Differential Down-Regulation of Aquaporin-2 in Rat Kidney Zones by Peripheral Nociceptin/Orphanin FQ Receptor Agonism and Vasopressin Type-2 Receptor Antagonism

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ABSTRACT

We previously showed that aquaresis induced by the peripherally acting nociceptin/orphanin FQ receptor agonist ZP120 is associated with a decreased protein level of aquaporin-2 (AQP2) in whole-kidney homogenates. We now examined the effects of Ac-RYYRWKKKKKKK-NH₂ (ZP120) (1 nmol/kg/min i.v. for 4 h) on renal regional expression (cortex/outer stripe of outer medulla, inner stripe of outer medulla, and inner medulla) and subcellular localization of aquaporin-2. Responses to ZP120 were compared to the effects of an equi-aquaretic dose of OPC31260 (32 nmol/kg/min), an allosterically acting nociceptin/orphanin FQ receptor agonist ZP120 that increased the AQP2 mRNA level in the inner medulla. We conclude that equi-aquaretic doses of ZP120 and OPC31260 produce different patterns of aquaporin-2 down-regulation, suggesting different signaling pathways.

During normal conditions the kidneys regulate renal water excretion to compensate for variations in water intake and extrarenal water loss. The key hormone for regulation of renal water excretion is arginine-vasopressin (AVP), which is released from the posterior pituitary gland in response to an increase in plasma osmolality or a decrease in intravascular volume. AVP regulates water permeability of renal collecting duct (CD) principal cells by both short- and long-term regulation via the Gₛ-coupled AVP type-2 receptor (V₂-receptor) (Nielsen et al., 1995; Marples et al., 1999). On short term, CD water permeability is increased through cAMP-mediated protein kinase A serine-256 phosphorylation of aquaporin-2 (AQP2), inducing trafficking from intracellular vesicles to the apical membrane (Deen et al., 2000). On long term, protein kinase A phosphorylates and activates the cAMP-responsive element-binding protein to increase AQP2 gene expression (Yasui et al., 1997).

In response to a decrease in plasma osmolality or an increase in intravascular volume, water excretion is normally increased through a decreased AVP plasma concentration
leading to AQP2 down-regulation. However, in conditions such as syndrome of inappropriate AVP release or congestive heart failure (Schrier and Martin, 1998; Feldman et al., 2005), a pathological overactivity of the AVP-AQP2 system leads to excessive water retention, which in the end may lead to life-threatening hyponatraemia. For its treatment, it is desirable to develop compounds that increase free water clearance (i.e., produce aquaresis).

Recently, the combined \( V_{1a}/V_2 \)-receptor antagonist con-vaptan was registered for treatment of congestive heart failure, representing the aquarectic mechanism of blocking AVP binding to its receptor in the CD (Doggrell, 2005). Another compound that counteracts the AVP signaling pathway is nociceptin, the endogenous ligand of the nociceptin/orphanin FQ receptor (NOP) (alternative name: opioid receptor-like 1). This peptide exerts a marked aquarexis when infused either i.e.v. or i.v. (Kapusta et al., 1997). It has been shown that nociceptin infused i.e.v. decreases the AVP plasma concentration, thus proposing an aquarectic mechanism mediated via the central nervous system (Kakiya et al., 2000). To address the mechanism for aquarexis produced by peripherally infused nociceptin, we recently published data obtained with the stable NOP agonist ZP120 (Hadrup et al., 2004). ZP120 produces NOP-mediated central nervous system effects on pain and locomotor activity when injected i.e.v. but not when injected i.v., suggesting that it does not cross the blood-brain barrier (Rizzi et al., 2002). With i.v. ZP120, we found a marked aquarectic effect through a mechanism that includes AQP2 down-regulation at the protein level in whole-kidney preparations (Hadrup et al., 2004). We identified NOP throughout the CD but solely colocalized with AQP2 at the cellular level in the inner medulla (IM), suggesting that peripheral ZP120 exerts its effect in this zone. However, because the IM only constitutes a minor part of the kidney, we found it necessary to investigate the exact localization of AQP2 down-regulation. Furthermore, in the present study, we compared the effect of peripheral NOP stimulation with that of \( V_2 \)-receptor antagonism. This was done because the effect of such blockade on AQP2 regulation has not yet been investigated in a steady-state setup where physiological antagonism is avoided by replacement of urinary volume losses. Moreover, although the AVP plasma concentration was not decreased in previous experiments (Hadrup et al., 2004) and although data on pain modulation and locomotor activity indicate that i.v. ZP120 does not cross the blood-brain barrier (Rizzi et al., 2002), bolus injection data suggest that i.v. nociceptin/ZP120 might have central effects on water homeostasis control (Kapusta et al., 1997, 2005). Therefore, we wanted additional data to address a possible AVP plasma concentration-lowering effect by peripheral NOP stimulation. We hypothesized that an effect of i.v. ZP120 exclusively on AVP would induce an AQP2 down-regulation pattern similar to blockade of the AVP receptor.

