Reduced renal expression of AQP2, p-AQP2 and AQP3 in haemorrhagic shock-induced acute renal failure

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Abstract

Background. The aims of this study were to investigate the changes in the expression levels of renal aquaporins (AQPs) in response to haemorrhagic shock (HS) in rats and whether a change in the expression of AQPs was associated with parallel changes in urinary concentration.

Methods. HS was induced by withdrawal of blood through the femoral artery in rats. A mean arterial blood pressure (MAP) of 40 mmHg was maintained for 1 h before blood was reinfused, and rats were kept in metabolic cages for urine measurements. Two days after HS, we examined the abundance of AQPs in kidney by semiquantitative immunoblotting.

Results. HS rats (n = 13) developed acute renal insufficiency (creatinine clearance was 5.5 ± 0.4 vs 6.9 ± 0.3 ml/min/kg in sham-operated rats, n = 13, P < 0.05) and decreased urine osmolality (888 ± 88 vs 1799 ± 110 mosmol/kg H2O, P < 0.05). Consistent with this, semiquantitative immunoblotting revealed that the abundance of AQP2, phosphorylated (Ser256) AQP2 (p-AQP2) and AQP3 in whole kidney was significantly decreased after 2 days to 33 ± 4, 41 ± 9 and 35 ± 14% of sham levels, respectively (P < 0.05).

Conclusions. The expression of the collecting duct water channel AQP2, p-AQP2 and AQP3 was significantly downregulated after HS, which may play an important role in the impaired urinary concentrating ability in HS-induced acute renal failure.

Keywords: acute renal failure; aquaporin; haemorrhagic shock; urinary concentration

Introduction

Haemorrhagic shock (HS), secondary to major blood loss, frequently precedes multiple organ dysfunction. Multiple organ failure (MOF) following various states of shock remains a major clinical problem [1]. Thus, numerous investigators have studied the changes in the renal function and haemodynamics following severe haemorrhage, trauma and sepsis [1,2]. The initial cardiovascular response after severe haemorrhage is a decrease in cardiac output and a consequent depression in blood flow. These haemodynamic changes of hypoperfusion and ischaemia induce dysfunction of various organs including the kidney. In general, renal dysfunction can be recognized at an early stage after haemorrhage from a morphological and functional perspective [2]. Since the kidney is one of the key organs involved in haemorrhage-induced MOF, it is important to investigate the underlying mechanisms responsible for the altered renal functions (including renal regulation of water balance) following severe HS. However, the exact molecular mechanisms that result in the impairment in renal function in response to HS are not well understood. Moreover, to our knowledge, the renal water handling has not been characterized in a clinical rat model of HS.

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 these segments and its role in urinary concentration [3]. In the collecting duct, three AQPs (AQP2, AQP3 and AQP4) are known to be expressed and participate in...
the vasopressin-regulated water reabsorption. AQP2 is the apical water channel of collecting duct principal cells and is the chief target for regulation of collecting duct water permeability by vasopressin [4]. A series of studies have demonstrated that altered expression and apical targeting of AQP2 play a critical role in water balance disorders [3]. Water transport across the basolateral plasma membrane of collecting duct principal cells is thought to be mediated by AQP3 [5] and AQP4 [6]. Therefore, alterations in the expression of major renal AQPs may play a critical role in the development of water balance disorder in response to HS-induced acute renal failure (ARF).

Recently, we reported that experimental ARF induced by complete obstruction of the renal arteries for 30–60 min followed by reperfusion in rats has characteristic structural alterations in renal tubule epithelia in association with decreased urine concentration [7]. And this was associated with a significantly decreased abundance of AQPs in the collecting duct as well as in the proximal tubule [7]. However, such an experimental model with complete renal artery occlusion followed by reperfusion only partly mimics ARF in human patients, >70% of which is of circulatory nature and results from hypoxic injury to the kidney [8], including occurrence following hypovolaemia or HS. In the present study, we used an experimental model for HS/resuscitation established by temporary withdrawal of blood through the femoral artery leading to a mean arterial blood pressure (MAP) of 40 mmHg, and the blood was then reinjected in 1 h. In this model, kidney perfusion is still present but with a marked decrease in glomerular filtration. Thus, this model is very different from the one involving complete temporary occlusion of renal arteries with a complete arrest in organ perfusion.

