Dehydration reverses vasopressin antagonist-induced diuresis and aquaporin-2 downregulation in rats

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The present study was designed to test the effect of thirsting on urine output and aquaporin-2 expression in rats. The results demonstrated that OPC-31260 caused a modest decrease in AQP2 expression and that this could be reversed by water deprivation for 12 h. In combination

THE ANTI DIURETIC HORMONE vasopressin causes a rapid increase in the water permeability of the mammalian collecting duct, allowing water reabsorption out of the tubule and hence the production of a concentrated urine. It has recently been demonstrated that this increase in water permeability is brought about by the transfer of specific aquaporin-2 (AQP2) water channels from a store in intracellular vesicles to the apical plasma membrane of the collecting duct principal cells (15, 18, 21). When vasopressin levels decline, the water channels are retrieved endocytically, although their subsequent fate remains to be determined. This acute response, originally proposed as the membrane shuttle hypothesis (26), occurs within a time frame of a few minutes and is triggered by the binding of vasopressin to V2 receptors. These receptors are linked via a heterotrimeric G protein to adenylate cyclase and consequently cause an increase in cytosolic cAMP, which acts as the second messenger in the acute response.

It is becoming clear that the regulation of water permeability brought about by the shuttling of water channels is modulated by longer-term changes in the total number of AQP2 water channels available in the cells. The mechanism(s) by which these changes of expression are controlled remain to be determined, but several previous studies (1, 9, 10, 22, 28) have shown changes in AQP2 mRNA levels in association with changes in protein levels, suggesting that changes in protein synthesis are at least part of the answer. A putative cAMP-responsive element has been identified in the 5'-untranslated region of the AQP2 gene (25), suggesting that vasopressin, via V2 receptors and cAMP production, might also be responsible for the long-term changes in AQP2 expression, by increasing mRNA production during periods of prolonged dehydration. However, evidence from a number of previous studies has indicated that changes in AQP2 expression can occur independent of changes in vasopressin activity (1, 9, 11, 21), that the effect of OPC-31260 on reducing AQP2 expression is mediated partly, but not exclusively, via V2 receptors.

aquaporin-2; vasopressin; antidiureis; antidiuretic hormone
METHODS

Experimental Protocols

Male Wistar rats were obtained from Møllegaard Breeding Centre Denmark.

Protocol 1. Rats were placed in metabolic cages for 24 or 48 h prior to the start of treatment with OPC-31260 to allow them to acclimate to the cages and provide information on baseline urine excretion and water intake. While in metabolic cages, rats were given 5 g of powdered food twice daily. This ensured that all food was eaten, and food was usually consumed within 1–2 h of administration. Water drinking and urine output were measured every 12 h. OPC-31260 was administered mixed with the food, 5 mg/rat, given twice daily. During the first 48 h of OPC-31260 treatment, animals had free access to water. In the subsequent 12 h, one-half of the animals had access to water and the other half did not. Controls were given 5 g of food without additives twice daily and had free access to water throughout.

Protocol 2. To test whether the effects of thirsting observed during protocol 1 were due to raised circulating vasopressin levels, rats were treated as described in protocol 1 except that during the final 12-h period they were not thirsted but were given a subcutaneous injection of 0.5 ml normal saline containing 0.2 or 20 ng DDAVP (desmopressin).

Protocol 3. For the administration of furosemide, osmotic minipumps (Alzet, Palo Alto, CA) were filled with furosemide solution buffered with ethanolamine (1.7%), to give a dose of 12 mg/day. Animals were anesthetized with intraperitoneal pentobarbital sodium (75 mg/kg), the pump was placed subcutaneously along the animal’s back, and the incision was closed with 4–0 Mersilene. Controls were treated identically, except that furosemide was omitted from the solution in the pumps. Animals were then returned to metabolic cages and monitored for 5 days, with free access to food, fresh water, and salt water.

Protocol 4. To test whether OPC-31260 administration could cause a diuresis in thirsted rats, animals were thirsted for 24h, while having free access to food. Animals were briefly anesthetized with halothane by inhalation, and OPC-31260 was then administered by gavage, in 2 ml of water. Control animals received water only. Urine was collected during the 12-h periods before and after treatment.

