Reduced abundance of aquaporins in rats with bilateral ischemia-induced acute renal failure: prevention by α-MSH

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1Department of Cell Biology, Institute of Anatomy, University of Aarhus; 2Department of Clinical Physiology, Aarhus University Hospital and Institute of Experimental Clinical Research, DK-8000 Aarhus, Denmark; and 3Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20892

Kwon, Tae-Hwan, Jørgen Frøkiaer, Patricia Fernández-Llama, Mark A. Knepper, and Søren Nielsen. Reduced abundance of aquaporins in rats with bilateral ischemia-induced acute renal failure: prevention by α-MSH. Am. J. Physiol. 277 (Renal Physiol. 46): F413–F427, 1999.—We examined the effect of temporary renal ischemia (30 min or 60 min) and reperfusion (1 day or 5 days) on the expression of renal aquaporins (AQPs) and urinary concentration in rats with bilateral ischemia-induced acute renal failure (ARF). Next, we tested whether reducing ischemia/reperfusion (I/R) injury by treatment with α-melanocyte stimulating hormone (α-MSH) affects the expression of AQPs and urine output. Rats with ARF showed significant renal insufficiency, and urinary concentration was markedly impaired. In rats with mild ischemic injury (30 min), urine output increased significantly to a maximum at 48 h, and then nearly normalized within 5 days. Consistent with this, semiquantitative immunoblotting revealed that kidney AQP1 and AQP2 abundance was significantly decreased after 24 h to 30 ± 5% and 40 ± 11% (n = 8) of controls (n = 9), respectively (P < 0.05). Five days after ischemia, AQP2 abundance was not significantly decreased and urine output was normalized. In contrast, severe ischemic injury (60 min) resulted in a marked reduction in urine output at 24 h, despite a significant decrease in urine osmolality and solute-free water reabsorption, TcH2O. AQP1 and AQP2 abundance was markedly decreased to 51 ± 5% and 31 ± 9% (n = 10) of controls (n = 8) at 24 h (P < 0.05). After 5 days, the rats developed gradually severe polyuria and had very low AQP2 and AQP1 levels [11 ± 4% and 6 ± 2% (n = 5) of controls (n = 8), respectively; P < 0.05]. A similar reduction was observed for AQP3. The reduction in AQP expression in the proximal tubule and inner medullary collecting duct was confirmed by immunocytochemistry. Next, we found that intravenous α-MSH treatment of rats with ARF significantly reduced the ischemia-induced downregulation of renal AQPs and reduced the polyuria. In conclusion, the I/R injury is associated with markedly reduced expression of the collecting duct and proximal tubule AQPs, in association with an impairment of urinary concentration. Moreover, α-MSH treatment significantly prevented the reduction in expression of AQPs and renal functional defects. Thus decreased AQP expression is likely to contribute to the impairment in urinary concentration in the postischemic period.

acutetubularnecrosis; α-melanocyte stimulating hormone; collecting duct; proximal tubule; urinary concentration mechanism

EXPERIMENTAL ACUTE RENAL failure (ARF) induced by ischemia and reperfusion in rats is known to cause characteristic structural alterations in renal tubule epithelia in association with an impairment of urinary concentrating mechanism (4). The straight portion of proximal tubule (S3 segment) and thick ascending limb (TAL), which both are located in the outer medulla of the kidney, are marginally oxygenated under normal conditions and suffer the most severe and persistent hypoxia after an ischemic injury (28). Therefore, the proximal tubule (S3 segment) and TAL are generally viewed as the main sites of ischemic insult in the kidney (6, 51). In contrast, collecting ducts are generally considered to be relatively invulnerable to ischemic injury.

Urinary concentration and dilution depends on the presence of a discrete segmental distribution of transport properties along the renal tubule (26). Concentration of the urine requires 1) establishment and maintenance of a hypertonic medullary interstitium and 2) vasopressin-regulated water transport across the collecting duct epithelium for osmotic equilibration. Thus defects in any of these mechanisms would be predicted to be associated with urinary concentrating defects.

Several studies have shown defects both in collecting duct water reabsorption and proximal tubule water reabsorption, as well as defects in solute handling in postischemic kidneys (19, 22, 48, 51). In an isolated tubule micropuncture study of the rabbit, it was observed that water reabsorption in the proximal tubule and cortical collecting duct was significantly reduced following ischemia (19). Moreover, it has been demonstrated that there are no differences in either basal or vasopressin-induced CAMP levels in outer or inner medulla in rats with ARF compared with sham-operated rats (2), supporting the view that there are defects in collecting duct water reabsorption in postischemic kidneys. It is also well known that kidneys subjected to injury by ischemia are unable to establish or maintain a high medullary solute content (3). Moreover, a decreased ability of the TAL to lower perfusate chloride ion concentration was observed as well (19).

The studies suggest that there are defects in both countercurrent multiplication and collecting duct water permeability in response to ischemic damage. The aquaporins (AQPs) are a family of membrane proteins that function as water channels. AQP1 is highly abundant in the proximal tubule and descending thin limb and several studies have emphasized its important role in the constitutive water reabsorption in

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these segments and its role for urinary concentration (1, 29, 41). In the kidney collecting duct at least three AQP s are known to be expressed, and they participate in the vasopressin-regulated water reabsorption. AQP2 (17) is the apical water channel of collecting duct principal cells and is the chief target for regulation of collecting duct water permeability by vasopressin (38, 39, 43, 45). Water transport across the basolateral plasma membrane of collecting duct principal cells is thought to be mediated by AQP3 (9, 21) and AQP4. A series of studies have demonstrated that altered expression and apical targeting of AQP2 play a significant role in water balance disorders (25, 40).

