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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Running Title Page

a) Running title: NOP and $V_2$-receptor mediated regional AQP2 down-regulation

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d) AQP2  aquaporin-2,
AVP   Arginine vasopressin
CD    Collecting duct
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e) Recommended section assignment: Renal
Abstract

We previously showed that aquarestis induced by the peripherally acting nociceptin/orphanin FQ receptor agonist ZP120 is associated with a decreased protein level of aquaporin-2 in whole kidney homogenates. We now examined the effects of ZP120 (1 nmol/kg/min i.v. for 4 hours) on renal regional expression (cortex/outer stripe of outer medulla, inner stripe of outer medulla, and inner medulla) and sub-cellular localization of aquaporin-2. Responses to ZP120 were compared to the effects of an equi-aquaretic dose (~40% inhibition of distal water reabsorption) of the vasopressin type-2 receptor antagonist OPC31260 (32 nmol/kg/min). ZP120 decreased the aquaporin-2 protein level in the rat cortex/outer stripe of outer medulla; and decreased apical plasma membrane localization of aquaporin-2 in the cortex (P=0.002) and in the inner medulla (P=0.06). These effects were not accompanied by a decrease in the aquaporin-2 mRNA level. OPC31260-induced aquarestis was associated with a decreased aquaporin-2 protein level in both the cortex/outer stripe of outer medulla and in the inner stripe of outer medulla. Apical localization of aquaporin-2 was decreased throughout all kidney zones and OPC31260 decreased 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.
Introduction

During normal conditions the kidneys regulate renal water excretion to compensate for variations in water intake and extra-renal 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 (PKA) serine-256 phosphorylation of aquaporin-2 (AQP2), inducing trafficking from intracellular vesicles to the apical membrane (Deen, et al., 2000). On long term, PKA 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 over-activity of the AVP-AQP2 system leads to excessive water retention that in the end may lead to life threatening hyponatremia. For the treatment of this, it is desirable to develop compounds that increase free water clearance (i.e. produce aquaresis).

Recently the combined V1A/V2-receptor antagonist Conivaptan was registered for treatment of congestive heart failure, representing the aquaretic 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 aquaresis when infused either intracerebroventricularly (i.c.v.) or intravenously (i.v.) (Kapusta, et al., 1997). It has been shown that nociceptin infused i.c.v. decreases the AVP plasma concentration, thus proposing an aquaretic mechanism mediated via the CNS (Kakiya, et al., 2000). To address the mechanism for aquaresis produced by peripherally infused nociceptin, we recently published data obtained with the stable NOP agonist ZP120 (Hadrup, et al., 2004). ZP120 produces NOP mediated CNS effects on pain and locomotor activity when injected i.c.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 aquaretic 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 co-localized 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. Additionally in the present study, we also compared the effect of peripheral NOP stimulation with that of V2-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; Kapusta, et al., 2005). We therefore wanted further 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 V₂-receptor antagonist OPC31260 were infused i.v. for four hours in doses that produced similar increases in fractional distal water excretion (V/CLi). In parallel with this, we employed a one hour infusion model without volume replacement enabling investigation of AQP2 regulation during onset of aquareisis with concomitant volume loss and physiological antagonism.
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 ºC) and moisture (40-70%) controlled room with a 12 hour light-dark cycle (light on from 6:00 A.M. to 6:00 P.M.). The rats were given free access to tap water and a diet containing ~140 mmol/kg of 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 N₂O/O₂ 1:1. The rats were then allowed to recover for one week (to relieve post-operative pain buprenorphine (0.05 mg/kg) was administrated s.c. twice daily for two 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, 6.3 mM NaCl, 2.6 mM LiCl, and tracer amounts of ^3[H]-inulin (Amersham, Buckinghamshire, UK) at 2 ml/hour. The arterial catheter was kept open by infusion of 150 mM glucose containing 10 international units/ml heparin at 0.5 ml/hour. After a 90 min equilibration period, urine was collected during two 30 min control periods. Then i.v. infusion of ZP120 (Ac-RYYRWKKKKKKK-NH₂) (1 nmol/kg/min) or OPC31260 (5-dimethylamine-1-[4-(2-methylbenzoylamino)benzoyl]-2,3,4,5-tetrahydro-1H-benzapine)
(32 nmol/kg/min) was started and urine was sampled in consecutive 30 min periods during the following four hours. Total body water content was kept constant during ZP120 or OPC31260 treatment by servo-controlled i.v. replacement of urine 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; Jonassen, et al., 2000b). At the end of the experiment the rats were anaesthetized with 4% (induction) followed by 2% (maintenance) isoflurane in N₂O/O₂ 1:1. The right kidney was removed and divided into 1) cortex/outer stripe of outer medulla (Ctx/OSOM), 2) inner stripe of outer medulla (ISOM) and 3) inner medulla (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 Ctx/OSOM: 79±2%, ISOM: 19±2%, and IM: 3±0.2% (mean ± SD, n=6). The left kidney was in situ perfused as described below, and the rats were euthanized.

