Unique endothelin receptor binding in kidneys of ET₉ receptor deficient rats

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Submitted 20 September 2002; accepted in final form 31 October 2002

Taylor, Traci A., Cheryl E. Gariepy, David M. Pollock, and Jennifer S. Pollock. Unique endothelin receptor binding in kidneys of ET₉ receptor deficient rats. Am J Physiol Regul Integr Comp Physiol 284: R674–R681, 2003. First published November 7, 2002; 10.1152/ajpregu.00589.2002.—Gariepy and colleagues (Gariepy CE, Williams SC, Robinson PA, and Yanagisawa M. J Clin Invest 102: 1092–1101, 1998.) developed rescued spotting-lethal rats that carry a naturally occurring deletion of the endothelin (ET) type B receptor gene resulting in a lack of functional renal ET₉ receptor expression. It has been shown that rats homozygous (sl/sl) for the deletion have elevated plasma ET-1 levels; thus, the purpose of this study was to determine whether this deletion would result in a downregulation of ET₉ receptors in renal tissue. ET-1 and ET-3 binding experiments were performed with cortex, outer medullary, and inner medullary membranes of heterozygous (sl/+ ) and sl/sl ET₉ receptor-deficient rats. ¹²⁵I-labeled ET-1 binding in sl/sl cortex and outer medulla was significantly lower than cortex and outer medulla from sl/+ rats. In contrast to sl/+ rats, [¹²⁵I]ET-3 binding was not detected in the cortex and outer medulla of sl/sl rats, indicating a lack of ET₉ receptor expression. The inner medulla of sl/+ rats also demonstrated an abundance of ET₉ receptors. Surprisingly, however, we also observed significant [¹²⁵I]ET-3 binding in the sl/sl inner medulla. Furthermore, ET-3 binding in the inner medulla could be blocked with an ET₉ receptor antagonist in sl/sl rats but not in tissue from sl/+ rats. These studies indicate that rats deficient in ET₉ receptors have decreased renal cortical and outer medullary ET₉ receptor number, most likely in response to elevated plasma ET-1 levels. In addition, homozygous ET₉-deficient rats express a novel inner medullary ET₉ receptor family.

endothelin B receptor; kidney; receptor binding; spotting-lethal rats

IN 1988, YANAGISAWA ET AL. (33) isolated a potent vasoconstrictor produced by endothelial cells that was named endothelin (ET). Inoue and colleagues (14) determined through DNA analysis that there are three distinct genes encoding three ET isopeptides (ET-1, ET-2, and ET-3). G protein-coupled receptors were discovered and then characterized by their selective affinity to each of the ET isopeptides, location, and their biological function (18). Endothelin A (ETₐ) receptors bind ET-1 with higher affinity than ET-2 or ET-3, are localized to vascular smooth muscle cells, and cause vasoconstriction. Endothelin B (ET₉) receptors are found on endothelial cells and have been shown to produce vasodilation and bind all three ET isopeptides with equal affinity. Additionally, there is evidence that ET₉ receptors in pulmonary endothelial cells “clear” ET-1 from the circulation (7). However, this classification of ET receptors may be oversimplified, with increasing evidence suggesting additional receptor subtypes. It is now known that there exist two pharmacologically distinct subtypes of the ET₉ receptor, ET₉₁ and ET₉₂, whose biological activity is a function of their localization. ET₉₁ are located on the vascular endothelium and produce vasodilation upon agonist binding, whereas ET₉₂ are found on vascular smooth muscle cells and mediate non-ETₐ vasoconstriction.

Within the kidney, both ET receptor subtypes have been identified in various regions differing both in number and function. In both cortex and medulla, ET₉ receptors are more abundant than ETₐ receptors (24). The ET₉ receptor has been localized to renal arterioles, glomeruli, proximal convoluted tubule, medullary thick ascending loop, and collecting duct (29). ET₉ receptors in the renal vasculature couple to nitric oxide and prostaglandin synthesis, leading to vasorelaxation (6), whereas ET₉ receptors on renal tubular cells promote natriuresis and diuresis (8, 20). ET₉ receptors localized in arterioles and proximal tubules mediate vasoconstrictor (5, 20) and antinatriuretic effects (18, 26).

