ET_B receptor-deficient rats exhibit reduced contraction to ET-1 despite an increase in ET_A receptors

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Several disease states, including hypertension, are associated with elevations in plasma endothelin-1 (ET-1) and variable changes in vascular contraction to ET-1. The spotting lethal (sl) rat carries a deletion of the endothelin-B (ET_B) receptor gene that prevents expression of functional ET_B receptors, resulting in elevated plasma ET-1. On a normal diet, these rats are normotensive and thus provide an opportunity to study the vascular effects of chronically elevated ET-1 in the absence of hypertension. Studies were performed in rats homozygous for the ET_B deficiency (sl/sl; n = 8) and in transgenic rats heterozygous for the ET_B deficiency (sl/+; n = 8). Plasma ET-1 was elevated in sl/sl rats (3.85 ± 0.55 pg/ml) compared with sl/+ rats (0.31 ± 0.11 pg/ml). Mean arterial blood pressure in conscious unrestrained sl/sl and sl/+ rats was 101 ± 5 and 107 ± 6 mmHg, respectively. Concentration-dependent contractions to ET-1 (10⁻¹¹–10⁻⁸ M) were reduced in mesenteric small arteries (150–250 μm) from sl/sl rats, as indicated by an ~10-fold increase in EC₅₀. A selective ET_A antagonist, A-127722 (30 nM), abolished contraction to ET-1 in both groups, whereas a selective ET_A antagonist had no effect. Also, ET_A agonists (IRL-1620 and sarafotoxin 6c) produced neither contraction nor relaxation in either group, indicating that contraction to ET-1 is exclusively ETA dependent. De-endothelization, dog and rat pulmonary circulation, porcine skin, rabbit saphenous vein, and the guinea pig mesenteric vein (7, 15, 23, 24, 28, 33). ET_B receptors are also located on the vascular endothelium and mediate vasodilation through release of nitric oxide and prostacyclin (5). Intravenous administration of ET-1 in rats causes a transient decrease in arterial pressure, mediated by ET_B receptors, followed by a sustained increase in arterial pressure, mediated predominantly by ETA receptors (4, 24). Another important action attributed to ET_B receptors is clearance of ET-1 from the circulation (9).

The spotting lethal (sl) rat carries a naturally occurring deletion in the ET_B receptor gene that prevents expression of a functional ET_B receptor (10). The null mutation of the ET_B receptor gene in spotting lethal rats causes aganglionic megacolon and death by ~1 mo of age (10). However, spotting lethal rats can be rescued using a dopamine β-hydroxylase (DBH) promoter to direct ET_B transgene expression to support normal enteric nervous system development (12). The rescued ET_B receptor-deficient rat has been used in several studies (11, 18, 26) investigating the pathophysiological roles of ET_B receptors. ET_B-deficient rats exhibit high levels of plasma ET-1, presumably due to the lack of clearance of ET-1 by ET_B receptors (11).

ET-1 has been proposed to be a local rather than a systemic regulating factor. However, increases in plasma ET-1 have been reported in a wide variety of pathophysiological conditions including hypertension (27). Reports on vascular contraction to ET-1 in the presence of elevated ET-1 in pathophysiological condi-

ENDOTHELIN-1 (ET-1) is a potent vasoconstrictor peptide thought to contribute to several cardiovascular dis-

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tions have been inconsistent. Contraction to ET-1 was shown to be increased in rat hearts during ischemia-reperfusion and in the rat pulmonary circulation in pulmonary hypertension (2, 8, 21), decreased in mesenteric arteries of deoxycorticosterone acetate (DOCA)-salt hypertensive rats (6, 13), and unchanged in the aorta of spontaneously hypertensive rats (3). Because hypertension does not develop in the ETB receptor-deficient rat on a normal diet, this is a good model for studying the effects of chronically elevated plasma ET-1 on vascular contraction in the absence of hypertension. Studies were designed to determine the influence of ETB receptor deficiency and the associated chronic elevations in plasma ET-1 levels on ET-1-induced contraction of mesenteric small arteries [150- to 250-μm intraluminal diameter (ID)]. ET-1 concentration-response curves were performed in the absence and presence of selective ETA and ETB receptor antagonists, and ETα receptor protein expression was determined in mesenteric small arteries from rats homozygous (sl/sl) and heterozygous for the ETB deficiency (sl/+).

