Myocardial, smooth muscle, nephron and collecting duct gene targeting reveals
the organ sites of endothelin A receptor antagonist fluid retention

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Running title: Site of endothelin A receptor antagonist fluid retention

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Nonstandard abbreviations:
CD – collecting duct
Cre – Cre recombinase
ECV – extracellular fluid volume
ETA – endothelin A receptor
ETB – endothelin B receptor
ET-1 – endothelin-1
Floxed – loxP-flanked
KO – knockout
rtTA – reverse tetracycline transactivator
TBW – total body water
VSMC – vascular smooth muscle cell

**Recommended section assignment:** Cardiovascular
Endothelin-1 binding to endothelin A receptors (ETA) elicits pro-fibrogenic, pro-inflammatory and proliferative effects that can promote a wide variety of diseases. While ETA antagonists are approved for the treatment of pulmonary hypertension, their clinical utility in several other diseases has been limited by fluid retention. ETA blocker-induced fluid retention could be due to inhibition of ETA activation in the heart, vasculature and/or kidney, consequently the current study was designed to define which of these sites are involved. Mice were generated with absence of ETA specifically in cardiomyocytes (heart), smooth muscle, the nephron, the collecting duct or no deletion (control). Administration of the ETA antagonist ambrisentan or atrasentan for two weeks caused fluid retention in control mice on a high salt diet as assessed by increases in body weight, total body water and extracellular fluid volume (using impedance plethysmography), as well as decreases in hematocrit (hemodilution). Mice with heart ETA knockout retained fluid in a similar manner as controls when treated with ambrisentan or atrasentan. Mice with smooth muscle ETA knockout had substantially reduced fluid retention in response to either ETA antagonist. Mice with nephron or collecting duct ETA disruption were completely prevented from ETA blocker-induced fluid retention. Taken together, these findings suggest that ETA antagonist-induced fluid retention is due to a direct effect of this class of drug on the collecting duct, is partially related to their vascular action, and is not due to alterations in cardiac function.
INTRODUCTION

Endothelin-1 (ET-1) likely plays a role in a wide variety of diseases, including arterial hypertension, pulmonary artery hypertension, atherosclerosis, myocardial infarction, cancer, systemic sclerosis, chronic proteinuric kidney disease, diabetes and others (Battistini et al. 2006). These effects of ET-1 are mediated through activation of endothelin A receptors (ETA) and endothelin B receptors (ETB). The relative contribution of ETA and ETB to disease pathology is incompletely understood, particularly in humans. However, the majority of experimental animal and in vitro studies suggest that ETA is the primary mediator of the detrimental effects of ET-1. ET-1 binding to ETA elicits a wide range of pathophysiologic responses, including proliferation, hypertrophy, extracellular matrix accumulation, fibrosis, increased vascular resistance, vascular calcification, insulin resistance, inflammation, and others (Barton and Yanagisawa 2008). Based on these pre-clinical findings, numerous clinical trials have been conducted using ET receptor antagonists in a variety of disorders, including congestive heart failure, pulmonary artery hypertension, arterial hypertension, systemic sclerosis, subarachnoid hemorrhage, chronic kidney disease (particularly diabetic nephropathy), and others (Barton and Yanagisawa 2008). Despite this, ET receptor blockers are approved for only pulmonary artery hypertension and prevention of digital ulcers in patients with systemic sclerosis (Barton and Kohan 2011).

One of the key reasons why ET receptor blockers are not more widely utilized is their propensity to cause fluid retention. Indeed, such fluid retention was the cause for discontinuation of a large trial on progression in patients with diabetic nephropathy.
(Mann et al., 2010) as well as the likely failure of several trials using ET receptor blockers in patients with congestive heart failure (Barton and Kohan 2011). In addition, ET receptor antagonist fluid retention occurs with relatively high frequency in studies in other patient populations, particularly when higher doses of these agents are employed (Barton and Kohan 2011). Initial studies with ET receptor antagonists utilized agents that had significant effects on the ETB receptor; given that this receptor has been clearly implicated in inhibiting Na and water reabsorption in the nephron (Kohan et al. 2011b), it is not surprising that its blockade was associated with edema and fluid overload. Subsequent trials with ET receptor antagonists with more ETA selectivity were also associated with fluid retention, however it was possible that, at the doses employed, some ETB blockade occurred (Barton and Kohan 2011). More recently, a number of clinical studies using highly selective ETA antagonists at doses that should have no effect on ETB have also noted fluid retention. Notably, administration of zibotentan, an ETA antagonist with no detectable ETB binding even at extremely high concentrations, caused up to a 48% incidence of edema compared to 10% in the placebo group in patients with prostate cancer (James et al. 2010). Thus, despite the possible therapeutic benefit of using an ETA selective antagonist over a non-selective ET receptor blocker, it appears that blocking ETA alone does not provide a clear benefit with regard to mitigating the fluid retention seen with this class of drugs (although a head-to-head comparison between ETA vs. ETB antagonist-induced fluid retention in humans has not been conducted).