Elevated plasma levels of ET-1 have been implicated in the pathogenesis of many disease models, including pulmonary hypertension (2) and DOCA salt hypertension (27). Although plasma ET-1 levels are high in these disease models, there is still conflicting evidence as to the regulation of ET receptors. Studies by Bauer et al. (2) have shown increased mRNA transcripts...
encoding the ET<sub>B</sub> receptor with no difference in ET<sub>A</sub> mRNA levels in pulmonary arterial tissue taken from patients with pulmonary hypertension. However, in endothelium-denuded saphenous veins from African-American patients with essential hypertension, elevated plasma ET-1 levels may be responsible for a decrease in ET<sub>A</sub> mRNA expression together with increased ET<sub>B</sub> mRNA levels (13). In DOCA-salt hypertensive rats, Pollock et al. (27) observed increased ET<sub>B</sub> receptor number with an apparent decrease in ET<sub>A</sub> receptor number in the renal medulla. Conversely, a transient elevation of plasma ET-1 levels induced by adenovirus gene transfer led to systemic hypertension but no change in renal ET receptor number (28).

The spotting-lethal rat carries a natural 301-bp deletion in the ET<sub>B</sub> receptor that abrogates expression of functional ET<sub>B</sub> receptors. Rats that are homozygous (sl/sl) for this mutation exhibit a lethal phenotype of congenital intestinal aganglionic and are commonly used as a model for Hirschsprung's disease (9). In 1998, Gariepy et al. (11) used a dopamine-β-hydroxylase promoter to direct transgenic ET<sub>B</sub> receptor expression in adrenergic tissue that "rescued" the rats from the intestinal defect. The sl/sl animals have elevated plasma ET-1 levels. Therefore, the purpose of this study was to determine whether ET<sub>B</sub> receptor deficiency and elevated plasma ET-1 levels are associated with changes in ET receptor density in the kidney.

**METHODS**

**Membrane preparations.** All of the procedures carried out on the rats were approved by the Medical College of Georgia Animal Care and Use Committee and followed the guidelines of the American Physiological Society. Rats (300–350 g) heterozygous and homozygous for the ET<sub>B</sub> receptor deficiency from our breeding colony and male Sprague-Dawley (Harlan) rats (300–350 g) were anesthetized, and kidneys were excised and separated with a razor blade on ice into cortex, outer medulla, and inner medulla and frozen at −80°C. Tissue was weighed, pulverized, and then homogenized in buffer containing 250 mM sucrose, 50 mM Tris-HCl, 5 mM EDTA, and 15 μM phenylmethylsulfonyl fluoride (PMSF), pH 7.4, as described (27).

Briefly, homogenized samples were then centrifuged at 1,000 g for 30 min. The supernatant was further centrifuged at 30,000 g at 4°C for 45 min, and the resulting pellet was resuspended in one-half of the starting volume of homogenization buffer and frozen at −80°C. The protein concentration of the membrane preparations was determined by the Bradford method (Bio-Rad, Hercules, CA).

**Saturation binding curves.** Receptor binding curves were performed to quantify the amount of ET receptors in the cortex, outer medulla, and inner medulla of sl/+ or sl/sl rats for the ET<sub>B</sub> receptor deficiency. Sprague-Dawley rat renal tissue was also used in preliminary experiments to serve as a comparison to both previously published data and the transgenic animals. Membrane preparations from each of the three renal tissue sections were bound to wheat germ agglutinin scintillation proximity beads as previously described (27). Briefly, membrane preparations of sl/+ or sl/sl ET<sub>B</sub> receptor-deficient rats from renal cortex (20 μg), outer medulla (20 μg), or inner medulla (5 μg) were added to each well of a 96-well microtiter plate (Optiplate; Packard Instruments, Meridian, CT). Wheat germ agglutinin polyvinyltoluene beads (scintillation proximity beads; Amersham Life Science, Arlington Heights, IL) were suspended in binding buffer (40 mg/ml), with 1 mg added to each well. Binding buffer consisted of 20 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, and 3 mM EDTA, pH 7.4, with additional 0.1 mM PMSF, 5 μg/ml peptatin A, 0.025% bacitracin, and 0.2% BSA. The plate was covered and shaken for 3 h at room temperature to allow for coupling of the protein to the beads. After this precoupling, 25 μl of binding buffer were added to cells for total binding determination, whereas 2 μl of ET-1 or ET-3 was added to determine nonspecific binding. [125I]ET-1 or [125I]ET-3 was diluted in binding buffer and added to each well with a final concentration of 0.03, 0.1, 0.3, 1, or 3 nM. The plate was sealed and shaken for 18 h at room temperature. The plate was then centrifuged for 5 min at 1,000 g before being counted on a Packard TopCount microplate scintillation counter. Our laboratory had previously determined optimum incubation times and procedures (27). All measurements were performed in duplicate, and all dilutions of radioactive and nonradioactive peptides were made in siliconized tubes.

