Myocardial, Smooth Muscle, Nephron, and Collecting Duct Gene Targeting Reveals the Organ Sites of Endothelin A Receptor Antagonist Fluid Retention

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ABSTRACT

Endothelin-1 binding to endothelin A receptors (ETA) elicits profibrogenic, proinflammatory, and proliferative effects that can promote a wide variety of diseases. Although 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 2 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 the vascular action of the drugs, 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 preclinical 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 used 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 of other patient populations, particularly when higher doses of these agents are used (Barton and Kohan, 2011). Initial studies with ET receptor antagonists used agents that had significant effects on the ETB receptor; given that this receptor has been clearly implicated in inhibiting sodium 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 used, 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 with 10% in the placebo group in patients with prostate cancer (James et al., 2009). Thus, despite the possible therapeutic benefit of using an ETA selective antagonist over a nonselective 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 of ETA versus 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 sodium 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 National Institutes of Health 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: loxP-flanked (floxed) ETA, heart ETA knockout (KO), vascular smooth muscle cell (VSMC) ETA KO, collecting-duct ETA KO, and nephron ETA KO (Fig. 1). All mouse lines were maintained on C57/BL6 background. Floxed ETA mice are homozygous for 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 kilobases of the mouse aquaporin-2 gene 5′-flanking region driving expression of Cre recombinase (Cre). 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 α-myosin heavy chain 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-reverse tetracycline transactivator (rtTA) and LC-1 (bicistronic luciferase/Cre) transgenes with mice containing floxed ETA, as previously described (Stuart et al., 2012). The Pax8 (paired box gene 8)-rtTA transgene contains 4.3 kilobases of the Pax8 gene promoter along with exon 1, intron 1, exon 2, and part of intron 2 driving expression of the rtTA, whereas 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 physiologic studies were conducted. 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 polymerase chain reaction amplified, and the following primers were used for genotyping: ETA: F 5’-CCCATGCTTAGACACAACCATG-3’ and R 5’-GATGACAACCAAGCAGAACAGACAG-3’, which yields a 364-bp product for the floxed EDNRA gene.

![Fig. 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 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 rtTA and transgenic for a heptamerized Tet07 (heptamerized tetracycline operator)–minimal 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.](https://jpet.aspetjournals.org/doi/fig/10.1124/jpet.117.246139/supplmedia)
EDNRA gene (includes loxP site) and a 324-bp product for the wild-type EDNRA gene; F9-1: T 5'-CCATGCTCTGAGAAGCAGA-3' and R 5'-CATCAATGTATCTTTATCGTGG-3', which yields a 600-bp product; LC-1: F 5'-TCGTCGATCCACGGGATGTCG-3' and R 5'-CCATGAGTGAACGAACCTGGTCG-3', which yields a 480-bp product; AQP2 (aquaporin-2-Cre: F 5'-CTCTGCGAGAATTGGTGCTGG-3' and R 5'-GCCGAACACCTTCGTTACTCGG-3', which yields a 671-bp product between the mouse AQP2 promoter and the Cre gene; myosin heavy chain-Cre: F 5'-GCCATACCGCTCCGCAGTGGACGT-3' and R 5'-TTAGCAACCTCAGCCACCTTAC-3', which yields an 800-bp product; and SM22-Cre: F 5'-CCAGACACCGAGGTCATCCTCC-3' and R 5'-GCCATAACCGAGTAAAAGGACCTTG-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 were monitored during the baseline day and during the days 1 and 2 weeks after starting drug or vehicle treatment. All mice were fed a high-sodium (3.2%) diet throughout the monitoring periods.

**Plasma Volume.** Mice were anesthetized, and a catheter was placed in the jugular vein. Twenty-five microliters 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 was calculated according to standard dye-dilution formulae.

**Fluid Retention Analysis.** All mice were fed a high-sodium (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 intercostal 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 2 weeks while being maintained on the same high-sodium 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 of Purina LD101 food (Purina TestDiet, Richmond, IN), 1 package of Knox brand gelatin (Kraft Foods, Northfield, IL), and 110 ml of 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 ensuring complete and accurate drug delivery.

**Statistical Analysis.** Data are presented as the mean ± S.E. 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 from 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 with the other genotypes (Table 2). All baseline measurements were conducted after mice were fed a 3.2% sodium 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-hour urine volume and urinary sodium excretion in wild-type mice after 1, 2, or 4 weeks of treatment on either a normal (0.3% sodium) or high-salt (3.2%) diet using 50, 100, 200, or 300 mg/kg body weight per day ambrisentan. No effect of ambrisentan on these urine parameters was detected (unpublished data). Studies were then conducted under the same conditions, but using the more sensitive measures of changes in body fluid volume. Increases in body weight (Fig. 2A), TBW (Fig. 3A), and extracellular fluid volume (Fig. 4A), as well as decreases in hematocrit (Fig. 5A), were most evident at 2 weeks of drug treatment, required being on a high-sodium diet, and were first evident at the 100 mg/kg/day dose (data for each dose and time point not shown). To minimize the chances of blocking

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Baseline (pre-drug treatment) parameters in floxed and cell-specific ETA knockout mouse lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>All mice were fed a 3.2% sodium diet for 7 days prior to baseline determinations.</td>
<td></td>
</tr>
<tr>
<td><strong>Floxed ETA</strong></td>
<td><strong>Heart KO</strong></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>25.3 ± 0.5</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>48.3 ± 0.6</td>
</tr>
<tr>
<td>ECV (g)</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>TBW (g)</td>
<td>14.3 ± 1.0</td>
</tr>
<tr>
<td>Plasma volume (% BW)</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>N</td>
<td>16</td>
</tr>
</tbody>
</table>