METHODS

Animals. Gariepy et al. (12) developed DBH-ETβ transgenic rats as previously described. We established a colony of transgenic rats from which the sl/sl and sl/+ rats were obtained through sl/sl and sl/+ breeding pairs originally obtained from Drs. Gariepy and Yanagisawa (University of Texas Southwestern Medical Center, Dallas, TX). In the present study, male sl/+ and sl/sl rats (240–260 g and 11–12 wk old) were used. Heterozygous rats have pigmented heads, backs, and tails, whereas homozygous rats have pigmented coats only in small spots on their heads.

Telemetry measurements of mean arterial blood pressure. Telemetry transmitters (Data Sciences; St. Paul, MN) were implanted in the abdominal aorta according to the manufacturer's specifications while rats were under pentobarbital sodium anesthesia (65 mg/kg ip, Abbott Laboratories; North Chicago, IL) as previously described (25). After the transmitters were implanted, rats were allowed to recover from surgery and returned to individual housing for at least 1 wk before data acquisition was initiated.

Plasma ET-1. Plasma ET-1 concentrations were determined by luminometric ELISA (R&D Systems; Minneapolis, MN). Rats were anesthetized with pentobarbital sodium (65 mg/kg ip), and arterial blood samples (~1 ml) were immediately collected from the abdominal aorta into one tube with EDTA as the anticoagulant. Plasma (100 μl/well) was used in the assay with no extraction procedure needed. The sensitivity of the assay is 0.16 pg/ml. The intra-assay variation is 2.5%, and the inter-assay variation is 8.5%. The assay recognizes both natural and synthetic human and rat ET-1. There is limited cross-reactivity to ET-2 (27.4%) and ET-3 (7.8%), and no cross-reactivity to the other ET peptides.

Vascular reactivity. After arterial blood samples were collected, a section of the small intestine (~2 cm below the stomach was clamped and removed with the mesentery intact. Tissue was placed in chilled oxygenated (20% O2-5% CO2-balance N2) Krebs-Ringer bicarbonate solution [composed of (in mM) 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11.1 dextrose]. Mesenteric small arteries (150–250 μM) were isolated for continuous measurement of ID at a constant intraluminal pressure of 40 mmHg, and vascular contraction was assessed with a video dimension analyzer (Living Systems Instrumentation) as previously described (13). In most experiments, vessels were isolated within 0.5 h of removal of the mesentery. For comparison, some experiments were performed in vessels isolated 24 h after removal of the mesentery. Concentration-response curves to ET-1 (3 × 10–12–3 × 10–8 M, Sigma; St. Louis, MO) were performed in the absence or presence of the selective ETA receptor antagonist A-127722 (30 nM, Abbott Laboratories) (24) or the selective ETB receptor antagonist A-196261 (30 nm, Abbott Laboratories) (22). Antagonists were added to the vessel bath 30 min before the concentration-response curve to ET-1 was performed. At the end of the concentration-response curve to ET-1, the endothelium-dependent vasodilator acetylcholine (ACH; 10–5 M, Sigma) was added to assess endothelial function. If contraction to ET-1 was not observed, KCl (50 mM, Sigma) was added at the end of the concentration-response curve to verify vascular contractility. Vascular contraction to the selective ETβ receptor antagonists IRL-1629 (10–10–10–6 M, American Peptide; Sunnyvale, CA) and sarafotoxin 6c (S6c; 10–12–10–9 M, American Peptide) was assessed in untreated vessels, whereas vascular relaxation to the same agents was assessed in vessels precontracted 35–45% of baseline ID of the isolated vessel with an intraluminal pressure of 40 mmHg with phenylephrine (10–6 M, Sigma). Only one experiment was performed per vessel, and each experiment was performed only once per rat.