For these purposes, we investigated AQP2 protein level, mRNA level, subcellular localization, and phosphorylation status, using two different infusion models. First, we conducted renal clearance experiments in chronically catheterized rats in a setup where renal water losses were replaced by servo-controlled infusion of 50 mM glucose to avoid physiological antagonism (Burgess et al., 1993). In this model, ZP120 and the \( V_2 \)-receptor antagonist OPC31260 were infused i.v. for 4 h in doses that produced similar increases in fractional distal water excretion (\( V/CL_{\text{i,d}} \)). In parallel with this finding, we employed a 1-h infusion model without volume replacement, enabling investigation of AQP2 regulation during onset of aquarexis with concomitant volume loss and physiological antagonism.

### Materials and Methods

**Animals.** Male Wistar rats (300–400 g) (Charles River, Sulzfeld, Germany) were used for the experiments. The rats were housed in a temperature- (between 22 and 24°C) and moisture-controlled (40–70%)-room with a 12-h light/dark cycle (light on from 6:00 AM to 6:00 PM). The rats were given free access to tap water and a diet containing −140 mmol/kg sodium, −275 mmol/kg potassium, and 23% protein. All rat procedures followed the guidelines for the care and handling of laboratory animals established by the Danish government and were done in accordance with the Declaration of Helsinki.

**Aquaretic Infusion with Volume Replacement.** Renal function was examined in conscious chronically catheterized rats. Permanent venous, arterial, and bladder catheters were implanted during anesthesia with 4% (induction) followed by 2% (maintenance) isoflurane in \( N_2/O_2 \) (1:1). The rats were then allowed to recover for 1 week; to relieve postoperative pain, buprenorphine (0.05 mg/kg) was administrated s.c. twice daily for 2 days. Two days before the experiment, the rats were switched to a diet supplemented with lithium (12 mmol/kg). The rats were accustomed to the experimental restraining cages by two sessions of training on different days. On the experimental day, the rats were placed in the restraining cages and infused i.v. with a fluid containing 150 mM glucose, 6.5 mM NaCl, 2.6 mM LiCl, and tracer amounts of \( [\text{H}]\text{inulin} \) (GE Healthcare, Buckinghamshire, UK) at 2 ml/h. The arterial catheter was kept open by infusion of 150 mM glucose containing 10 IU/ml heparin at 0.5 ml/h. After a 90-min equilibration period, urine was collected during two 30-min control periods. Intravenous infusion of ZP120 (1 nmol/kg/min) or OPC31260 (32 nmol/kg/min) then was started, and urine was sampled in consecutive 30-min periods during the next 4 h. Total body water content was kept constant during ZP120 or OPC31260 treatment by servo-controlled i.v. replacement of urinary losses with 50 mM glucose (Burgess et al., 1993; Hadrup et al., 2004). The mean arterial pressure was measured throughout the study, and arterial blood samples (0.3 ml) were drawn every hour. The plasma concentration of AVP (Kjaer et al., 1994) was measured in 1-ml blood samples drawn before the first control period and at the termination of the study. All blood samples were immediately replaced with heparinized blood from a donor rat (Jonassen et al., 1998, 2006b). At the end of the experiment, the rats were anesthetized with 4% (induction) followed by 2% (maintenance) isoflurane in \( N_2/O_2 \) (1:1). The right kidney was removed and divided into 1) the cortex/outer stripe of outer medulla (Ctx/OSOM), 2) the inner stripe of outer medulla (ISOM), and 3) the IM. The kidney zones were frozen in liquid nitrogen and stored at −80°C until processing for AQP2 mRNA and protein level measurements. The weight proportions of these zones were 79 ± 2% for Ctx/OSOM, 19 ± 2% for ISOM, and 3 ± 0.2% for IM (mean ± S.D., \( n = 6 \)). The left kidney was perfused in situ as described below, and the rats were euthanized.