It remains unclear how ETA antagonists promote edema and fluid overload. ETA blockade can cause vasodilation that in turn promotes fluid retention, however this
possibility has not been directly examined. In addition, activation of cardiomyocyte ETA increases cardiac contractility, hence it is possible that ETA blockade could be negatively inotropic and facilitate fluid retention. Although controversial, ETA receptors have been implicated in mediating inhibition of renal tubular Na reabsorption (Kohan et al. 2011b), thus ETA blockade might elicit fluid retention through a direct effect on the nephron. Given the uncertainty in this area, the current study was undertaken to identify the organ site(s) of ETA antagonist-induced fluid retention.

MATERIALS AND METHODS

Animal use assurance

All animal use and welfare adhered to the NIH Guide for the Care and Use of Laboratory Animals following protocol reviews and approval by the Institutional Laboratory Animal Care and Use Committee of the University of Utah Health Sciences Center.

Generation of cell-specific ETA knockout mice

Five lines of mice were developed: Floxed ETA, heart ETA KO, vascular smooth muscle cell (VSMC) ETA KO, collecting duct ETA KO, and nephron ETA KO (Figure 1). All mouse lines were maintained on C57/BL6 background. Floxed ETA mice are homozygous for loxP-flanked (floxed) exons 6-8 of the EDNRA gene (Ge et al. 2005). Collecting duct ETA KO mice were achieved by breeding floxed ETA mice with mice containing the 11 kb of the mouse aquaporin-2 gene 5′ flanking region driving
expression of Cre recombinase. These mice express Cre selectively within principal cells in the renal collecting duct; collecting duct ETA KO mice have an approximately 90-95% reduction in principal cell ETA mRNA content (Ge et al. 2005). Heart ETA KO mice were made by breeding floxed ETA mice with mice containing the □-myosin heavy chain (MHC) promoter driving Cre recombinase; this approach has been demonstrated to yield cardiomyocyte-selective targeting of ETA with approximately an 80% reduction of cardiac ETA mRNA and a marked reduction in binding of ET-1 to ETA in heart membrane preparations (Kedzierski et al. 2003). Mice with inducible nephron-specific ETA KO were achieved by breeding mice containing the Pax8-rtTA and LC-1 transgenes with mice containing floxed ETA as previously described (Stuart et al. 2012). The Pax8-rtTA transgene contains 4.3 kb of the Pax8 gene promoter along with exon 1, intron 1, exon 2 and part of intron 2 driving expression of the reverse tetracycline transactivator (rtTA) while the LC-1 transgene encodes tetracycline-inducible bicistronic Cre recombinase and luciferase. To obtain nephron ETA KO, mice were given 2 mg/ml doxycycline in 2% sucrose drinking water for 11 days, followed by 4 days off doxycycline (recovery period) before conducting physiologic studies. The nephron ETA KO mice have been demonstrated to have nephron-wide EDNRA gene recombination (Stuart et al. 2012). Vascular smooth muscle cell ETA KO mice were made by breeding floxed ETA mice with mice transgenic for the smooth muscle SM22 promoter driving Cre recombinase, as previously described (Kedzierski et al. 2003). These mice have Cre-mediated recombination in all smooth muscle cells, including those in the vasculature. All four cell-specific ETA KO mouse lines were homozygous for the floxed ETA allele and hemizygous for the transgenes.
Genotyping

Tail DNA was PCR amplified and the following primers were used for genotyping:

ETA - F 5’-CCCATGCTTAGACACAACCATG-3’ and R 5’-
GATGACAAACCAAGCAGAAAGACAG-3’ which yield a 364 bp product for the floxed
EDNRA gene (includes loxP site) and a 324 bp product for the wild type EDNRA gene;
Pax8-rtTA - F 5’-CCATGTCTAGACTGGACAAGA-3’ and R 5’-
CATCAATGTATCTTATCATGTCTGG-3’ which yields a 600 bp product; LC-1 - F 5’-
TCGCTGATTACCGTGGATGC-3’ and R 5’-CCATGAATGTAACGACCTGGTGGTGG-3’
which yields a 480 bp product; AQP2-Cre - F 5’-CCTCTGCAGGAACGACCTGGTGG-3’
and R 5’-GCGAACATCTTTCCAGTCTGG-3’ which yields a 671-bp product between
the mouse AQP2 promoter and the Cre gene; MHC-Cre - F 5’-
CGCATAACCATGAGAAACAGCATTGC-3’ and R 5’-
TTAGCAACCTAGCGACACTTACCCTAC-3’ which yields an 800 bp product; and SM22-Cre
- 5’-CAGACACCGAAGCTACTCTTCTTCC-3’ and R 5’-
CGCATAACCATGAGAAACAGCATTG-3’ which yields a 600 bp product.

Blood pressure monitoring

Blood pressure was monitored in all mouse lines by radiotelemetry (TA11-PAC10,
Data Sciences International, St. Paul, MN) with catheters inserted into the right carotid
artery. The mice were allowed to recover for 1 week after surgery. Blood pressure and
heart rate and were monitored during the baseline day and during the days one and two
week after starting drug or vehicle treatment. All mice were fed a high Na (3.2%) throughout the monitoring periods.

**Plasma volume**

Mice were anaesthetized and a catheter was placed in the jugular vein. Twenty-five µl of 2 mg/ml Evans blue was infused and 7 minutes later, approximately 600 µl of blood was withdrawn from the heart. Plasma was separated and absorbance determined at 620 nm. Plasma Evans blue concentrations were determined according to a standard curve generated by a serial dilution of the 2 mg/ml Evans blue-saline solution and plasma volume calculated according to standard dye-dilution formulae.

**Fluid retention analysis**

All mice were fed a high Na (3.2%) diet for 7 days. On the next day, body weight, hematocrit and body compartment fluid volumes were measured. Animals were weighed and an ~ 20 µl blood sample was obtained for determination of hematocrit. Body compartment fluid volume was determined by impedance plethysmography as previously described (Chapman et al. 2010). Briefly, mice were anesthetized and measured for length and width. Four needles were inserted under the skin at the base of the tail, the intercept between the front of the ears and the longitudinal midline, and 0.5 cm from these sites toward the tip of the tail and the nose, respectively. Leads from the needles were attached to the ImpediVet Vet BIS1 system (ImpediMed, San Diego, CA) which analyzes whole body bioimpedance data to determine total body water.
(TBW), extracellular fluid volume (ECV) and intracellular fluid volume. A resistance coefficient equal to 10% of that for rats was used for all mice studies.

All mice were then given either placebo or drug (ambrisentan or atrasentan) for two weeks while being maintained on the same high Na diet. Body weight, hematocrit, and body fluid compartment volumes were determined after 1 and 2 weeks of ETA receptor antagonist administration. Mice were given drugs daily by oral gel. Gel was made fresh daily and consisted of a ratio of 65.7 g Purina LD101 food, 1 package Knox brand gelatin, and 110 ml water. Drugs were dissolved in the gel to deliver 50-300 mg/kg/day ambrisentan (provided by Gilead Sciences, Foster City, CA) or 5-10 mg/kg/day atrasentan (provided by Abbott Laboratories, Abbott Park, IL) in 1 ml of gel (gel is placed into small plastic vials which are placed into the cage). Mice immediately consumed all of the gel, thereby assuring complete and accurate drug delivery.

**Statistical Analysis**

Data are presented as mean ± SE. Data from all genotypes were compared using Mood’s median test to control for a non-normal distribution. To determine if a specific genotype was different than floxed controls, the Mann-Whitney test was used which also does not assume a normal distribution. The criterion for significance was P < 0.05.