We hypothesized that HS-induced ARF is associated with an altered expression of renal AQPs and that this may play an important role in the development of water balance disorder following HS. Therefore, we examined (i) whether experimental HS affects urinary concentration; (ii) whether HS is associated with changes in the expression of collecting duct AQPs [AQP2, phosphorylated AQP2 (p-AQP2) and AQP3]; and (iii) whether there are changes in the expression of proximal tubule and descending limb AQP1. The results demonstrate a significant downregulation of the expression of collecting duct AQP2. p-AQP2 and AQP3 protein levels, whereas there were no changes in the expression levels of AQP1. Thus, the decreased urine concentration observed following HS is likely to be in part due to downregulation of both apical and basolateral collecting duct AQPs.

Subjects and methods

Experimental animals

Experiments were performed on 26 male Munich-Wistar rats, weighing 250–350 g. The animals were maintained on standard rat chow (Altromin, Lage, Germany) and had free access to food and water throughout the study. After a period of acclimatization, the animals were randomized into two groups matched for body weight: an HS group (n = 13) and a sham-operated group (n = 13). During the entire study, rats were kept in individual metabolic cages with a 12 h artificial light/dark cycle, a temperature of 21 ± 2°C and humidity of 55 ± 2%.

HS/resuscitation model

Rats were anaesthetized with an intraperitoneal injection of 100 mg/kg ketamine hydrochloride and 5 mg/kg aecpromazine maleate. PE-50 catheters were introduced into the left femoral artery for recording blood pressure (Sirecust 961 and Siredoc 220; Siemens, Munich, Germany) and withdrawal of blood and into the left femoral vein for blood reinfusion. Immediately after the catheter insertion, 200 U of heparin (LEO, Glostrup, Denmark) was administered. The rectal temperature was maintained throughout the study at 37.5°C with a heating pad. HS was induced by withdrawing blood via a heparinized syringe until the MAP fell to 40 mmHg. During the following 1 h, extracting or infusing small amounts of blood was performed to maintain a constant low MAP in the range of 35–45 mmHg. All reserved blood was returned to the animals after 1 h of hypovolaemia and hypotension. The sham-operated rats underwent a similar surgical procedure, but they were not bled.

Clearance studies

Urine osmolality, creatinine concentration and water intake were measured throughout the study. Plasma was collected from the abdominal aorta at the time of sacrifice for measurement of osmolality, creatinine and urea nitrogen concentration.

Primary antibodies

For semiquantitative immunoblotting, previously characterized polyclonal antibodies were used and summarized in the following: AQP1 (RA3391/2352AP), an affinity purified polyclonal antibody to AQP1 has been characterized previously [7]; AQP2 (LL127AP), an affinity purified polyclonal antibody to AQP2 has been characterized previously [4]; p-AQP2 (AN244-pp-AP), an affinity purified rabbit polyclonal antibody to phosphorylated AQP2, which is raised against a peptide that was phosphorylated in the protein kinase A (PKA) phosphorylation consensus site (i.e. Ser256), has been characterized previously [9]; AQP3 (RA3040/1592AP), an affinity purified polyclonal antibody to AQP3 has been characterized previously [7]; NaPi-2, an affinity purified polyclonal antibody was used as described previously [10]; Na,K-ATPase, a monoclonal antibody against the α-1 subunit of Na,K-ATPase was used [10]; BSC-1 (NKCC2), an affinity purified polyclonal antibody to BSC-1 has been characterized previously [10].
using an ultra-turrax T8 homogenizer (IKA Laborteknik, Staufen, Germany) at maximum speed for 20 s, and the homogenate was centrifuged in an Eppendorf centrifuge at 4000 g for 15 min at 4 °C to remove whole cells, nuclei and mitochondria [7]. 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. Gel samples (Laemmli sample buffer containing 2% SDS) were made of this pellet.

Electrophoresis and immunoblotting

Samples of membrane fractions from either whole kidney or inner medulla were run on 12% polyacrylamide minigels (Bio-Rad Mini Protean II). For each gel, an identical gel was run in parallel and subjected to Coomassie staining to ensure identical loading. 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 primary antibodies (P448, diluted 1:3000; DAKO, Glostrup, Denmark) by using an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biosciences, Little Chalfont, Buckinghamshire, UK).

