Opioid receptor-like 1 stimulation in the collecting duct induces aquarexia through vasopressin-independent aquaporin-2 downregulation

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than nociceptin and does not produce centrally mediated responses after peripheral administration (35). In the present study, we demonstrate that ORL1 receptors are present within the renal collecting ducts and that selective ORL1 receptor stimulation with ZP120C selectively increases renal water excretion (i.e., produces aquaresis) by a novel AVP-independent mechanism that has not been described previously.

MATERIALS AND METHODS

Experimental animals. Male Sprague-Dawley rats (250 g, Charles River, Sulzfeld, Germany) were used for the experiments. The animals were housed in a temperature (22–24°C) and moisture (40–70%)-controlled room with a 12:12-h light-dark cycle (lights on from 6 AM to 6 PM). The animals were given free access to tap water and a diet with ~140 mmol/kg sodium, ~275 mmol/kg potassium, and 23% protein. All animal procedures followed the guidelines for the care and handling of laboratory animals established by the Danish government.

Immunohistochemistry. The rats were anesthetized with 4% (induction) followed by 1% (maintenance) halothane in 1:1 N2O-O2. The kidneys were in situ perfused through the aorta with 0.1 M sodium cacodylate buffer, pH 7.4, containing 4% paraformaldehyde. A section containing all kidney zones was paraffin embedded. Two-micrometer-thick slices were blocked in 1% BSA, 0.2% gelatin, 0.05% saponin in PBS, washed in 0.1% BSA, 0.2% gelatin, and 0.05% saponin in PBS, and incubated overnight at 4°C with a goat polyclonal anti-ORL1 receptor antibody (sc-9759, Santa Cruz Biotechnology, Santa Cruz, CA) together with a polyclonal rabbit anti-AQP2 antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) and preabsorption control sections, the ORL1 receptor antibody was combined with a polyclonal rabbit anti-AQP2 antibody together with swine anti-rabbit FITC-conjugated antibody (Molecular Probes) for 60 min at room temperature. After being rinsed with PBS, the sections were mounted in glycerol mounting medium and examined using a Leica TCS SP2 laser confocal microscope (11, 30). In preabsorption control sections, the ORL1 receptor antibody was incubated with a fivefold (by weight) excess of the peptide to which it was raised (sc-9759 P, Santa Cruz Biotechnology). The sections were blocked and washed as described above and incubated overnight at 4°C before horseradish peroxidase immunohistochemistry was conducted (11, 30).

Induction of CHF. Male Sprague-Dawley rats were anesthetized with 4% (induction) followed by 2% (maintenance) isoflurane in 1:1 N2O-O2. CHF was then induced by left coronary artery ligation (LCAL) (30, 38). Two weeks later, the rats were instrumented with permanent catheters in the femoral artery and vein and with a servo-controlled intravenous replacement of urine losses with 50 mM glucose (2, 38). The mean arterial pressure was measured throughout the study. Arterial blood samples were drawn once every hour. The plasma concentration of AVP (21) was measured in blood samples drawn before the first control period and at the termination of the study. All blood drawn was immediately replaced with blood from a donor rat (15, 16). We had to exclude clearance data from one animal in each of the two time control groups (1 CHF-time control and 1 Sham-time control) due to lack of blood samples related to failure of the permanent arterial catheters. However, the rats received baseline intravenous infusion with drug throughout the experiment, and the kidneys were removed at the end according to our protocol. Therefore, these kidneys were not excluded from Western blot analyses.

Experimental groups. The following experimental groups took part in the study: Sham-time control (vehicle-treated sham-operated rats; n = 5); Sham-ZP120C (sham-operated rats treated with 1 nmol kg–1 min–1 ZP120C; n = 8); CHF-time control (vehicle-treated CHF rats; n = 5); and CHF-ZP120C (CHF rats treated with 1 nmol kg–1 min–1 ZP120C; n = 10).