Tissue Preparation

For removal of kidneys, rats were anesthetized with intraperitoneal pentobarbital sodium (75 mg/kg), and the right kidney was removed for preparation of membrane fractions. The left kidney was either removed for the isolation of total RNA or perfusion-fixed via the aorta as previously described (13). Briefly, the inner medulla was isolated, minced finely, and homogenized in 10 ml of dissection buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 µM leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.2), with a motor-driven Potter-Elvehjem homogenizer. This homogenate was centrifuged in a Beckman L8M centrifuge at 4,000 g for 15 min at 4°C to remove nuclei and mitochondria. The supernatant was centrifuged at 200,000 g for 1 h. The resultant pellet, containing a mixture of plasma membranes and intracellular vesicles, was resuspended in ~100 µl of dissecting buffer and assayed for protein concentration using the method of Lowry. Gel samples (in 2% SDS) were made from this membrane preparation to a final concentration of 1 µg protein/µl.

Electrophoresis and Immunoblotting

Samples of membrane fractions from kidney inner medulla (1 µg/lane) were run in duplicate on 12% polyacrylamide minigels (Bio-Rad Mini Protein II). One gel was Coomassie stained, to ensure that loading in the lanes was consistent (6), while the other 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, and 0.1% Tween 20, pH 7.5) for 1 h and incubated with affinity-purified anti-AQP2 (40 ng IgG/µl). The labeling was visualized with horseradish peroxidase (HRP)-conjugated secondary antibody (P448, diluted 1:3,000; Dako, Glostrup, Denmark) using an enhanced chemiluminescence system (ECL, Amersham International). Controls were replaced by primary antibody with antibody preabsorbed with immunizing peptide, or with nonimmune IgG, were without labeling.

Preparation of RNA and Northern Blotting

Northern blotting was performed using a 32P-labeled AQP2 cDNA probe or a digoxigenin-11-dUTP-labeled AQP2 RNA probe. Total RNA was prepared from kidney inner medulla using two different methods. As previously described (6), the inner medulla was homogenized in a guanidinium thiocyanate-containing solution and subjected to a double extraction using water-saturated phenol and CHCl3:isooamyl alcohol (49:1). Isoopropanol precipitation was also repeated twice followed by wash in 70% ethanol. Using a second method, total RNA was prepared from kidney inner medulla using a RNaseasy Mini Kit (Qiagen). Total RNA was electrophoresed on a 1% agarose gels followed by blotting on a nylon membrane filter (Hybond-N; Amersham Life Science, Buckinghamshire, UK). To ensure efficient transfer of RNA from the gel to the blot, the gels were inspected under ultraviolet light after blotting. This revealed absence of ethidium bromide-stained bands.

The blots were probed with an [α-32P]dCTP-labeled human AQP2 cDNA probe (full-length cDNA of human AQP2, kindly provided by Dr. Peter Deen, Nijmegen University, The Netherlands). cDNA probe labeling was performed using random-primed method (Prime-it II random primer labeling kit, Stratagene). AQP2 labeling was visualized by exposing the blot to high-performance autoradiography film. Alternatively, the blots were hybridized with a digoxigenin-labeled AQP2 RNA probe. RNA probe labeling was performed by in vitro transcription (MAXIscript In Vitro Transcription kit, Ambion, Austin, TX). The bands were visualized using a chemiluminescent substrate, CSPD (Boehringer, Mannheim, Germany). To determine the size of the AQP2 mRNA transcript, a digoxigenin-labeled RNA molecular weight marker (Boehringer) was used.

AQP2, THIRSTING, AND ADH-RECEPTOR ANTAGONIST

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Quantitation of AQP2 Protein or mRNA Expression

ECL films with bands within the linear range were scanned using a Umax Vistascan scanner and Adobe Photoshop Software. As described previously there is a good relationship between optical density and protein levels using ECL, when optimal background subtraction is performed (13). Thus bands were scanned at the upper end of the linear range.

Samples from OPC-31260-treated or furosemide-treated animals were run with samples from kidneys from appropriate control animals.

Immunoblotting. Both the 29-kDa and the 35- to 50-kDa bands (corresponding to nonglycosylated and the glycosylated forms of AQP2) were scanned, as previously described (13). Following densitometry, AQP2 labeling in the samples from the experimental animals was calculated as a fraction of the mean control value for that gel, as previously described (13). Values were corrected by densitometry of Coomassie-stained preliminary gels (to correct for minor variability in loading).

Northern blotting. The band of ~1.6 kb corresponding to AQP2 mRNA on the autoradiography film or ECL film was scanned. Values were corrected for potential differences in loading of total RNA in the specific lanes by densitometry of 18S and 28S rRNA bands, which were visualized by ethidium bromide on the same gel.

