frontiers in
PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 18 April 2012
doi: 10.3389/fphys.2012.00103

Antidiuretic effects of the endothelin receptor antagonist avosentan

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Several clinical studies have investigated the potential benefits of endothelin receptor antagonism in chronic pathologies such as diabetic kidney disease. However, fluid retention and edema have been identified as major side effects of endothelin receptor antagonists. In the present study we hypothesized that avosentan which was described as a predominant ET_A receptor antagonist would produce fluid retention at high concentrations where non-specific blockade of ET_B receptors may occur. Incremental doses of the predominant ET_A receptor antagonist SPP301 (0.003; 0.03; 3 mg/kg) were administered intravenously to anesthetized Sprague-Dawley rats undergoing saline diuresis. Diuresis, glomerular filtration rate, and blood pressure (BP) were monitored. SPP301 decreased urine output (5.6; 34.8; 58.8% decrease from vehicle) and fractional excretion of water (5.7; 31.7; 56.4% decrease from vehicle) in a concentration-dependent manner. Glomerular filtration rate was unchanged while BP was reduced by 10 mmHg only by the highest dose of SPP301. Administration of the ET_B selective receptor antagonist BQ-788 (3 mg/kg) following SPP301 3 mg/kg did not further decrease urine output or water excretion and was without effect on glomerular filtration rate. These data indicate that increasing concentrations of SPP301 may also block ET_B receptors and cause antidiuresis. This effect could explain why fluid retention and edema occur during treatment with predominant ET_A receptor blockers.

Keywords: endothelin receptor antagonist, fluid retention, diuresis, renal

INTRODUCTION

Endothelin-1 (ET-1) is a 21 amino acid peptide derived from the vascular endothelium that has potent vasoactive properties (Yanagisawa et al., 1988). ET-1 induces its effect by acting on endothelin A and B receptors (ET_A and ET_B). Chronically elevated ET-1 is involved in the pathophysiology of pulmonary arterial hypertension, heart failure, systemic hypertension, renal dysfunction, and atherosclerosis (Haynes and Webb, 1998; Schneider et al., 2007). Elevated plasma levels of ET-1 can be found in several pathological states, including diabetes mellitus (De Mattia et al., 1998; Bruno et al., 2002). The discovery of several compounds acting as endothelin receptor antagonists has prompted research toward their use in clinical practice (Anand et al., 2004; Packer et al., 2005; McMurray et al., 2007).

Avosentan (SPP301) is a predominant ET_A receptor antagonist which was in development for the treatment of diabetic nephropathy (Mann et al., 2010). Administration of SPP301 on top of standard care [including angiotensin-converting enzyme inhibitors (ACEIs) or high dose angiotensin receptor blockers (ARBs)] has been shown to result in a clinically relevant decrease in proteinuria in patients with diabetic nephropathy. In the Randomized, Double Blind, Placebo Controlled, Parallel Group Study to Assess the Effect of the Endothelin Receptor Antagonist Avosentan on Time to Doubling of Serum Creatinine, End Stage Renal Disease or Death in Patients With Type 2 Diabetes Mellitus, and Diabetic Nephropathy (ASCEND) study, 50% of patients with diabetic nephropathy and well-controlled blood pressure (BP) showed a 40–50% reduction in proteinuria with the addition of SPP301 to standard care (Mann et al., 2010). However, the ASCEND trial was stopped due to the adverse effects of SPP301. The most commonly reported adverse effects were consistent with those previously reported for the endothelin receptor antagonist class; namely edema, headache, and anemia (Mann et al., 2010). Edema appears to be dose-related and occurs more frequently with SPP301 than standard care alone. In patients with renal failure, SPP301 can aggravate existing edema.

