensin II AT1 receptor blockade

Sophie C. Lütken,1,2 Soo Wan Kim,1,3 Thomas Jonassen,4 David Marples,5 Mark A. Knepper,6 Tae-Hwan Kwon,1,7 Jørgen Frøkiær,1,8 and Søren Nielsen1,2

1Water and Salt Research Center and 2Institute of Anatomy, University of Aarhus, Aarhus C, Denmark; 3Department of Internal Medicine, Chonnam National University Medical School, Gwangju, Korea; 4Department of Pharmacology, Panum Institute, University of Copenhagen, Denmark; 5Institute of Membrane and Systems Biology, University of Leeds, Leeds, United Kingdom; 6Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland; 7Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Taegu, Korea; and 8Institute of Clinical Medicine, Aarhus University Hospital, Aarhus N, Denmark

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Lütken SC, Kim SW, Jonassen T, Marples D, Knepper MA, Kwon TH, Frøkiær J, Nielsen S. Changes of renal AQP2, ENaC, and NHE3 in experimentally induced heart failure: response to angiotensin II AT1 receptor blockade. Am J Physiol Renal Physiol 297: F1678–F1688, 2009. First published September 23, 2009; doi:10.1152/ajprenal.00010.2009.—Heart failure (HF) was induced by ligation of the left anterior descending artery (LAD). Left ventricular end-diastolic pressure (LVEDP) >25 mmHg (at day 23 after LAD ligation) was the inclusion criterion. The rats were divided into three groups: sham-operated (Sham, n = 23, LVEDP: 5.6 ± 0.6 mmHg), HF (n = 14, LVEDP: 29.4 ± 1.4 mmHg), and candesartan (1 mg·kg−1·day−1) (Can, n = 9, LVEDP: 29.2 ± 1.2 mmHg). After 7 days (i.e., 29 days after LAD ligation) semi-quantitative immunoblotting revealed increased abundance of inner medulla aquaporin-2 (AQP2) and AQP2 phosphorylated at Ser256 (p-AQP2) in HF. There was also markedly enhanced apical targeting of AQP2 and p-AQP2 in inner medullary collecting duct (IMCD) in HF compared with Sham rats, shown by immunocytochemistry. Candesartan treatment significantly reversed the increases in both AQP2 and p-AQP2 expression and trafficking. In contrast, there were only modest changes in other collecting duct segments. Semiquantitative immunoblotting revealed increased expression of type 3 Na+/H+ exchanger (NHE3) and Na+/K+-2Cl− cotransporter (KCC2) in kidney tissues. AQP2 expression was not affected by candesartan treatment. The protein abundance of α-epithelial sodium channel (α-ENaC) was increased while β-ENaC and γ-ENaC expression was decreased in the cortex and outer stripe of the outer medulla in HF compared with Sham rats, which was partially reversed by candesartan treatment. These findings strongly support an important role of angiotensin II in the pathophysiology of renal water and sodium retention associated with HF.

Role of AQP2 in HF, because of its various effects on vascular tone, kidneys, adrenal glands, brain, heart, and the autonomic nervous system (for review, see Ref. 53). Thus in HF activation of RAAS may become a maladaptive response to arterial underfilling, introducing a vicious cycle leading to increasing morbidity and mortality (17). As a consequence, a variety of pharmaceutical efforts have targeted the RAAS. However, the underlying mechanisms in the impairment of body water and sodium handling in HF are not fully clarified, and whether activated sodium and water transport can be reversed by the blockade of ANG II receptor remains undefined.

Renal water retention in HF is in part mediated through the release of AVP, which acts on the AVP-dependent water channel aquaporin-2 (AQP2) in the collecting duct (CD) (49, 56, 62). Briefly, AVP binds to the vasopressin V2 receptor at the basolateral membrane of the principal cells, increasing the intracellular levels of cAMP via adenylyl cyclase and thereby activating protein kinase A (PKA). The AQP2 monomers contain a consensus site for PKA phosphorylation at Ser256 near the COOH terminal. Phosphorylation of 3 or 4 monomers of the homotramers is necessary for trafficking to the apical plasma membrane (10, 20, 29). AVP-induced water permeability, mediated by the translocation of AQP2 from intracellular vesicles to the apical plasma membrane in the CD principal cells, is well characterized, and there is an extensive axial heterogeneity in the subcellular localization of AQP2 along the connecting tubule (CNT) and CD in different physiological settings (9).

Future investigations need to focus on defining the changes of subcellular localization and segmental changes in the expression of AQP2 and AQP2 phosphorylated at Ser256 (p-AQP2) in HF, which may play a major role in the expansion of extracellular fluid volume, and whether such changes in expression and trafficking can be inhibited by ANG II type 1 (AT1) receptor blockade.