Comparisons between groups were made by unpaired t-test. P < 0.05 was considered significant.

Preparation of Tissue for Immunocytochemistry

After in situ perfusion fixation, kidneys were postfixed in the same fixative for 2 h. Tissue blocks were then prepared from the inner medulla and were infiltrated for 30 min with 2.3 M sucrose containing 2% paraformaldehyde, mounted on holders, and rapidly frozen in liquid nitrogen (19). For light microscopy, the frozen tissue blocks were cryosectioned (0.8 µm, Reichert Ultracut S Cryoultramicrotome), sections were incubated with affinity-purified anti-AQP2 antibodies (LL127AP), and the labeling was visualized with HRP-conjugated secondary antibody (P448, diluted 1:100), followed by incubation with diaminobenzidine. For immunoelectron microscopy, ultrathin (40–80 nm) Lowicryl sections were cut (18), incubated with affinity-purified anti-AQP2 (LL127AP), and the labeling was visualized with goat-anti-rabbit IgG conjugated to 10-nm colloidal gold particles (GAR.EM10; BioCell Research Laboratories, Cardiff, UK).

RESULTS

Treatment with OPC-31260 Causes Substantial Polyuria

Animals were kept in metabolic cages for 48 h before and 60 h after the start of OPC-31260 treatment. Urine output and osmolality were monitored throughout this period. The results are shown in Fig. 1. Average urine production rose from 13 ± 1 ml/day in controls to 56 ± 4 ml/day during treatment with OPC-31260. There was marked diurnal variation in the urine production, with greater water intake and urine production during the night, which is normal for rats, and this persisted in the OPC-31260-treated animals. There were reciprocal changes in urine osmolality, which dropped from 1,227 ± 163 to 313 ± 48 mosmol/kgH₂O following treatment with OPC-31260.

AQP2 Protein Levels Fall in Response to OPC-31260 Treatment

To assess the effect of OPC-31260 on AQP2 protein levels, membrane fractions were prepared from kidney inner medulla after animals had been treated for 60 h with OPC-31260. AQP2 levels were assessed by immunoblotting and densitometry of both the nonglycosylated (29 kDa) and glycosylated (35–50 kDa) bands. The results are shown in Fig. 2. Following OPC-31260 treatment, AQP2 expression decreased to 52 ± 11% of control levels (n = 12, P < 0.05). No significant change in AQP2 protein was seen after only 24 h treatment with OPC-31260 [n = 4, not significant (NS), data not shown].
AQP2 mRNA Levels Fall in Response to OPC-31260 Treatment

To investigate whether the decrease in AQP2 protein levels during OPC-31260 treatment reflects decreased AQP2 mRNA levels or increased protein degradation, AQP2 mRNA levels were measured in inner medulla. The results are summarized in Fig. 3.

After 24-h treatment with OPC-31260, mRNA levels fell to 60 ± 15% of control levels (n = 5, P < 0.05) and after 48 h were 30 ± 10% of controls (n = 9, P < 0.01). These results indicate that mRNA levels decline significantly and to a slightly greater degree than protein expression, consistent with the reduction in AQP2 protein levels being a consequence of decreased AQP2 mRNA levels.

Immunocytochemistry Confirms the Decrease in Expression

Thin cryosections were cut from kidney inner medulla of control rats and animals treated with OPC-31260 for 60 h and labeled for AQP2 using the immunoperoxidase method. As shown in Fig. 4, there is a moderate decrease in labeling after OPC-31260 treatment, consistent with the results of immunoblotting (Fig. 2). The labeling of the apical plasma membrane domains also appeared reduced following OPC-31260 treatment. To confirm this observation, we carried out immunogold labeling at the electron microscopic level. This also demonstrated significantly reduced AQP2 labeling of the apical plasma membrane after 60-h treatment with OPC-31260 (Fig. 5), as would be expected with blockade of V2 receptors, and hence of the acute vasopressin-regulated AQP2 shuttling, consistent with our earlier reports on the acute action of OPC-31260 (17). As expected from the Western blotting.