ET_A receptors are mainly localized in vascular smooth muscle, vasa recta, and arcuate arteries and glomerulus (mesangial cells and podocytes) and their stimulation or activation induce vasoconstriction, salt and water retention, vascular proliferation, inflammation, and fibrosis (Jandeleit-Dahm and Watson, 2012). There is now large evidence that ET_A endothelin receptor blockade confers renoprotection in several progressive renal disorders (Davenport and Maguire, 2011; Gagliardini et al., 2011; Jandeleit-Dahm and Watson, 2012). Preclinical and clinical data demonstrates that of ET_A receptor blockade is protective in chronic kidney disease through several effects including vasodilation, attenuation

of proteinuria, increase of diuresis and natriuresis, inhibition of inflammation, oxidative stress, and fibrosis (Dhaun et al., 2011; Jandeleit-Dahm and Watson, 2012). ET_B receptors are expressed in vascular and glomerular endothelial cells, mesangial cells, convoluted tubules and collecting duct epithelial cells, and their stimulation or activation cause vasodilation, increase sodium excretion, and inhibit vascular proliferation, inflammation, and fibrosis (Schneider et al., 2007). Recent data indicate that renal ET_B receptor antagonism or knockout may cause sodium retention (Ge et al., 2006; Guo and Yang, 2006). Furthermore, ET_B receptor deficiency is associated with renal injury and an impaired ability to excrete a sodium load (Ohkita et al., 2005). Thus, compounds with predominant selectivity against ET_A receptors may produce fluid retention at concentrations where non-specific blockade of ET_B receptors may occur.

The objectives of our study were to (1) test the hypothesis that the ET_A receptor antagonist SPP301 at high concentrations may cause fluid retention, and (2) identify the concentration of SPP301 that inhibits ET_B receptor-mediated effects and consequently leads to acute fluid retention in anesthetized rats. It is thought that SPP301 at high doses may antagonize the ET_B receptors and induce fluid retention.

MATERIALS AND METHODS

All procedures complied with guidelines from the American Physiological Society, and the study was approved by a local review board.

RAT STRAINS

Male Sprague-Dawley (SD) rats were obtained from Harlan, Indianapolis (12–14 weeks of age). Following acclimatization and a health examination, rats were housed in groups of three in standard cages with wire mesh tops and standardized softwood bedding, synchronized to a 12-h light–dark cycle, at ambient temperature 23 ± 2°C. A standard rat diet and tap water were supplied *ad libitum*.

STUDY DESIGN: EFFECT OF SPP301 ON RENAL EXCRETORY FUNCTION

The purpose of the experimental protocols was to examine the acute effect of SPP301 on renal excretory function in a model of volume expansion/saline diuresis (Guo and Yang, 2006).

After induction of anesthesia with Inactin (80 mg/kg i.p. thiobutabarbital sodium, Sigma Chemical, St. Louis, MO, USA), SD rats were instrumented with catheters in: (1) the femoral artery for measurement of BP by a pressure transducer connected to a computerized data-acquisition system (PowerLab, ADInstruments, Colorado Springs, CO, USA; Campos et al., 2004); (2) the femoral vein for a constant infusion of artificial rat plasma and to administer study drugs via bolus infusion; and (3) the jugular vein for a constant infusion of ^{3}H-inulin solution. A catheter was also inserted in the left ureter for urine collection. Artificial rat plasma [bovine serum albumin (BSA 2.5 g/100 mL, Sigma Chemical, St. Louis, MO, USA) + bovine immunoglobulins (2.5 g/100 mL, Sigma Chemical, St. Louis, MO, USA) in 0.9% NaCl] was infused (12.5 mL/kg/h for 45 min, thereafter 1.5 mL/kg/h) to maintain euvolemia throughout the experiment (Reckelhoff et al., 1992).

Rats were divided into two groups of six animals each (Group A and Group B). In both groups, Na_2CO_3 0.1 M 1 mL/kg was administered as a vehicle during the baseline period.