Studies in HF patients and in experimentally induced rat models have revealed dysregulation of some of the renal sodium transporters indicating enhanced sodium reabsorption in the proximal tubule (59) and thick ascending limb (TAL) of Henle (17, 55). After bulk transport in the proximal tubule and TAL, the distal nephron, including renal CNT and CD, is responsible for the final regulation of urinary sodium excretion. Active transepithelial sodium reabsorption is achieved by principal cells under the control of aldosterone, AVP, and ANG II when sodium enters the cell through apical amiloride-sensitive
epithelial sodium channel (ENaC) and leaves it via the basolateral Na-K-ATPase (5, 8, 14). We demonstrated in previous studies that nephrotic syndrome (35, 38) and liver cirrhosis (37, 39) were associated with increased abundance and apical targeting of ENaC subunits, which were proposed to play a role in the development of sodium retention. However, it remains to be elucidated whether there are changes in expression and plasma membrane targeting of ENaC subunits and whether such a potential dysregulation may play a role in sodium retention in HF. Further studies may help the understanding of molecular mechanisms of sodium retention in HF.

Thus the purpose of the present study was to examine whether there are changes in the expression and the subcellular localization of AQP2 or p-AQP2 along the CNT and CD in rats with HF; to investigate whether there are changes in expression of the major renal sodium transporters [type 3 Na+/H+ exchanger (NHE3), bumetanide-sensitive Na+/K+2Cl- cotransporter type 1 (NKCC2), and ENaC] and type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2) in HF; and to examine whether these changes can be modulated by AT1 receptor antagonist treatment.

MATERIALS AND METHODS

Experimental animals. Wistar rats obtained from Harlan Netherlands with an initial weight of 250 g were given free access to tap water and standard rat chow (Altromin 1324, Altromin, Lage, Germany). The rats were housed under controlled temperature (21 ± 2°C) and humidity (55 ± 2%) in a 12:12-h light-dark cycle. The animal protocols were approved by the board at the Institute of Clinical Medicine, University of Aarhus according to the licenses for use of experimental animal issued by the Danish Ministry of Justice.

Animal preparations. HF was induced by free wall myocardial infarction following ligation of the left anterior descending artery (LAD) (51, 54, 55). Rats were anesthetized with 2% isoflurane and artificially ventilated with a rodent ventilator (Ugo Basile catalog no. 7025). LAD occlusion was ascertained by paling of the left ventricular lateral wall before closure of the chest wall. The thorax was closed in 7025). LAD occlusion was ascertained by paling of the left ventricular lateral wall before closure of the chest wall. The thorax was closed in artificial ventilation with a rodent ventilator (Ugo Basile catalog no. 7025). LAD occlusion was ascertained by paling of the left ventricular lateral wall before closure of the chest wall. The thorax was closed in artificial ventilation with a rodent ventilator (Ugo Basile catalog no. 7025). LAD occlusion was ascertained by paling of the left ventricular lateral wall before closure of the chest wall. 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Denmark, Odense. ANG II in plasma was determined by radioimmunoassay using a modification of the method originally described by Kappelgaard et al. (30). The antibody was obtained from the Department of Clinical Physiology, Glostrup Hospital, Glostrup, Denmark. The minimal detection level was 2 pmol/l plasma. ANG II analysis was performed at the Laboratory of Biological Psychiatry, Psychiatric Hospital, Aarhus, Denmark.