Free water clearance. \( (C_{H₂O}) \) was calculated as urine volume (\( V \)) – osmolar clearance (\( C_{osm} \)) in which \( C_{osm} = V \ast U_{osm}/P_{osm} \) (\( U_{osm} \) = urine osmolarity; \( P_{osm} \) = plasma osmolarity). Lithium clearance \( (C_{Li}) \) was used as a marker for the output of isotonic fluid from the proximal tubules (Thomsen and Shirley, 1997). Fractional lithium clearance \( (FE_{Li}) \) was calculated as \( C_{Li}/GFR \) and indicates the fraction of GFR being excreted from the proximal tubules. Fractional distal water excretion \( (FE_{H₂O \ dist}) \) was calculated as \( FE_{H₂O \ dist} = \)
V/CLi and indicates the percentage of fluid delivery from the proximal tubules into the distal tubules being excreted as urine. GFR was measured as the clearance of $^3$H-inulin, and urinary sodium excretion ($U_{Na}V$) was calculated as the urine concentration of sodium * V and indicates the amount of sodium excreted per time unit. Fractional distal sodium excretion ($FE_{Na\text{ distal}}$) is the fraction of distal sodium delivery which is excreted. $FE_{Na\text{ distal}}$ is calculated as: $C_{Na}/C_{Li}$. In which: can = ($V \times U_{Na}$) / $P_{Na}$, ($U_{Na}$ = urine sodium concentration; $P_{Na}$ = 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$_2$O/O$_2$ 1:1. One week later the rats were accustomed to the experimental restraining cages by two sessions of training. On the day of the experiment, the animals were randomized and placed in restraining cages. After one hour of vehicle infusion (150 mM NaCl, 0.5 ml/hour) the animals were infused i.v. with 0.5 ml/hour ZP120 (1 nmol/kg/min), OPC31260 (32 nmol/kg/min) or vehicle for one hour. Then the rats were anaesthetized by i.v. injection of pentobarbitone (40 mg/kg), 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 ortho-vanadate, 1 mg/ml sodium fluoride and 82 µg/ml okadeic acid (Sigma, St Louis, USA). The protein concentration was assessed with Pierce BCA (Pierce Biotechnology, Rockford, IL, USA) 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 DTT and 0.9 mM bromephenol 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 electro-transferred to polyvinylidene difluoride blotting membranes (Millipore Corporation, Bedford, MA, USA) that were blocked for 30 min in 0.5% Tween-20 in PBS-T (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 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, CA). This antibody recognizes a sharp band at 29 kDa representing the nonglycosylated immature form of the protein and a broad band at 35-50 kDa representing mature glycosylated AQP2 (DiGiovanni, et al., 1994). Subsequently, the membranes were washed in PBS-T, and incubated for one hour 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 (Amersham, Buckinghamshire, UK) 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 specific AQP2 TaqMan probe. The probe was designed to span an intron to avoid measurement of genomic DNA and the primers were designed according to this. AQP2 probe: 5’-FAM-CCT GGG CCA CCT CCT TGG GAT CTA –TAMRA-3’ (Applied Biosystems, Foster City, CA). Forward AQP2 primer:
CCCTCTCCATTGGTTTCTCTGTT, Reverse AQP2 primer: TGGATTCCATGGAGCAACCG. 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 (Gibco BRL, Gaitherburg, MD). The kidneys were dissected into zones and the tissue was crushed in a mortar in liquid nitrogen, then TRIzol 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 is limited, glycogen (Invitrogen, Carlsbad, CA) was included as RNA carrier and DNA was sheared by two passes 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 reversely transcribed into cDNA with the Geneamp RNA PCR Kit (Applied Biosystems, Foster City, CA) 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 (Roche, Indianapolis, IN). The MgCl₂ 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 (Roche, Indianapolis, IN) using the following lightcycler program: Activation of the TAQ polymerase 95 °C for five min, 45 cycles of: 95 °C for 0 s, 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 RNA. 