In Group A, the selective ET_B receptor antagonist BQ-788 (Sigma Chemical, St. Louis, MO, USA) was administered alone as a 3-mg/kg dose by intravenous (i.v.) bolus in order to demonstrate its effects on ET_B receptors compared with the vehicle, as described by Okada and Nishikibe (2002; **Figure 1**). Urine and blood samples were collected over 20-min clearance periods during the baseline period and 10 min after the i.v. bolus administration. The protocol was to administer vehicle, followed by BQ-788, then two more administrations of vehicle. The concentration of BQ-788 used was the highest concentration that could be obtained by dissolution in the same vehicle as SPP301. Since this concentration had the expected physiological effects during single administration, it was used as final positive control at the end of SPP301 administration experiments (Group B of study).

Group B received increasing concentrations of SPP301 (0.003; 0.03; 3 mg/kg, determined following completion of a dose-ranging study; Speedel, Basel, Switzerland) administrated as an i.v. bolus at 30-min intervals (**Figure 1**). The choice of bolus vs. infusion was justified by the relatively long half-life of the compound (4 h) compared to the length of our acute experimental protocol. It was also preferred in order to minimize the length of time required for loading and maintenance infusions. A 3-mg/kg dose of the selective ET_B receptor antagonist BQ-788 was administered 30 min after the highest dose of SPP301 in order to test whether any additional effects of blocking ET_B receptors would be observed (Okada and Nishikibe, 2002). Urine and blood samples were collected over a total of five 20-min clearance periods during the baseline period and 10 min after i.v. bolus administration of each study drug (**Figure 1**).

During each clearance period, urine was collected throughout the 20-min clearance period and an arterial blood sample (two hematocrit tubes – approximately 60 μL) was collected from the femoral artery catheter at the middle of the clearance period. Blood samples were centrifuged and the resulting plasma used for further analysis.

PLASMA AND URINE ELECTROLYTE MEASUREMENT

At the end of the experiment, sodium and potassium concentration in the urine was determined for each clearance period. The rate of sodium and potassium excretion was calculated from these values and the volume of urine per 20 min as mEq/min. In addition, ^{3}H-Inulin concentration in the plasma and urine samples was measured (using a scintillation counter) and used to calculate GFR. GFR for each clearance period was calculated as [Urine volume (mL)/min] × [Urine concentration (cpm/mL)]/[Plasma concentration (cpm/mL)] and expressed as mL/min.

STATISTICAL ANALYSIS

Statistical differences were determined with a one-way analysis of variance (ANOVA), followed by Tukey's range test for dose-ascending studies using GraphPad software. $p < 0.05$ was considered to be statistically significant. Data are expressed as means ± SE.