Additional experiments were performed using whole kidney homogenate or separated renal inner medulla from sl/+ and sl/sl animals that were homogenized as described above. From whole kidney, homogenates from whole kidney, homogenates from the inner medulla (5 μg) were added to wells followed by precoupling to scintillation proximity beads. Unlabeled ET-1 (2 μM) was added to wells to determine nonspecific binding, whereas the total binding wells received buffer. Radiolabeled ET-1 (1 nM) was added to all wells, and the plate was covered, shaken for 18 h at room temperature, and then counted on the microplate scintillation counter.

**Competition binding curves.** Competition binding curves were performed using selective ETA and ETB receptor antagonists. Preliminary experiments were performed using inner medullary tissue preparations from Sprague-Dawley animals. Membrane preparations from the inner medulla (5 μg) of sl/+ or sl/sl animals were precoupled to scintillation proximity beads similar to the same receptor binding protocol described above. For generation of competition binding curves, either the ETA receptor antagonist A-127722 (0.01 μM-0.5 mM), the ETB receptor antagonist A-192621 (100 μM-10 mM), or unlabeled ET-1 (10 μM-1 μM) was added to separate wells followed by the addition of [125I]ET-1 (1 nM) to every well. The plate was shaken for 18 h and then counted on the microplate scintillation counter.

**Determination of maximal binding and dissociation constant values.** Experiments using Chinese hamster ovary cells stably transfected with either the ETA or ETB receptor have shown that the ETA receptor preferentially binds ET-1 over ET-3 (IC<sub>50</sub> values of 0.28 and 475 nM, respectively), whereas the ETB receptor binds both ET-1 and ET-3 with similar affinity (IC<sub>50</sub> values of 0.14 and 0.08 nM, respectively; see Ref. 19). Thus, in our receptor binding experiments, [125I]ET-3 receptor binding was used to determine maximal binding (B<sub>max</sub>) values for ETB receptor number. To determine ETA receptor number, B<sub>max</sub> values for [125I]ET-3 binding were subtracted from [125I]ET-1 B<sub>max</sub> values.

**Materials.** Wheat germ agglutinin scintillation proximity assay beads were purchased from Amersham Life Sciences. [125I]ET-1 (2,200 Ci/mmol) was obtained from New England Nuclear (Boston, MA), and [125I]ET-3 (2,000 Ci/mmol) was acquired from Amersham Life Sciences. ET-1 and ET-3 were purchased from American Peptide (Sunnyvale, CA). A-127722 (trans-2-[4-(N,N-dibutylamino)carboxymethyl]pyrrolidine-3-carboxylic acid) and A-192621 [2R-(2α,3α,4α)-4-(1,3-benzodioxol-5-yl)-1-[2-(2.6 diethylphenyl)amino]-2-oxoethyl]-2-(4-propoxypyrrolid-3-ylpyrrolidinecarboxylic acid) were obtained from Dr. Jerry Wessale at Abbott Laboratories (Abbott Park, IL). PMSF,
pepstatin A, and bacitracin were purchased from Sigma Chemical (St. Louis, MO). All other agents were acquired from Bio-Rad Laboratories.

Data and statistical analysis. Receptor binding data were analyzed by nonlinear regression using the one-site model of the binding isotherm except for the Scatchard analysis of [125I]ET-1 binding in inner medulla of sl/sl animals in which a two-site model was utilized (Prism; GraphPad Software, San Diego, CA). Statistical differences in the mean values for all binding data were determined by ANOVA, and Fisher’s protected least-significant difference test was used to determine differences between individual means (StatView, Abacus Concepts, Berkley, CA). Values are reported as means ± SE, with P < 0.05 and P < 0.001 considered significant.