**Semiquantitative immunoblotting.** The kidney was dissected into renal cortex including the outer stripe of the outer medulla (OSOM), the inner stripe of the outer medulla (ISOM), and the inner medulla (IM). These sections were individually homogenized (Ultra-Turrax T8 homogenizer, IKA Laborteknik, Staufen, Germany) in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.2. The homogenates were centrifuged at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria, and the supernatant was pipetted off and kept on ice. The total protein concentration was measured (Pierce BCA protein assay reagent kit, Pierce, Rockford, IL). All samples were adjusted with isolation solution to reach the same final protein concentrations, solubilized at 65°C for 15 min in Laemmli sample buffer, and then stored at −20°C. To confirm that protein loading of the gels was equal, preliminary 12% polyacrylamide gels were stained with Coomassie blue (Fig. 2), as previously described (7). Coomassie blue staining and densitometry with an image analysis system (Amersham Pharmacia Bio-tech). The band densities were quantitated by scanning densitometry, adjusted for equal loading of proteins to each lane (generally, <5% variation relative to the mean) (7, 58). SDS-PAGE was performed on 9% or 12% polyacrylamide gels. Each lane was loaded with ~15 μg of protein from samples from a different rat. The proteins were transferred from the gel electrooretically (Bio-Rad Mini Protein II) to nitrocellulose membranes (Hybond ECL RP13032D, Amersham Pharmacia Biotech, Little Chalfont, UK). After transfer the blots were blocked with 5% milk in PBS-T (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with primary antibodies. The sites of antibody-antigen reaction were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (P447 or P448, diluted 1:3,000; DAKO, Glostrup, Denmark) for 1 h at room temperature. After three 10-min rinses with PBS wash buffer, the bands of antibody-antigen reaction were visualized with a brown chromogen produced for 10 min at room temperature by incubation with 0.05% 3,3′-diaminobenzidine tetrachloride (Kem-en Tek, Copenhagen, Denmark) dissolved in distilled water with 0.1% H2O2. Mayer’s hematoxylin was used for counterstaining, and after dehydration coverslips were mounted with hydrophobic medium (Eukitt, O. Kindler, Freiburg, Germany). Light microscopy was carried out with Leica DMRE (Leica Microsystems). For immunoelectron microscopy, small tissue blocks were trimmed from the perfusion-fixed kidneys from the intermediate one-third of the inner medulla (IM2), cryoprotected with 2.3 M sucrose, mounted on holders, and frozen in liquid nitrogen as previously described in detail (47). Ultrathin Lowicryl HM20 sections (50 nm) were incubated with affinity-purified anti-AQP2, and labeling was visualized with goat-anti-rabbit immunoglobulin, DAKO P448, DAKO (goat anti-rabbit immunoglobulin, DAKO P448, DAKO) for 1 h at room temperature. After three 10-min rinses with PBS wash buffer, the bands of antibody-antigen reaction were visualized with a brown chromogen produced for 10 min at room temperature by incubation with 0.05% 3,3′-diaminobenzidine tetrachloride (Kem-en Tek, Copenhagen, Denmark) dissolved in distilled water with 0.1% H2O2. Mayer’s hematoxylin was used for counterstaining, and after dehydration coverslips were mounted with hydrophobic medium (Eukitt, O. Kindler, Freiburg, Germany). Light microscopy was carried out with Leica DMRE (Leica Microsystems). For immunoelectron microscopy, small tissue blocks were trimmed from the perfusion-fixed kidneys from the intermediate one-third of the inner medulla (IM2), cryoprotected with 2.3 M sucrose, mounted on holders, and frozen in liquid nitrogen as previously described in detail (47). Ultrathin Lowicryl HM20 sections (50 nm) were incubated with affinity-purified anti-AQP2, and labeling was visualized with goat-anti-rabbit IgG conjugated to 10-nm colloidal gold particles (GAR.EM10, BioCell Research Laboratories, Cardiff, UK). Examination was performed with a FEI Morgagni 268D transmission electron microscope.

**Computational analysis of apical labeling.** For computational analysis of apical labeling, the Morphscale program (version 5; D. Marples) was used. Immunocytochemical light microscopy pictures were taken at ×63 magnification. Sham (n = 5), HF (n = 4), and HF + Can (n = 5) inner medullary collecting duct (IMCD) were divided into IMCD1-3, IMCD1 being closest to OSOM, and two pictures were taken from each zone at random, converted into grayscale with Corel Photo-Paint version 9, and imported to the Morphscale program. The inner and outer borders of CD principal cells were marked manually, and the IMCD principal cells were divided into IMCD1-3, IMCD1 being closest to OSOM, and two pictures were taken from each zone at random, converted into grayscale with Corel Photo-Paint version 9, and imported to the Morphscale program. The inner and outer borders of CD principal cells were marked manually, and the IMCD principal cells were...
automatically subdivided by the program into nine equally sized subcellular segments from the apical to the basolateral part of the cells, allowing Morphscale to perform pixel value statistics to determine the grayscale density within each segment. The apical domain was represented in subcellular segment 1. Apical labeling was determined as the p-AQP2 grayscale density in the apical plasma membrane compared to that of the whole cell. An increase in the percentage reflects increased apical labeling of p-AQP2.

Statistical analysis. Data are expressed as means ± SE. Statistical significance between sham-operated rats and HF groups was estimated with one-way analysis of variance (ANOVA) followed by the Tukey-Kramer method for unequal sample sizes to test all possible pairwise differences of means to determine whether at least one difference was significantly different from 0. P values <0.05 were considered significant.

RESULTS

Effect of AT₁ receptor antagonist treatment on heart failure and renal water, sodium, and potassium excretion. Pretreatment LVEDP was significantly increased in LAD-ligated rats compared with Sham rats. Furthermore, these rats had decreased contractility determined by a decrease in maximum change in pressure over time (dP/dt,max) and decreased compliance determined by increase in minimum change in pressure over time (dP/dt,min). These findings are consistent with impaired cardiac pumping ability with increased myocardial stiffness and shifting of the pressure-volume curve to the right (6, 51). There was a significant decrease of MAP in HF rats compared with sham-operated rats but no significant difference in MAP between the two LAD-ligated groups (HF vs. HF + Can, Table 1).