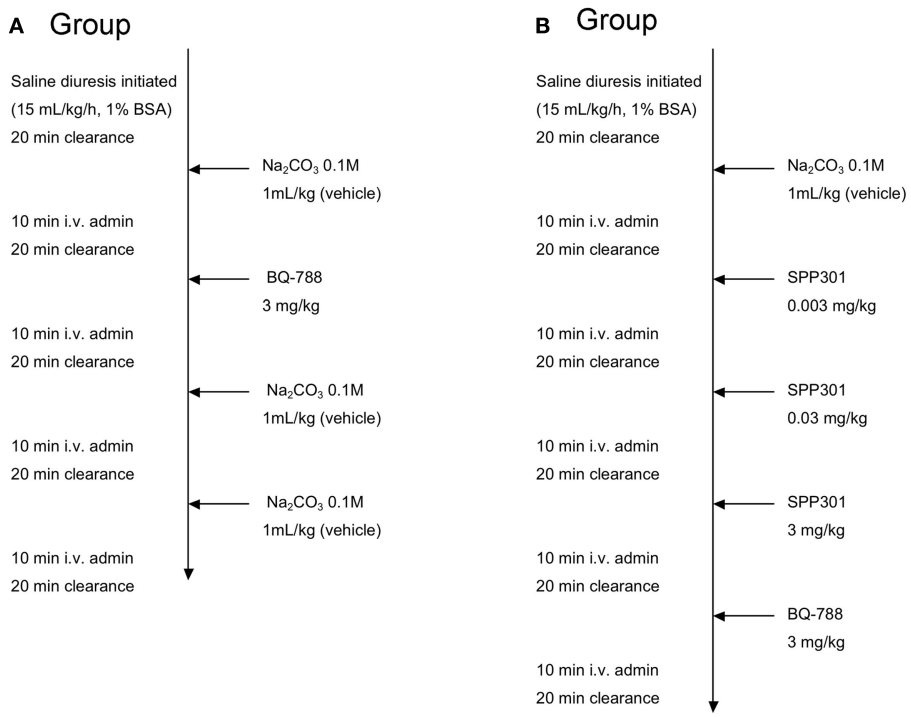

FIGURE 1 | Study design. Group A study design: i.v. administration of BQ-788 (3 mg/kg) to anesthetized male SD rats undergoing saline diuresis. Group B study design: i.v. administration of incremental doses of SPP301 (0.003; 0.03; 3 mg/kg) followed by BQ-788 (3 mg/kg) to anesthetized male SD rats undergoing saline diuresis.

RESULTS

SELECTIVE BLOCKADE OF ET_B RECEPTORS WITH BQ-788 AT CONCENTRATIONS THAT DO NOT AFFECT GLOMERULAR HEMODYNAMICS (GFR) LEADS TO ANTIDIURESIS

In this study, BQ-788 decreased urine output (115.4 ± 15 vs. 150.1 ± 21 μL/min, $p < 0.05$) and fractional excretion of water (5 ± 0.3 vs. $7.7 \pm 1\%$, $p < 0.05$) compared with the vehicle, while urinary excretion rates and plasma concentrations of sodium and potassium were not altered. BQ-788 increased BP by 6 ± 1 mmHg ($p < 0.05$), but did not affect GFR (2.2 ± 0.1 vs. 1.9 ± 0.1 mL/min, $p = 0.2$) compared with the vehicle. These alterations returned rapidly to normal values when vehicle was further administered, demonstrating that the effects of BQ-788 are not only reversible after acute administration but also provide a time-course effect of vehicle.

SPP301 INDUCES ANTIDIURESIS AT HIGH CONCENTRATION

In this study, SPP301 decreased urine output and fractional excretion of water in a concentration-dependent manner (**Figures 2A,B**). Sodium excretion was initially increased but returned to control values after the highest dose of SPP301 (**Figures 2C,D**). GFR was unchanged (**Figure 3**) while mean arterial pressure (MAP) was reduced only by the highest dose (3 mg/kg) of SPP301 (**Figure 4**). Administration of BQ-788 (3 mg/kg) after SPP301 3 mg/kg did not further decrease urine output or water excretion (**Figures 2A,B**) and was without effect on GFR (**Figure 3**). The data indicates that after the highest concentration of SPP301, the effects persist beyond the 20-min clearance period, when BQ-788 does not have an additional effect. This indicates a full occupancy of ET_B receptors by SPP301 before the BQ-788 administration. Hematocrit was significantly ($p < 0.05$) decreased by the higher doses of SPP301 (0.03; 3 mg/kg) and with BQ-788 (**Figure 5**). Repetitive administration of the vehicle did not cause the effects seen with SPP301 or BQ-788.

DISCUSSION

The present study demonstrates that SPP301 has antidiuretic effects at high doses and causes subsequent hemodilution (indicated by a decrease in hematocrit). These effects of SPP301 appear independent of changes in GFR or BP. Following administration of high dose (3 mg/kg) SPP301, ET_B receptor blockade with BQ-788 did not cause further reductions in fractional water excretion in the presence of SPP301.