In a recent study using unilateral ischemia-induced ARF in combination with contralateral nephrectomy, we found a reduction in the expression of AQP1, AQP2, and AQP3 (11). In the present study, we use another well-characterized model (bilateral ischemia-induced ARF), which has other advantages and allows us to extend the previous studies with regard to the role of aquaporins in the urinary concentrating defects associated with ischemia-induced ARF. The chief advantages of this model are: 1) The urinary concentration can be directly monitored and compared with the expression of renal aquaporins. 2) It is possible to make observations during both nonoliguric and oliguric phases of ischemia-induced ARF. 3) It obviates the problem that exists with our previous model (unilateral ischemia with contralateral nephrectomy; Ref. 11), that there might be adaptive changes in epithelial morphology, glomerular perfusion, and filtration rates due to nephrectomy alone (12). 4) It provides an appropriate setting for the evaluation of the potential role of aquaporins in the preventive effect of α-melanocytic stimulating hormone (α-MSH) in ischemia-induced injury, using the same model as employed previously for initial descriptions of α-MSH effects (8). 5) Most importantly, the bilateral renal ischemia model may share a spectrum of clinical features in oliguric and nonoliguric forms of ARF in humans.

In the present study, we examined 1) whether temporary bilateral renal ischemia (30 min or 60 min) and reperfusion (1 day or 5 days) affects the expression of renal aquaporins (AQP1, -2, and -3) and affects the urinary concentration; 2) whether changes in aquaporin expression correlate with the impairment of urinary concentration encountered in both the oliguric phase and the polyuric phase of ARF; and 3) whether reducing ischemia/reperfusion injury by treatment with an anti-inflammatory agent (i.e., α-MSH) (8) affects the expression levels of aquaporins and affects the changes in urinary concentration.

METHODS

Experimental Animals

Studies were performed on adult Munich-Wistar rats initially weighing 236 ± 6 g (Møllegard Breeding Centre, Eiby, Denmark). The rats were maintained on a standard rodent diet (Altromin, Lage, Germany) with free access to water.

Induction of Ischemia-Induced ARF in Rats

After a period of acclimation to the metabolic cages, experimental ARF was induced by occlusion of both renal arteries for 30 min or 60 min (Fig. 1). During surgery, rats were anesthetized with halothane (Halocarbon Laboratories).
and were placed on a heated table to maintain rectal temperature at 37–38°C. Both kidneys were exposed through flank incisions, mobilized by being dissected free from the perirenal fat. A small portion of the renal artery was gently dissected from the vein (34). The renal arteries were occluded with a smooth surfaced vascular clip (60 g pressure; World Precision Instruments, UK) for 30 min or 60 min. Total ischemia was confirmed by observing blanching of the entire kidney surface. During the period of ischemia, the wound was closed temporarily to maintain body temperature. After the clips were removed, the kidneys were observed for additional 2–5 min to ensure color change, indicating blood reflow, and the wound was closed with 3–0 silk and surgical metal clamps. The rats were returned to metabolic cages, and daily 24 h urine output and water intake were measured according to the protocols depicted in Fig. 1.

As a control group, rats were subjected to sham operations identical to the ones used for ARF rats without occlusion of both renal arteries. Sham-operated rats were monitored in parallel with rats with ARF. All rats were killed under light halothane anesthesia, and kidneys were rapidly removed and processed for membrane fractionation and immunoblotting at the same day.

Clearance Studies

The rats were maintained in the metabolic cages, allowing quantitative urine collections and measurements of water intake (Fig. 1). Urine volume, osmolality, creatinine, and sodium and potassium concentration were measured. Plasma was collected from abdominal aorta at the time of death for measurement of sodium and potassium concentration, creatinine, and osmolality.

Experimental Protocols

The following protocols were performed (Fig. 1).

Protocol 1. This protocol involved 1) rats with ARF established by bilateral renal ischemia for 30 min and monitored for additional 24 h (n = 8, ARF 30/1d) and 2) sham-operated rats (n = 9).

Protocol 2. This protocol involved 1) rats with ARF established by bilateral renal ischemia for 60 min and monitored for additional 24 h (n = 10, ARF 60/1d) and 2) sham-operated rats (n = 8).

Protocol 3. This protocol involved 1) rats with ARF established by bilateral renal ischemia for 30 min and monitored for additional 5 days (n = 6, ARF 30/5d) and 2) sham-operated rats (n = 5).

Protocol 4. This protocol involved 1) rats with ARF established by bilateral renal ischemia for 60 min and monitored for additional 5 days (n = 5, ARF 60/5d) and 2) sham-operated rats (n = 5).

Protocol 5. For immunocytochemistry, kidneys of rats with ARF [protocol identical to protocols 1 (n = 4), 2 (n = 4), 3 (n = 3), and 4 (n = 3)] and of sham-operated rats [protocol identical to protocols 1 (n = 2), 2 (n = 2), 3 (n = 2), and 4 (n = 2)] were perfusion fixed (see below, not depicted in Fig. 1).

Protocol 6. This involved 1) rats with ARF established by bilateral ischemia for 40 min and monitored for additional 2 days. The animals were divided into two groups: ARF rats not treated with α-MSH (n = 9) and α-MSH-treated ARF rats (n = 8). α-MSH (50 µg iv; Phoenix Pharmaceutical, Mountain View, CA) was given at the midpoint of the ischemic period, at 6 and 18 h postperfusion, and then every 24 h thereafter, as previously described (8); and 2) sham-operated rats were treated with vehicle (n = 9).