Western blotting. For the clearance experiments, the rats were isoflurane anesthetized and the kidneys were rapidly removed and the protein levels of AQP2 and serine 256 phosphorylated AQP2 (pAQP2) were measured by Western blotting. Six kidneys from each of the four groups were used for Western blotting. In regard to the groups where more than six clearance experiments were conducted, we homogenized the kidney from the first six animals. Whole kidneys were homogenized in 9 ml buffer containing 300 mM sucrose, 25 mM imidazole, 1 mM EDTA, 0.1 mg/ml leupeptin, 184 μg/ml sodium orthovanadate, 1 mg/ml sodium fluoride, and 82 μg/ml okadecic acid (Sigma, St. Louis, MO). The protein concentrations were assessed with Pierce 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 DTT, and 0.9 mM bromophenol blue. The samples were then heated for 10 min at 60°C. The samples were run on 12% polyacrylamide gels. The proteins were then electrotransferred to polyvinylidene difluoride blotting membranes (Millipore, Bedford, MA) and blocked for 1 h in 5% milk 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 anti-AQP2 or anti-pAQP2 antibody (5, 6). The blots were then washed in PBS-T and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Dako). After final washing in PBS-T, the proteins were visualized with the ECL plus chemiluminescence system (Amersham).

The inner medullas from three rats were cut out for detection of the ORL1 receptor by Western blotting on microdissected inner medullary collecting ducts. The inner medullas were minced and pooled in aerated (5% CO2-95% O2) Hanks' buffer. Collecting ducts were released by collagenase A treatment (0.12 U/ml Hanks' buffer, cat.
no. 1088 793, Roche, Mannheim, Germany) for 4 min at 37°C, followed by 30 s of vortexing and 1 min of settling. The supernate with isolated collecting ducts was removed and kept at 4°C. The procedure was repeated until all tissue was dissolved. The collecting duct pools were separated from collagenase A by centrifugation (2 × 2 min, 3,000 g). The collecting ducts were then further isolated by sorting under a microscope. Sample buffer was added, and Western blotting was conducted as described above using three affinity-purified polyclonal antibodies raised against different epitopes on the ORL1 receptor (NH2-terminal epitope: sc-9759; COOH-terminal epitope: sc-9760; and internal epitope: sc-15309, Santa Cruz Biotechnology). AVP-stimulated cAMP levels in isolated inner medullary collecting ducts. Inner medullary collecting ducts from three rats were isolated by the method described above except that sorting under a microscope was omitted as the V2 receptor is solely found in collecting ducts. Resuspended collecting ducts in an amount corresponding to 6/ H9262 C for 60 min. Subsequently, AVP or vehicle was added to the collecting ducts for 20 min. The reactions were then immediately stopped by freezing on dry ice. The cAMP level was measured with an enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI).

RESULTS

Renal localization of the ORL1 receptor. A possible mechanism responsible for the described aquaretic effects of nociceptin could be direct stimulation of renal tubular ORL1 receptors. However, whether ORL1 receptors are expressed in the kidneys has never been investigated. We therefore conducted immunofluorescence histochemistry of the collecting ducts using a polyclonal goat anti-ORL1 receptor antibody and found that ORL1 receptors were present in the apical membrane throughout the cortex, the outer medulla, and the inner medulla (Fig. 1A). The collecting ducts have two cell types: 1) the principal cells responsible for transepithelial water and sodium reabsorption, which express AQP2; and 2) intercalated cells involved in the regulation of the acid-base status. We conducted double-immunofluorescence labeling with the ORL1 receptor antibody together with a polyclonal rabbit anti-AQP2 antibody to investigate in which cell types the ORL1 receptor is expressed and found coexpression of the ORL1 receptor and AQP2 in the inner medullary collecting ducts. Interestingly, the ORL1 receptor was expressed in the apical membrane of the principal cells in the inner medulla, whereas in the cortex and outer medulla the ORL1 receptor was only expressed in the intercalated cells. ORL1 receptor labeling was abolished by preabsorption with the peptide to which the antibody was raised (Fig. 1B). Furthermore, ORL1 receptor expression in the inner medullary collecting duct was confirmed by Western blotting on microdissected inner medullary collecting ducts using three affinity-purified polyclonal antibodies raised against different epitopes on the ORL1 receptor (Fig. 1C). These findings provide a structural basis to link ORL1 receptor stimulation in the inner medullary collecting ducts to the aquaretic effect of nociceptin previously described by Kapusta et al. (18–20).