Fig. 2. A: immunoblot, labeled for AQP2, of membrane fractions of kidney inner medulla from 4 controls, 4 OPC-31260-treated (OPC), and 4 OPC-31260-treated and thirsted (OPC + Thirst) animals. Antibody recognizes both the 29-kDa nonglycosylated and the 35- to 35-kDa glycosylated forms of AQP2. B: summary of densitometry results from the whole series of animals. Treatment with OPC-31260 caused a decrease in AQP2 to 52 ± 11% of control levels. This decrease was reversed following 12-h thirsting. * P < 0.05.

Fig. 3. A: Northern blot of AQP2 mRNA in inner medulla from kidneys of rats treated with OPC-31260 (OPC) for 48 h. Digoxigenin-labeled human AQP2 RNA probe labels a band of ~1.6 kb, consistent with the predicted size of AQP2 mRNA. B: ribosomal 18S and 28S rRNA bands visualized by ethidium bromide on the same gel, as a loading control. C: densitometric analysis of expression of AQP2 mRNA from all experiments. Compared with levels in control rats, expression is reduced in OPC-31260-treated animals. Densitometry of all samples reveals significant reduction (P < 0.01, n = 9) after 48 h of OPC-31260 treatment. Densitometric values are presented as fraction of controls.
results and the light microscopy, vesicular labeling also appeared to be reduced, consistent with the decrease in total AQP2 levels.

Chronic Treatment with Furosemide Does Not Significantly Alter AQP2 Expression

As shown in Fig. 6, furosemide treatment caused even more severe polyuria than seen with OPC-31260, averaging 82 ± 23 ml/day compared with 11 ± 2 ml/day in the controls. Immunoblotting demonstrated no significant change in AQP2 levels (128 ± 20% of control levels; n = 6 in each group, NS; Fig. 6). Thus furosemide does not cause a decrease in AQP2 levels, demonstrating that neither increased urine flow nor medullary osmotic washout causes changes in AQP2 expression.

Thirsting for 12 h Reverses the Polyuria and Decrease in AQP2

Animals thirsted for the last 12 h (overnight) of treatment with OPC-31260 had a reduced urine output, which was not significantly different from that of non-OPC-31260-treated, nonthirsted controls (Fig. 1A). After thirsting of OPC-31260-treated animals, urine osmolality rose to 1,191 ± 91 mosmol/kgH2O, which was not significantly different from the values obtained from the (nonthirsted) control animals (Fig. 1B). In the animals thirsted for 12 h, AQP2 expression in the inner medulla returned to 95 ± 16% of the value seen in nonthirsted controls (100 ± 23%, n = 12, NS; Fig. 2).

To ensure that OPC-31260 is able to provide an adequate blockade even during dehydration known to induce high circulating levels of vasopressin, two series of control experiments were performed. In the first, rats were thirsted for 24 h and subsequently treated by gavage with OPC-31260 (40 mg/kg in 2 ml water). The data are presented in Table 1. Even in these thirsted animals, OPC-31260 treatment was able to cause a marked increase in urine production and decrease in urine osmolality, confirming effective receptor blockade. In the second series, rats were treated orally with OPC-31260 for 60 h, then with DDAVP treatment.
during the last 12 h of this period (subcutaneous injection of 2 or 20 ng in 0.5 ml normal saline; controls received only saline). Even at the high dose of DDAVP, OPC-31260 continued to produce a marked polyuria (Table 2).

DISCUSSION

The results presented here demonstrated that the vasopressin V₂ receptor antagonist OPC-31260 produced a marked diuresis and that this was associated with a decrease in AQP2 expression in the rat kidney inner medulla, but only to about one-half the control level. Furthermore, OPC-31260 treatment decreased the amount of AQP2 in the apical plasma membrane, consistent with blockade of the V₂ receptors known to mediate the vasopressin-induced transfer of AQP2 water channels from intracellular vesicles to the apical plasma membrane. These results confirm a previous report (10). In addition, we have investigated whether a relatively short (12 h) period of water deprivation during continued treatment with OPC-31260 was able to modify the effect of OPC-31260 on AQP2 expression. The results demonstrated that AQP2 expression returns to control level during this time, in conjunction
with the production of a concentrated urine. Control experiments ensured that there was effective receptor blockade during thirsting or high-dose DDAVP treatment. In combination with previous studies, the modest downregulation of AQP2 and the ability of thirsting to reverse the effect despite continued blockade of the V2 receptors support the view that modulation of AQP2 expression is mediated partly, but not exclusively, via V2 receptors.