Protocol 7. For immunocytochemistry, kidneys of rats with ARF, either treated (n = 5) with α-MSH or not treated (n = 5) with α-MSH, and of sham-operated rats treated with vehicle (n = 5) were perfusion fixed (see below, not depicted in Fig. 1).

Protocol 8. Normal rats (n = 5) were treated with α-MSH (50 µg iv; Phoenix Pharmaceutical) using same protocol as protocol 6 (3 injections every 12 h). Control rats (n = 6) received only vehicle intravenously.

Membrane Fractionation for Immunoblotting

The kidneys were homogenized (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing 8.5 µM leupeptin, 1 mM phenylmethylsulfonyl fluoride) using an Ultra-Turrax T8 homogenizer (IKA Labortechnik), at maximum speed for 10 s, and the homogenate was centrifuged in an Eppendorf centrifuge at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria. The supernatant was then centrifuged at 200,000 × g for 1 h to produce a pellet containing membrane fractions enriched for both plasma membranes and intracellular vesicles (31, 32). Gel samples (Laemmli sample buffer containing 2% SDS) were made of this pellet.

Electrophoresis and Immunoblotting

Samples of membrane fractions from total kidney were run on 12% polyacrylamide minigels (Bio-Rad Mini Protein II). For each gel, an identical gel was run in parallel and subjected to Coomassie staining to assure identical loading (49). The other gel was subjected to immunoblotting. After transfer by electroelution to nitrocellulose membranes, 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 with AQP1 immune serum (LL266, diluted as 1:2,000), or with anti-AQP2 immune serum (LL127, diluted as 1:1,200) (38, 39), or with affinity-purified anti-AQP3 (LL178AP, 0.5 µg/ml) (9). The labeling was visualized with horseradish peroxidase (HRP)-conjugated secondary antibody (P448, diluted as 1:3,000; DAKO, Glostrup, Denmark) using an enhanced chemiluminescence system (ECL, American International).

Quantitation of Kidney AQP1, AQP2, and AQP3 Density

ECL films with bands within the linear range were scanned (32) using an AGFA scanner (ARCUS II) and Corel Photo-Paint Software to control the scanner. For AQP1 and AQP2, both the 29-kDa and the 35- to 50-kDa bands (corresponding to nonglycosylated and the glycosylated species) were scanned (31, 49). For AQP3, both the 27-kDa and the 33- to 40-kDa bands (corresponding to nonglycosylated and the glycosylated species) were scanned (9, 49). The labeling density was quantitated (31) of blots where samples from ARF kidneys were run on each gel with control kidneys from sham-operated animals. The labeling density was corrected by densitometry of Coomassie-stained gels.

Statistical Analyses

Values are presented as means ± SE. Comparisons between groups were made by unpaired t-test. P < 0.05 was considered significant.

Preparation of Tissue for Immunocytochemistry

The kidneys from ARF rats and sham-operated rats were fixed by retrograde perfusion via the abdominal aorta with periodate-lysine-paraformaldehyde (PLP; 0.01 M NaO2, 0.075 M lysine, 2% paraformaldehyde, in 0.0375 M NaH2PO4 buffer, pH 6.2). Kidneys were postfixed for 1 h, and tissue blocks were infiltrated for 30 min with 2.3 M sucrose containing 2% paraformaldehyde, mounted on holders, and rapidly frozen in liquid nitrogen. For light microscopy, the frozen tissue blocks were cryosectioned (0.8–1 µm, Reichert Ultratut S Cryoultra-
Table 1. Changes in plasma creatinine, urine output, and urinary osmolality

<table>
<thead>
<tr>
<th></th>
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<td>P&lt;sub&gt;Cr&lt;/sub&gt;, µmol/l</td>
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<td>(U/P&lt;sub&gt;osm&lt;/sub&gt;)&lt;sub&gt;ur&lt;/sub&gt;</td>
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<td>6.3 ± 0.5</td>
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<td>5.4 ± 0.7</td>
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<tr>
<td>T&lt;sub&gt;H2O&lt;/sub&gt;, µl·min&lt;sup&gt;-1&lt;/sup&gt;·kg&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>60 ± 13*</td>
<td>146 ± 16</td>
<td>16 ± 2*</td>
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Values are means ± SE from rats with acute renal failure (ARF) and sham-operated controls; n = number of rats. C<sub>Cr</sub>, creatinine clearance; P<sub>Cr</sub>, plasma creatinine; T<sub>H2O</sub>, solute-free water reabsorption; (U/P<sub>osm</sub>)<sub>ur</sub>, urine-to-plasma osmolality ratio. *P < 0.05 for ARF rats compared with sham-operated rats.

Microscopy Immunolabeling was performed on ultrathin Lowicryl HM20 sections (60–80 nm), which were incubated overnight at 4°C with affinity-purified anti-AQP2 (LL127AP) diluted in PBS with 0.1% BSA or 0.1% skim milk. The labeling was visualized with goat-anti-rabbit IgG conjugated to 10-nm colloidal gold particles (GAR.EM10; BioCell Research Laboratories, Cardiff, UK) diluted 1:50 in PBS with 0.1% BSA. The sections were stained with uranyl acetate and lead citrate before examination in a Phillips CM100 or Philips 208 electron microscopes.