Free water clearance (\( C_{\text{wq}} \)) was calculated as urine volume (V) – osmolar clearance (\( C_{\text{osm}} \)) in which \( C_{\text{osm}} = V \times U_{\text{osm}}/P_{\text{osm}} \), where \( U_{\text{osm}} = \) urine osmolality and \( P_{\text{osm}} = \) plasma osmolality. Lithium clearance (\( C_{\text{Li}} \)) was used as a marker for the output of isotonic fluid from the proximal tubules (Thomsen and Shirley, 1997). Fractional lithium clearance (\( F_{\text{Li}} \)) was calculated as \( C_{\text{Li}}/GFR \) and indicated the fraction of GFR excreted from the proximal tubules. Fractional distal water excretion was calculated as \( F_{\text{Li20}}\text{dist} = V/CL_{\text{i,d}} \), and the percentage of fluid delivery from the proximal tubules into the distal tubules excreted as urine. GFR was measured as the clearance of \( [\text{H}]\text{inulin} \) and urinary sodium excretion (\( U_{\text{Na}}V \)) was calculated as the urine concentration of sodium \( \times V \) and indicated...
the amount of sodium excreted per time unit. Fractional distal sodium excretion (\( FE_{Na\; distal} \)) is the fraction of distal sodium delivery, which is excreted. \( FP_{Na\; distal} \) is calculated as \( C_{Na}/C_{Li} \), in which \( C_{Na} = V \times U_{Na}/V_{Na} \), where \( U_{Na} \) is urine sodium concentration and \( P_{Na} \) is plasma sodium concentration.

Aquaretic Infusion without Volume Replacement. Permanent venous catheters were implanted during anesthesia with 4% (induction) followed by 2% (maintenance) isoflurane in \( N_2O/O_2 \) (1:1). One week later, the rats were accustomed to the experimental re-straining cages by two sessions of training. On the day of the experiment, the animals were randomized and placed in restraining cages. After 1 h of vehicle infusion (150 mM NaCl, 0.5 ml/h), the animals were infused i.v. with 0.5 ml/h ZP120 (1 nmol/kg/min), OPC31260 (32 nmol/kg/min), or vehicle for 1 h. The rats then were anesthetized by i.v. injection of pentobarbitone (40 mg/kg), and the kidneys were rapidly removed and divided into Ctx/OSOM, ISOM, and IM. The kidney zones were frozen in liquid nitrogen and stored at −80°C until processing for Western blotting and mRNA measurement.

AQP2 Protein Level Measurement. Kidney zones were homogenized in buffer containing 300 mM sucrose, 25 mM imidazole, 1 mM EDTA, 0.1 mg/ml Pefabloc, 4 μg/ml leupeptin, 184 μg/ml sodium orthovanadate, 1 mg/ml sodium fluoride, and 82 μg/ml okadaic acid (Sigma, St. Louis, MO). The protein concentration was assessed with BCA (Pierce Biotechnology, Rockford, IL) and adjusted to the same level. Sample buffer was added to a final concentration of 485 mM Tris HCl, 8.7% glycerol, 104 mM SDS, 20 mM dithiothreitol, and 0.9 mM bromphenol blue. The samples were then heated for 10 min at 60°C and stored at 4°C. Subsequently, the samples were heated for 6 min at 90°C and run on 12% polyacrylamide gels, and the proteins were then electrotransferred to polyvinylidene difluoride blotting membranes (Millipore Corporation, Bedford, MA) that were blocked 6 min at 90°C and run on 12% polyacrylamide gels, and the proteins were then electrotransferred to polyvinylidene difluoride blotting membranes (Millipore Corporation, Bedford, MA) that were blocked with 5% BSA (Pierce Biotechnology, Rockford, IL) and adjusted to the same level. Sample buffer was added to a final concentration of 485 mM Tris HCl, 8.7% glycerol, 104 mM SDS, 20 mM dithiothreitol, and 0.9 mM bromphenol blue. The samples were then heated for 10 min at 60°C and stored at 4°C. Subsequently, the samples were heated for 6 min at 90°C and run on 12% polyacrylamide gels, and the proteins were then electrotransferred to polyvinylidene difluoride blotting membranes (Millipore Corporation, Bedford, MA) that were blocked for 30 min in 0.5% Tween 20 in PBS-T (80 mM Na\(_2\)HPO\(_4\), 20 mM NaH\(_2\)PO\(_4\), 100 mM NaCl, 0.1% Tween 20, pH 7.5). The membranes were then washed and incubated overnight at 4°C with a C-terminal anti-AQP2 antibody at a concentration of 1:2000 (C17; Santa Cruz Biotechnologies, Santa Cruz, CA). This antibody recognizes a sharp band at 29 kDa, representing the nonglycosylated immature form of the protein, and a broad band at 35 to 50 kDa, representing mature glycosylated AQP2 (DiGiovanni et al., 1994). Subsequently, the membranes were washed in PBS-T and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody at a concentration of 1:3000 (Dako, Glostrup, Denmark). After final washing in PBS-T, the proteins were visualized with the ECL Plus chemiluminescence system (GE Healthcare) and quantitated by use of a Fluor S Max charge-coupled device camera and the Quantity One software (Bio-Rad, Hercules, CA).