Endothelin is known to have an important role in the regulation of salt and water excretion by the kidney (Kohan et al., 2011). For example, endothelin inhibits the action of vasopressin in the isolated inner medullary collecting ducts (IMCD) of the kidneys, an effect mediated by ET_B receptors (Nadler et al., 1992; Edwards et al., 1993). Medullary ET_B receptor activation induces diuretic and natriuretic responses through a nitric oxide synthase 1 (NOS1) and cyclic guanylate monophosphate (cGMP) pathway (Nakano et al., 2008). Intramedullary infusion of an ET_B antagonist has been shown to reduce urine flow rate to 68% at 30 min. Urinary sodium excretion starts to decline only after 20–30 min (Guo and Yang, 2006). Collecting duct-specific knockout of ET-1 increases urine concentrating effects of arginine vasopressin (AVP), while

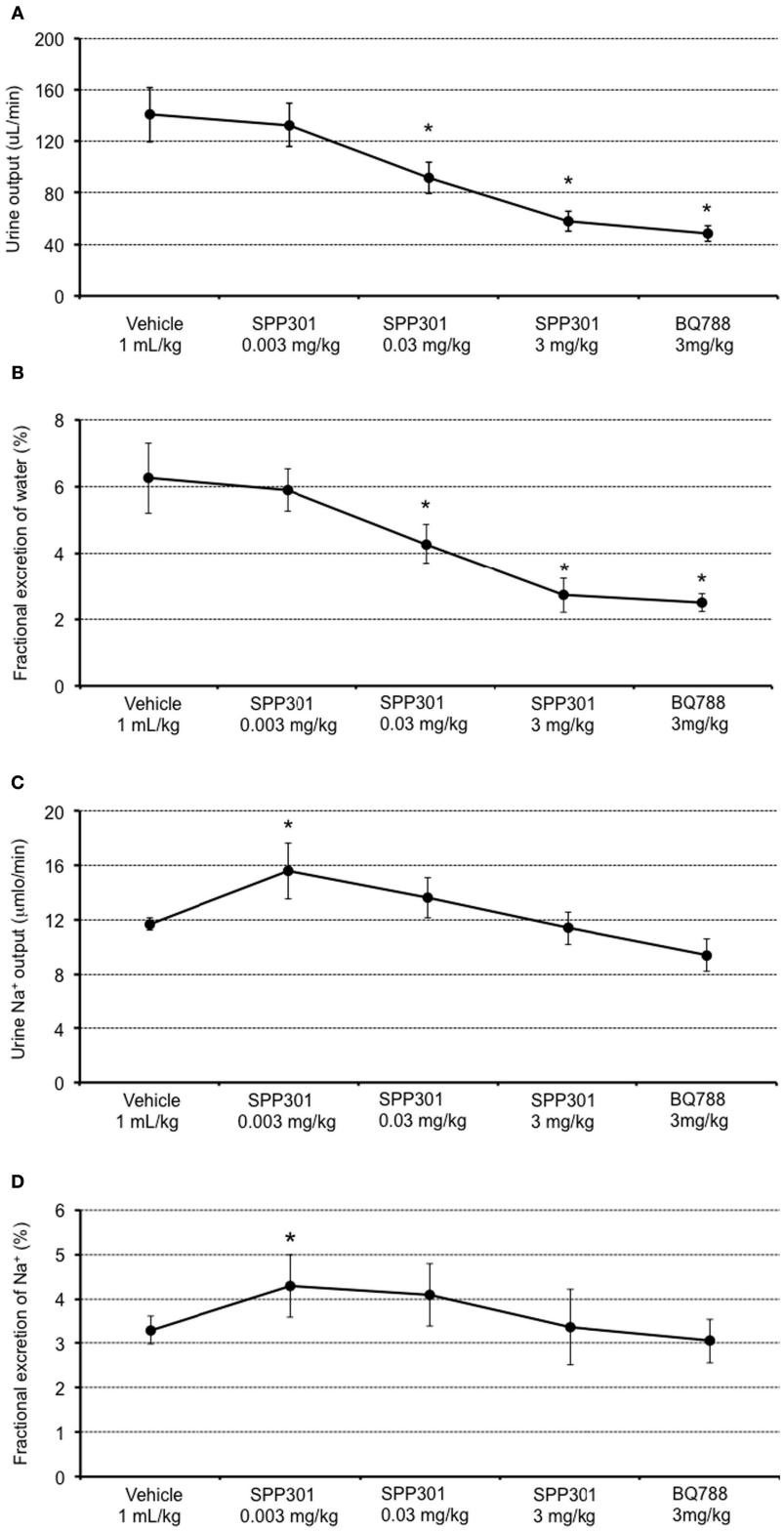