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 hours and another five male Wistar rats (250 g) were given access to water containing 300 mM sucrose. Subsequently, the animals were anaesthetized 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 *in situ* perfused 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 thin sections were cut with a microtome. The sections were deparaffinised, and blocked in 1% BSA, 0.2% gelatin, 0.05% saponin in PBS, washed in 0.1% BSA, 0.2% gelatin, 0.05% saponin in PBS and incubated overnight at 4°C with a rabbit polyclonal anti-AQP2 antibody (LL-127, Søren Nielsen, The Water and Salt Research Center, University of Aarhus, Denmark) at a concentration of 1:10,000 or AN244 polyclonal rabbit anti-serine-256 phosphorylated AQP2 antibody (Søren Nielsen) (also 1:10,000). The sections were then washed again and incubated with horse radish peroxidase conjugated anti-goat or anti rabbit IgG (Dako, Glostrup, Denmark) for 60 min at room temperature. Diaminobenzidine was added to mark the localization of horse radish 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 vs*
ZP120; and 2) Vehicle vs OPC31260. The sections were blinded by an independent observer and then, by microscopical investigation, divided into two groups: A) A group with predominantly apical AQP2 labeling; or B) A group with predominantly non-apical labeling. Then 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 in 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 ± standard deviation (SD), with \( P \)-values less than 0.05 considered significant, except when more than one comparison were done ANOVA. In these instances, Bonferroni corrected \( P \)-values were used. The statistical package used was Graph Pad Prism by GraphPad Software (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., 2000b; Jonassen, et al., 2000a), marked increases in diuresis (ZP120 $\Delta V$: 34 ± 16 $\mu$l/min/100 g b.w. and OPC31260 $\Delta V$: 55 ± 21 $\mu$l/min/100 g b.w., where $\Delta$ is the mean value of the 60 min control period subtracted from the mean value of the last 90 min of the experiment, ± SD)(Fig.1A), and marked increases in aquareesis measured as free water clearance (ZP120 $\Delta C_{H2O}$: 41±16 $\mu$l/min/100 g b.w. and OPC31260 $\Delta C_{H2O}$: 57±17 $\mu$l/min/100 g b.w., ± SD) (Fig.1B). The fractional distal water excretion can be estimated from the ratio between the diuresis and the lithium clearance ($V/C_{Li}$). Both OPC31260 and ZP120 increased $V/C_{Li}$ to ~40% (OPC31260: 40.4 ± 7.0% and ZP120: 39.2 ± 9.0%, mean ± SD) showing that $V_2$-receptor blockade and NOP stimulation produced a comparable blockade on distal water reabsorption (Fig. 1C). MAP 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., 2000b; Jonassen, et al., 2000a), 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. $FE_{Li}$ was not different when comparing ZP120 with control rats. However, we measured a significant difference when comparing OPC31260 (24±9%, mean last 1½ hours, ± SD, Table 1) with ZP120 (16±3%) or control (17±4%) indicating that the fractional proximal sodium reabsorption was decreased by OPC31260. In regard to $U_{Na}V$, $C_{Na}$ and $FE_{Na}$ distal, ZP120 displayed antinatriuresis when 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, n.s.).