RESULTS

Saturation binding of [125I]ET-1 in renal tissue. Total and nonspecific binding was determined for [125I]ET-1 over a wide range of renal cortical, outer medullary, and inner medullary membrane protein concentrations. Maximum binding for sl/+ and sl/sl cortex and outer medulla preparations was achieved at 20 µg, whereas only 5 µg were needed for maximum binding in the inner medulla (data not shown). Maximum binding of [125I]ET-3 in the inner medulla required only 0.5 µg for maximum binding (data not shown). Figure 1 shows saturation binding curves of cortex (20 µg), outer medulla (20 µg), or inner medulla (5 µg) of membrane preparations from sl/+ or sl/sl rats incubated with [125I]ET-1 (1 nM). Specific binding of ET-1 was similar and saturable in both the renal cortex (Fig. 1A) and renal outer medulla (Fig. 1B) of sl/+ ETβ receptor-deficient rats. Additionally, ET-1 binding in cortex and outer medullary preparations from sl/sl rats was less than one-half that observed in sl/+ rats. Specific binding of ET-1 in the inner medulla was eightfold higher in both sl/sl and sl/+ rats compared with the cortex and outer medulla.

Scatchard analysis of [125I]ET-1 saturation binding curves. To visualize Bmax and dissociation constant (Kd) differences between sl/+ and sl/sl animals, Scatchard analyses were performed on nonlinear regression results from [125I]ET-1 saturation binding curves (Table
1). Scatchard analyses from both sl/+ and sl/sl renal cortex and outer medulla were linear (data not shown), revealing a one-site model of receptor binding, which was expected. Figure 2 depicts Scatchard plots of inner medullary saturation binding curves of radiolabeled ET-1. Figure 2A shows inner medullary binding results from the sl/+ ETB receptor-deficient rat that are linear, indicating a single binding site. However, the Scatchard plot of [125I]ET-1 for inner medullary tissue from sl/sl rats (Fig. 2B) reveals a two-site model of receptor binding, each with a distinct $K_d$. There appears to be one site that has very high affinity (0.08 ± 0.03 nM) but relatively few binding sites (14.2 ± 2.8 fmol/mg protein), whereas the other has lower affinity (5.72 ± 0.79 nM) but is greater in abundance (347.5 ± 25.4 fmol/mg protein).

Saturation binding of [125I]ET-3 in renal tissue. Figure 3 represents saturation binding curves using [125I]ET-3 incubated with either renal cortex (Fig. 3A), renal outer medulla (Fig. 3B), or renal inner medulla (Fig. 3C) membrane preparations. Specific binding of [125I]ET-3 was similar and saturable in the cortex and outer medulla of the sl/+ ETB-deficient rats. The inner medulla of the sl/+ ETB-deficient rat exhibited an almost 10-fold greater binding of [125I]ET-3 compared with the cortex and outer medulla, indicating a greater number of ET B receptors in this renal section. Predictably, there was no detectable [125I]ET-3 specific binding in the renal cortex and outer medulla from sl/sl ETB-deficient rats. However, within the renal inner medulla of the

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**Fig. 3.** Saturation binding curves were performed using [125I]ET-3 incubated with 20 μg membrane preparations from sl/+ (●) or sl/sl (□) ETB receptor-deficient rat renal cortex (A), outer medulla (B, n = 4–6 animals/group), or 0.5 μg inner medulla membrane preparations (C, n = 3 animals/group). Values are means of nonspecific binding subtracted from total binding ± SE.

**Fig. 4.** Because the ETB receptor has similar affinity for both ET-1 and ET-3, whereas ETA receptors preferentially bind ET-1, [125I]ET-3 receptor binding was used to determine ETB receptor number. Maximal binding ($B_{max}$) values for [125I]ET-3 were subtracted from [125I]ET-1 $B_{max}$ values to determine ETA receptor number. ETA receptor number (●) and ETB receptor number (□) were calculated for sl/+ and sl/sl renal cortex (A), outer medulla (B), and inner medulla (C). Values represent the means ± SE of $B_{max}$ calculations from 3–6 animals/group. Statistical analysis was performed using Student’s $t$-tests with *$P < 0.05$ and **$P < 0.001$. 