**RESULTS**

**Characteristics of mouse lines**
All mice (homozygous floxed ETA, heart ETA KO, VSMC ETA KO, nephron ETA KO and collecting duct ETA KO) gained weight at the same rate, had no gross morphologic abnormalities and lived to at least 1 year of age. All mice were studied at 3-4 months of age and care was taken to use an equal distribution (50:50) of males and females of each genotype. Nephron ETA KO mice were generated at 3-4 months of age and studied immediately after the doxycycline treatment protocol. Baseline weight, hematocrit and body fluid volume compartments were determined in each mouse line (Table 1). There were no significant differences between groups in baseline weight, hematocrit, ECV, TBW or plasma volume. Baseline mean, systolic and diastolic pressures were significantly reduced in VSMC ETA KO mice as compared to the other genotypes (Table 2). All baseline measurements were conducted after mice were fed a 3.2% Na diet for 7 days.

**Fluid retention analysis in floxed ETA mice**

Pilot studies were performed to determine whether ETA antagonists caused fluid retention in mice. Studies were first conducted with ambrisentan, an ETA antagonist with ETA:ETB selectively estimated at ~260:1 (Battistini et al., 2006). Initial studies measured the effect of ambrisentan on 24 hr urine volume and UNaV in wild type mice after 1, 2 or 4 weeks of treatment on either a normal (0.3% Na) or high (3.2%) salt diet using 50, 100, 200 or 300 mg/kg BW/d ambrisentan. No effect of ambrisentan on these urine parameters was detected (data not shown). Studies were then conducted under the same conditions, but using the more sensitive measures of changes in body fluid volume. Increases in body weight (Figure 2A), TBW (Figure 3A) and ECF (Figure 4A),
as well as decreases in hematocrit (Figure 5A), were most evident at 2 weeks of drug treatment, required being on a high Na diet, and were first evident at the 100 mg/kg/d dose (data for each dose and time point not shown). To minimize the chances of blocking the ETB receptor, this lowest dose of drug that caused detectable fluid retention was selected for further analysis. Note that all studies compared mice given ambrisentan with those given vehicle, thereby controlling for time, any stress associated with manipulation of the cage, or effects of high Na feeding. Finally, mean, systolic and diastolic pressures had a trend to decrease in all genotypes over the two week period of treatment with vehicle or ambrisentan (Table 2). Ambrisentan did not decrease arterial pressure compared to that seen with vehicle alone.

Evans blue dye dilution determination of plasma volume was assessed after 1, 2 or 4 weeks of 100 mg/kg/day ambrisentan for 2 weeks. No effect of ambrisentan on plasma volume in floxed ETA mice was observed (5.4 ± 0.3% of body weight at baseline and 5.6 ± 0.4% of body weight after 2 weeks of ambrisentan, N=10 each data point). Since the plasma volume studies required sacrificing the mice, they did not permit paired analysis and therefore reduced sensitivity of detecting changes in volume. Consequently, changes in fluid volume status were determined in subsequent studies without assessing plasma volume.

In order to support the specificity of the fluid retention due to ETA antagonism, a second ETA blocker, atrasentan, was utilized. This agent has a reported ETA:ETB specificity of ~1860:1 (Battistini et al. 2006). Initial studies were conducted as for ambrisentan, using 5-10 mg/kg/day atrasentan in floxed ETA mice. Atrasentan-induced fluid retention was first evident when mice were fed a high Na diet and treated with 10
mg/kg/day atrasentan for 2 weeks, hence this regimen was used in ensuing studies. Atrasentan induced increases in body weight (Figure 2B), TBW (Figure 3B) and ECV (Figure 4B), as well as decreases in hematocrit (Figure 5B) as compared to treatment with vehicle alone. Atrasentan did not reduce arterial pressures compared to that seen with vehicle alone (Table 2).

**Effect of cell-specific ETA knockout on ETA antagonist fluid retention**

The effects of 2 weeks of treatment with 100 mg/kg/day ambrisentan, 10 mg/kg/day atrasentan or their respective vehicles on fluid retention in mice with heart, vascular, nephron or collecting duct ETA KO fed a high Na diet was assessed by examining changes in body weight, TBW, ECV and hematocrit.