AQP2 mRNA Measurement. For the measurement of the AQP2 mRNA level, we established a real-time PCR LightCycler method. We used specific AQP2 primers and a fluorescence-labeled and -specific AQP2 TaqMan probe. The probe was designed to span an intron to avoid measurement of genomic DNA, and the primers were designed accordingly: AQP2 probe, 5′-FAM-CCT GGG CCA CCT CCT TGG GAT CTA-TAMRA-3′ (Applied Biosystems, Foster City, CA); forward AQP2 primer, 5′-CCCTCTCCATTGTTTCCTGTGTT-3′; and reverse AQP2 primer, 5′-TGGATTCATGGAGCAACCG-3′. The primers were purchased as solids (TAG Copenhagen, Copenhagen, Denmark). The primer product was checked on an agarose gel by evaluating the size of the product. RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). The kidneys were dissected into zones, and the tissue was crushed in a mortar in liquid nitrogen. TRIzol then was added, and the RNA was isolated with chloroform and isopropyl alcohol according to the recommendations from the manufacturer. For RNA isolation from the IM, in which the tissue amount was limited, glycerogen (Invitrogen) was included as RNA carrier and DNA was sheared by two passages through a 26-gauge needle. The quality of the RNA isolation method was checked by running isolated RNA on a 1% agarose gel to monitor the integrity of the ribosomal RNA bands. The RNA was reverse-transcribed into cDNA with the GeneAmp RNA PCR kit (Applied Biosystems) using random hexamer primers. The RT-PCR reaction was run for 10 min at 25°C for the random hexamers to bind the RNA, followed by 30 min at 42°C for reverse transcription to occur and 5 min at 95°C for denaturation of the protein components. The cDNA was stored at −20°C until use. The real-time PCR LightCycler reaction was conducted with LightCycler-FastStart DNA master hybridization probes from Roche (Indianapolis, IN). The MgCl\(_2\) concentration, primer concentration, and probe concentration were optimized, and a standard curve was established. The level of the AQP2 cDNA was then measured in the Roche LightCycler using the following program: activation of the Taq polymerase at 95°C for 5 min, 45 cycles of 95°C for 5 s, and 60°C for 30 s followed by single fluorescence measurement and cooling at 40°C for 30 s. The AQP2 mRNA results were normalized to 18S ribosomal DNA. To verify the method, we measured the AQP2 mRNA level in water-deprived and water-loaded male rats. Five male Wistar rats weighing 250 g were water-deprived for 48 h, and another five male Wistar rats (250 g) were given water access to contain 300 mM sucrose. Subsequently, the animals were anesthetized with isoflurane, as described above, and the kidneys were taken out and frozen in liquid nitrogen. Liver tissue was used as a negative AQP2 mRNA control, and AQP2 mRNA was not detectable in this organ with the present method.

Immunohistochemistry. The left kidney was perfused in situ through the aorta bifurcation with 3% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Slices of the perfused kidneys were paraffin-embedded, and two micrometer-thick sections were cut with a microtome. The sections were deparaffinized and blocked in 1% bovine serum albumin, 0.2% gelatin, and 0.05% saponin in PBS; washed in 0.1% bovine serum albumin, 0.2% gelatin, and 0.05% saponin in PBS; and incubated overnight at 4°C with a rabbit polyclonal anti-AQP2 antibody (LL-127; Sørøe Nielsen, The Water and Salt Research Center, University of Aarhus, Aarhus, Denmark) at a concentration of 1:10,000 or AN244 polyclonal rabbit anti-serine 256-phosphorylated AQP2 antibody (Sørøe Nielsen) (also 1:10,000). The sections were then washed again and incubated with horseradish peroxidase-conjugated anti-goat or anti-rabbit IgG (Dako) for 60 min at room temperature. Diaminobenzidine was added to mark the localization of horseradish peroxidase antibodies. The sections were then rinsed with PBS, dehydrated, and mounted in Eukitt mounting medium (Bie and Berntsen, Copenhagen, Denmark). For evaluation of whether AQP2 displayed apical localization, we made the following comparisons: 1) vehicle versus ZP120 and 2) vehicle versus OPC31260. The sections were blinded by an independent observer and then, by microscopic investigation, divided into two groups: A) a group with predominantly apical AQP2 labeling or B) a group with predominantly nonapical labeling. The blinding was broken, and it was statistically evaluated whether the localization corresponded to the treatment. The different kidney zones were examined separately (Ctx, OM, and IM).

Statistical Analysis. In the renal clearance studies, two-way analysis of variance (ANOVA) was used to assess differences among infusion groups with regard to diuresis, aquaresis, and fractional distal water excretion graphs. In the Western blotting and mRNA experiments, data were analyzed by unpaired two-tailed t test. Fisher’s exact test was applied to the immunohistochemical data. Data are presented as mean ± S.D., with P values less than 0.05 considered significant, except when more than one comparison was made through ANOVA. In these instances, Bonferroni’s corrected P values were used. The statistical package used was Prism (GraphPad Software Inc., San Diego, CA).