Western immunoblotting. In some rats, a section of mesentery adjacent to that obtained for isolation of mesenteric small arteries for vascular reactivity was removed and placed in ice-cold homogenizing buffer [composed of (in mM) 2 EDTA, 2 EGTA, 250 sucrose, and 50 MOPS; pH 7.4]. Mesenteric small arteries of similar size as those used for vascular reactivity were isolated and placed in homogenizing buffer. The vessels were homogenized with a glass/glass homogenizer in the presence of protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM leupeptin, 0.1% aprotonin, and 1 μM pepstatin A; Sigma]. The homogenate was centrifuged at 100,000 g for 30 min at 4°C. The precipitated proteins were homogenized and resuspended in buffer [composed of (in mM) 50 Tris·HCl, 0.1 EDTA, 0.1 EGTA, 250 sucrose, 0.1% β-mercaptoethanol (BME), 10% glycerol, and protease inhibitors; pH 7.4]. The protein concentration was determined by the Bradford method (Bio-Rad protein assay). Sample buffer [625 mM Tris (pH 6.8), 20% SDS, 25% glycerol, 0.15% bromphenol blue, and 5% BME] was added (1.5 dilution) to 60 μg of protein for loading on a 10% SDS-polyacrylamide gel for electrophoresis after electroblotting onto a polyvinylidene difluoride membrane. The blots were blocked with Tris-buffered saline (TBS; 10 mM Tris and 150 mM NaCl; pH 7.4)-0.1% Tween 20–5% nonfat milk and incubated overnight in TBS-Tween 20–5% nonfat milk with an anti-ETα receptor polyclonal antibody (1:200 dilution, Alamone Labs; Jerusalem, Israel) (22). This site-specific antibody was directed against amino acids 413–426 of the rat ETα receptor. The secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:4,000 dilution, Amersham Pharmacia Biotech; Piscatway, NJ). Additional Western blot analysis was performed using the presence of a competing peptide for the site-specific ETα receptor antibody (Alamone Labs). The competing peptide was preincubated with the ETα receptor antibody (1 μg antibody to 0.75 μg competing peptide) for 1 h at room temperature before overnight incubation of blots. The blots were developed using enhanced chemiluminescence, as recommended by the manufacturer (Amersham Pharmacia Biotech).
Data analysis. ID measurements obtained from mesenteric small arteries were expressed in micrometers. Vasoconstrictor and vasodilatory responses in isolated vessels were expressed as percent contraction or percent relaxation, respectively. EC50 values were calculated from each concentration-response curves using Prism GraphPad (San Diego, CA). All data are reported as means ± SE. Statistical differences were determined by analysis of variance for repeated measures followed by Student’s modified t-test with Bonferroni correction for multiple comparisons.

RESULTS

Plasma ET-1 and mean arterial blood pressure. Plasma ET-1 levels were elevated~10-fold in sl/sl rats compared with sl/+ rats (Table 1). Mean arterial blood pressure (averaged over a 24-h period) and baseline mesenteric small artery ID (in vitro at an intraluminal pressure of 40 mmHg) were not significantly different between sl/sl and sl/+ rats (Table 1).

Vascular reactivity. ET-1 (3 × 10⁻¹²–3 × 10⁻⁸ M) produced a concentration-dependent contraction of mesenteric small arteries isolated from sl/+ and sl/sl rats (Fig. 1). The maximum contraction to ET-1 was similar between groups (71 ± 3% vs. 65 ± 8% in vessels from sl/+ and sl/sl rats, respectively). However, the EC50 was significantly increased in vessels from sl/sl rats (18 ± 6 × 10⁻¹⁰ M) compared with sl/+ rats (1.8 ± 0.5 × 10⁻¹⁰ M). Addition of ACh (10⁻⁵ M) at the end of the concentration-response curve to ET-1 resulted in a 73 ± 7% and 78 ± 5% relaxation of vessels from sl/+ and sl/sl rats, respectively. Concentration-response curves were also performed in the presence of selective ETₐ and ETₕ receptor antagonists. The selective ETₐ receptor antagonist A-127722 abolished contraction to ET-1 in vessels from both groups of animals (Fig. 1). However, contraction to KCl (50 mM) was unchanged in the presence of A-127722 in vessels from sl/+ (65 ± 6%) and sl/sl rats (72 ± 5%) (data not shown). In contrast, the selective ETₕ antagonist A-196261 had no effect on ET-1-mediated contraction in vessels from either group of animals (Fig. 2). Neither A-127722 nor A-196261 altered baseline ID before the addition of ET-1.

To determine if desensitization to ET-1 contributed to the decreased responsiveness to ET-1 in vessels from sl/sl rats, concentration-response curves to ET-1 were performed in small mesenteric arteries isolated from sl/sl rats. Values are means ± SE.