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*AJP-Regul Integr Comp Physiol* • VOL 284 • MARCH 2003 • www.ajpregu.org
sl/sl ETB-deficient rat, there was an unexpected [125I]ET-3 binding. Bmax and Kd values. By definition, ET-1 binds both ET_A and ET_B receptors, and ET-3 is selective for ET_B receptors at low concentrations; Bmax values for [125I]ET-3 binding were subtracted from [125I]ET-1 Bmax values to determine ET_A receptor number in each of the renal sections from sl/+ and sl/sl ETB-deficient rats. [125I]ET-3 receptor binding was used to determine ET_B receptor number. Figure 4 represents Bmax values from renal cortical (Fig. 4A), outer medullary (Fig. 4B), and inner medullary (Fig. 4C) tissue from both sl/+ and sl/sl membrane preparations. ET_A receptor number was significantly lower (P < 0.001) in sl/sl cortical (19.95 ± 0.61 fmol/mg protein) and outer medullary (23.75 ± 0.36 fmol/mg protein) membrane preparations compared with the sl/+ cortex (33.40 ± 1.20 fmol/mg protein) and outer medulla (41.49 ± 1.30 fmol/mg protein) preparations. Additionally, there were no detectable ET_B receptors in the sl/sl cortex or outer medulla. ET_A receptor number was not significantly different in the inner medulla between sl/+ and sl/sl animals (60.20 ± 39.89 and 76.00 ± 50.31 fmol/mg protein, respectively). There was a significantly (P < 0.05) lower number of ET_B receptors in the sl/sl inner medulla (211.50 ± 25.36 fmol/mg protein) compared with the sl/+ inner medulla (309.40 ± 26.58 fmol/mg protein); however, the observation that significant [125I]ET-3 binding was detected in the inner medulla of sl/sl ETB-receptor-deficient rats was surprising. 

Bounding of [125I]ET-1 in whole kidney. Experiments were also performed to explore the possibility that the use of whole kidney homogenate may mask or prevent the detection of ET-3 binding sites in the inner medulla of sl/sl ETB-deficient rats. Figure 5 shows [125I]ET-1 specific binding using inner medulla and whole kidney membrane preparations from either sl/+ or sl/sl ETB-deficient rats. [125I]ET-1 specific binding in the inner medulla of sl/+ ETB-deficient rats was not significantly different compared with [125I]ET-1 specific binding in the whole kidney (1,356 ± 200 and 1,447 ± 354 counts·min⁻¹·(cpm)/µg protein⁻¹, respectively). However, when inner medullary membrane preparations from sl/sl ETB-deficient rats were incubated with [125I]ET-1, significantly more specific binding occurred compared with that observed in whole kidney membrane preparations (832 ± 74 vs. 72 ± 38 cpmp/µg protein, respectively).

Competition binding curves with selective ET_A and ET_B receptor antagonists. Competitive binding experiments were conducted to ascertain whether the novel ET-3 binding in the sl/sl inner medulla could be inhibited by ET_A or ET_B receptor antagonists. Competition binding curves were generated using various concentrations of the ET_A receptor selective antagonist A-127722, the ET_B receptor selective antagonist A-192621, or unlabeled ET-1. Results previously reported using Chinese hamster ovary cells stably transfected with either the ET_A or ET_B receptor report IC50 values using A-127722 as 0.09 nM for the ET_A receptor and 128 nM for the ET_B receptor, whereas the IC50 of ET-1 for the ET_A receptor is 0.28 and 0.14 nM for the ET_B receptor (31). Results from von Geldern and colleagues (30a) report that the ET_B receptor selective antagonist has an IC50 of 1.10 µM for the ET_A receptor and 0.7 nM for ET_B receptor. Thus, in sl/+ inner medullary membrane preparations (Fig. 6A), ET-1 was found to have the highest affinity (IC50 0.16 ± 0.24 nM; Table 2) followed by the ET_B receptor antagonist A-192621 (IC50 0.12 ± 0.05 µM; Table 2), whereas
A-127722, the ETA receptor antagonist, had the lowest affinity (IC$_{50}$ 7.3 ± 0.11 nM; Table 2). However, competition binding curves performed on inner medullary membrane preparations from sl/sl rats were quite different (Fig. 6B). The ETB receptor antagonist A-192621 displaced the [125I]ET-1 with similar concentrations in the sl/sl rats (IC$_{50}$ 0.11 ± 0.15 nM; Table 2), as it did in the sl/+ animals. Additionally, ET-1 was less able to compete for binding sites as [125I]ET-3 (IC$_{50}$ 1.8 ± 0.14 nM; Table 2), and most strikingly the ETA receptor antagonist was extremely potent, with an IC$_{50}$ of 1.2 ± 0.15 pM (Table 2). Additional competition binding experiments performed on Sprague-Dawley inner medullary tissue preparations revealed that the IC$_{50}$ for ET-1 was 0.18 ± 0.16 nM (Table 2), similar to that of the sl/+ rat. The ETB receptor antagonist was next highest in affinity for the receptor (IC$_{50}$ 0.10 ± 0.04 nM; Table 2), again similar to results from the sl/+ rat, whereas the ETA receptor antagonist had the least affinity of the three ligands, with an IC$_{50}$ value of 20.7 ± 0.69 nM (Table 2). Taken together, results from the saturation binding curves suggest that there is an ET-3 binding site in the inner medulla of sl/sl rats and that competition binding curves suggest that this site binds the ETB receptor antagonist with very high affinity.