Results
Infusion of ZP120 (1 nmol/kg/min) or OPC31260 (32 nmol/kg/min) with replacement of urinary volume losses produced, as previously shown (Hadrup et al., 2004; Jonassen et al., 2004).
2000a,b), marked increases in diuresis (ZP120 ΔV: 34 ± 16 μl/min/100 g b.wt.; and OPC31260 ΔV: 55 ± 21 μl/min/100 g b.wt., where Δ is the mean value of the 60-min control period subtracted from the mean value of the last 90 min of the experiment, ± S.D.) (Fig. 1A) and marked increases in aquaresis measured as free water clearance (ZP120 ΔC\text{H2O}F: 41 ± 16 μl/min/100 g b.wt.; and OPC31260 ΔC\text{H2O}F: 57 ± 17 μl/min/100 g b.wt., ± S.D.) (Fig. 1B). The fractional distal water excretion can be estimated from the ratio between the diuresis and the lithium clearance (V/CLi). Both OPC31260 and ZP120 increased V/CLi to ~40% (OPC31260: 40.4 ± 7.0% and ZP120: 39.2 ± 9.0%, mean ± S.D.) showing that V2-receptor blockade and NOP stimulation produced a comparable blockade on distal water reabsorption (Fig. 1C). Mean arterial pressure was unchanged in all groups throughout the study (Table 1). In contrast to previous studies conducted with the same setup (Hadrup et al., 2004; Jonassen et al., 2000a,b), ZP120 and OPC31260 infusion coincided with a reduction in GFR (Table 1). We note that, although no protocol or equipment abnormalities were detected and steady-state diuresis was obtained, minute volume depletion could explain the GFR effects. F\text{E}_{\text{Li}} was not different when comparing ZP120 with control rats. However, we measured a significant difference when comparing OPC31260 (24 ± 9%, mean last 1½ h, ± S.D.; Table 1) with ZP120 (16 ± 3%) or control (17 ± 4%), indicating that the fractional proximal sodium reabsorption was decreased by OPC31260. With regard to U\text{Na}, C\text{Na}, and F\text{E}_{\text{Na} \text{ distal}, ZP120 displayed antinatriuresis compared with control and OPC31260 (Fig. 2). Neither OPC31260 nor ZP120 treatment induced significant changes in the circulating concentrations of AVP (Table 1), and in line with previously reported data (Hadrup et al., 2004), we found no correlation between changes in diuresis and changes in the AVP plasma concentration (correlation coefficients, ZP120: −0.06, n.s.; OPC31260: −0.5, not significant).

We have previously shown that ZP120-induced aquaresis is associated with a decreased AQP2 protein level in whole-kidney preparations. In the present study, we investigated the zonal localization of this down-regulation compared with the V2-receptor antagonist OPC31260. ZP120-induced steady-state aquaresis in the volume replacement model was associated with a decreased AQP2 protein level in the Ctx/OSOM and an unchanged level in the ISOM and the IM (Fig. 3A). The down-regulation of AQP2 in the Ctx/OSOM was also present after 1 h of infusion without volume replacement. The AQP2 protein level was unchanged in the ISOM, whereas the AQP2 protein level within the IM was increased without volume replacement (Fig. 4A). There were no differences in the effects of ZP120C on glycosylated and nonglycosylated AQP2 in the Ctx/OSOM, respectively (statistics not included). In the steady-state animal model with volume replacement, OPC31260-induced aquaresis was associated with a down-regulated AQP2 protein level in both the Ctx/OSOM and ISOM and an unchanged level in the IM (Fig. 3B). In contrast, OPC31260 infusion in the 1-h model without volume replacement was associated with a slight up-regulation of the AQP2 protein level in the Ctx/OSOM (Fig. 4B).

Water is reabsorbed across the CD epithelium only when AQP2 is located in the apical principal cell membrane. We investigated whether ZP120- and OPC31260-induced aquaresis was associated with a decreased apical localization of AQP2 in different kidney zones. At the end of the steady-state study with volume replacement, the left kidney was perfusion-fixed in vivo and removed for AQP2 immunohistochemistry. ZP120-induced aquaresis was associated with a decreased apical AQP2 labeling in Ctx (P = 0.002) (Fig. 5). ZP120 displayed no significant decrease in apical AQP2 localization in the OM (P = 0.24), whereas there was a strong tendency to decreased apical labeling in the IM (P = 0.06). With regard to serine 256-phosphorylated AQP2, the P values for decreased apical labeling were 0.24 for Ctx, 1.0 for OM, and 0.24 for IM (pictures not shown). OPC31260-induced aquaresis was associated with a significantly decreased apical labeling in Ctx (P = 0.002) and OM (P = 0.002), whereas decreased apical labeling was only near-significant in the IM (P = 0.08) (Fig. 5). With regard to serine 256 phosphorylation, there were significant decreases in apical labeling by OPC31260 in all kidney zones (pictures not shown).