Table 1. Plasma ET-1, MABP, and mesenteric small artery ID in sl/+ and sl/sl rats

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<th>sl/+ Rats</th>
<th>sl/sl Rats</th>
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<td>Plasma ET-1, pg/ml</td>
<td>0.31 ± 0.11</td>
<td>3.85 ± 0.55*</td>
</tr>
<tr>
<td>MABP, mmHg</td>
<td>107 ± 6</td>
<td>101 ± 5</td>
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<tr>
<td>Mesenteric small artery ID, μm</td>
<td>219 ± 12</td>
<td>223 ± 19</td>
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Values are means ± SE. ET-1, endothelin-1; MABP, mean arterial blood pressure; ID, intraluminal diameter; sl/+ rats, transgenic rats heterozygous for ET₁ receptor deficiency; sl/sl rats, rats homozygous for ET₁ receptor deficiency. *P < 0.05 vs. sl/+ rats.

Fig. 1. Concentration-response curve to endothelin-1 (ET-1) in the presence or absence of the selective ETₐ receptor antagonist A-127722 (30 nM). Percent contraction from baseline intraluminal diameter (ID) at a constant intraluminal pressure of 40 mmHg from heterozygous (sl/+ ) and homozygous (sl/sl) rats is shown. Values represent means ± SE. *Significant difference from sl/+ rats (P < 0.05).

Fig. 2. Concentration-response curve to ET-1 in the presence or absence of the selective ETₕ receptor antagonist A-196261 (30 nM) in mesenteric small arteries from sl/+ (A) and sl/sl (B) rats. Values represent means ± SE.
mesentery that had been stored in cold Krebs buffer in the absence of ET-1 for 24 h. As shown in Fig. 3, contraction to ET-1 was not altered under these conditions. Similar experiments were performed in sl/+ rats to confirm that storage for 24 h did not alter contraction to ET-1. There were no significant differences in the contraction to ET-1 in sl/+ vessels dissected at 0.5 or 24 h (data not shown).

**ETB receptor-mediated contraction and relaxation of small mesenteric arteries** was measured with the selective ETB receptor agonists S6c and IRL-1620 in vessels from sl/+ and sl/sl rats. Neither S6c (10^{-12}–10^{-9} M) nor IRL-1620 (10^{-10}–3 \times 10^{-6} M) produced significant contraction of mesenteric small arteries from sl/+ or sl/sl rats. However, contraction to KCl (50 mM) remained intact in both groups. These results are summarized in Fig. 4A. After precontraction with phenylephrine (10^{-6} M), neither S6c (10^{-12}–10^{-9} M) nor IRL-1620 (10^{-10}–3 \times 10^{-6} M) produced significant relaxation of vessels from sl/+ or sl/sl rats, whereas relaxation to ACh (10^{-5} M) remained intact (Fig. 4B).

**Western immunoblotting.** After membrane protein from mesenteric small arteries of sl/+ and sl/sl rats was isolated, ET_A receptor protein density was determined with Western blot analysis. A typical blot, shown in Fig. 5A, demonstrates elevated ET_A receptor protein expression in vascular membrane protein from sl/sl rats compared with sl/+ rats. The a-actin protein band on the same blot was similar between the two groups, demonstrating equal protein loading. Furthermore, a competing peptide for the ET_A receptor antibody completely abolished the ET_A receptor band, as indicated on a separate blot, demonstrating specificity of the antibody (data not shown). Densitometry was performed and showed that ET_A receptor protein density was increased by 64 \pm 14\% in sl/sl rats compared with sl/+ rats (P < 0.05; Fig. 5B).

**DISCUSSION**

The spotting lethal rat carries a naturally occurring 301-bp deletion in ETB receptors that causes intestinal obstruction, leading to death shortly after birth due to a lack of ETB-mediated development of enteric neurons (10). A transgenic rat was developed in which the D\betaH promoter was used to direct ETB expression in the neural crest-derived enteric nervous system precursors. These “rescued” rats, which develop normal enteric nervous systems and live into adulthood, were used in the present study (12). Rats homozygous for the ETB deficiency with the D\betaH-ETB transgene express ETB receptors in adrenergic tissues but exhibit an absence of detectable ETB mRNA in the glomeruli, renal tubules, and vasculature of the kidney and in the pulmonary vasculature (11, 14). Additionally, expression of ETB receptors was dramatically reduced in the brain and intestine of sl/sl rats (10).