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 in comparison 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 one hour 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 non-glycosylated 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 one hour 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 \( (P=0.06) \). In regard to serine-256 phosphorylated AQP2, the \( P \) values for decreased apical labeling were Ctx: 0.24; OM 1.0 and IM: 0.24 (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). In regard to serine-256 phosphorylation, there were significant decreases in apical labeling by OPC31260 in all kidney zones (pictures not shown).

We investigated whether the decreased AQP2 protein levels in the CD’s 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. 6B, C). Actually the AQP2 mRNA level was up-regulated in the outer medulla after infusion of ZP120 for one hour 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 one hour infusion model without volume replacement (Fig. 6B, 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 CD’s, 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 is then solely mediated 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 in regard to 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; and our results on this zone are in line with those previously reported by others (Christensen, et al., 1998; Hayashi, et al., 1994; Marples, et al., 1998). In 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 M, et al., 1996). Echevarria et al found no effect of V₂-receptor antagonism when investigating mRNA level in Ctx and medulla in water deprived rats with increased plasma concentrations of AVP (Murillo-Carretero, et al., 1999). In rats with experimentally induced congestive heart failure and increased plasma AVP plasma concentration, Xu and coworkers showed a decreased mRNA level in Ctx, and decreased AQP2 protein level in the Ctx membrane fraction (Xu, et al., 1997). 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 one hour 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 in regard to protein down-regulation, compared to ZP120 treatment. In 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. 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 (one hour) compared with the employed 60 hour period (Marples, et al., 1998). 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 and in regard to principal cells only in the IM (Hadrup, et al., 2004). This causes a discrepancy between the AQP2 down-regulation pattern and the NOP expression pattern in regard to the Ctx/OSOM, because AQP2 is only found in principal cells. Speculative explanations for this are: 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.

In regard to sodium handling, ZP120 displays an antinatriuretic effect compared to control and OPC31260 (Fig. 2). To determine tubular localization of this effect, we measured sodium handling in the proximal (FE_Li) and distal tubules (FE_Na_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 OPC3160 was significantly different from the control group, indicating that this compound by increasing fractional proximal excretion was responsible for the difference. ZP120 decreased FE_Na_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 is comprised of 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⁺/Cl⁻-co-
transporter 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 in regard to water homeostasis. In relation to sodium homeostasis both ligands increase reabsorption. AVP has been described to increase Na+-K+-2Cl co-transporter and epithelial sodium channel protein levels (Ecelbarger, et al., 2001; Gimenez and Forbush, 2003).

From a pharmacological point of view ZP120 and OPC31260 have somewhat different profiles. The slightly larger aquarexis produced by OPC31260 in comparison with ZP120 (fig 1B) could be attributed to an effect on all three kidney zones compared to 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 one hour infusion model suggesting an effect of ZP120 more persistent to physiological antagonism. Moreover, for potential treatment of hyponatremia, the antinatriuretic 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.
Acknowledgments

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References


Footnotes


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Legends for Figures

Fig. 1. ZP120 and OPC31260 induce diuresis and aquareasis by a distal mechanism.
After a 60 min control period, ZP120 or OPC31260 were infused for four hours 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 nmol/kg/min); ■, OPC31260 (32 mmol/kg/min). A) Diuresis (V). B) Aquareasis expressed as free water clearance (C\textsubscript{H2O}). C) Fractional distal water excretion (V/CL\textsubscript{I}). Control n=5, OPC31260 n=6, ZP120 n=6. Data represent means, bars are SD. #P < 0.025 (Bonferroni corrected P-value) compared to time control vehicle (§ compared to OPC31260) by two-way ANOVA.

Fig. 2. Effects of ZP120 and OPC31260 infusion on sodium handling. After a 60 min control period, ZP120 or OPC31260 were infused for four hours 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 nmol/kg/min); ■, OPC31260 (32 mmol/kg/min). A) Urinary sodium excretion (U\textsubscript{Na}V). B) Sodium clearance (C\textsubscript{Na}). C) Fractional distal sodium excretion (FE\textsubscript{Na \textsubscript{distal}}). Control n=5, OPC31260 n=6, ZP120 n=6. Data represent means, bars are SD. #P < 0.025 (Bonferroni corrected P-value) ZP120 compared to time control vehicle (§ ZP120 compared to OPC31260) by two-way ANOVA.