Semiquantitation of kidney AQP levels

ECL films with bands within the linear range were scanned using an AGFA scanner (ARCUS II) and Corel Photopaint Software to control the scanner. The labelling density was quantitated with blots, whereby samples from the kidney of HS rats were run on each gel with samples from the kidney of sham-operated rats. The labelling density was corrected by densitometry of Coomassie-stained gels.

Statistical analysis

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

Results

HS was associated with renal dysfunction

HS resulted in significant rises in the plasma levels of creatinine and urea nitrogen compared with sham levels (43 ± 2.1 vs 35 ± 1.2 µmol/l and 7.1 ± 0.3 vs 6.0 ± 0.3 mmol/ l, respectively, P < 0.05; Table 1). This demonstrates that HS induced acute renal insufficiency. Parallel to this, HS rats had significantly decreased creatinine clearance (5.5 ± 0.4 vs 6.9 ± 0.3 ml/min/kg, P < 0.05; Table 1), further indicating that HS induced acute renal insufficiency.

Renal water handling was impaired

To our knowledge, renal water handling had not been characterized in this rat model of HS. Therefore, we monitored the urine in both HS and sham-operated rats for 2 days after the operation. In the basal period before the operation, urine osmolality was not different between the two groups. After the operation, the urine osmolality in all HS rats was consistently and significantly decreased (888 ± 88 mosmol/kg H2O) compared with sham levels (1799 ± 110 mosmol/kg H2O, P < 0.05; Figure 1), indicating a decrease in urinary concentration in HS rats. Consistent with the changes in urine osmolality, the urine-to-plasma osmolality ratio was reduced to 3.9 ± 0.4 (vs 5.6 ± 0.3, P < 0.05; Table 1) and solute-free water reabsorption was significantly decreased to 107 ± 12 µl/min/kg (vs 142 ± 7, P < 0.05; Table 1). The markedly decreased urine osmolality, urine-to-plasma osmolality ratio and solute-free water reabsorption indicate that HS is associated with reduced urinary concentrating ability.

AQP2 and p-AQP2 abundance was decreased

The anti-AQP2 antibodies recognized the 29 and 35–50 kDa bands (Figure 2), which correspond to the non-glycosylated and glycosylated forms of AQP2, respectively. As shown in Figure 2A and B and Table 2, semiquantitative immunoblotting revealed a marked decrease in the abundance of AQP2 in whole kidney to 33 ± 4% of sham levels (100 ± 6%, P < 0.05). The abundance of AQP2 in inner medulla was also significantly reduced to 36 ± 8% of sham levels (100 ± 5%, P < 0.05; Figure 2C and D, Table 2). Moreover, semiquantitative immunoblotting with antibodies that selectively recognize AQP2 (p-AQP2), which is phosphorylated in the PKA phosphorylation consensus site (Ser256) [9], demonstrated that the abundance of p-AQP2 was also markedly decreased in HS rats (Figure 3). Densitometric analysis revealed a decrease in the abundance of p-AQP2 in whole kidney to 41 ± 9% of sham levels (100 ± 12%, P < 0.05; Figure 3A and B, Table 2) and in inner medulla to

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<th>Table 1. Changes in renal function after haemorrhagic shock (HS)</th>
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<td>Number of rats (n)</td>
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Values are means ± SE.

*P < 0.05, compared with sham levels.
39 ± 10% of sham levels (100 ± 7%, *P* < 0.05; Figure 3C and D, Table 2).

The reduced expression of AQP2 and p-AQP2 therefore indicates that downregulation of the vasopressin-regulated water channel AQP2 may contribute to the reduced urinary concentration seen in rats following HS.

**AQP3 abundance was decreased**

AQP3 is expressed in the basolateral plasma membrane of collecting duct principal cells, and a number of studies have suggested that AQP3 functions as an exit channel for water reabsorbed apically via AQP2 [5,11]. To investigate whether HS is associated with changes in the abundance of AQP3, semiquantitative immunoblotting of membrane fractions from whole kidney and inner medulla was performed. The abundance of AQP3 was significantly decreased in HS rats. Semiquantitative immunoblotting revealed that the abundance of AQP3 in both whole kidney and inner medulla was significantly decreased compared with sham levels (B and D). HS (open bars), rats with haemorrhagic shock; sham (filled bars), sham-operated rats. *P* < 0.05.