Mean body weight was unchanged among groups. Plasma osmolality (mosmol/kgH₂O) was significantly increased in HF rats and HF + Can rats compared with Sham rats. Measurement of plasma urea revealed a significant increase in the HF + Can rats compared with Sham and HF rats. Urine osmolality and urine-to-plasma osmolality ratio were unchanged among groups as were urinary sodium excretion [urine sodium (UNa) × urine output (UO)] and fractional excretion of sodium (FENa). Creatinine clearance was not changed among the three groups (Table 2).

Plasma levels of renin and angiotensin II were increased in HF compared with Sham rats. Candesartan treatment normalized plasma levels of ANG II, while it increased plasma renin levels significantly (Table 3).

Table 1. Changes in heart function

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>HF</th>
<th>HF + Can</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>23</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>5.6±0.6</td>
<td>29.4±1.4*</td>
<td>29.2±1.2*</td>
</tr>
<tr>
<td>dP/dt_{max}, mmHg/s</td>
<td>-6,379±175</td>
<td>-3,157±174*</td>
<td>-3,140±299*</td>
</tr>
<tr>
<td>dP/dt_{min}, mmHg/s</td>
<td>7,073±182</td>
<td>4,149±183*</td>
<td>3,987±342*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>102±4</td>
<td>87±3*</td>
<td>92±3</td>
</tr>
</tbody>
</table>

Values are means ± SE for n rats. These values were measured at day 21 after operation before allocation to candesartan treatment at day 22. Sham, sham operated; HF, heart failure; Can, candesartan; LVEDP, left ventricular end-diastolic pressure; dP/dt_{max}, maximum value of the first derivate of left ventricular pressure over time; dP/dt_{min}, minimum value of the first derivate of left ventricular pressure over time; MAP, mean arterial pressure. *P < 0.001 vs. Sham.

Table 2. Changes in renal function

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>HF</th>
<th>HF + Can</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>23</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Median body weight, g</td>
<td>310±7</td>
<td>296±6</td>
<td>299±7</td>
</tr>
<tr>
<td>UO, μl/min kg⁻¹</td>
<td>23.2±1.2</td>
<td>19.1±1.0</td>
<td>22.5±2.6</td>
</tr>
<tr>
<td>U-Osm, mosmol/kgH₂O</td>
<td>2,049±85</td>
<td>2,122±123</td>
<td>1,967±206</td>
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<tr>
<td>P-Osm, mosmol/kgH₂O</td>
<td>299±0.5</td>
<td>302±1*</td>
<td>304±2*†</td>
</tr>
<tr>
<td>U/P-Osm</td>
<td>6.9±0.3</td>
<td>7.0±0.4</td>
<td>6.5±0.7</td>
</tr>
<tr>
<td>U-Prea, mmol/l</td>
<td>6.1±0.3</td>
<td>6.5±0.3</td>
<td>9.1±0.9†</td>
</tr>
<tr>
<td>FENA, %</td>
<td>30.0±0.2</td>
<td>29.0±0.2</td>
<td>28.0±0.3</td>
</tr>
<tr>
<td>UNa × UO, mmol</td>
<td>4.8±0.1</td>
<td>4.4±0.2</td>
<td>4.2±0.2</td>
</tr>
<tr>
<td>P-Na, mmol/l</td>
<td>139.0±0.1</td>
<td>140±0.4*</td>
<td>138±0.4‡</td>
</tr>
<tr>
<td>P-K, mmol/l</td>
<td>4.5±0.1</td>
<td>4.8±0.2</td>
<td>4.4±0.1</td>
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<tr>
<td>P-Cr, μmol/l</td>
<td>27±1</td>
<td>28±1</td>
<td>32±2*</td>
</tr>
<tr>
<td>Ccr, ml/min</td>
<td>2.1±0.1</td>
<td>1.8±0.1</td>
<td>1.7±0.1</td>
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</table>

Values are means ± SE for n rats. These values were measured at last day of experiment. UO, urine output; U-Osm, urine osmolality; P-Osm, plasma osmolality; U/P Osm, urine-to-plasma osmolality ratio; P-Urea, plasma urea; FENA, fractional excretion of sodium into urine; UNa × UO, rate of urinary potassium excretion; P-Na, plasma sodium; P-K, plasma potassium; P-Cr, plasma creatinine; Ccr, creatinine clearance. *P < 0.05 vs. Sham, †P < 0.05 vs. HF.