RESULTS

Ischemia-Induced ARF is Associated with Severe Acute Renal Insufficiency

Rats with ARF induced by temporary bilateral renal ischemia for 30 min or 60 min had significantly increased plasma creatinine levels and decreased creatinine clearance, indicating acute renal insufficiency (Tables 1 and 2). Especially, rats with ARF induced by bilateral ischemia for 60 min that were followed for an additional 1 day (ARF60/1d, protocol 2) demonstrated the most severe degree of renal insufficiency (plasma creatinine levels, 213 ± 43 in ARF vs. 43 ± 3 µmol/l in sham controls; P < 0.05).

Urine Output in Rats With ARF Varies According to the Degree of Renal Insufficiency

Rats with ARF (ARF30/1d, protocol 1) showed significantly increased urine output 1 day after release of bilateral renal artery occlusion (57 ± 10 in ARF vs. 28 ± 2 µl·min<sup>−1</sup>·kg<sup>−1</sup>) in sham-operated controls; P < 0.05). In contrast, rats with ARF (ARF60/1d, protocol 2) that had severe acute renal insufficiency
showed markedly decreased urine output 1 day after release of bilateral renal artery occlusion (17 ± 6 in ARF vs. 48 ± 7 μl·min⁻¹·kg⁻¹ in sham controls; P < 0.05) (Fig. 3C).

Next, we have monitored the urine output in rats with ARF 5 days after release of 30-min (ARF30/5d, protocol 3) or 60-min bilateral renal artery occlusion (ARF60/5d, protocol 4; Fig. 4C). In rats with mild ARF (ARF30/5d), the urine output was significantly increased 1 day after release of renal ischemia, and then normalized progressively within 5 days. In contrast, the rats with severe ARF (ARF60/5d) demonstrated a marked polyuria during the following 5 days after the ischemic insult (Fig. 4C).

Renal Water Handling is Impaired in Rats With ARF

Rats with ARF (ARF30/1d) demonstrated significantly decreased urine osmolality (676 ± 85 in ARF vs. 1,897 ± 148 mosmol/kgH₂O in sham controls, P < 0.05) 1 day after renal ischemia, in association with the marked increase in urine output (Fig. 2, C and D). Moreover, rats with severe ischemic renal injury (ARF60/1d) also showed a significant reduction in urine osmolality (659 ± 104 in ARF vs. 1,650 ± 200 mosmol/kgH₂O in sham controls; P < 0.05), despite a marked decrease in urine output (17 ± 6 in ARF vs. 48 ± 7 μl·min⁻¹·kg⁻¹ in sham controls; P < 0.05) (Fig. 3, C and D). This suggests that urinary concentration is significantly decreased in rats with both oliguric and nonoliguric ARF. Consistent with this, rats with ARF were associated with a significant decrease in urine-to-plasma osmolality ratio [(U/P)_{osm}] and solute-free water reabsorption (T'H₂O) (Table 1).

\[ \text{Next, we have monitored the urine osmolality for 5 days in rats with ARF and in sham-operated rats. In rats with mild ARF (ARF30/5d), the urine osmolality was significantly decreased for 3 days after release of renal ischemia and then normalized within day 4 and 5. Consistent with this, the (U/P)_{osm} and T'H₂O measured at day 5 were normalized in rats with ARF (ARF30/5d, Table 2). In contrast, the rats with severe ARF (ARF60/5d) demonstrated that the urine osmolality was significantly and persistently decreased during the 5-day experimental period. Consistent with this, the (U/P)_{osm} and T'H₂O were also significantly decreased at day 5 (Table 2), indicating an impairment of urinary concentration in ARF.}

Postischemic Kidneys are Associated With Reduced AQP2 Levels

Semiquantitative immunoblotting using membrane fractions prepared from the whole kidney of ischemia-induced ARF rats and sham-operated rats revealed that ARF was associated with a markedly reduced AQP2 expression 1 day after release of 30 min or 60 min ischemic injury (Figs. 2 and 3, and Table 3). Both AQP2 bands (29 kDa and 35–50 kDa) were decreased proportionally. Densitometric analysis revealed a significant decrease in AQP2 expression in rats with ARF30/1d to 40 ± 11% of sham levels (100 ± 7%, P < 0.05). Furthermore, the AQP2 protein levels were markedly decreased in rats with ARF60/1d to 31 ± 9% of sham levels (100 ± 9%, P < 0.05), consistent with significant reductions in urine osmolality, (U/P)_{osm} and T'H₂O (Table 1).

Next, we examined the changes of AQP2 expression levels at the fifth day after release of 30 min or 60 min renal ischemia. In rats with ARF30/5d, AQP2 expression levels were not significantly different (data not shown), consistent with unchanged levels of urine output, urine osmolality, (U/P)_{osm} and T'H₂O (Table 2). In contrast, rats with severe ARF (ARF60/5d) showed that AQP2 expression was markedly decreased to 11 ± 4% of sham levels (100 ± 14%, P < 0.05; Fig. 4A and B).
Consistent with this, the rats were polyuric and had significantly decreased urine osmolality, (U/P) osm, and TcH₂O (Table 2).

Postischemic ARF is Associated With Reduced Renal AQP3 Levels

Semiquantitative immunoblotting demonstrated that ARF was also associated with significant reduction in AQP3 levels 1 day after release of 30- or 60-min bilateral renal artery occlusion (Table 3). AQP3 expression levels in ARF30/1d were 53 ± 18% of sham levels (100 ± 22%, P < 0.05). Furthermore, after severe ischemic insults (ARF60/1d and ARF60/5d), AQP3 levels were also significantly decreased at day 1 to 21 ± 4% of sham levels (100 ± 17%, P < 0.05) and at day 5 to 9 ± 4% of sham levels (100 ± 18%, P < 0.05).