In mice with heart ETA KO, as compared to vehicle, ambrisentan increased body weight (Figure 2), TBW (Figure 3), and ECV (Figure 4), while it decreased hematocrit (Figure 5). The magnitude of these changes was similar to those seen in floxed ETA mice treated with ambrisentan. Similarly, in heart ETA KO mice, atrasentan, as compared to vehicle, increased body weight (Figure 6), TBW (Figure 7), and ECV (Figure 8), while it decreased hematocrit (Figure 9). The magnitude of these changes was similar to those seen in floxed ETA mice treated with atrasentan. Neither ambrisentan nor atrasentan reduced arterial pressure in heart ETA KO mice as compared to vehicle alone (Table 2).

In mice with VSMC ETA KO, ambrisentan had an insignificant effect on body weight, TBW, ECV or hematocrit as compared to vehicle (Figures 2-5, respectively). However, in the case of each of these parameters, the numerical change tended to be
greater in the ambrisentan-treated mice as compared to vehicle treated mice. Similarly, in VSMC ETA KO mice, atrasentan did not significantly alter body weight, TBW, ECV or hematocrit as compared to vehicle (Figures 6-9, respectively). The numerical change in hematocrit and TBW tended to be greater in the atrasentan-treated as compared to the vehicle-treated mice. Neither ambrisentan nor atrasentan reduced arterial pressure in VSMC ETA KO mice as compared to vehicle alone, although these values remained lower than those seen in floxed ETA mice (Table 2).

In mice with nephron duct ETA KO, as compared to vehicle, ambrisentan failed to alter body weight, TBW, ECV or hematocrit (Figures 2-5, respectively). Similarly, in nephron ETA KO mice, atrasentan, as compared to vehicle, did not change body weight, TBW, ECV or hematocrit (Figures 6-9, respectively). Further, there was no trend for either ambrisentan or atrasentan to cause fluid retention in these mice. Neither ambrisentan nor atrasentan reduced arterial pressure in nephron ETA KO mice as compared to vehicle alone (Table 2).

To potentially localize the nephron site of ETA antagonist-induced fluid retention, the effect of ETA blockers in collecting duct ETA KO mice was studied. In mice with collecting duct ETA KO, as compared to vehicle, ambrisentan did not affect body weight, TBW, ECV or hematocrit (Figures 2-5, respectively). Similarly, in collecting duct ETA KO mice, atrasentan, as compared to vehicle, did not change body weight, TBW, ECV or hematocrit (Figures 6-9, respectively). There was no trend for either ETA antagonist to induce fluid retention in these mice. Finally, neither ambrisentan nor atrasentan reduced arterial pressure in collecting duct ETA KO mice as compared to vehicle alone (Table 2).
DISCUSSION

The current study demonstrates that ETA antagonism in mice causes fluid retention as evidenced by an increase in body weight, total body water and extracellular fluid volume, as well as a fall in hematocrit. We found in pilot studies that measuring urinary Na and water excretion or assessing plasma volume by Evans blue dye dilution were insufficiently sensitive to detect changes in body volume homeostasis in mice, hence a combination of complementary methods were utilized to assess longitudinal alterations in fluid balance. Our findings are in agreement with numerous trials documenting weight gain, edema and/or decreased hematocrit in humans receiving ETA antagonists (Kohan et al. 2012). Of note, the fluid retention seen with this class of drugs is most evident within the first week or two of therapy (Kohan et al. 2011a; Kohan et al. 2012), hence the time period of drug exposure used in the current study (2 weeks) fits well with the expected time course of occurrence of this adverse event. The fall in hematocrit is typically about 5% and is seen within 1-2 weeks of initiation of ETA blocker therapy in humans, similar to the changes observed in the current study. Discontinuation of the drug in patients results in a prompt normalization of hematocrit (Kohan et al. 2011a; Kohan et al. 2012), indicating that the anemia is hemodilutional and therefore likely related to fluid retention. We also observed that mice had to ingest a high Na diet in order to manifest ETA antagonist-induced fluid retention, raising the possibility that use of a low Na diet may be beneficial in reducing or preventing this adverse event in patients. Finally, the fluid retention seen in the current study was
similar between ambrisentan and atrasentan, supporting the notion that such fluid retention is a class effect of ETA antagonists.