Increased abundance of inner medullary AQP2 protein in HF rats was reversed by candesartan administration. A previously characterized affinity-purified polyclonal antibody against AQP2 (AQP2; LL127 AP), which recognizes both nonphosphorylated and phosphorylated AQP2 peptides, was used (48). Semiquantitative immunoblotting revealed that AQP2 protein abundance was significantly increased in the IM of rats with HF, which was counteracted by candesartan treatment (Fig. 3). Immunoblotting of p-AQP2 was performed with antibodies that selectively recognize p-AQP2, which is phosphorylated at the PKA consensus site (Ser256) (10). Semiquantitative immunoblotting also revealed that p-AQP2 abundance in the IM was increased in HF compared with Sham rats, which was reversed by candesartan administration, similar to the changes in total AQP2 abundance (Fig. 3).

Increased apical targeting of AQP2 and p-AQP2 in inner medulla of HF rats was reversed by candesartan treatment. Immunoperoxidase microscopy in IMCD revealed increased labeling of AQP2 and p-AQP2 in the apical plasma domains of principal cells, whereas candesartan treatment redistributed the AQP2 protein evenly to intracellular domains (Fig. 4). To investigate the subcellular localization of p-AQP2 in IMCD principal cells, a computational analysis was performed in which apical labeling was determined as the ratio of p-AQP2 density in the apical plasma membrane domain to that of the whole cell (Fig. 5, Table 4). Increase in percent values reflects increased apical labeling. The apical labeling of p-AQP2 in total IMCD was significantly increased in HF compared with
Sham rats, whereas candesartan treatment reversed this. This was most prominent in IMCD2 compared with IMCD1 and IMCD3 (Table 4).

To further validate the immunoperoxidase labeling seen with light microscopy, immunoelectron microscopic examination was carried out to examine whether the increased apical labeling of total AQP2 in IMCD2 was associated with the plasma membrane in rats with HF. In HF rats immunogold labeling of AQP2 was mainly associated with the apical plasma membrane, and only modest labeling was seen in the intracellular compartments (Fig. 6A), whereas in Sham rats, AQP2 immunogold labeling was mainly associated with intracellular vesicles (Fig. 6B).

AQP2 and p-AQP2 protein expression was increased in cortex/OSOM and ISOM but no change in subcellular localization. As depicted in Fig. 7, HF induced increased protein expression of p-AQP2 in cortex/OSOM and ISOM; however, in cortex the AQP2 expression was comparable with that of Sham rats. The increases in AQP2 and p-AQP2 were partly reversed by candesartan administration.

There were no apparent changes in the subcellular localization of AQP2 and p-AQP2 protein observed in the cortical and outer medullary CD (Fig. 8, A and B and C and D, respectively) and CNT (not shown) between HF and Sham rats. The abundance of NKCC2 protein was increased in cortex and outer medulla in HF. A previously characterized affinity-purified polyclonal antibody against the NKCC2 cotransporter (LL320 AP) was used (16).

The immunoblot was reacted with anti-bumetanide-sensitive NKCC2 and revealed an ~161-kDa band. Densitometry of protein samples from cortex/OSOM showed that NKCC2 abundance was increased in HF rats compared with Sham rats. This increase was markedly reversed in HF + Can rats. Similarly, abundance of NKCC2 protein was increased in the ISOM of rats with HF compared with Sham rats; however, candesartan treatment failed to reverse the increased abundance in this segment (Table 5).

Abundance of NHE3 was increased in cortex and outer medulla in HF. A previously characterized affinity-purified polyclonal antibody against NHE3 (LL546 AP) was used (19). Semiquantitative immunoblotting revealed a single 87-kDa band of the NHE3 protein. NHE3 abundance in the cortex/OSOM was significantly increased in the HF rats compared with Sham rats, which was reversed by candesartan administration. Similarly, NHE3 abundance in the ISOM was increased in HF compared with Sham rats, which was reversed by candesartan treatment (Table 5).

α-ENaC was increased whereas β-ENaC and γ-ENaC were decreased in HF. Previously characterized affinity-purified polyclonal antibodies against the α-, β-, and γ-ENaC subunits were used (45). Semiquantitative immunoblotting revealed a single 85-kDa band corresponding to the α-ENaC protein. As previously described, the anti-ENaC antibodies are peptide-derived polyclonal antibodies against the carboxy tail of the β- and γ-ENaC protein and against the amino tail of the α-ENaC subunit (45). Densitometric analysis of α-ENaC in the cortex/OSOM revealed a significant increase in protein abundance in HF rats compared with Sham rats, which was reversed in the HF + Can group (Fig. 9). In contrast, the abundance of β-ENaC protein at the 85-kDa band was slightly, but not significantly, downregulated in the HF group. Semiquantitative immunoblotting revealed a narrow 85-kDa band and a broader 70-kDa band corresponding to the mass of the γ-ENaC protein. Densitometric analysis from γ-ENaC also revealed a significant decrease in HF compared with Sham rats, which was reversed by candesartan administration (Fig. 9).