FIGURE 2 | Urine and sodium excretion measured by: (A) urine output (μL/min); (B) fractional excretion of water (%); (C) urine sodium output (μmol/min); and (D) fractional excretion of sodium (%); in male SD rats treated with incremental doses of SPP301 (0.003; 0.03; 3 mg/kg) followed by BQ-788 (3 mg/kg). Fractional excretion of water (percentage of water excreted by the kidney from the total amount filtered) = urine output/GFR. Fractional excretion of sodium (percentage of Na^+ excreted by the kidney from the total amount filtered) = urine Na^+ output/[Plasma (Na^+) × GFR]. *$p < 0.05$ compared with control (vehicle).

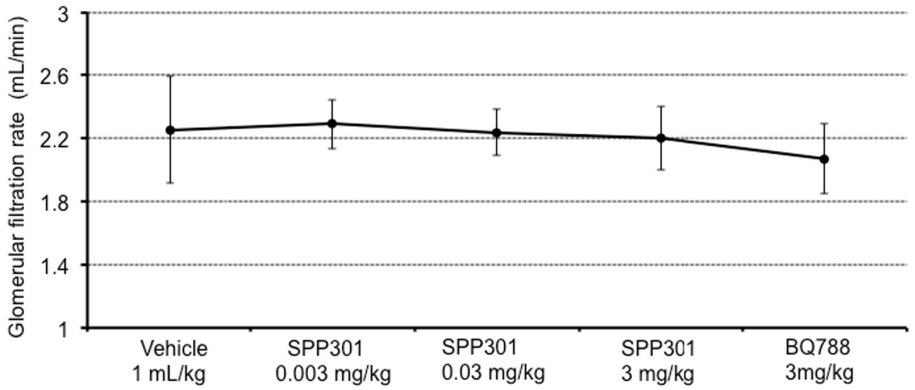

FIGURE 3 | Glomerular filtration rate (mL/min) in male SD rats treated with incremental doses of SPP301 (0.003; 0.03; 3 mg/kg) followed by BQ-788 (3 mg/kg).

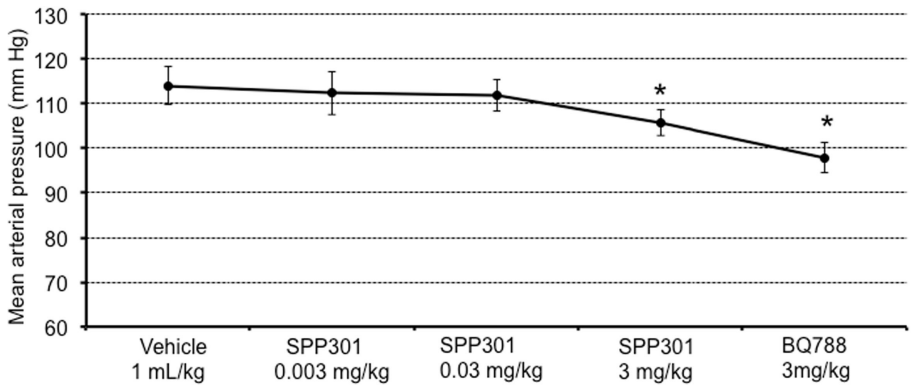

FIGURE 4 | Mean arterial pressure (mm Hg) in male SD rats treated with incremental doses of SPP301 (0.003; 0.03; 3 mg/kg) followed by BQ-788 (3 mg/kg). *$p < 0.05$ compared with control (vehicle).