Recent studies have indicated that, in addition to the acute increase in water permeability brought about by the insertion of AQP2 water channels into the apical plasma membrane in response to vasopressin, there is a longer-term modulation of collecting duct water permeability (12). This modulation reflects changes in total AQP2 expression (10, 19). These studies provide a mechanistic understanding of the long-known phenomenon that chronic dehydration results in an increased maximal concentrating ability, whereas prolonged water loading impairs it (5). Such a phenomenon also seems to underlie multiple forms of acquired nephrogenic diabetes insipidus (NDI), in which we have demonstrated decreases in AQP2 expression, which can be very severe (7, 13, 14). Similarly, two conditions in which there is water retention, congestive heart failure and hepatic cirrhosis, have been shown to be associated with increased AQP2 expression, consistent with a role in the water retention in these conditions (9, 27). It has been suggested (27) that water retention in these conditions is a consequence of increased vasopressin release, consistent with the early studies showing that infusion of vasopressin into Brattleboro rats (which lack endogenous vasopressin) increases AQP2 expression (2). Thus this long-term modulation of AQP2 levels may be important in a number of clinically important conditions, as well as providing part of the normal physiological response to changes in hydration status.

The simplest mechanism for the long-term modulatory phenomenon would be that it is also a response to

Table 1. Effect of oral OPC-31260 in rats thirsted for 24 h prior to treatment with OPC-31260 and for 12 h afterwards

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urine Volume, ml/12 h</th>
<th>Urine Osmolality, mosmol/kg H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPC-31260 (n = 6)</td>
<td>0.7 ± 0.1</td>
<td>2,960 ± 10</td>
</tr>
<tr>
<td>Water (n = 5)</td>
<td>0.9 ± 0.2</td>
<td>2,688 ± 173</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of experiments. Urine volumes and osmolalities for the 12 h before and 12 h after treatment are shown. OPC-31260 causes over a 3-fold increase in urine output and a substantial drop in urine osmolality in these animals.

Table 2. Effect of dDAVP in rats treated for 60 h with oral OPC-31260, with DDAVP injected subcutaneously at the beginning of the last 12 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urine Volume, ml/12 h</th>
<th>Urine Osmolality, mosmol/kg H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDAVP, 20 ng (n = 4)</td>
<td>15 ± 1</td>
<td>396 ± 54</td>
</tr>
<tr>
<td>DDAVP, 2 ng (n = 4)</td>
<td>18 ± 6</td>
<td>508 ± 167</td>
</tr>
<tr>
<td>Saline (n = 4)</td>
<td>15 ± 3</td>
<td>508 ± 55</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of experiments. Urine volumes and osmolalities for the 12 h before and 12 h after treatment are shown. Neither dose of desmopressin (DDAVP) caused a significant change in urine production or osmolality, indicating that this dose of OPC-31260 is producing effective receptor blockade.
elevated circulating vasopressin levels, acting via an increased cytosolic cAMP concentration. Consistent with this, a putative cAMP-responsive element has been described in the 5'-untranslated part of the AQP2 gene (25), and preliminary studies reveal low AQP2 levels in mice with reduced collecting duct cAMP due to a congenitally high level of phosphodiesterase activity (20). However, a number of studies have demonstrated that changes in AQP2 expression can occur independent of changes in vasopressin activity. Recently, a clear-cut study showed that water loading decreases AQP2 levels markedly in rats fitted with osmotic minipumps that maintained a high circulating level of DDAVP (3). Furthermore, we have shown that in lithium-treated rats, which have a profound downregulation of AQP2 in conjunction with extreme polyuria, DDAVP can be given at a sufficiently high dose to restore moderate antidiuresis but which only gives a small increase in AQP2 over the lithium-treated levels. In these animals, the AQP2 was efficiently targeted to the apical plasma membrane. In contrast, thirsting of lithium-treated animals for 48 h resulted in a much greater increase in AQP2 but less efficient antidiuresis, with little AQP2 in the plasma membrane (13). These results strongly suggest that a signal other than vasopressin must be involved in the regulation of AQP2 expression and even that vasopressin itself might be relatively unimportant. Studies of NDI induced by hypokalemia (14) or ureteric obstruction (7) also indicated that the signals inducing translocation of AQP2 to the plasma membrane and those regulating AQP2 expression are different, because in both cases there was decreased AQP2 expression but continued presence of the majority of AQP2 in the apical part of the cell.