Increased type 2 11β-hydroxysteroid dehydrogenase abundance in cortex/OSOM in HF was reversed by candesartan treatment. A commercial polyclonal antibody against 11β-HSD2 was used (Chemicon). Semiquantitative immunoblotting revealed a single 44-kDa band of 11β-HSD2. Protein samples prepared from cortex/OSOM showed a significant increase in the HF compared with Sham rats, which was reduced by candesartan treatment (Fig. 9).

DISCUSSION

Changes of AQP2 and p-AQP2 and response to AT1 receptor blockade in HF. The present results demonstrate that HF is associated with dysregulation of AQP2 in the renal medullary collecting duct. Three important findings were observed: 1) an increase in abundance of AQP2 and p-AQP2 water channel
protein in medullary CD principal cells, 2) a marked redistribution of AQP2 and p-AQP2 to the apical plasma membrane, and 3) reversed abundance of AQP2 and p-AQP2 protein as well as redistribution of AQP2 and p-AQP2 from apical to intracellular domains by treatment with candesartan. The first two findings are both thought to play an important role in the development of water retention (49, 62).

Importantly, previous studies demonstrated that circulating AVP levels and V2 receptor mRNA expression are elevated in HF (22, 61), which is likely to contribute to the increased AQP2 trafficking and abundance. AVP regulates the water permeability by acting on the V2 receptor in short-term and long-term manners. The short-term regulation occurs within minutes because of trafficking of AQP2 bearing vesicles to the

![Fig. 5. Changes in the subcellular localization of p-AQP2 in IMCD. Apical labeling was determined as the ratio of p-AQP2 density in the apical plasma membrane to that of the whole cell. Only principal cells were included. Increase in the % values reflects increased apical labeling. Apical labeling of p-AQP2 in IMCD was significantly increased in HF compared with Sham rats, which was reversed by candesartan treatment (HF + Can). *P < 0.05 vs. Sham; #P < 0.05 vs. HF.](image)

<table>
<thead>
<tr>
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<th>Sham (N = 5)</th>
<th>HF (N = 4)</th>
<th>HF + Can (N = 5)</th>
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<tr>
<td>IMCD (total)</td>
<td>15.2±0.7</td>
<td>19.1±0.8*</td>
<td>15.9±0.3†</td>
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<td>IMCD1</td>
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<td>17.4±1.3</td>
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<td>IMCD2</td>
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<td>19.8±1.1*</td>
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<tr>
<td>IMCD3</td>
<td>17.7±0.9</td>
<td>18.6±1.9</td>
<td>19.4±1.2</td>
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</tbody>
</table>

Values are mean ± SE % of total density for n rats. Apical labeling was determined as the ratio of phosphorylated AQP2 (p-AQP2) density in the apical membrane to that in the whole cell fraction. Increase in % values reflects increase in apical labeling. *P < 0.001 vs. Sham, †P < 0.001 vs. HF.

Fig. 4. Immunoperoxidase microscopy of AQP2 and p-AQP2 in inner medullary collecting duct (IMCD). A: in Sham rats, AQP2 labeling in IMCD showed a substantial cytoplasmatic staining and a less prominent apical staining. B: in HF rats, AQP2 labeling was predominantly associated with the apical plasma membrane domains and only marginal labeling of cytoplasmatic vesicles was seen. C: HF + Can rats revealed less immunolabeling in the apical plasma membrane, indicating that apical AQP2 labeling was as least in part reversed by candesartan treatment. D: immunocytochemical analysis revealed intracellular distribution of p-AQP2 in Sham rats, as seen in the AQP2 immunolabeling. E: correspondingly, in HF rats there was a marked increase in apical p-AQP2 immunolabeling compared with Sham rats (A), which was at least partly reversed by candesartan treatment (F). Magnification: ×630.
apical membrane. The long-term regulation induces increased transcription of the AQP2 gene with a concomitant rise in AQP2 mRNA (15, 61). This is believed to be mediated through phosphorylation of cAMP response element-binding protein (CREB) and increased c-Fos expression in response to elevated cAMP or intracellular calcium (63). Moreover, plasma ANG II levels were increased in HF rats. In addition to the proximal tubule, previous studies have demonstrated that ANG II has an important effect on the TAL and CD, where ANG II receptor mRNA and protein are present (25, 57). In particular, Wong and Tsui (61) demonstrated that elevated ANG II in cardiomyopathic hamsters increased V2 receptor mRNA expression. We recently demonstrated (42) that ANG II per se plays a role in the regulation of AQP2 targeting to the plasma membrane in primary cultured IMCD cells through AT1 receptor activation and potentiates the effect of 1-desamino-8-D-arginine vaso-

Fig. 6. Electron micrograph of ultrathin Lowicryl section of IMCD2 cell from Sham rat (A) and HF rat (B). A: AQP2 immunogold labeling in the Sham rat principal cell was mainly associated with intracellular vesicles (arrowheads). B: in the HF rat abundant AQP2 immunogold labeling was associated with the apical plasma membrane (arrows), and only modest labeling of intracellular vesicles (arrowheads) was seen. L, lumen; M, mitochondria. Magnification: ×36,000.