Postischemic ARF is Associated With Reduced Renal AQP1 Levels

Semiquantitative immunoblotting revealed a significant decrease in AQP1 levels in rats with ARF30/1d to 30 ± 5% of sham levels (100 ± 15%, P < 0.05) (Fig. 5, A and B; and Table 3). Furthermore, with severe ischemic insults (ARF60/1d), AQP1 expression levels were also significantly decreased to 51 ± 5% of sham levels (100 ± 14%, P < 0.05; Fig. 6, A and B). Five days after 60-min bilateral renal ischemia, AQP1 levels remained markedly reduced to 6 ± 2% of sham levels (100 ± 13%, P < 0.05; Fig. 6, C and D). Consistent with the reduced AQP2, AQP3, and AQP1 expression levels, rats with ARF (ARF60/5d) had a markedly increased urine output and a significant urinary concentration defect (Fig. 4C; Table 2).

Morphological Features of Kidneys From Rats With Ischemia-Induced ARF

One day after 30- or 60-min bilateral renal ischemia, light microscopical examination demonstrated tubular damage and cell death after renal ischemia and reperfusion. Tubular necrosis was most pronounced in the proximal tubule (Fig. 7B). Necrotic cells were detached from the tubular basement membrane and were seen in the tubular lumen. Furthermore, disruption of the brush border (arrows in Fig. 7B) and focal loss of brush border were observed.

Table 2. Changes in plasma creatinine, urine output, and urinary osmolality

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<td>TcH₂O, µl·min⁻¹·kg⁻¹</td>
<td>156 ± 3</td>
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Values are means ± SE; n = number of rats. *P < 0.05 for ARF rats compared with sham-operated rats.

Table 3. Changes in expression of AQP1, AQP2, and AQP3 in ischemia-induced ARF rats

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Values are percent changes expressed as means ± SE; numbers of rats are in parentheses. AQP, aquaporin. *P < 0.05 for ARF rats compared with sham-operated rats.
individual tubular cells in the proximal tubule were also seen. Characteristically, vascular congestion was noted in the outer stripe of outer medulla in rats with ARF (not shown), and were much more pronounced in the postischemic kidneys with severe renal insufficiency (ARF60/1d), consistent with previous observations (34).

Immunocytochemistry Confirms the Reduced Expression of Renal Aquaporins Levels After Ischemic Insults

Immunocytochemistry showed abundant labeling of AQP1 associated with apical (arrows in Fig. 7A) and basolateral plasma membranes (arrowheads in Fig. 7A) of proximal tubule and descending thin limbs (not shown) of sham-operated rat kidneys. In contrast, AQP1 labeling was substantially weaker in postischemic kidneys (ARF30/1d) with proximal tubules displaying significantly reduced labeling of the brush border as well as of basolateral plasma membrane infoldings (Fig. 7B). In sham-operated rats, strong AQP2 labeling was associated with the apical plasma membrane domains (arrows in Fig. 7C) and with intracellular vesicles in the cytoplasm in collecting duct principal cells. In kidneys from rats with ARF (ARF30/1d), there was a marked reduction in the AQP2 staining of collecting duct principal cells, and part of the remaining labeling was associated with apical plasma membrane domains of collecting duct principal cells (arrows, Fig. 7D). This suggests that AQP2 trafficking is not severely impaired in the kidneys with ischemic insults. Immunoelectron microscopy also confirmed that there was an overall decrease in AQP2 expression in kidneys from ARF rats (not shown). Abundant AQP3 labeling was associated with the basolateral plasma membrane of collecting duct principal cells in sham-operated rat kidneys (Fig. 7E). As seen with AQP2, AQP3 labeling was also reduced in kidneys of rats with ARF (Fig. 7F).

α-MSH Treatment Reduces the Ischemia-Induced Defects in Urinary Concentration

α-MSH, which is known to inhibit both inflammatory and nitric oxide (NO) pathways (7), has recently been demonstrated by Star and colleagues (8) to be effective in reducing the ischemia/reperfusion injury in rats.
This has been suggested to be ascribed, in part, to reduced neutrophil migration and inhibited production of neutrophil chemokines (8). We therefore tested whether α-MSH treatment affects the expression of aquaporins and the changes in the urinary concentration in postischemic ARF, with the same protocols described by Star and colleagues (8).

Rats with ARF (ARF40/2d, protocol 6) showed significantly acute renal insufficiency, compared with sham-operated rats (Table 4). α-MSH treatment markedly reduced the severity of renal insufficiency, since ARF rats that were not treated with α-MSH demonstrated significantly higher plasma creatinine levels (194 ± 45 µmol/l, P < 0.05) compared with ARF rats treated with α-MSH (68 ± 15 µmol/l). Thus α-MSH markedly inhibits the decline in renal functions in response to renal ischemia and reperfusion, consistent with previous observations (8). Furthermore, α-MSH treatment significantly reduced the degree of polyuria that was encountered in the postischemic period (Table 4; Fig. 8C). Consistent with this, urine osmolality, (U/P) osm, and TcH2O in α-MSH-treated ARF rats were significantly improved, compared with α-MSH nontreated ARF rats (Table 4; Fig. 8D).