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**Fig. 1.** Time courses of the changes in urine osmolality in HS rats (*n* = 13) and sham-operated rats (*n* = 13). Urine osmolality was significantly decreased in HS rats, whereas there was no change in sham-operated rats. HS (open bars), rats with haemorrhagic shock; sham (filled bars), sham-operated rats. Day –2 and day –1, the days before the operation as baseline period; day 1 and day 2, the days after the operation. *P* < 0.05.

**Fig. 2.** Semiquantitative immunoblotting of membrane fractions of whole kidney (A and B) and inner medulla (C and D). The immunoblots were reacted with anti-AQP2 antibodies and revealed 29 and 35–50kDa bands, which correspond to the non-glycosylated and glycosylated forms of AQP2, respectively (A and C). Densitometric analysis revealed that the abundance of AQP2 in both whole kidney and inner medulla was significantly decreased compared with sham levels (B and D). HS (open bars), rats with haemorrhagic shock; sham (filled bars), sham-operated rats. *P* < 0.05.
Table 2). Thus, together with the reduction in the abundance of AQP2, the reduced abundance of AQP3 in the collecting duct is also likely to participate in the impaired urinary concentration.

**AQP1 expression was not changed**

AQP1 is abundantly expressed in the proximal tubule, descending thin limb and descending vasa recta [3], and functional studies in AQP1 gene knockout mice have revealed an essential role of AQP1 in proximal nephron water reabsorption [12,13]. In contrast to the downregulation of AQP2 and AQP3, there were no significant changes in the abundance of AQP1 in whole kidney (81 ± 13 vs 100 ± 27%, not significant; Figure 5A and B, Table 2) or in inner medulla (104 ± 10% vs 100 ± 24%, not significant; Figure 5C and D, Table 2).

Since defects in the proximal tubule are often seen in response to ischaemia-induced ARF, we investigated whether the expression of proximal tubule sodium transporters was reduced. As shown in Table 2, the protein expression of the sodium phosphate cotransporter type 2 (NaPi-2) was significantly reduced, indicating a defect in the proximal tubule. In support of this, the expression of the α-subunit of the Na,K-ATPase was also significantly reduced, whereas the expression of the thick ascending limb Na-K-2Cl cotransporter BSC-1 or NKCC2 was not reduced (Table 2).

**Discussion**

We examined the abundance of the proximal nephron AQP1 and the collecting duct AQP2, p-AQP2 and AQP3 in an animal model of HS in parallel with the determination of functional parameters and urine concentration. This model, unlike bilateral complete renal arterial occlusion, mimics to a higher degree the clinical situation of ARF in human patients which occurred mainly due to conditions with temporary reduction of blood pressure and hypovolaemia.

HS-induced ARF is characterized by significantly elevated levels of plasma creatinine and urea nitrogen. Moreover, the rats developed a urinary concentrating...
The expression of AQPs was determined by semiquantitative immunoblotting of membrane fractions from whole kidney or inner medulla. We demonstrated that the abundance of apical AQP2 and p-AQP2 and basolateral AQP3 in collecting duct principal cells was significantly decreased in response to HS. In contrast, the abundance of proximal nephron water channel AQP1 was not significantly changed. The results suggest that downregulation of the collecting duct water channels of HS rats may play a significant role in the decreased urinary concentration encountered in the experimental HS-induced ARF.

**HS induces renal dysfunction and water balance disorder**

In the present study, HS was induced in rats by withdrawing blood to maintain the MAP at
Dysregulation of renal AQP5s in haemorrhagic shock

The number of water channels in cells that can be thereby modulating the acute response by changing that alter the total abundance of AQP2 protein, Long-term regulation of AQP2 involves mechanisms and this involves phosphorylation of AQP2 in the intracellular vesicles and apical plasma membrane, vasopressin-regulated trafficking of AQP2 between duct water reabsorption has been shown to involve in several water balance disorders [3]. Thus, the decrease in the abundance of AQP2 and p-AQP2 is likely to play an important role in the development of impairment of urinary concentration after HS. It should be emphasized that previous studies have demonstrated an increase in plasma vasopressin associated with HS [14–16]. Thus, the reduction in the expression of AQP2, p-AQP2 and AQP3 is likely to be vasopressin independent, consistent with observations in experimental NDI [3].