**DISCUSSION**

The present study was conducted to determine if any changes in ETA receptor binding in the kidney would occur under conditions of ETB receptor deficiency that is associated with chronic elevations in circulating ET-1 levels. We hypothesized that long-term exposure to ET-1 would cause a downregulation of ETA receptor expression. Indeed, results showed that ETA receptor binding was reduced significantly in renal tissue of sl/sl compared with sl/+ rats. These observations are consistent with a compensatory change in ETA receptor expression when ETB receptor function is lost and plasma levels of ET-1 are increased. An unexpected observation was evidence for a novel ET-3 binding site in the renal inner medulla of ET$_B$ receptor-deficient animals.

Saturation binding curves using radiolabeled ET-1 reveal an expected decrease in overall ligand-receptor binding interactions in all three regions of kidneys from sl/sl compared with sl/+ rats. Consistent with previous studies, B$_{max}$ values determined from ET-1 binding were ~10-fold greater in the inner medulla compared with the cortex and outer medulla of both sl/+ and sl/sl rats, highlighting the abundance of ET receptors in this region (16). However, when ET-1 saturation binding results were plotted using Scatchard analysis, the inner medulla from sl/sl rats revealed a surprising two-site model, whereas a linear one-site model was detected in the sl/+ inner medulla. This two-site model found in the sl/sl rat indicated that there was another ET binding site that was different from both ETA and ETB Receptors. When the results from both the ET-1 and ET-3 saturation curves were plotted as a function of receptor number, the total amount of ET receptors was significantly greater in the inner medulla of both sl/+ and sl/sl animals compared with their respective cortex and outer medulla, with the majority of receptors representing the ETB subtype. However, B$_{max}$ values were substantially lower in both sl/+ and sl/sl rats compared with published values from Sprague-Dawley rats (24).

We performed additional experiments to compare previously published ET-1 binding data using whole kidney homogenates with similar whole kidney preparations from sl/+ and sl/sl rats (11). When sl/+ whole kidney or inner medulla membranes were used, ligand-receptor binding was similar between the two preparations. However, the same experiment performed on sl/sl preparations indicates that significant ET-1 binding occurs only when the inner medulla was separated from the cortex and outer medulla, and this binding is “diluted out” when included in a whole kidney preparation. This led us to examine the nature of this binding site through competition binding curves using specific ETA and ETB receptor antagonists. Findings from competition binding curves using radiolabeled ET-1 in sl/+ inner medullary preparations indicated that the ETB receptor antagonist had 0.12 ± 0.05 nM.