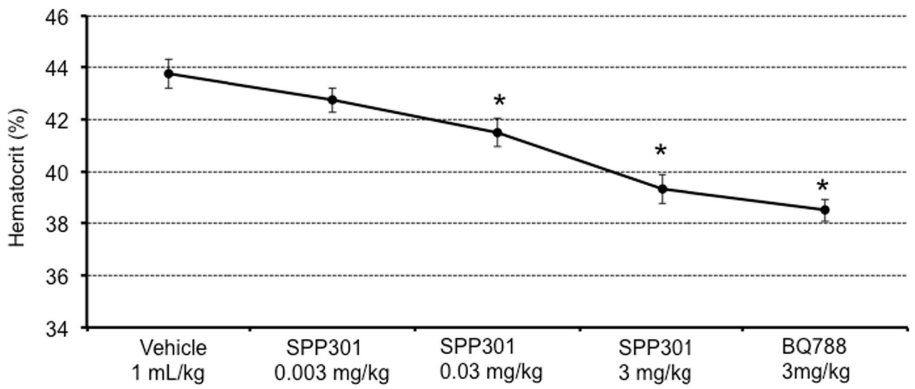

FIGURE 5 | Hematocrit (%) in male SD rats treated with incremental doses of SPP301 (0.003; 0.03; 3 mg/kg) followed by BQ-788 (3 mg/kg). *$p < 0.05$ compared with control (vehicle).

collecting duct-specific ET_A receptor knockout decreases renal sensitivity to AVP (Ge et al., 2005). These findings suggest that ET_B receptor activation in the collecting duct has diuretic effects and ET_A receptor activation has antidiuretic effects. These effects are mediated through altered renal sensitivity to the urinary concentrating effects of AVP.

ET_B receptor activation is thought to play an important role in the renal actions of some naturally occurring hormones. For example, relaxin has been localized in close proximity to the endothelin receptors in the systemic and renal vasculature as well as in the tubules of the kidney (Teichman et al., 2010). Relaxin binding to its receptor, RXFP1, results in activation of the ET_B receptor on endothelial cells. Activation of the ET_B receptor in the tubules of the kidney by relaxin may inhibit sodium/potassium ATPase, facilitating sodium and water secretion (Danielson et al., 1999; Dschietzig et al., 2003; Bogzil and Ashton, 2009). Accordingly, relaxin has been shown to increase clearance and urinary excretion of sodium in preclinical rat studies (Bogzil et al., 2005). Moreover, in the Pre-RELAX acute heart failure study, dyspnea relief correlated with BP reductions and increased weight loss in the relaxin treatment groups, supporting the hypothesis that relaxin may mediate natriuretic/diuretic effects (Teerlink et al., 2009).

Our results show that bolus i.v. administration of an ET_B receptor antagonist (BQ-788) reduces urine output and fractional excretion of water within 30 min of administration, but does not significantly alter urine sodium output or fractional excretion of sodium. These data indicate that endogenous ET-1 has a tonic diuretic effect mediated via renal ET_B receptors and may explain why fluid retention occurs during treatment with non-selective endothelin receptor blockers. Similarly, administration of a predominant ET_A receptor antagonist (SPP301) dose-dependently reduced urine output, fractional excretion of water and hematocrit. Since this effect does not appear to be mediated through blockade of ET_A receptors (which should promote diuresis), SPP301 may produce dose-dependent antidiuresis through non-specific blockade of ET_B receptors. Moreover, since one of the actions ascribed to the ET_B receptor is the clearance of ET-1 from circulation, its blockade may mediate an increased accessibility of ET-1 to ET_A receptors.