Fig. 1. ZP120 and OPC31260 induce diuresis and aquaresis by a distal mechanism. After a 60-min control period, ZP120 or OPC31260 was infused for 4 h in a computer-controlled servo model in which ZP120- and OPC31260-induced urine losses were replaced with 50 mM glucose to avoid physiological antagonism. , time control; ■, ZP120 (1 mmol/kg/min); ▼, OPC31260 (32 mmol/kg/min). A, diuresis (V). B, aquaresis expressed as C\text{H2O}F. C, fractional distal water excretion (V/CLi). Control, n = 5; OPC31260, n = 6; ZP120, n = 6. Data represent means ± S.D. #, P < 0.025 (Bonferroni’s corrected P value) compared to time control vehicle (§, compared to OPC31260) by two-way ANOVA.
After a 60-min control period, ZP120 or OPC31260 was infused for 4 h in a computer-controlled servo model in which ZP120- and OPC31260-induced urine losses were replaced with 50 mM glucose to avoid physiological antagonism. With regard to MAP, GFR, \(C_{\text{Li}}\), and \(\text{FE}_{\text{Li}}\), baseline values are mean values for the first two 30-min periods, and vehicle/compound values are mean values for the last three 30-min periods. AVP was measured in blood samples drawn just before the first control period and just before the termination of the study. Control, \(n = 5\); OPC31260, \(n = 6\); ZP120, \(n = 6\). Data represent mean ± S.D.

**TABLE 1**

<table>
<thead>
<tr>
<th>Time Control</th>
<th>Vehicle</th>
<th>OPC31260</th>
<th>ZP120</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>110 ± 10</td>
<td>109 ± 15</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>GFR (µl/min/100g b.w.)</td>
<td>1005 ± 223</td>
<td>1049 ± 222</td>
<td>1006 ± 304</td>
</tr>
<tr>
<td>(C_{\text{Li}}) (µl/min/100g b.w.)</td>
<td>174 ± 61</td>
<td>191 ± 69</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>(\text{FE}_{\text{Li}}) (%)</td>
<td>18 ± 6</td>
<td>22 ± 7</td>
<td>21 ± 3</td>
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<tr>
<td>AVP (pg/ml)</td>
<td>0.5 ± 0.2</td>
<td>1.3 ± 0.9</td>
<td>0.6 ± 0.3</td>
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</table>

MAP, mean arterial pressure; GFR, glomerular filtration rate; \(C_{\text{Li}}\), lithium clearance; \(\text{FE}_{\text{Li}}\), fractional lithium excretion; AVP, vasopressin plasma concentration.

\(P < 0.025\) (Bonferroni’s corrected \(P\) value) compared to control \(^\dagger\); \(P < 0.025\) compared to OPC31260 by two-way ANOVA.

We investigated whether the decreased AQP2 protein levels in the CDs of ZP120- and OPC31260-infused rats were associated with a decreased AQP2 mRNA level, which would suggest that down-regulation happened through decreased production rather than increased degradation. We established a real-time PCR method using specific AQP2 primers and a specific AQP2 TaqMan probe to measure the AQP2 mRNA level. We confirmed the method by showing that we could measure an increased AQP2 mRNA level in water-deprived rats compared with water-loaded rats (Fig. 6A). ZP120-induced diuresis was not associated with down-regulation of the AQP2 mRNA level in any of the kidney zones (Fig. 6, B and C). Actually, the AQP2 mRNA level was up-regulated in the outer medulla after infusion of ZP120 for 1 h without volume replacement. With OPC31260, no changes were found in the Ctx/OSOM and ISOM; whereas a significant reduction of AQP2 mRNA was found in the IM in the 1-h infusion model without volume replacement (Fig. 6, B and C).

**Discussion**

The fact that we solely found ZP120-induced decreases in AQP2 protein level in the Ctx/OSOM is consistent with previous results showing down-regulation when measured in whole-kidney homogenates (Hadrup et al., 2004). The Ctx/OSOM constitutes 80% of the rat kidney, and in addition to CDs, AQP2 is also expressed in connecting tubules in the Ctx. Moreover, the down-regulation was seen both with and without volume replacement, suggesting a persistent effect not counteracted by volume loss. A decreased protein level means lower availability of water channels for osmotic water reabsorption. However, substantial water reabsorption through AQP2 in the apical membrane may still occur if remaining water channels are being targeted to this site. Therefore, we investigated apical AQP2 localization by immunohistochemistry. We found that the AQP2 protein down-regulation was accompanied by a decreased apical AQP2 localization in the Ctx, indicating that the remaining AQP2 is not involved in such sustained water reabsorption. There was also a strong tendency to a decreased apical AQP2 labeling by ZP120 in the IM. However, in this zone, the AQP2 level was not decreased accordingly and was even up-regulated when ZP120 was infused without volume replacement. This suggests that, in the IM, a decreased protein level is not a prerequisite to decreased water reabsorption, which then is mediated solely by retrieval of AQP2 from the membrane.
We then addressed whether the mechanism underlying the decreased AQP2 protein level in the Ctx/OSOM involved decreased production or increased degradation. We measured mRNA to determine whether this regulated step in production was down-regulated. However, we did not find the AQP2 mRNA level to be decreased upon ZP120 infusion, indicating that the AQP2 protein level is down-regulated by increased degradation or urinary excretion rather than by decreased production.