**Table 1.** $B_{max}$ and $K_d$ of [125I]ET-1 and [125I]ET-3 binding in membrane preparations

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<th>$B_{max}$, fmol/mg</th>
<th>$K_d$, nM</th>
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<tr>
<td></td>
<td>sl/+</td>
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<tr>
<td><strong>Cortex</strong></td>
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<tr>
<td>ET-1</td>
<td>46.51 ± 1.20</td>
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<tr>
<td>ET-3</td>
<td>13.11 ± 0.24</td>
<td>ND</td>
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<tr>
<td><strong>Outer Medulla</strong></td>
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<tr>
<td>ET-1</td>
<td>46.25 ± 1.30</td>
<td>23.75 ± 0.36</td>
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<tr>
<td>ET-3</td>
<td>4.76 ± 0.60</td>
<td>ND</td>
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<td><strong>Inner Medulla</strong></td>
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<tr>
<td>ET-1</td>
<td>369.60 ± 39.89</td>
<td>ND</td>
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<tr>
<td>ET-3</td>
<td>309.40 ± 26.58</td>
<td>211.50 ± 12.24</td>
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Values are means ± SE; $n = 3$–6 animals/group. Maximum binding ($B_{max}$, fmol/mg) and dissociation constants ($K_d$, nM) of 125I-labeled endothelin (ET)-1 and 125I-ET-3 binding in membrane preparations from sl/+ and sl/sl ET type B-deficient rat renal cortex, outer medulla, and inner medulla. ND, not detected. *Two-site model: $B_{max1} = 14.20 ± 2.78$ and $B_{max2} = 347.50 ± 25.36$ fmol/mg. †Two-site model: $K_{d1} = 0.08 ± 0.03$ and $K_{d2} = 5.72 ± 0.79$ nM.

**Table 2.** IC$_{50}$ values of ET receptor ligands against [125I]ET-1 binding to inner medullary preparations

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<th>Sprague Dawley</th>
<th>sl/+</th>
<th>sl/sl</th>
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<tr>
<td>ET-1, nM</td>
<td>0.18 ± 0.16</td>
<td>0.16 ± 0.24</td>
<td>1.77 ± 0.14</td>
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<tr>
<td>A-127722, μM</td>
<td>20.79 ± 0.69</td>
<td>7.31 ± 0.11</td>
<td>1.22 ± 0.15</td>
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<tr>
<td>A-192621, μM</td>
<td>0.10 ± 0.04</td>
<td>0.12 ± 0.05</td>
<td>0.11 ± 0.15</td>
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Values are means ± SE; $n = 3$ animals/group. Shown are IC$_{50}$ values of ET receptor ligands against 125I-ET-1 binding to inner medullary preparations from sl/+ and sl/sl, and Sprague-Dawley rats calculated from competition binding curves. Values represent $n = 3$ animals per group. Statistical analysis was performed using one-way ANOVA with $*P < 0.05$ and $†P < 0.001$ comparing values from Sprague-Dawley control, sl/+ and sl/sl animals. †Units are pM.
lesser affinity compared with the natural ligand, ET-1 but more affinity for the receptor than the ETA receptor antagonist. However, within the sl/sl inner medulla, the ETA receptor antagonist had very high affinity, greater than either ET-1 or the ETB antagonist.

The sl/sl animal is devoid of ETB receptor apart from adrenergic tissue where its expression in these regions is essential for survival (9). Because plasma ET-1 levels are elevated, these animals have had to adapt to the loss of renal ETB receptor function in the face of high ET-1 levels. Plasma ET-1 levels in sl/sl rats are significantly elevated compared with sl/+ rats, and results from this colony of rats have been published previously (25). Consistent with our original hypothesis, the sl/sl rat appears to downregulate ETA receptors in renal cortical and outer medullary tissue. However, the situation in the renal inner medulla appears more complex. Within the inner medulla, there is no a downregulation of ETA receptors. In fact, ETA receptor number is similar to those found in the sl/+ inner medulla. Additionally, our studies provide evidence for the existence of a unique binding site that has the novel characteristics of having a high affinity for ET-3 than either ET-1 or the ETA antagonist. We can only speculate, at this point, that this novel binding site represents some form of compensation for the lack of functional ETB receptors in this region of the kidney. More exhaustive biochemical studies will be needed to determine whether this novel binding site is actually a putative ET receptor along with its estimated size, biochemical behavior, and its pharmacological profile against multiple ET-related ligands are clearly warranted. In addition, to determine whether this binding site is unique to the renal inner medulla, receptor binding experiments will need to be performed using tissue from the sl/sl animals that normally are enriched with ETB receptors, such as the lungs and the cerebellum.