α-MSH Treatment Reduces the Ischemia-Induced Downregulation of Renal Aquaporins

Figure 8 shows the effects of α-MSH treatment on the kidney AQP2 levels, with changes in urine output and urine osmolality in ARF and sham-operated rats. Rats with ARF that were not treated with α-MSH during reperfusion period demonstrated markedly decreased AQP2 levels (13 ± 3% of sham levels, P < 0.05) compared with sham-operated rats (100 ± 15%; Fig. 8, A and B). Importantly, α-MSH treatment prevented the downregulation of AQP2. ARF rats treated with α-MSH had almost sevenfold higher AQP2 expression levels compared with untreated ARF rats (Fig. 8, A and B).

Next, we examined whether the downregulation of AQP3 and AQP1 was also prevented by α-MSH treatment. Similar to the observations on AQP2 expression (Fig. 8A), AQP3 expression was also approximately sevenfold higher in the α-MSH-treated rats compared with untreated rats (Fig. 9), and this expression was not different from the levels in sham-operated rats. Also AQP1 expression was markedly higher (2-fold) in the α-MSH-treated ARF rats compared with untreated ARF rats (Fig. 9). This supports the view that reduced expression of aquaporins may play a role in the functional defects demonstrated to be associated with experi-

Table 4. Urine output and urinary concentrating ability 2 days after release of 40 min bilateral renal ischemia with or without α-MSH treatment

<table>
<thead>
<tr>
<th></th>
<th>ARF</th>
<th>ARF + MSH</th>
<th>Sham</th>
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<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Pcr, µmol/l</td>
<td>194 ± 45†</td>
<td>68 ± 15*</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Ccr, ml/min</td>
<td>0.4 ± 0.1†</td>
<td>0.8 ± 0.1*</td>
<td>1.3 ± 0.06</td>
</tr>
<tr>
<td>Plasma urea nitrogen, mmol/l</td>
<td>36 ± 5*</td>
<td>12 ± 3*</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Urine output, µl·min⁻¹·kg⁻¹</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline period</td>
<td></td>
<td></td>
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<tr>
<td>Day – 2</td>
<td>44 ± 1</td>
<td>35 ± 3</td>
<td>44 ± 4</td>
</tr>
<tr>
<td>Day – 1</td>
<td>40 ± 3</td>
<td>36 ± 4</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>After operation</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 1</td>
<td>94 ± 13*</td>
<td>67 ± 9*</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>Day 2</td>
<td>104 ± 12*</td>
<td>71 ± 8*</td>
<td>51 ± 2</td>
</tr>
<tr>
<td>Urine osmolality, mosmol/kgH₂O</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day – 2</td>
<td>1,356 ± 94</td>
<td>1,602 ± 150</td>
<td>1,503 ± 96</td>
</tr>
<tr>
<td>Day – 1</td>
<td>1,435 ± 99</td>
<td>1,439 ± 135</td>
<td>1,315 ± 97</td>
</tr>
<tr>
<td>(U/P)osm</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 1</td>
<td>443 ± 51*</td>
<td>530 ± 49*</td>
<td>1,587 ± 138</td>
</tr>
<tr>
<td>Day 2</td>
<td>557 ± 89*†</td>
<td>824 ± 80*</td>
<td>1,297 ± 74</td>
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<tr>
<td>TcH₂O, µl·min⁻¹·kg⁻¹</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline period</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day – 2</td>
<td>1.7 ± 0.3†</td>
<td>2.7 ± 0.3*</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Day 1</td>
<td>51 ± 16*</td>
<td>118 ± 18*</td>
<td>163 ± 10</td>
</tr>
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Values are means ± SE; n = number of rats. α-MSH, α-melanocortin-stimulating hormone. *P < 0.05 for ARF rats, which were either treated or not treated with α-MSH, compared with sham-operated rats. †P < 0.05 for ARF rats treated with α-MSH compared with ARF rats treated with α-MSH. (U/P)osm and TcH2O were measured at day 2.

Immunocytochemistry Confirms That α-MSH Treatment Has Protective Effects in Response to Renal Ischemia and Reperfusion

Immunocytochemistry confirmed that there was an overall decrease in AQP2 expression in inner medullary collecting duct: principal cells from ARF rats (40/2d) without α-MSH treatment, compared with α-MSH-treated ARF rats or sham-operated control rats (Fig. 10, A–C). The marked reduction in AQP2 labeling in inner medulla was consistent with a significant decreased density observed by immunoblotting (Fig. 8, A and B). The part of the remaining labeling was associated with apical plasma membrane domains of collecting duct principal cells, as demonstrated in Fig. 10A.

Fig. 7. Immunoperoxidase localization of AQP1, AQP2, and AQP3 in 0.8–1 µm cryosections of kidney cortex and inner medulla from sham-operated rats (A, C, and E) and rats with ARF (30/1d, B, D, and F). A: abundant labeling of AQP1 is associated with apical (arrows) and basolateral plasma membranes (arrowheads) of proximal tubules in sham-operated rat kidneys. B: in contrast, AQP1 labeling is weaker in postischemic kidneys. Disruption of brush border (arrows) in the proximal tubule is also seen. C: AQP2 labeling is associated with the apical plasma membrane domains (arrows) and with intracellular vesicles in the cytoplasm of collecting duct cells in kidneys from sham-operated rats. D: in kidneys from rats with ARF (30/1d), there is a marked reduction in the AQP2 staining, and the remaining labeling is partly associated with apical plasma membrane domains of collecting duct principal cells (arrows). E: abundant AQP3 labeling is associated with the basolateral plasma membrane of collecting duct principal cells in kidneys from sham-operated rat kidneys. F: AQP3 labeling is reduced in kidneys of rats with ARF. ×1,100.
In α-MSH-treated ARF rats, AQP2 labeling was unchanged compared with sham-operated rats, demonstrating α-MSH treatment inhibited the decreased expression of AQP2 in response to renal ischemia and reperfusion injury (Fig. 10C). The labeling of AQP3 and AQP4 in inner medullary collecting duct from α-MSH-treated ARF rats were also unchanged (Fig. 10, D–I). This confirmed that α-MSH treatment during reperfusion period after renal ischemic insults is effective in inhibiting decreased expression of aquaporins in the inner medullary collecting duct.