BW, body weight; CD, collecting duct.
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-sodium feeding. Finally, mean, systolic, and diastolic pressures had a trend to decrease in all genotypes over the 2-week period of treatment with vehicle or ambrisentan (Table 2). Ambrisentan did not decrease arterial pressure compared with that seen with vehicle alone.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean pressure (mm Hg)</th>
<th>Systolic pressure (mm Hg)</th>
<th>Diastolic pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floxed ETA</td>
<td>112 ± 5</td>
<td>129 ± 3</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>Heart KO</td>
<td>114 ± 2</td>
<td>128 ± 4</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>VSMC KO</td>
<td>104 ± 3*</td>
<td>114 ± 2*</td>
<td>96 ± 3*</td>
</tr>
<tr>
<td>Nephron KO</td>
<td>119 ± 5</td>
<td>129 ± 5</td>
<td>109 ± 4</td>
</tr>
<tr>
<td>CD KO</td>
<td>118 ± 3</td>
<td>125 ± 4</td>
<td>110 ± 6</td>
</tr>
</tbody>
</table>

CD, collecting duct; MAP, mean arterial pressure.
*P < 0.05 versus floxed ETA.

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** Effect of ambrisentan (100 mg/kg/day) (A) or atrasentan (10 mg/kg/day) (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 between baseline and after 2 weeks of drug treatment. N = 14–16 per data point. *P < 0.05 versus vehicle within genotype.
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.

To support the specificity of the fluid retention due to ETA antagonism, a second ETA blocker, atrasentan, was used. 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-sodium 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 (Fig. 2B), TBW (Fig. 3B), and ECV (Fig. 4B), as well as decreases in hematocrit (Fig. 5B), as compared with treatment with vehicle alone. Atrasentan did not reduce arterial pressures compared with 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-sodium diet were assessed by examining changes in body weight, TBW, ECV, and hematocrit.

In mice with heart ETA KO, as compared with vehicle, ambrisentan increased body weight (Fig. 2), TBW (Fig. 3), and ECV (Fig. 4), whereas it decreased hematocrit (Fig. 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 with vehicle, increased body weight (Fig. 2), TBW (Fig. 3), and ECV (Fig. 4), whereas it decreased hematocrit (Fig. 5). 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 with vehicle alone (Table 2).

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

In mice with neprhon duct ETA KO, as compared with vehicle, ambrisentan failed to alter body weight, TBW, ECV,
or hematocrit (Figs. 2–5, respectively). Similarly, in nephron ETA KO mice, atrasentan, as compared with vehicle, did not change body weight, TBW, ECV, or hematocrit (Figs. 2–5, 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 with 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 with vehicle, ambrisentan did not affect body weight, TBW, ECV, or hematocrit (Figs. 2–5, respectively). Similarly, in collecting-duct ETA KO mice, atrasentan, as compared with vehicle, did not change body weight, TBW, ECV, or hematocrit (Figs. 2–5, 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 with 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 decrease in hematocrit. In pilot studies, we found that measuring urinary sodium and water excretion and 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 used 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, 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 decrease 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, 2012), indicating that the anemia is hemodilutional and therefore likely related to fluid retention. We also observed that mice had to ingest a high-sodium diet to manifest ETA antagonist–induced fluid retention, raising the possibility that use of a low-sodium 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 used 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 with mice with intact ETA, although there was a trend toward 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 decrease 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 decrease in blood pressure.

Nephron ETA KO has been reported to cause mild fluid retention in mice eating a high-sodium 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 promote sodium and water retention. Previous studies have clearly demonstrated that ET-1 can inhibit proximal tubule, thick ascending limb, and collecting-duct sodium 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 used. 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–induced 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 do not manifest alterations in blood pressure or urinary sodium excretion, although small changes in volume regulation could have been missed (Ge et al., 2005). Mice with collecting-duct

Fig. 5. Effect of ambrisentan (100 mg/kg/day) (A) or atrasentan (10 mg/kg/day) (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 between baseline and after 2 weeks of drug treatment. $N = 14–16$ per data point. $^*P < 0.05$ versus vehicle within genotype.
ETB KO are modestly hypertensive and retain sodium (Ge et al., 2006), whereas combined collecting-duct ETA and ETB KO mice are significantly more hypertensive and retain more sodium 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 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 sodium flux in vitro microperfused mouse collecting duct is prevented by ETA antagonist (Lynch et al., 2013). Thus, collecting-duct ETA may well be capable of inhibiting sodium and water transport. How such ETA regulation occurs may be complex—it is worth noting that viewing ETA versus 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, β1-Pix, phosphoinositides, and other mechanisms (Kohan et al., 2011b), hence ETB receptor interaction with ETA receptors could potentially affect fluid reten-
tion. In addition, ET isoform binding to ETA may be polyvalent, whereas antagonists, which may act in an allosteric manner, might differently affect receptor function (De Mey 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 antig-
aint–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.

Acknowledgments

The authors gratefully acknowledge Masashi Yanagisawa at the University of Texas Southwestern for providing the floxed ETA mice, Ralph Shohet at the University of Hawaii for the cardiomyocyte-specific ETA knockout mice, and Robert Koesters at INSERM/Université Pierre et Marie Curie for the Pax8-rtTA/LC-1 mice.

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