Aquaretic effect of the novel nociceptin analog ZP120C. We tested the renal effects of administration of ZP120C (1 nmol·kg−1·min−1·iv) in conscious, chronically instrumented rats with CHF induced by LCAL. Sham-operated rats were used as controls. To avoid any ZP120C-induced changes in fluid balance we used a computer-driven, servo-controlled intravenous volume-replacement system (2, 38). Vehicle-treated sham and CHF rats were used as time controls.

ZP120C displayed a marked aquaretic effect in both CHF and sham-operated rats (Fig. 2). Steady-state diuresis was reached after 2.5 h of constant intravenous infusion of ZP120C and was sustained throughout the 4-h study period. The ZP120C-induced changes in diuresis, free water clearance, fractional water excretion, and fractional distal water excretion were at the same magnitude as previously described effects of selective V2-receptor antagonists (14, 16, 38). Glomerular filtration rate, effective renal plasma flow, and mean arterial pressure were all unchanged throughout the study in both CHF and sham-operated rats (Table 1).

These results show that ZP120C displays marked aquaretic effects in both normal and CHF rats. One possible mechanism responsible for the aquaretics could be a suppression of the circulating concentrations of AVP by an action on the central nervous system. However, direct measurements of plasma AVP before and at the end of the 4-h ZP120C treatment period showed that there were no changes in plasma AVP concentrations within the experimental groups (Table 1). A negative correlation between change in AVP plasma concentrations and change in diuresis was not determined, as would be expected if a drop in the AVP plasma concentration caused the increase in diuresis. The correlation coefficients were 0.23 ( sham-operated rats) and 0.24 (CHF rats) (Fig. 3). These findings demonstrate that specific ORL1 receptor stimulation with ZP120C induces a marked aquaretic response in the absence of changes in the circulating concentration of AVP.

ZP120C-induced changes in the AQP2 protein level. We investigated whether the ZP120C-induced aquaretics was associated with changes in the renal protein level of the AQP2-regulated water channel AQP2, which is exclusively expressed in the collecting ducts. The kidneys were rapidly removed at the termination of the renal function study, and the right kidney was prepared for Western blotting on whole kidney preparations. The affinity-purified anti-AQP2 protein, antibody recognizes the 29- and the 35- to 50-kDa band, corresponding to nonglycosylated and glycosylated AQP2 protein, respectively (6) (Fig. 4). Densitometry showed that the AQP2 protein level was significantly increased in the CHF rats (Sham: 1.0 ± 0.18 vs. CHF: 1.71 ± 0.26, 0 < 0.05) (Fig. 4). Four hours of treatment with ZP120C decreased the AQP2 protein levels in both normal and CHF rats (Fig. 4) (Sham: 1.0 ± 0.16 vs. Sham-ZP120C: 0.47 ± 0.09, 0 < 0.05; CHF-ZP120C: 0.47 ± 0.12, 0 < 0.05).

ZP120C-induced effects on AQP2 phosphorylation. AVP stimulation induces PKA-mediated phosphorylation of serine 256 in AQP2, which is required for the translocation of AQP2 from intracellular stores to the apical plasma membrane (9). To examine the effects of ORL1 receptor stimulation on phosphorylated AQP2, we conducted Western blotting using an affinity-purified anti-pAQP2 protein antibody, raised against the pro-
tein PKA phosphorylation site on serine 256 in AQP2. In agreement with previous findings showing increased plasma membrane targeting of AQP2 in CHF rats (30), the pAQP2 protein level was significantly increased in the CHF rats (Sham: 1.0 ± 0.14 vs. CHF: 1.76 ± 0.26, P < 0.05; Fig. 4). Four hours of ZP120C infusion significantly decreased pAQP2 expression in rats with CHF (CHF: 1.0 ± 0.26 vs. CHF-ZP120C: 0.49 ± 0.07, P < 0.05; Fig. 4), but significant amounts of pAQP2 were still present. A similar picture was found in the Sham rats (Sham: 1.0 ± 0.21 vs. Sham-ZP120C: 0.61 ± 0.17; Fig. 4). These data show that specific ORL1 receptor stimulation for 4 h reduces the amount of AQP2 and pAQP2 in both normal and CHF rats.