Fig. 3. The effect of ZP120 and OPC31260 infusion with 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 vs. ZP120 (1 nmol/kg/min) B) Control vs. OPC31260 (32 mmol/kg/min) (in the IM one OPC31260 sample could not be measured because of tissue loss). Data represent means, bars are SD, *P<0.05 by unpaired t-test.

**Fig. 4. The effect of ZP120 and OPC31260 infusion for one hour without volume replacement on the AQP2 protein level.** The protein level of AQP2 was measured in kidney zones using a polyclonal anti-AQP2 antibody. A) The effect of ZP120 (1 nmol/kg/min) in Ctx/OSOM, ISOM and IM. B) The effect of OPC31260 (32 mmol/kg/min) in Ctx/OSOM, ISOM and IM. Data represent means, bars are SD, *P<0.05 by unpaired t-test.

**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 horse radish peroxidase antibody reacted with diaminobenzidine to produce a brown color. Pictures were acquired at a magnification of 63 times. a-c) AQP2 localization in the Ctx. AQP2 was located apically in control rats (arrows, a), and non-apically in OPC31260 (arrowheads, b) and ZP120 treated rats (arrowheads, c). d-f) AQP2 localization in the OM. AQP2 was located apically in control rats (arrows, d) and non-apically 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-i) AQP2 localization in the IM. AQP2 was located
apically in control rats (arrows, g) and non-apically in OPC31260 (P=0.08) (arrowheads, h) and ZP120 treated rats (P=0.06) (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 hours 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, bars are SD, *P<0.05.
### Table 1.

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**Table 1. Renal functional parameters.** After a 60 min control period, ZP120 or OPC31260 were infused for four hours in a computer controlled servo model in which ZP120 and OPC31260 induced urine losses were replaced with 50 mM glucose to avoid physiological antagonism. MAP: Mean arterial pressure, GFR: Glomerular filtration rate, C_{Li}: Lithium clearance, FE_{Li}: Fractional lithium excretion, AVP: Vasopressin plasma concentration. In regard to MAP, GFR, C_{Li} and FE_{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 ± SD, *P < 0.025 (Bonferroni corrected P-value) compared to control (**P < 0.025 compared to OPC31260) by two way ANOVA using all experimental time points.
Figure 1

A: 

$ZP120/OPC31260$ infusion

$V$ (µl/min/100 g bw)

Time (min)

- Control
- OPC31260
- ZP120

B:

$ZP120/OPC31260$ infusion

$C_{H2O}$ (µl/min/100 g bw)

Time (min)

- Control
- OPC31260
- ZP120

C:

$ZP120/OPC31260$ infusion

$V/C_Li$ (%)

Time (min)

- Control
- OPC31260
- ZP120

# #
Figure 2

A:

\[ U_{Na,V} \ \text{(nmol/min/100 g bw)} \]

Time (min)

B:

\[ C_{Na} \ \text{(μL/min/100g bw)} \]

Time (min)

C:

\[ FE_{Na} \text{ distal (％)} \]

Time (min)
Figure 3

A: Ctx/OSOM

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AQP2 protein level (Fraction of control)

B: ISOM

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AQP2 protein level (Fraction of control)

B: IM

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AQP2 protein level (Fraction of control)
Figure 4

A: Ctx/OSOM

35-50 kDa - 28 kDa -

Con 1-6  ZP120 1-7

AQP2 protein level (Fraction of control)

1.0

Con  ZP120

B: ISOM

35-50 kDa - 28 kDa -

Con 1-6  OPC31260 1-7

AQP2 protein level (Fraction of control)

1.0

Con  OPC31260

B: IM

Con  ZP120

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Figure 5

Control        OPC31260        ZP120

a              b              c

[Images with arrows indicating specific areas]

25 µm
Figure 6

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