The main goal of the current study was to define the organ sites of ETA blocker-induced fluid retention. Cardiomyocyte-specific disruption of ETA yields mice that grow normally, do not manifest cardiac pathology under normal conditions (Kedzierski et al. 2003), and in fact are resistant to hyperthyroid cardiac hypertrophy (Shohet et al. 2004). Administration of ambrisentan or atrasentan to these mice resulted in fluid retention virtually identical to that seen in control mice, suggesting that cardiomyocyte ETA are not involved in ETA antagonist-induced fluid retention. One might argue that longer periods of ETA blockade might adversely affect cardiac function and promote fluid retention, however this does not seem likely given that the heart ETA KO mice have normal cardiac function. Another possibility is that the function of ETA is altered in the setting of congestive heart failure; this is certainly a possibility, however our study was not designed to test this. In general, given the increased risk of fluid overload in patients treated with ET receptor antagonists and the failure of ET blocker in congestive heart failure trials, it is unlikely that this class of drugs will be utilized in patients with known congestive heart failure.

We found that mice with absence of ETA in smooth muscle had modestly reduced arterial pressure as previously reported (Kedzierski et al. 2003). We refer to these mice as VSMC ETA KO for simplicity, however it is recognized that ETA is absent in all smooth muscle cells. How such ETA deficiency impacts function in other organ systems is not known, however the model seemed a reasonable approach to investigate whether vascular smooth muscle was involved in ETA blocker-induced fluid
retention. Both ambrisentan and atrasentan had substantially reduced fluid retention as compared to mice with intact ETA, although there was a trend towards fluid retention particularly in the ambrisentan-treated group. This protective effect of VSMC ETA KO is most likely due to an inability of ETA antagonists to vasodilate. It is well known that vasodilators, and particularly those that act directly upon arterioles, cause fluid retention manifested by weight gain and hemodilution; the fluid retention is presumably secondary to a fall in renal perfusion. Notably, we did not observe that either ETA blocker reduced blood pressure in control mice (or for that matter in any of the mouse lines), however this does not mean that vasodilation did not occur – a large variety of compensatory mechanisms could have obviated detection of a fall in blood pressure.

Nephron ETA KO has been reported to cause mild fluid retention in mice eating a high Na diet, suggesting that nephron ETA exerts a natriuretic/diuretic effect (Stuart et al. 2012). The fluid retaining effect of ambrisentan or atrasentan was completely prevented in nephron ETA KO mice. This suggests that some region(s) of the nephron is/are acted upon by ETA antagonists to promoter Na and water retention. Previous studies have clearly demonstrated that ET-1 can inhibit proximal tubule, thick ascending limb, and collecting duct Na and/or water reabsorption (Kohan et al. 2011b), hence we sought to further define where in the nephron ETA blockers were acting to elicit fluid retention. For this purpose, mice with collecting duct ETA KO were utilized. Similar to the nephron ETA KO mice, ambrisentan or atrasentan failed to elicit any detectable fluid retention in collecting duct ETA KO mice. Thus, ETA antagonist-induced fluid retention critically depends upon the presence of collecting duct ETA.
The current study was solely intended to localize the sites of ETA antagonist-induce fluid retention; it was not intended to define the mechanism(s) by which this occurs. However, given that the collecting duct appears to be crucial, it is relevant to discuss what is known about ETA in this nephron segment. Mice with collecting duct-specific ETA KO mice do not manifest alterations in blood pressure or urinary Na excretion, although small changes in volume regulation could have been missed (Ge et al. 2005). Mice with collecting duct ETB KO are modestly hypertensive and retain Na (Ge et al. 2006), while combined collecting duct ETA and ETB KO mice are significantly more hypertensive and retain more Na than mice with collecting duct ETB KO alone (Ge et al. 2008). In addition, renal medullary administration of ET-1 to rats deficient in ETB causes a natriuresis and diuresis and this effect is prevented by an ETA antagonist (Nakano and Pollock 2009). Furthermore, ETA blockade in intact rats reduces the natriuretic and diuretic response to renal medullary infusion of hyperosmotic saline (Boesen and Pollock 2010). Finally, ET-1 inhibition of amiloride-sensitive transepithelial Na flux \textit{in vitro} microperfused mouse collecting duct is prevented by ETA antagonism (unpublished data in collaboration with Charles Wingo laboratory at University of Florida). Thus, collecting duct ETA may well be capable of inhibiting Na and water transport. How such ETA regulation occurs may be complex – it is worth noting that viewing ETA vs. ETB effects may be too simplistic. For example, ETA/ETB receptor heterodimerization can occur and potentially affects receptor signaling and internalization (Evans and Walker 2008). Activation of ETB receptors in the collecting duct inhibits salt and water reabsorption through modulation of nitric oxide, \(\beta_1\)-Pix, phosphoinositides and other mechanisms (Kohan et al. 2011b), hence ETB receptor
interaction with ETA receptors could potentially affect fluid retention. In addition, ET isoform binding to ETA may be polyvalent, while antagonists, which may act in an allosteric manner, might differentially affect receptor function (De May et al. 2011). Hence, much work remains to dissect out the mechanisms by which ETA antagonists exert their effects in the collecting duct as well as elsewhere.