**Effects of ORL1 receptor stimulation on AVP-induced cAMP formation in isolated collecting ducts.** To further examine the effects of ORL1 receptor stimulation on AVP-induced cAMP formation, which is responsible for PKA activation and AQP2 phosphorylation, we tested whether ORL1 receptor stimulation, in a time period corresponding to the onset of aquaresis (60 min), was able to inhibit V₂-receptor-mediated cAMP formation in the inner medullary collecting ducts (Fig. 5). AVP (10⁻⁸ M) significantly stimulated cAMP accumulation in isolated inner medullary collecting ducts. However, neither pre-treatment with ZP120C nor nociceptin inhibited AVP-stimulated cAMP formation.

**DISCUSSION**

Our present findings show that acute administration of the novel selective nociceptin analog ZP120C produces a decrease in the AQP2 protein level in the renal collecting ducts, which is associated with a marked and sustained aquaretic response in
ORL1 receptor stimulation induces aquaresis

The absence of changes in circulating AVP concentrations. The finding that ORL1 receptors are coexpressed with AQP2 in principal cells in the inner medullary collecting ducts suggests that ZP120C produces aquaresis by a direct renal tubular effect.

Fig. 2. Selective nociceptin analog ZP120C induces aquaresis in normal and congestive heart failure (CHF) rats. The study was conducted in conscious, chronically instrumented rats with CHF induced by ligation of the left anterior descending coronary artery or in sham-operated rats (Sham). Volume depletion during ZP120C treatment was prevented by intravenous volume replacement of urinary losses with 50 mM glucose. Values are means \( \pm \) SE; \( n = 5–10 \)/group. \( \circ \), Sham-time control; \( \bullet \), Sham-ZP120C; \( \square \), CHF-time control; \( \triangle \), CHF-ZP120C. A: effects of ZP120C or vehicle on urine flow rate. BW, body wt. B: free water clearance. C: fractional distal water excretion.

Kapusta and co-workers (18–20) originally described that nociceptin has aquaretic effects in vivo. They demonstrated that continuous intravenous infusion of nociceptin at a dose of 20 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) in conscious instrumented rats induces production of solute-free urine as well as a moderate decrease in mean arterial pressure (20). This study was followed by another study, where a 100-fold lower dose of nociceptin was used (0.2 \( \mu g \cdot kg^{-1} \cdot min^{-1} \)), showing that low-dose nociceptin produces aquaresis without changes in mean arterial blood pressure (19). The mechanism underlying this aquaretic effect of low-dose nociceptin infusion is unknown, but potentially nociceptin could have direct renal effects through binding to yet unidentified ORL1 receptors. Therefore, we conducted immunohistochemical studies with double labeling of the ORL1 receptor and AQP2. We found the ORL1 receptor in rat kidney collecting ducts. Colocalization with AQP2 was found in the principal cells within the inner medullary collecting ducts. The immunohistochemical results were verified by preabsorption studies and by Western blotting on isolated inner medullary collecting ducts, using three different antibodies raised against different epitopes on the ORL1 receptor.

A potential mechanism responsible for the aquaretic effect of intravenous nociceptin infusion could be that the peptide penetrated the blood-brain barrier and inhibited AVP release from the posterior pituitary (17). To avoid central effects in the in vivo studies, we used a new highly selective ORL1 receptor agonist (ZP120C), which lacks central effects when given intravenously (35). We found that intravenous infusion of this compound in volume-replete rats, at a dose that had no effect on systemic blood pressure, induced marked and sustained aquaresis in both control and CHF rats, in the absence of changes in plasma concentrations of AVP. This finding strongly indicates that the compound induces aquaresis through direct stimulation of ORL1 receptors within the renal collecting ducts. We therefore conducted Western blotting on kidneys that were removed during ZP120C-induced steady-state aquaresis. The Western blot analyses showed that 4 h of ZP120C treatment were associated with downregulation of AQP2 in both CHF and control rats. This finding could suggest that ORL1 receptor stimulation produces aquaresis by inhibiting the \( V_2 \)-receptor-mediated stimulation of collecting duct water reabsorption.