Fig. 7. Semiquantitative immunoblotting of kidney protein prepared from cortex/OSOM (A) and ISOM (B). A: immunoblot was reacted with anti-AQP2 (top) and anti-p-AQP2 (bottom). In cortex/OSOM the protein expression of AQP2 was not altered in HF rats compared with control rats and was decreased in HF + Can compared with Sham and HF rats. p-AQP2 abundance was increased in HF rats compared with control rats, which was reversed by candesartan treatment. B: in ISOM immunoblotting revealed an increased abundance of AQP2 (top) in HF compared with Sham rats, which was reversed by candesartan treatment. p-AQP2 abundance (bottom) was increased in HF and HF-Can compared with Sham rats. Open bars, Sham; filled bars, HF; gray bars, HF + Can. *P < 0.05 vs. Sham; #P < 0.05 vs. HF.
pressin (DDAVP) on cAMP production, p-AQP2 expression, and AQP2 targeting. Moreover, AT$_1$ receptor blockade with candesartan prevented upregulation of inner medullary AQP2 and p-AQP2 expression in response to long-term DDAVP administration in vivo (41), indicating that ANG II-stimulated AT$_1$ receptor activation plays an important role in regulation of CD AQP2 expression.

In addition, AT$_1$ receptor blockade by candesartan could improve hemodynamic changes in HF, leading to attenuation of the increased plasma AVP level and activated renin-angiotensin system. Consistent with this, plasma ANG II levels were significantly increased in HF, whereas candesartan treatment decreased ANG II levels, which could attenuate, at least in part, the upregulated AQP2 in HF.

In the cortex/OSOM AQP2 protein abundance remained unaltered. No change in subcellular AQP2 distribution was observed. In contrast, ISOM and IM revealed increased protein abundance of AQP2 and p-AQP2. Computational analysis from immunoperoxidase microscopy in IMCD demonstrated an increased ratio of p-AQP2 density in the apical plasma membrane domain in HF compared with Sham rats, which was reversed by candesartan treatment. The finding in IMCD2 that showed the most substantial changes by immunoperoxidase microscopy was consistent with immunoelectron microscopy of total AQP2, which was also found in LAD-ligated HF rats in a previous study by this group (49). This observed heterogeneity in p-AQP2 and total AQP2 between the proximal and distal sections of CDs could be explained by the fact that the concentration of the V$_2$ receptor in the medullary CD is higher than observed in the cortical part (43). Studies have suggested that ANG II receptor abundance is higher in the medulla than in cortex, and other studies have suggested that ANG II has a more substantial influence in medullary than cortical hemodynamics (26, 28). The IMCDs are responsible for the final fine regulation of the water excretion. Thus the increased abundance of AQP2 in the medullary CD may play a role in water retention in HF.

Interestingly, plasma urea was increased in candesartan-treated HF rats compared with Sham and HF rats, which would be able to induce the observed increase in plasma osmolality in this group because there was no change in urine output among groups. However, because urea is not an effective osmol this would not effect AVP levels (not measured) (52). As there was no change in urine osmolality and free water reabsorption, the state of water retention in this model could be questioned despite dysregulation of renal water and salt transporters. The lack of hyponatremia in this HF model despite highly increased LVEDP indicates the presence of compensated HF in which the compensating mechanisms are intact. This would explain that plasma sodium and plasma osmolality are within the normal range despite renal sodium and water channel dysregulation. However, there may have been a transient impairment of sodium

Table 5. Expression of NKCC2 and NHE3 as fraction of level in sham-operated rats

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<th></th>
<th>Sham</th>
<th>HF</th>
<th>HF + Can</th>
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<tbody>
<tr>
<td>Cortex/OSOM</td>
<td></td>
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<td>NKCC2</td>
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<td>1.37±0.11*</td>
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<tr>
<td>ISOM</td>
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<td>1.13±0.11*</td>
<td>1.30±0.06*</td>
</tr>
<tr>
<td>NKCC2</td>
<td>1.00±0.09</td>
<td>1.62±0.30*</td>
<td>1.08±0.07*</td>
</tr>
<tr>
<td>NHE3</td>
<td>1.00±0.09</td>
<td>1.62±0.30*</td>
<td>1.08±0.07*</td>
</tr>
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</table>

Values are means ± SE for n rats. NKCC2, Na$^+$/K$^+$/2Cl$^-$ cotransporter; NHE3, type 3 Na$^+$/H$^+$ exchanger; Cortex/OSOM, cortex including outer stripe of outer medulla; ISOM, inner stripe of outer medulla. *p < 0.05 vs. Sham, †p < 0.05 vs. HF.
and water excretion during the initial stage of HF, and the compensatory mechanisms may prevail in later stages.