**DISCUSSION**

We have demonstrated that the levels of aquaporins in the collecting duct as well as in proximal tubule were severely decreased in response to ischemia. Furthermore, the decreased levels of aquaporins were associ-
Fig. 10. Immunoperoxidase localization of AQP2, AQP3, and AQP4 in 0.8- to 1-µm cryosections of kidney inner medulla from ARF rats (ARF40/2d; A, D, and G), sham-operated rats (B, E, and H), and α-MSH-treated ARF rats (ARF40/2d; C, F and I). A: decrease in AQP2 labeling is seen in inner medullary collecting duct principal cells from ARF rats without α-MSH treatment. B: in sham-operated rats, AQP2 labeling is associated with the apical plasma membrane domains and with intracellular vesicles in the cytoplasm. C: in α-MSH-treated ARF rats, AQP2 labeling is unchanged. D: AQP3 labeling in basolateral membrane domains of collecting duct principal cells is reduced in kidneys from rats with ARF. E and F: in contrast, AQP3 labeling is unchanged in α-MSH-treated ARF rats, compared with sham-operated control rats. G: in kidneys from rats with ARF (without α-MSH treatment), AQP4 labeling in the basal plasma membrane of collecting duct principal cells is reduced. H and I: in contrast, in α-MSH-treated ARF rats, AQP4 labeling is unchanged compared with sham controls. Magnification, ×1,010.
ated with impaired urinary concentration in rats with oliguric or nonoliguric ARF. Both the reduced expression of aquaporins and reduced urinary concentration were significantly prevented by cotreatment with α-MSH. The results together suggest that decreased levels of aquaporins in both the proximal tubule and collecting duct in posts ischemic kidneys may play a significant role in the impairment of urinary concentration encountered in oliguric, maintenance, and polyuric phases of experimental ischemia-induced ARF.

Bilateral Renal Ischemia-Induced ARF in Rats

In the present study, bilateral renal ischemia-induced ARF was induced in rats by bilateral occlusion of the renal arteries for varying periods of time (30, 40, or 60 min) and reperfusion for 1–5 days to produce lesions resembling those of human ARF. Indeed, 30 min of bilateral renal ischemia reproduced an abbreviated and nonoliguric form of ARF in rats, similar to abbreviated ARF (pattern A), which is observed in humans after an isolated ischemic insult (36, 37). Although significant changes in renal function, seen by increased plasma creatinine levels and impairment of urinary concentration, were apparent, recovery of renal function followed promptly and was nearly complete within 5 days. These findings suggest that this protocol produced only a mild renal injury. In contrast, studies of bilateral renal ischemia for 60 min were associated with severe and sustained forms of renal insufficiency with high frequency of oliguric ARF. This model may share similarities with the overt or protracted ARF with severe and sustained forms of renal insufficiency or repeated episodes of hypotension (35, 37).

Collecting Duct Water Channel AQP2 and AQP3 are Severely Affected in Postischemic Kidneys

The proximal tubule (especially, in S3 segment) and TAL are known to be main sites of the ischemic insult, whereas collecting ducts are generally considered to be relatively invulnerable. Nevertheless, we demonstrated that ischemia-induced ARF is associated with markedly reduced collecting duct water channel AQP2 and AQP3 levels with decreased urine osmolality and T\textsuperscript{H2O} after the ischemic insult. Moreover, we demonstrated that α-MSH treatment in ARF rats significantly reduced the ischemia-induced decrease in AQP2 and AQP3 levels in collecting duct principal cells and the functional defects. Thus increased collecting duct aquaporin levels may partly contribute to the impairment in urinary concentration in the postischemic period.

The collecting duct represents the final site for the control of water excretion into the urine. Water permeability of the collecting duct is tightly regulated, under the control of the antidiuretic hormone, vasopressin, which causes a dramatic increase in collecting duct water permeability, allowing reabsorption of water from the tubular fluid down an osmotic gradient (25, 40). The acute vasopressin-induced increase in collecting duct water reabsorption has been shown to involve vasopressin-regulated trafficking of AQP2 between intracellular vesicles and the apical plasma membrane. Long-term regulation of AQP2 involves mechanisms that alter the total abundance of AQP2 protein, thereby modulating the acute response by changing the number of water channels in the cell that can be recruited for vasopressin-regulated trafficking (40).