To further explain the cellular mechanism in the inner medullary collecting ducts, we therefore investigated whether ORL1 receptor stimulation interacts with \( V_2 \)-receptor-mediated cAMP formation and, thereby, PKA-mediated regulation of AQP2. AVP acts through PKA activation as both a long-term transcriptional regulator of AQP2 (13, 43) and a short-term regulator of AQP2 plasma membrane translocation (24). PKA-mediated phosphorylation of serine 256 is necessary for membrane translocation and, thereby, activation of AQP2 (6, 9). Christensen et al. (5) showed that acute \( V_2 \)-receptor blockade causes an almost complete disappearance of serine 256 pAQP2 within 30–60 min, which is associated with a marked increase in solute-free urine production. In contrast to the pronounced inhibition of AQP2 phosphorylation observed during \( V_2 \)-receptor blockade, pAQP2 was still present after 4-h ORL1 receptor stimulation with ZP120C. Moreover, preincubation with ORL1 receptor agonists did not inhibit AVP-stimulated cAMP formation in isolated inner medullary col-
RENAL ORL1 RECEPTOR STIMULATION INDUCES AQUARESIS

Table 1. Hemodynamics, GFR, proximal tubular function, and AVP measurements during ZP120C-induced aquareasis

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<thead>
<tr>
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<th>Sham-Time Control</th>
<th>Sham-ZP120C</th>
<th>CHF-Time Control</th>
<th>CHF-ZP120C</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>Vehicle</td>
<td>Baseline</td>
<td>ZP120C</td>
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<tr>
<td>MAP, mmHg</td>
<td>109 ± 7</td>
<td>109 ± 6</td>
<td>108 ± 5</td>
<td>107 ± 5</td>
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<tr>
<td>ERPF, ml/min/1.73 m 2</td>
<td>2.73 ± 0.50</td>
<td>3.24 ± 0.18</td>
<td>3.28 ± 0.43</td>
<td>2.93 ± 0.18</td>
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<tr>
<td>GFR, ml/min·1.73 m 2</td>
<td>790 ± 146</td>
<td>907 ± 46</td>
<td>837 ± 135</td>
<td>892 ± 52</td>
</tr>
<tr>
<td>C_{Lr}, µl/min·1.73 m 2</td>
<td>148 ± 23</td>
<td>141 ± 13</td>
<td>196 ± 30</td>
<td>190 ± 32</td>
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<tr>
<td>Plasma AVP, pg/ml</td>
<td>2.9 ± 1.5</td>
<td>2.9 ± 0.7</td>
<td>2.9 ± 0.7</td>
<td>2.5 ± 0.6</td>
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<td>3.5 ± 1.0</td>
<td>3.7 ± 0.5</td>
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<td>4.1 ± 1.3</td>
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Values are means ± SE. Mean arterial pressure (MAP), effective renal plasma flow (ERPF), glomerular filtration rate (GFR), lithium clearance (C_{Lr}), and plasma vasopressin (AVP) concentration during control conditions (baseline) and during steady-state aquareasis induced by ZP120C treatment or vehicle (50 mM glucose) treatment in conscious, chronically instrumented rats. CHF, rats with congestive heart failure induced by ligation of the left anterior descending coronary artery; Sham, sham-ligated rats.

Collecting ducts. These findings indicate that, at least in inner medullary collecting ducts, renal ORL1 receptor stimulation with ZP120C decreases the protein level of AQP2 and produces a marked aquareasis by a mechanism that does not involve inhibition of AVP-induced PKA activation.