In summary, the current study suggests that ETA antagonist-induced fluid retention is primarily due to blockade of ETA in the collecting duct. In addition, ETA blocker-induced vasodilation also likely contributes to the fluid retention. The intent of this study was solely to identify the sites of ETA antagonist-induced fluid retention; our findings suggest that further examination of how ETA affects collecting duct function is warranted. In addition, our findings may ultimately be useful in defining diuretic choice when attempting to mitigate ETA blocker-induced fluid retention. Given that a number of clinical trials testing the efficacy of ETA blockers in a variety of disease are ongoing, the current study may particularly help in defining future studies that will ultimately inform how to best ameliorate or prevent fluid retention that is common to the use of all ETA antagonists.
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Authorship Contributions

Participated in research design: Stuart, Chapman, Kohan
Conducted experiments: Stuart, Chapman, Rees, Woodward
Contributed new reagents or analytical tools: Koesters
Performed data analysis: Stuart, Chapman, Kohan
Wrote or contributed to the writing of the manuscript: Kohan
References


Footnote:

This work was supported by grants from Abbott Laboratories and Gilead Sciences.

*Deborah Stuart and Mark Chapman shared equally in first authorship
Figure Legends

Figure 1. Schematic of gene targeted mice. Floxed ETA mice are homozygous for loxP-flanked exons 6-8 of the *EDNRA* gene. Vascular smooth muscle ETA knockout (KO), cardiomyocyte ETA KO, and collecting duct ETA KO mice were generated by breeding floxed ETA mice with mice transgenic for the smooth muscle SM22 promoter driving Cre recombinase, the cardiac myosin heavy chain (MHC) promoter driving Cre recombinase, or the aquaporin-2 promoter driving Cre recombinase, respectively. Nephron ETA KO mice were generated by breeding floxed ETA mice with mice transgenic for the Pax8 promoter driving the reverse tetracycline transactivator (rtTA) and transgenic for a heptamerized Tet07-mimimal cytomegalovirus promoter (CMV*) driving Cre recombinase; in the presence of doxycycline, rtTA binds to Tet07 and transactivates Cre expression in a nephron-wide manner. All mice that were studied were homozygous for the floxed *EDNRA* gene and hemizygous for the transgenes.

Figure 2. Effect of ambrisentan (100 mg/kg/day) (Panel A) or atrasentan (10 mg/kg/day) (Panel B) on body weight in floxed ETA mice and mice with cell-specific knockout of ETA in heart, collecting duct (CD), nephron or smooth muscle (vascular). Data are expressed as change in body weight comparing between baseline and after 2 weeks of drug treatment. N=14-16 per data point. *P < 0.05 vs. vehicle within genotype.

Figure 3. Effect of ambrisentan (100 mg/kg/day) (Panel A) or atrasentan (10 mg/kg/day) (Panel B) on total body water (TBW) in floxed ETA mice and mice with cell-specific
knockout of ETA in heart, collecting duct (CD), nephron or smooth muscle (vascular).

Data are expressed as change in TBW comparing between baseline and after 2 weeks of drug treatment. N=14-16 per data point. *P < 0.05 vs. vehicle within genotype.

Figure 4. Effect of ambrisentan (100 mg/kg/day) (Panel A) or atrasentan (10 mg/kg/day) (Panel B) on extracellular fluid (ECV) in floxed ETA mice and mice with cell-specific knockout of ETA in heart, collecting duct (CD), nephron or smooth muscle (vascular).

Data are expressed as change in ECV comparing between baseline and after 2 weeks of drug treatment. N=14-16 per data point. *P < 0.05 vs. vehicle within genotype.