Decreased AQP2 and p-AQP2 abundance

We demonstrated that HS is associated with a marked downregulation of collecting duct AQPs and decreased urinary concentration. The pathophysiological mechanisms for inducing ARF by complete bilateral renal arterial occlusion (i.e. zero perfusion and filtration) and reperfusion are likely to be, at least in part, different from those in this rat model of HS. In the HS model, renal blood flow is reduced, but not zero, whereas the glomerular filtration is very low or absent due to a blood pressure below the autoregulatory limit (ischaemic phase). Thus, it is important to investigate the molecular background for the urinary concentrating defect observed in response to HS.

The proximal tubule (especially the S3 segment) and the thick ascending limb are known to be the main susceptible sites of the ischaemia and reperfusion injury, whereas the collecting duct is generally considered to be relatively invulnerable. Nevertheless, we demonstrated that HS-induced ARF was associated with a markedly reduced abundance of the collecting duct water channel AQP2. The reduced expression of AQP2 was associated with decreased urine osmolality and solute-free water reabsorption, consistent with previous observations, e.g. in response to complete bilateral renal arterial occlusion induced ARF [7] and conditions with acquired nephrogenic diabetes insipidus (NDI) [3]. Thus, decreased collecting duct AQP2 levels may contribute, at least in part, to the impairment of urine concentration in HS-induced ARF. The acute vasopressin-induced increase in collecting duct water reabsorption has been shown to involve vasopressin-regulated trafficking of AQP2 between intracellular vesicles and apical plasma membrane, and this involves phosphorylation of AQP2 in the PKA phosphorylation consensus site (Ser256) [9]. 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 cells that can be recruited for vasopressin-regulated trafficking [3]. Semi-quantitative immunoblotting with antibodies that selectively recognize AQP2, which is phosphorylated in the PKA consensus site (Ser256), revealed that p-AQP2 was also markedly reduced after HS. It is likely that HS results in decreased urinary concentration by inhibition of (i) short-term regulation of vasopressin-regulated water channel AQP2 by decreasing phosphorylation at the PKA phosphorylation consensus site (Ser256) and (ii) long-term adaptive regulation by decreasing the overall abundance of AQP2. Importantly, multiple studies have demonstrated a critical role of AQP2 in several water balance disorders [3]. The presence of AQP2 and p-AQP2 have been shown to play an important role in the development of impairment of urinary concentration after HS. It should be emphasized that previous studies have demonstrated an increase in plasma vasopressin associated with HS [14–16]. Thus, the reduction in the expression of AQP2, p-AQP2 and AQP3 is likely to be vasopressin independent, consistent with observations in experimental NDI [3].

Decreased AQP3 abundance

Our data demonstrate a significant downregulation of AQP3 in the kidney of HS rats, and the reduced AQP3 may also play a significant role in the urinary concentrating defect associated with HS. This is based on studies showing an essential role of AQP3 in urinary concentration, including studies showing that AQP3 knockout mice have a severe urinary concentrating defect [11]. The mechanisms underlying the regulation of AQP3 expression are currently not well understood. AQP3 is localized in the basolateral plasma membrane domains of collecting duct principal cells [5]. Immunoelectron microscopy demonstrated a predominant labelling of AQP3 in the basolateral plasma membranes, with little labelling of intracellular vesicles [5], suggesting that AQP3 may be not regulated by vesicular trafficking (in contrast to AQP2). Immunoblotting evidence has shown that thirsting or dDAVP treatment of Brattleboro rats for 5 days induces a marked increase in the abundance of AQP3 [17]. Thus, AQP3 regulation is dependent on the plasma vasopressin levels. However, there are several examples where there is a decoupling of AQP2 and AQP3 expression, suggesting that other factors in addition to vasopressin may regulate one or the other AQP. This is seen in conditions such as hepatic cirrhosis, vasopressin escape and low-protein diet [3]. Moreover, we have recently demonstrated that the mineralocorticoid aldosterone regulates the abundance of AQP3 in rat kidney [18]. The mechanism causing AQP3 downregulation in HS-induced renal failure is currently not established, but it may share the same mechanisms seen in several forms of acquired NDI [3], where both AQP2 and AQP3 are downregulated.