As described above, Kapusta and co-workers (20) found that high-dose nociceptin, in addition to producing aquareasis, is associated with a moderate decrease in mean arterial pressure. Interestingly, the maximal hypotensive effect of nociceptin occurs within 10–15 min, whereas the aquarectic effect does not reach its maximum until after 40 min of nociceptin infusion. Our findings, showing that the onset of ORL1 stimulation in the collecting ducts is slow and does not inhibit cAMP production and, thereby, AQP2 phosphorylation, correspond well with this finding. It could therefore be suggested that ORL1 stimulation in the collecting ducts activates a slower signal transduction pathway, possibly involving protein synthesis and/or degradation. Ongoing studies are therefore examining the role of ORL1 stimulation in collecting duct AQP2 protein synthesis.

Another possible mechanism responsible for the aquaretic effect of ORL1 stimulation could involve increased AQP2 degradation. It was recently reported that AQP2 half-life is approximately 6 h in a cell line of mouse cortical collecting duct principal cells and that AQP2 degradation is mediated through both proteasomal and lysosomal pathways (12). Interestingly, it was reported that the well-known inhibitory effect of prostaglandin E2 on collecting duct water permeability, which involves the stimulation of the G_{i}-coupled prostaglandin E-prostanoid-3 receptor, is due to retrieval of AQP2 from the apical plasma membrane by mechanisms not involving dephosphorylation (44). Furthermore, van Balkom and co-workers (40) recently reported data supporting the hypothesis that PKC mediates endocytosis of AQP2 independently of the phosphorylation state of AQP2. Thus endocytosis and degradation of AQP2 could be regulated processes that could be subjected to pharmacological targeting. Further studies are warranted to examine whether ORL1 receptor stimulation affects AQP2 internalization and/or degradation.

It is generally recognized that distal water reabsorption occurs in both cortical outer and inner medullary segments of the collecting ducts, implying that a maximal aquaretic response most probably must involve inhibition of water reabsorption in all segments of the collecting ducts. In the present study, ZP120C induced a near-maximal aquareasis corresponding to the full diabetes insipidus observed in AVP-deficient rats (37). Furthermore, we show that ORL1 receptors are present in the intercalated cells, but not in the principal cells, in the cortical and the outer medullary part of the collecting ducts. It could therefore be speculated whether ORL1 receptor stimulation of the intercalated cells in the cortex and/or the outer medulla induces a paracrine response, which inhibits water reabsorption through the principal cells. Further studies are warranted to examine this hypothesis.

V_{2}-receptor antagonists have been shown to increase free water excretion in patients with severe CHF (25) and to normalize plasma sodium in patients with hypotension (41). However, these effects were reported to be associated with a marked increase in plasma AVP (31, 36), which indicates that V_{2}-receptor antagonist treatment may induce unopposed AVP type-1 receptor (V_{1} receptor)-mediated vasoconstriction. In the present study, ORL1 receptor stimulation with ZP120C produced a selective aquareasis without concomitant changes in systemic hemodynamics or changes in circulating concentrations of AVP. The study was conducted during servo-controlled volume replacement, which protects against volume depletion and, thereby, osmoreceptor-mediated AVP stimulation. However, whether servo-controlled volume replacement during V_{2}-receptor antagonist-induced water diuresis would
prevent increases in plasma AVP and thus any V₁-receptor-mediated vasoconstriction is unknown.

In summary, ORL1 receptors were present within the renal collecting ducts, and treatment with the novel nociceptin analog ZP120C produced a marked aquaresis in both normal and CHF rats by AQP2 downregulation through a mechanism not involving changes in the AVP plasma concentration, not involving inhibition of V₂-receptor-mediated cAMP production, and not involving total downregulation of serine 256 AQP2 phosphorylation. The retention of water and sodium is responsible for most symptoms in CHF. Ultimately, many CHF patients develop hyponatremia, due to excessive water retention. This is associated with a poor prognosis. The present results suggest that peripherally acting ORL1-receptor agonists may become a new class of therapeutics in the treatment of acute decompensated CHF.

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GRANTS

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