Figure 5. Effect of ambrisentan (100 mg/kg/day) (Panel A) or atrasentan (10 mg/kg/day) (Panel B) on hematocrit in floxed ETA mice and mice with cell-specific knockout of ETA in heart, collecting duct (CD), nephron or smooth muscle (vascular). Data are expressed as change in hematocrit comparing between baseline and after 2 weeks of drug treatment. N=14-16 per data point. *P < 0.05 vs. vehicle within genotype.
Table 1. Baseline (pre-drug treatment) parameters in floxed and cell-specific ETA knockout mouse lines. All mice were fed a 3.2% Na diet for 7 days prior to baseline determinations. ECV – extracellular fluid volume; BW – body weight; KO – knockout; CD – collecting duct; VSMC – vascular smooth muscle cell.

<table>
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<th>Floxed ETA</th>
<th>Heart KO</th>
<th>VSMC KO</th>
<th>Nephron KO</th>
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<td>Weight (g)</td>
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<td>5.0 ± 0.3</td>
<td>5.4 ± 0.2</td>
<td>5.5 ± 0.5</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>Total body water (g)</td>
<td>14.3 ± 1.0</td>
<td>14.1 ± 0.7</td>
<td>14.9 ± 0.8</td>
<td>15.2 ± 1.1</td>
<td>14.6 ± 0.9</td>
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<tr>
<td>Plasma volume (% BW)</td>
<td>5.5 ± 0.3</td>
<td>5.4 ± 0.3</td>
<td>5.7 ± 0.4</td>
<td>5.8 ± 0.4</td>
<td>5.5 ± 0.2</td>
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<tr>
<td>N</td>
<td>16</td>
<td>14</td>
<td>15</td>
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Table 2. Arterial pressure in floxed and cell-specific ETA knockout mouse lines. All mice were fed a 3.2% Na diet for 7 days prior to baseline determinations, then given ambrisentan for 2 weeks while on the 3.2% Na diet. MAP – mean arterial pressure; KO – knockout; CD – collecting duct; VSMC – vascular smooth muscle cell. *P < 0.05 vs. floxed ETA. N=7-8 each group.

<table>
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<tr>
<th></th>
<th>Floxed ETA</th>
<th>Heart KO</th>
<th>VSMC KO</th>
<th>Nephron KO</th>
<th>CD KO</th>
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<tr>
<td><strong>Mean pressure (mmHg)</strong></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>112 ± 5</td>
<td>114 ± 2</td>
<td>104 ± 3</td>
<td>119 ± 5</td>
<td>118 ± 3</td>
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<tr>
<td>1 week</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>108 ± 4</td>
<td>109 ± 3</td>
<td>100 ± 2*</td>
<td>114 ± 4</td>
<td>114 ± 4</td>
</tr>
<tr>
<td>Ambrisentan</td>
<td>109 ± 5</td>
<td>110 ± 4</td>
<td>101 ± 3</td>
<td>112 ± 5</td>
<td>114 ± 6</td>
</tr>
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<td>2 weeks</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Vehicle</td>
<td>105 ± 3</td>
<td>106 ± 5</td>
<td>98 ± 2*</td>
<td>108 ± 3</td>
<td>110 ± 5</td>
</tr>
<tr>
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<td>104 ± 3</td>
<td>96 ± 3*</td>
<td>109 ± 4</td>
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<tr>
<td><strong>Systolic pressure (mmHg)</strong></td>
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<td>Baseline</td>
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<td>128 ± 4</td>
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<td>123 ± 3</td>
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<tr>
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<td><strong>Diastolic pressure (mmHg)</strong></td>
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<tr>
<td>Baseline</td>
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<td>106 ± 3</td>
<td>96 ± 2</td>
<td>109 ± 6</td>
<td>110 ± 6</td>
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<tr>
<td>1 week</td>
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<tr>
<td>Vehicle</td>
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<td>102 ± 4</td>
<td>93 ± 3*</td>
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<td>2 weeks</td>
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<tr>
<td>Vehicle</td>
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<td>90 ± 2*</td>
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</table>
Figure 1

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Figure 2
Figure 3

A

- Floxed
- Heart KO
- CD KO
- Nephron KO
- VSMC KO

B

- Floxed
- Heart KO
- CD KO
- Nephron KO
- VSMC KO

Change in TBW (g)

Vehicle
Ambrisentan

VSMC KO

Change in TBW (g)

Vehicle
Atrasentan

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Figure 5