ARF, caused either by renal ischemia or nephrotoxic agents, is typically characterized by oliguria, a severe reduction in glomerular filtration rate, and a variable fall in renal blood flow. Characteristically, the urinary concentration ability is significantly decreased, and tubular reabsorption of sodium is markedly impaired. The pathophysiology of ischemic ARF is complex and is not well defined. Structural and biochemical changes in the postischemic kidney that results in vasoconstriction, desquamation of tubular cells, intraluminal tubular obstruction, and transtubular backleakage of the glomerular filtrate are pathophysiological mechanisms that have been reported (50). The mechanisms underlying intrarenal vasoconstriction and outer medullary hyperperfusion also remain incompletely defined but probably involve multiple factors including endothelin and NO imbalance (4). Furthermore, in the outer medulla, where tubules have high oxygen requirements, ischemic injury causes swelling of endothelial cells (33) as well as adherence of neutrophils to capillaries and venules. These changes may contribute to vascular congestions and hence decrease blood flow (5, 52). The ischemic damage to the outer medulla may lead to impaired function of the tubular cells that transverse this region of the kidney, especially with respect to the urinary concentrating ability (34).

Recent evidences demonstrated that leukocyte adhesion may play a significant role in renal ischemia (8, 20, 23, 24). Inflammatory cascades (i.e., interleukin-1, tumor necrosis factor-α, interleukin-8, intracellular adhesion molecule-1) that are activated in reperfusion period cause neutrophils to accumulate in the vasa recta in the outer stripe of the outer medulla (23, 44). This may contribute to obstruction of vasa recta and/or leukocyte-mediated increases in endothelial permeability (42) leading to erythrocyte aggregation and medullary congestion. Furthermore, this may sustain the ischemic injury in the outer medulla, even if total renal blood flow is restored.

NO, which is a chemical form of endothelium-derived relaxing factor, has important roles for renal hemodynamics and renal sodium and water metabolism (18, 47). Recently, it was shown directly that NO inhibits vasopressin-stimulated osmotic water permeability in isolated and perfused cortical collecting duct (18). The inducible form of NO synthase, which produces greater quantities of NO, is present in the rat proximal tubule and inner medullary collecting duct (30). In hypoxia/reoxygenation model, Yu et al. (53) demonstrated that NO production is stimulated by the hypoxic injury in epithelial cells, and its inhibition protects against hypoxic injury in rat proximal tubule cells. Therefore,
renal ischemic injury may be attenuated by inhibiting either inflammatory or NO pathways.

α-MSH is a potent antiinflammatory agent that inhibits neutrophil migration and production of neutrophil chemokines and NO (7). We demonstrate here that α-MSH treatment of rats with bilateral ischemia-induced ARF significantly reduced the downregulation of AQP2 and AQP3 levels and that this was paralleled by functional changes. This is consistent with previous observations that α-MSH treatment in mice and rats with ischemia-induced ARF significantly reduces the neutrophil plugging and erythrocyte congestion in the medullary region, in association with a marked improved renal function (8). Thus α-MSH-induced inhibition of NO production and decreased production of chemokines in response to ischemic injury may be involved in preventing AQP downregulation and renal functional defects. The exact role and signaling pathways involved for NO, endothelin, and chemokines in altering AQP expression remain to be identified. However, several of these mediators also have been implicated to play a role in the urinary concentrating defects associated with ureteral obstruction, a condition which also has been shown to be associated with reduced expression of AQP1–3 (14, 15). This raises the possibility that these mediators may play a significant role in the dysregulation of tubular water and sodium metabolism associated with several common pathological conditions. In the present study, immunocytochemistry confirmed that AQP2 and AQP3 levels are significantly decreased in the inner medullary collecting duct principal cells after bilateral ischemic insults. However, part of remaining AQP2 labeling was associated with apical plasma membrane domains despite a significant reduction in AQP2 abundance, suggesting that AQP2 trafficking is not severely impaired in kidneys with ischemic insults. Reduction in AQP2 levels with maintained AQP2 targeting was also observed in another ARF model using rats with unilateral ischemia with contralateral nephrectomy (11). Furthermore, in different experimental model using the unilateral ureteral obstruction where AQP2 levels were significantly decreased in the obstructed kidney (14), it was also demonstrated that there was a maintained AQP2 targeting to the apical plasma membrane domains. Therefore, this further supports the view that common signal transduction pathways may be affected in these settings.

Proximal Tubule Water Channel AQP1 is Severely Affected in Postischemic Kidneys

We demonstrated that the expression of proximal tubule and descending thin limb water channel AQP1 is significantly decreased in response to ischemic injury. ATP is known to be required for metabolic transformations such as protein synthesis and maintenance of cellular structures. The proximal tubule is largely dependent on oxidative metabolism for generation of ATP, whereas distal nephrons are able to synthesize ATP under conditions of limited oxygen availability (46). Therefore, energy-dependent functions of the proximal tubule may be likely to be very susceptible to hypoxic conditions. The role of reduced AQP1 levels in ARF remains to be characterized, although the observed defect in urinary concentration in mice that lack AQP1 (29) suggests that reduced AQP1 may play a significant role in urinary concentration defects in ARF.

Summary

The ischemic insult is associated with decreased expression of the aquaporins in the collecting duct as well as in the proximal tubule, coinciding with the impairment of urinary concentration. α-MSH treatment markedly reduce the ARF-induced decrease in urinary concentration and the decrease in AQP expression levels. Therefore, we conclude that decreased aquaporin expression may contribute to the impairment in urinary concentration in the postischemic period. Further studies are warranted to test whether there are alterations in the expression of sodium transporters in bilateral ARF that may play a critical role in the altered tubular sodium handling, the increased fractional excretion of sodium, and, together with reduced AQP expression, play a role for the decreased urinary concentration ability in ARF. Furthermore, trials to modulate the expression of water channel proteins and sodium transporters may potentially provide relevant therapeutic modalities in ischemia-induced ARF of human patients.

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