In addition to the induction of fluid retention by renal mechanisms discussed here, it has been hypothesized that endothelin receptor antagonists induce edema through increased capillary permeability and consequent extravasation of fluids. Results of a related study in anesthetized bi-nephrectomized male Wistar rats show that SPP301 blunted a decrease in hematocrit in a concentration-dependent manner compared with a control, with a plateau at 1 μM ($p < 0.05$ for the trend). These observations suggest that SPP301 induces a concentration-dependent vascular leakage of fluid in this model (Maillard et al., 2008). Together, these findings help to elucidate the mechanisms by which the predominant ET_A receptor antagonist SPP301 induces fluid retention.

High doses of SPP301 (25, 50 mg/day) have previously been reported to result in fluid overload and congestive heart failure in patients with diabetic nephropathy (Mann et al., 2010). In a follow-up explanatory mechanistic study in healthy subjects, a dose-dependent median reduction in the fractional renal excretion of sodium was found (up to 8.7% withSPP301 50 mg); this reduction was paralleled by a dose-related increase in proximal sodium reabsorption (Smolander et al., 2009). These clinical data together with our preclinical results suggest thatSPP301 may cause non-specific blockade of renal ET_B receptors at high concentrations, thereby causing fluid retention.

The results of this study indicate that increasing concentrations of SPP301 may block ET_B receptors in addition to ET_A receptors and cause antidiuresis, which could explain the occurrence of fluid retention and the abolishment of benefits mediated by ET_A receptor blockade observed in clinical trials (Smolander et al., 2009; Mann et al., 2010). Further studies are needed to clarify whether lower doses of ET_A receptor blockers may be associated with fewer renal adverse effects in clinical practice. In addition, further studies may help to clarify the potential therapeutic value of ET_B receptor activation.

ET_A receptor antagonists show promise in trials for the treatment of chronic pathologies associated with tissue remodeling, including diabetic kidney disease (Wenzel et al., 2009; Saleh et al., 2011). However whilst demonstrating benefits, these drugs also have adverse effects on fluid retention, leading to an altered risk–benefit ratio and limiting their clinical value (Kohan et al., 2011). Indeed, efficacy and safety are the main issues responsible for the high attrition rate in phase III clinical drug development (van Gool et al., 2010). Studying the complex mechanisms of drug actions and corroborating them in an integrative physiological context are necessary for a valid translation of medical research into clinical practice. Our study represents a concrete example of translational research, demonstrating that drug development is a two-way street requiring efficient interactions between preclinical and clinical research, and between pharmaceutical and academic scientists. These data, corroborated with our colleagues' data (Smolander et al., 2009), support further investigation of the antiproteinuric effect of SPP301 at adjusted doses.

ACKNOWLEDGMENTS

We would like to thank Jennifer Pollard of ACUMED® for providing writing assistance. Source of Funding: this work was supported by Speedel Holding AG and the São Paulo Research Foundation [Grant 2011/50078-0].

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Conflict of Interest Statement: Christoph Schumacher and Pat Louie are employed by Novartis Pharma AG, Basel, Switzerland. Radu Iliescu has received significant honoraria and Jane Reckelhoff has received a significant research grant from Speedel Holding AG, Basel, Switzerland. The remaining authors have no conflicts of interests.

Received: 06 March 2012; paper pending published: 27 March 2012; accepted: 02 April 2012; published online: 18 April 2012.

Citation: Baltatu OC, Iliescu R, Zaugg CE, Reckelhoff JF, Louie P, Schumacher C and Campos LA (2012) Antidiuretic effects of the endothelin receptor antagonist avosentan. Front. Physio. ***3****:103. doi: 10.3389/fphys.2012.00103*

This article was submitted to Frontiers in Integrative Physiology, a specialty of Frontiers in Physiology.