Nevertheless, the signals affecting the downregulation of collecting duct AQPs in HS-induced renal failure are currently unknown. A number of events are
known to take place in HS. HS initiates an inflammatory cascade that includes the production of cytokines and the recruitment of neutrophils and may progress to organ failure [19]. Similar events are also a key feature of ARF caused by renal ischaemia and reperfusion after complete renal artery occlusion [20]. In the clinical setting, HS is associated with reduced cardiac output and the reduction of renal blood flow. Importantly, the renal blood flow often remains depressed despite blood transfusion or adequate resuscitation [2]. Structural, inflammatory and biochemical changes in the post-ischaemic kidney that result in vasoconstriction, desquamation of tubular cells, intraluminal tubular obstruction and transtubular back-leakage of the glomerular filtrate are pathophysiological mechanisms that have been reported [2]. The mechanisms underlying intrarenal vasoconstriction and outer medullary hypoperfusion remain incompletely defined, but they probably involve multiple factors including endothelin and nitric oxide imbalance. In outer medulla, where tubules have high oxygen requirements, ischaemic injury causes swelling of endothelial cells and adherence of neutrophils to capillaries and venules [21]. These changes may contribute to vascular congestions and hence decrease blood flow [22]. The ischaemic damage to the renal medulla may lead to impaired function of tubular cells that are essential for the urinary concentrating ability [23]. Nitric oxide has important roles for renal haemodynamics and renal water metabolism, and it has been shown directly that nitric oxide inhibits vasopressin-stimulated osmotic water permeability in isolated and perfused cortical collecting duct [24]. Whether nitric oxide, endothelin and chemokines are involved in inducing downregulation of collecting duct AQPs in response to HS is unknown, and further studies are necessary to investigate the signalling pathways involved in the downregulation of AQP2 and AQP3.

Unchanged AQP1 abundance
In contrast to the decreased abundance of AQP2 and AQP3, semiquantitative immunoblotting revealed that the abundance of proximal nephron water channel AQP1 in both whole kidney and inner medulla was not altered in HS rats. This is different from the result of our previous studies of ischaemia/reperfusion-induced ARF, where ischaemia and reperfusion injury was associated with a marked decrease in the abundance of AQP1 and of AQP1 labelling in the proximal tubule of rat kidney [7]. This raises the possibility that the change of AQP1 expression is related to the severity of the insult. It should be emphasized that the observed reduction in the expression of NaPi-2 and Na,K-ATPase demonstrates that there is some dysfunction of the proximal tubule, consistent with previous evidence from other models involving complete renal artery occlusion [10]. Thus, there is a dysfunction of the proximal tubule with reduction in NaPi-2 and Na,K-ATPase but not of AQP1.

Summary
Our study illustrates a relatively new approach to the study of complex pathophysiological processes involved in the dysregulation of renal water balance in animals using antibodies to major renal AQPs (AQP1, AQP2 and AQP3). The antibodies were employed to survey the nephron and collecting duct with regard to the changes in the abundance of AQPs using semiquantitative immunoblotting. The results demonstrate that the abundance of AQP2, p-AQP2 and AQP3 in the collecting duct is significantly decreased in HS and coincides with a markedly decreased urine concentration. This suggests that downregulation of both apical and basolateral collecting duct AQPs (AQP2 and AQP3) may contribute to the impairment in urinary concentration in HS. In contrast, there was no significant reduction in AQP1 expression. These results provide new insights into the renal dysfunction associated with HS.

Acknowledgements. The authors thank Mette Vistisen, Lotte V. Holbech and Gitte Kall for expert technical assistance. The Water and Salt Research Center at the University of Aarhus was established by, and is supported by, the Danish National Research Foundation (Dansmarks Grundforskingsfond). This study was supported by a grant to H.G. from the Danish Medical Research Council (innovative postdoctoral grant) and Action Pharma Aps. Support for this study was also provided by the Karen Elise Jensen Foundation, Human Frontier Science Program, Novo Nordic Foundation, University of Aarhus Research Foundation, the University of Aarhus, the Dongguk University and the Commission of the European Union (QRLT 2000 00778 and QRLT 2000 00987).

Conflict of interest statement. None declared.

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Received for publication: 3.2.03
Accepted in revised form: 23.5.03