In the light of these findings, we investigated the effect of V2-receptor blockade on AQP2 expression, to determine whether vasopressin and possibly other ligands such as oxytocin were acting via this receptor pathway. The results presented here demonstrate that this is indeed the case. The very pronounced diuresis produced in response to OPC-31260 demonstrated that there was efficient blockade of the V2 receptors and hence of the acute antidiuretic action of vasopressin. This was confirmed by the immunocytochemistry, which showed that AQP2 labeling of the apical plasma membrane was markedly reduced following OPC-31260 treatment. Thus there was a marked effect of the V2-receptor antagonist treatment on the subcellular distribution of AQP2. In association with this, there was a modest decrease in AQP2 expression, to about one-half the level seen in control animals. Thus blockade of V2 receptors does appear to cause a decrease in AQP2 expression. However, it is interesting that the decrease in AQP2 seen after OPC-31260 treatment is smaller than that seen in association with several forms of acquired NDI that show a more modest diuresis, indicating that changes in expression are not directly linked with the degree of diuresis (which presumably also reflects blockade of the acute transfer of AQP2 from intracellular vesicles to the apical plasma membrane) and consistent with the other evidence that signals other than changes in cAMP due to V2-receptor activity play a role in the modulation of AQP2 levels.

The reduced AQP2 mRNA levels seen in response to treatment with OPC-31260 (Fig. 3) are consistent with previous studies on the short-term effect of OPC-31260 (17). These results indicate that the changes in mRNA abundance precede the decrease in AQP2 protein levels. Therefore, the decline in AQP2 protein after 60 h of treatment with OPC-31260 (Fig. 2) is likely to reflect decreased synthesis in the presence of ongoing or possibly increased degradation or excretion of AQP2. During treatment with OPC-31260, AQP2 is found in intracellular vesicles (Figs. 4 and 5) and also in multivesicular bodies (present study, not shown, and Ref. 17), thought to be lysosomal in nature, consistent with degradation of AQP2 internalized from the plasma membrane. Such degradation may be the default pathway following internalization of AQP2. However, other work using cultured cells has suggested that under some circumstances endocytosed AQP2 may be available for re-use (11). Delivery to degradative compartments may occur when vasopressin activity is low or, as in this case, when blockade at V2 receptors prevents its induction of cAMP production. Such degradation may be a significant factor in allowing the amount of AQP2 to decline during water loading, as required for the long-term modulation of the acute hydromotic response to vasopressin.

To exclude the possibility that the decrease in AQP2 expression was a consequence of the increased tubular flow rate itself (directly or indirectly) or of washout of the medullary hypertonicity, the effect of chronic furosemide treatment on AQP2 levels was investigated. This was important because of recent evidence that a putative hypertonicity response element has been found in association with the AQP2 gene (16). The results presented here demonstrate that even after 5 days treatment with furosemide, with urine outputs very similar to those seen during OPC-31260 treatment, there was no decrease in AQP2 expression. These results confirm and extend earlier experiments in which the effects of treatment with furosemide for 24 h were studied (14). It is clear that changes in medullary tonicity cannot be the major factor causing changes in AQP2 expression and that even prolonged diuresis does not, per se, result in decreased expression. Thus it is very likely that the decrease in expression seen after treatment with OPC-31260 is a direct consequence of its action at V2 receptors.

Following 12 h of water deprivation, urine output and AQP2 levels returned to levels not significantly different from those in control animals (which were not thirsted or OPC-31260 treated), despite continued OPC-31260 administration. This is a surprisingly rapid increase in AQP2 expression. However, the thirsting period in this study was overnight, and rats drink relatively little during the day, when their activity is also low. Furthermore, because of the treatment with OPC-31260, which caused high urine output, the rats were probably relatively dehydrated compared with...
control rats even before the thirsting period. Consequently, the effect of 12-h water deprivation in these animals may be similar to the effects of 24-h deprivation in control animals, which we have found to cause a significant increase in AQP2 expression (Nielsen et al., unpublished observations). It remains a possibility that thirsting may inhibit degradation of AQP2 and thus that the apparent increase in AQP2 expression may reflect decreased degradation with a constant rate of synthesis.