In the ISOM, there was no effect of ZP120 with regard to AQP2 protein level.

**Fig. 4.** The effect of ZP120 and OPC31260 infusion for 1 h without volume replacement on the AQP2 protein level. The protein level of AQP2 was measured in Ctx/OSOM, ISOM, and IM using a polyclonal anti-AQP2 C-terminal antibody. A, control versus ZP120 (1 nmol/kg/min). B, control versus OPC31260 (32 mmol/kg/min) (in the IM, one OPC31260 sample could not be measured because of tissue loss). Data represent means ± S.D., *P < 0.05 by unpaired t test.
decreases in protein level or apical localization, suggesting that this zone is not involved in the aquaretic effect of this compound.

Effects of V2-receptor antagonism on AQP2 regulation have been extensively investigated in the IM; our results on this zone are in line with those previously reported by others (Hayashi et al., 1994; Christensen et al., 1998; Marples et al., 1998). With regard to the Ctx and OM, it has been reported that AQP2 was diffusely stained in CD by V2-receptor antagonism following OPC31260 infusion (Hayashi et al., 1996). Echevarria and colleagues (Murillo-Carretero et al., 1999) found no effect of V2-receptor antagonism when investigating

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Fig. 5. The effect of ZP120 and OPC31260 on AQP2 localization. At the termination of the aquaretic infusion study with volume replacement, the left kidney was removed for immunohistochemistry. AQP2 was labeled with a polyclonal anti-AQP2 antibody and visualized with a secondary anti-goat horseradish peroxidase antibody reacted with diaminobenzidine to produce a brown color. Pictures were acquired at a magnification of 63×. a to c, AQP2 localization in the Ctx. AQP2 was located apically in control rats (arrows, a) and nonapically in OPC31260- (arrowheads, b) and ZP120-treated rats (arrowheads, c). d to f, AQP2 localization in the OM. AQP2 was located apically in control rats (arrows, d) and nonapically in OPC31260-treated rats (arrowheads, e). In ZP120-treated rats, no difference was encountered in comparison with control rats (arrows mark cells with apical labeling, f). g to i, AQP2 localization in the IM. AQP2 was located apically in control rats (arrows, g) and nonapically in OPC31260- (arrowheads, h) and ZP120-treated rats (arrowheads, i).

Fig. 6. AQP2 mRNA measurements. The AQP2 mRNA level was measured by real-time PCR and normalized to 18S ribosomal RNA. A, AQP2 mRNA control study. Rats water-loaded (by access to 300 mM sucrose in the drinking water) for 48 h were compared with rats water-deprived for 48 h (n = 5 in each group). B, the AQP2 mRNA level in the volume replacement study (n = 5–6 in each group). C, the AQP2 mRNA level without volume replacement (n = 6–7 in each group). Data represent means ± S.D. *, P < 0.05.
mRNA level in Ctx and medulla in water-deprived rats with increased plasma concentrations of AVP. In rats with experimentally induced congestive heart failure and increased plasma AVP plasma concentration, Xu et al. (1997) showed a decreased mRNA level in Ctx and decreased AQP2 protein level in the Ctx membrane fraction. In the present study, we employed a volume replacement model to investigate AQP2 down-regulation during steady-state diuresis induced by V₂-receptor antagonism. We found a decreased AQP2 protein level in both the Ctx/OSOM and ISOM. The apical labeling of AQP2 was down-regulated throughout the Ctx and OM and, to a strong extent, IM. In addition, supporting these data, serine 256-phosphorylated AQP2 was significantly down-regulated throughout all kidney zones by OPC31260 (data not shown). In contrast to rats receiving volume replacement and in contrast to infusion with ZP120, we did not find a decreased AQP2 protein level in animals infused with OPC31260 for 1 h without volume replacement. This suggests that the effects of V₂-receptor antagonism on AQP2 were masked by physiological antagonism induced by volume loss and/or that OPC31260 has a slower onset of action with regard to protein down-regulation compared with ZP120 treatment. With regard to mRNA, we only found a decrease in IM, suggesting that the down-regulatory effect on AQP2 in the Ctx/OSOM and ISOM did not happen through decreased production in the Ctx and OM but rather through increased degradation or increased urinary excretion. In IM, we found a decreased mRNA level but not a decreased protein level. This is in contrast to findings by Marples et al. (1998), and the absence of a decrease in AQP2 protein level to accompany the decreased mRNA level can be explained by the shorter V₂-receptor antagonist treatment period (1 h) compared with the employed 60-h period. This finding suggests that a decrease in AQP2 mRNA level does not rapidly result in a decreased protein level, perhaps due to low AQP2 turnover in this zone.