In the present study, plasma ET-1 levels were increased by \sim 10\text{-fold}, whereas arterial pressure was not elevated, in homozygous rats. This confirms previous reports (11, 26) showing that plasma ET-1 levels are elevated in homozygous rats, whereas arterial blood pressure was not altered.

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**Fig. 3.** Concentration-response curve to ET-1 performed in mesenteric small arteries from sl/sl rats kept in cold Krebs buffer for 0.5 or 24 h after removal of the mesentery. Values represent means \pm SE.

**Fig. 4.** A: percent contraction of mesenteric small arteries from sl/+ and sl/sl rats produced by the selective ETB agonists IRL-1620 (3 \times 10^{-6} M) or sarafatoxin 6c (S6c; 10^{-9} M) or by KCl (50 mM). B: percent relaxation of mesenteric small arteries, precontracted 35–45% with phenylephrine, produced by the selective ETB agonists IRL-1620 (3 \times 10^{-6} M) or S6c (10^{-9} M) or by acetylcholine (ACh; 10^{-5} M, n = 8). Values represent means \pm SE.
In the present study, an absence of ETB-mediated contraction was observed in mesenteric small arteries from heterozygous rats compared with homozygous rats. Previously, Pollock et al. (26) reported an enhanced pressor response to ET-1, associated with a loss of the ETB-mediated depressor response, in rats homozygous for the ETB deficiency. The discrepancy in the findings observed in the intact animal (increased pressor response to ET-1) and the isolated vessel (decreased contraction to ET-1) may be explained by our finding that contraction to ET-1 was exclusively ETA mediated in the isolated mesenteric small artery of homozygous and heterozygous rats, whereas an ETB-mediated depressor response was observed in the intact heterozygous but not homozygous rats.

Removal of mesenteric small arteries from exposure to high circulating levels of plasma ET-1 for 24 h did not alter contraction to ET-1. This finding does not support a role for desensitization to ET-1 that could be reversed acutely in the decreased responsiveness found in vessels from sl/sl rats. We observed that ETA receptor protein expression was increased in mesenteric small arteries from homozygous rats compared with heterozygous rats. A similar finding was observed in rats with acute renal failure induced by ischemia-reperfusion, in which plasma and urine levels of ET-1 were increased and 125I-labeled ET-1-binding affinity and receptor number on the renal vasculature were increased (16, 20). While one might have predicted enhanced contraction to ET-1 under these conditions, there are several possible explanations for our finding. The location or conformation of the ETA receptors in intact tissue could be altered in a manner that would reduce receptor binding.

In summary, ETB receptor deficiency associated with high plasma ET-1 causes a decrease in ETA-dependent contraction to ET-1 in mesenteric small arteries. This effect occurs in the presence of an increase in ETA receptor protein, suggesting the possibility of uncoupling of receptor expression and functional effects. In pathophysiological conditions that are associated with elevated plasma ET-1 levels, the vascular response to ET-1 is variable. We (13) reported previously in DOCA-salt hypertensive rats that contraction to ET-1 is reduced in mesenteric small arteries. Similar effects were observed by others in DOCA-salt hypertensive rats (6). In other models of disease, such as rat pulmonary hypertension and rat coronary ischemia-reperfusion, in which plasma ET-1 levels are elevated, ET-1-induced vasodilation was lost, leading to enhanced contraction to ET-1 (2, 8, 21). In a recent study (14) in ETB receptor-deficient rats, lungs of sl/sl rats lacked ETB mRNA in the pulmonary vasculature, had minimal ETB receptors, and lacked ET-1 mediated pulmonary vasodilation. The sl/sl rats had higher pulmonary arterial pressure and vasopressor responses to ET-1 (14). These findings suggest that ETB Receptors play an important role in modulating resting pulmonary vascular tone and that impaired ETB receptor activity can contribute to pulmonary hypertension.
The findings that pulmonary vascular contraction to ET-1 is enhanced in sl/sl rats, whereas mesenteric small artery contraction to ET-1 is reduced in sl/sl rats, is likely due to the presence or absence of ET<sub>B</sub> receptor-mediated vasodilation under normal conditions in the pulmonary vasculature and mesenteric small artery, respectively. On the basis of the findings of our study and others, it is suggested that alterations of vascular contraction to ET-1, associated with elevated plasma ET-1, may be partially dependent on the types of ET receptors present and their role in mediating contraction to ET-1.

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