The question arises as to whether the relative increase in AQP2 expression back to control levels may reflect an increase in circulating vasopressin to levels where it can overcome the competitive block caused by OPC-31260. However, the dose of OPC-31260 used in this study (40 mg·kg\(^{-1}\)·day\(^{-1}\) in divided doses) is greater than that shown to be effective in opposing the antidiuresis induced by continuous infusion of DDAVP in rats at rates up to 5 ng/h (8, 9, 24) and induced in humans by thirsting for 14 h (23). Tsuboi et al. (24) showed that OPC-31260 could induce a profound diuresis even in rats thirsted for 24 h when given orally at a similar dose (100 mg/kg). Moreover, when Brattleboro rats, with subcutaneous infusion of 1 ng/h DDAVP (which markedly decreased urine output to 12.6 from 148.7 ml/day), were treated with OPC-31260 (30 mg/kg), this resulted in a promptly increased urine volume to levels similar to those before the administration of DDAVP (24). This strongly supports the view that OPC-31260 given at the same doses as in the present study effectively can block V\(_2\) effects even with vasopressin or DDAVP at high supraphysiological doses in rats. Furthermore, although urine output dropped in the thirsted rats (Fig. 1), it fell only to the level seen in nonthirsted controls; normally urine output would fall further in response to the rise in vasopressin induced by water deprivation. This indicates that there was still at least partial blockade of the V\(_2\) receptors mediating the antidiuretic response to vasopressin. These results indicate that, at worst, the OPC-31260 is likely to have provided an efficient blockade of the effect of circulating vasopressin during the majority of the 12-h thirsting period. To ensure that this was the case, we performed experiments to test that OPC-31260, in the dose used in this study, could both cause a diuresis even in thirsted animals and prevent antidiuresis in the presence of high levels of vasopressin. When OPC-31260 was given to rats previously thirsted for 24 h, it produced a substantial increase in urine volume (more than 3-fold relative to controls which were also thirsted), together with a reciprocal drop in urine osmolality. It is clear, therefore, that OPC-31260 could effectively oppose the rise in circulating vasopressin induced by thirsting and cause a diuresis. The net effect of this was to produce a urine output very similar to that seen in nonthirsted controls. Thus the results of giving OPC-31260 to thirsted rats was identical to the effect of thirsting OPC-31260-treated rats. The experiments in which rats were given DDAVP after prolonged OPC-31260 treatment confirm that there is efficient blockade of the receptors and that, when adequately hydrated, the rats continue to produce a profound diuresis even when given large exogenous doses of antidiuretic hormone, many times greater than the maximum physiological dose. Our results indicate that thirsting provides a powerful stimulus for increased AQP2 expression that is likely to be at least partly independent of the effect of circulating vasopressin. Our results also provide a further demonstration that dehydrated animals can reduce their urine output via a vasopressin-independent pathway; this has previously been clear from experiments with Brattleboro rats, which can respond to dehydration by increasing their urine osmolality to values very similar to those seen here (i.e., ~800 mosm/kgH\(_2\)O) (4).

An interesting side issue raised by this study is the persistence of the diurnal variation in urine output and osmolality during treatment with OPC-31260. Because this persists despite continuing treatment with OPC-31260, it must be mediated by factors other than vasopressin acting via V\(_2\) receptors. Presumably, the variation is a reflection of the pattern of water intake and activity shown by the animals, together with the non-vasopressin-mediated factors involved in the thirsting response discussed above. These may include decreased glomerular filtration and increased medullary tonicity.

Conclusions

Our results confirm that blockade of V\(_2\) receptors with OPC-31260 causes a decrease in AQP2 expression in rat kidney collecting ducts and that this decrease is preceded by a decline in mRNA production, consistent with the hypothesis that the effect is due to decreased gene transcription as a consequence of reduced activity at the cAMP-responsive element identified in the promoter region of the AQP2 gene. The decrease is not a consequence of the increased diuresis, because prolonged furosemide-induced polyuria did not cause a decrease in expression. However, this decrease in expression can be reversed by a 12-h period of water deprivation in the continued presence of OPC-31260, indicating that thirsting, in addition to its presumed effects via increased circulating vasopressin levels, provides a stimulus for increased AQP2 expression other than that mediated by V\(_2\) receptors.

We thank Mette Vistisen, Trine Møller, Gitte Christensen, and Annette Blak Rasmussen for expert technical assistance.

Support for this study was provided by the Novo Nordisk Foundation, Karen Elise Jensen Foundation, the Danish Medical Research Council, University of Aarhus, Leeds University, and the Intramural budget of the National Heart, Lung, and Blood Institute. The OPC-31260 compound was kindly provided by Otsuka Pharmaceutical Co., Tokyo, Japan.

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Received 9 December 1997; accepted in final form 8 June 1998.

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