We previously mapped NOP in the CD by immunohistochemistry and Western blotting and found it in intercalated cells in the Ctx and OM with regard to principal cells only in the IM (Hadrup et al., 2004). This caused a discrepancy between the AQP2 down-regulation pattern and the NOP expression pattern with regard to the Ctx/OSOM, because AQP2 is only found in principal cells. Speculative explanations for this discrepancy could be multiple NOP isoforms or indirect signaling via paracrine or endocrine factors. This raises the question of whether i.v. ZP120 actually displays its effect on water homeostasis by decreasing the AVP plasma concentration, either through receptors on the blood side of the blood-brain barrier or by traversing into areas of AVP secretion control. We did not find a correlation between the urine flow rate and the AVP plasma concentration during ZP120 infusion, but note that it is difficult to measure changes in AVP plasma concentration in normal hydrated rats due to basal levels being close to detection limits. However, we found the pattern of AQP2 down-regulation by V₂-receptor blockade in the kidney to be different from the pattern induced by NOP stimulation, adding circumstantial evidence that peripheral NOP stimulation, in addition to a possible central component, also has an AVP-independent component.

With regard to sodium handling, ZP120 displays an antinatriuretic effect compared with control and OPC31260 (Fig. 2). To determine tubular localization of this effect, we measured sodium handling in the proximal (FE\textsubscript{Na}\text{distal}) and distal tubules (FE\textsubscript{Na}\text{distal}). We found that the proximal sodium excretion was different between the two compounds. This difference was mediated through a slight increase by OPC31260 combined with a slight decrease by ZP120. However, only OPC31260 was significantly different from the control group, indicating that this compound by increasing fractional proximal excretion was responsible for the difference. ZP120 decreased FE\textsubscript{Na}\text{distal}, indicating that the antinatriuretic effect of this compound was mediated via the distal part of the nephron or in the CD.

It makes sense that the antinatriuretic effect of NOP stimulation comprises combined sodium and water channel regulation in the distal tubules. When sodium reabsorption is increased by NOP stimulation, there will be an increased osmotic driving force for water reabsorption through AQP2 localized in the apical membrane. Therefore, unless AQP2 is down-regulated, the antinatriuretic effect would potentially be converted into a combined sodium and water-retaining effect. The fact that AQP2 is down-regulated in the Ctx/OSOM indicates involvement of sodium transporters, such as the Na\textsuperscript{+}/Cl\textsuperscript{−} cotransporter and/or the epithelial sodium channel both located in this zone. It is noticeable that the endogenous V₂-receptor ligand AVP and the endogenous NOP ligand nociceptin only counteract each other with regard to water homeostasis. In relation to sodium homeostasis, both ligands increase reabsorption. AVP has been described to increase Na\textsuperscript{+}/K\textsuperscript{+}/Cl\textsuperscript{−} cotransporter and epithelial sodium channel protein levels (Ecelbarger et al., 2001; Giménez and Forbush, 2003).

From a pharmacological point of view, ZP120 and OPC31260 have somewhat different profiles. The slightly larger aquarexia produced by OPC31260 in comparison with ZP120 (Fig. 1B) could be attributed to an effect on all three kidney zones compared with the more selective effect of ZP120. This indicates that OPC31260, at the dose used (32 nmol/kg/min), has a stronger aquaretic effect than ZP120 (1 nmol/kg/min). However, in contrast to OPC31260, the effect of ZP120 on AQP2 protein level down-regulation was not masked by volume loss in the 1-h infusion model, suggesting an effect of ZP120 more persistent to physiological antagonism. Moreover, for potential treatment of hyponatremia, the antinatriuretic effect of ZP120 may be an advantage to quickly restore the sodium plasma concentration.

We conclude that equi-aquaretic doses of the peripherally acting NOP agonist ZP120 and the selective AVP type-2 receptor antagonist OPC31260 produce differential renal regional effects on AQP2 down-regulation, suggesting that these compounds employ different signaling pathways. Future investigations on NOP-mediated renal effects should be focused at the Ctx/OSOM and the IM, whereas V₂-receptor antagonism acts in all kidney zones.

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References


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