The cortical CDs have been shown to play a more substantial role in water reabsorption than the inner medullary section. Two-thirds of the filtered water that enters the CD is reabsorbed in the cortical segments (2). Consistent with this, AQp3-null mice showed a marked polyuria and urine concentration defect, which supports the notion that the majority of the filtered water entering the distal tubules is extracted in the proximal segments where the concentration of AQP3 is most prominent (36). Thus it could also be speculated that the lack of marked AQP2 and p-AQP2 dysregulation as well as apical labeling in the cortical and outer medullary segments of the CD may in part account for the lack of water retention in this model.

Changes of NHE3 and NKCC2 in HF rats in response to AT1 antagonism in HF. AVP is thought to play an important role in the regulation of NKCC2 and NHE3, probably with cAMP as the second messenger (16, 21, 23, 33). In addition, ANG II-treated rats increased the abundance of NKCC2 as well as NHE3 in medullary TAL (mTAL) (40). In the present study the abundance of the TAL salt transporter NKCC2 was increased in HF rats consistent with the observations reported by other investigators (50). Staahltoft et al. (55) found this increase in female mild HF rats to be normalized by inhibition of the AT1 receptor; surprisingly, in our study normalization of NKCC2 protein abundance was only observed in the cortex/OSOM, whereas AT1 receptor blockade failed to normalize the NKCC2 abundance in ISOM. An explanation for this heterogeneity could be sex differences or greater cardiac impairment measured as LVEDP in the present study compared with previous data (55). However, it cannot be ruled out that ANG II plays a role in the regulation of NKCC2, and this needs to be investigated in future studies. In the present study NHE3 abundance was increased in HF rats in cortex as well as the ISOM. HF rats showed increased levels of ANG II compared with sham-operated rats, and this upregulation was reversed in candesartan-treated HF rats. Indeed, several studies have shown that ANG II stimulates NHE3 (4, 32, 41). In addition Kwon et al. reported (40, 41) that ANG II-treated rats revealed increased abundance of NHE3 in TAL, and candesartan decreased the NHE3 abundance in mTAL. Consistent with this, AT1 antagonism significantly inhibited the rate of proximal convoluted tubule bicarbonate and volume absorption (4). It has been demonstrated that increased levels of ANG II are associated with significantly increased renal nerve activity in HF rats and increased sodium reabsorption (11). Moreover, the stimulatory effect of renal sympathetic nerves seems to be proportional to the density of the renal tubular innervation, being greatest in the TAL (3). Thus it may be possible that ANG II and renal sympathetic nerve overactivity may coordinate play a role in NHE3 and NKCC2 regulation in TAL in HF.

HF-induced changes of ENaC subunits and 11β-HSD2. Even though sodium transport occurs along the entire length of the tubule, the fine regulation in sodium secretion is primarily seen in the CNT and CDs. ENaC is a heterooligomer consisting of three subunits: α, β, and γ (8). Both protein abundance and apical plasma membrane targeting of the ENaC subunits are under the coordinate control of hormones such as aldosterone, AVP, and ANG II (5, 8, 13, 45). In our study HF rats had decreased levels of cortical β- and γ-ENaC protein, whereas α-ENaC abundance was increased. A similar pattern has been reported after stimulation with ANG II (5, 7, 46). Interestingly, AVP has been reported to upregulate β- and γ-ENaC protein independently of the increase of α-ENaC (12, 14). Conversely, in our study, candesartan-treated HF rats showed a substantial upregulation of β- and γ-ENaC but a likewise downregulation of α-ENaC. These data supports the view that β- and γ-ENaC are regulated independently of α-ENaC (5, 14, 60). We demonstrated that 11β-HSD2 was upregulated in HF associated with increased ANG II levels. This novel finding is consistent with the observations that ANG II stimulates the activity of 11β-HSD2 (27, 31) and our finding that candesartan administration induced a marked downregulation of 11β-HSD2 in HF.
rats. In HF aldosterone, AVP, and ANG II are elevated, and the ENaC subunits and 11β-HSD2 are altered in the same directions as seen with ANG II treatment. Thus it could be speculated that ANG II activity overrules the aldosterone-induced signal in respect to trafficking and subunit regulation of ENaC in HF.

In summary, LAD ligation-induced HF was associated with increased targeting and protein expression of AQP2, upregulation of NKCC2 and NHE3, and altered expression of ENaC subunits and 11β-HSD2. These changes were, at least in part, reversed by AT1 receptor antagonism, indicating an important role of ANG II in the development of water and salt retention seen in HF.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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