The physiological significance of this novel ET binding site has yet to be determined, but we can speculate that it could be of importance in models of hypertension that have elevated circulating ET levels. In the DOCA-salt rat model of hypertension, it has been shown that plasma ET-1 levels are elevated compared with placebo-treated controls (1). Additionally, it has been shown that there is an upregulation of ETB receptors in the renal medulla from DOCA-salt rats whose function is thought to promote sodium and water excretion (27). Thus the DOCA-salt model of hypertension is associated with elevated ET-1 levels, and the kidney responds by upregulating renal medullary ETB receptors. In rats that lack functional ETB receptors, there are also elevated plasma ET-1 levels, and these animals have elevated blood pressures (10); we would expect the kidney to respond by upregulating medullary ETB receptors. However, because there is a mutation in the ETB receptor gene, we speculate that the sl/sl animal tries to compensate by expressing a renal inner medullary ETB receptor that has a unique binding profile different from that of the ETA and ETB receptors. We can speculate further that it may influence blood pressure because of its ETA receptor-like sensitivity to the ETA antagonist but might also function to promote natriuresis because of its ETB receptor-like binding of ET-3. Future studies are required to determine if this renal inner medullary binding site has functional significance under normal physiological conditions.

There is anecdotal evidence for the existence of a third class of ET receptors, named ETc. By definition, the ETc receptor has a rank order of affinity of ET-3 > ET-1 in radioligand binding and functional studies. In 1993, Karne et al. (17) cloned an ETc-like receptor that has high affinity for ET-3 from Xenopus dermal melanophores. However, no ETc receptor has yet been cloned from mammalian sources (17). The possibility arises that the ET-3 binding site is unmasked in the renal inner medulla of the sl/sl ETB-deficient animal and could be the mammalian counterpart to the ETc receptor found in Xenopus melanophores. In addition, Zeng and colleagues (34) have cloned and characterized a novel ET receptor type B-like gene enriched in the brain that encodes an ET receptor type B-like protein. This could explain the ET-3 binding found in the inner medulla of the sl/sl animals. Additionally, there is the possibility of a splice variant of this ET receptor type B-like gene or even a splice variant of the ETA receptor.

In recent years, evidence has accumulated supporting the notion of alternative splice variants of the ETB receptor. Cheng et al. (4) identified a splice variant of the ETB receptor in rat brain. More recently, Nambi and colleagues (24) demonstrated the existence of a splice variant of the ETB receptor in porcine cerebellum that differs in the last 32 amino acids of the COOH-terminal tail from that of the wild-type receptor but that binding and stimulation of inositol phosphate production are identical to that found in the wild type. Radioligand binding data also indicated that the splice variant and the wild-type ETB receptor are expressed at the same level in the porcine brain. Additionally, Tsutsumi et al. (30) provide evidence of a novel ETB receptor transcript that could potentially generate a new receptor. It should be noted that the dopamine-beta-hydroxylase ETB receptor transgene was constructed using the ETB cDNA, that is, it does not contain ETB gene introns, making splice variants from the transgene unlikely.

The sl/sl ETB-deficient animal has a 301-bp deletion in the gene encoding for the first two membrane-spanning regions of the receptor, and this shortened transcript does not encode functional ETB receptors (11), normally, the part of the NH2-terminal domain that is in close proximity to the first transmembrane region required for ligand binding. Through proteolytic truncation experiments, it has been shown that ET-1 binds to a 29-amino-acid sequence in the NH2-terminal region of the ETB receptor near the first transmembrane-spanning domain (18, 31). Thus we can speculate that ET-3 binding in the sl/sl inner medulla must be to an ET-like receptor that contains an NH2-terminal region of 29 amino acids near the first transmembrane segment. Future studies will need to be completed to
address whether this binding site has an alteration at the COOH-terminal region of the receptor.

In summary, our data suggest that there is a reduction in ETA receptor number in the cortex and outer medulla of rats deficient of functional ETB receptor consistent with a downregulation related to elevated circulating ET-1 levels. Furthermore, we provide evidence for an ET-3 binding site within the inner medulla of ETB receptor-deficient rats that binds the ETA antagonist, A-127722, with very high affinity. We speculate that this binding site is a novel ET receptor that is unmasked during ETB receptor deficiency.

We thank Dr. Masashi Yanagisawa for efforts in providing us with breeders for our ETB receptor-deficient colony. We also thank Dr. Jerry Wessalle for the ETA and ETB receptor antagonists used in this study. This work was supported in part by National Heart, Lung, and Blood Institute Research Grants HL-60653 and HL-64776 and by Scientist Development Grants from the American Heart Association.

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