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Combined knockout of collecting duct endothelin A and B receptors causes hypertension and sodium retention

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Ge Y, Bagnall A, Stricklett PK, Webb D, Kotelevtsev Y, Kohan DE. Combined knockout of collecting duct endothelin A and B receptors causes hypertension and sodium retention. Am J Physiol Renal Physiol 295:F1635–F1640, 2008. First published September 10, 2008; doi:10.1152/ajprenal.90279.2008.—The collecting duct (CD) endothelin (ET) system regulates blood pressure (BP) and Na excretion. CD-specific knockout (KO) of ET-1 causes hypertension. CD-specific KO of the ETA receptor does not alter BP, while CD-specific KO of the ETB receptor increases BP to a lesser extent than CD ET-1 KO. These findings suggest a paracrine role for CD-derived ET-1; however, they do not exclude compensation for the loss of one ET receptor by the other. To examine this, mice with CD-specific KO of both ETA and ETB receptors were generated (CD ETA/B KO). CD ETA/B KO mice excreted less urinary Na than controls during acute or chronic Na loading. Urinary aldosterone excretion and plasma renin concentration were similar during Na intake and both fell comparably during Na loading. On a normal sodium diet, CD ETA/B KO mice had increased BP, which increased further with high salt intake. The degree of BP elevation during normal Na intake was similar to CD ET-1 KO mice and higher than CD ETB KO animals. During 1 wk of Na loading, CD ETA/B KO mice had higher BPs than CD ETB KO, while BP was less than CD ET-1 KOs until the latter days of Na loading. These studies suggest that 1) CD ETA/B deficieny causes salt-sensitive hypertension, 2) CD ETA/B KO-associated Na retention is associated with failure to suppress the renin-angiotensin-aldosterone system, and 3) CD ETA and ETB receptors exerts a combined hypotensive effect that exceeds that of either receptor alone.

ET-1 plays an important role in controlling BP and facilitating Na excretion in response to expanded extracellular fluid volume.

ET-1 interacts with two receptors, ETA and ETB. Evidence exists that both receptors modulate CD function. CDAs have very high ETB receptor expression (24); stimulation of the ETB receptor in A6 cells inhibits apical Na entry (18). ETB-deficient rats have salt-sensitive hypertension that is partially normalized by amiloride, suggesting that the ETB receptor regulates the CD apical Na channel in vivo (7). More direct evidence for a role of the CD ETB receptor comes from studies in which this receptor was selectively disrupted in CD (8). CD ETB KO mice, like CD ET-1 KO animals, are hypertensive on a normal Na diet and this hypertension is exacerbated by Na loading. However, the degree of hypertension is ~50% less in CD ETB KO compared with CD ET-1 KO mice. This suggests that ETB receptors partially, but not completely, mediate the natriuretic and hypotensive effects of CD-derived ET-1. CD also expresses ETA receptors, although in appreciably lower levels compared with ETB receptors (9). CD ETA receptors do have functional significance in that CD ETA KO causes decreased water reabsorption associated with lower vasopressin-stimulated CD cAMP accumulation (9). However, these mice have normal BP and urinary Na excretion regardless of Na intake. Hence, the CD ETA KO and CD ETB KO studies suggest that CD-derived ET-1 reduces BP, in part, but not completely, through autocrine actions. Hence, how CD-derived ET-1 could exert its full antihypertensive and natriuretic effects remains an open question. Several possibilities exist, including paracrine actions on neighboring cells. Such paracrine effects might include inhibition of renin release (21, 22), medullary vasodilation (4, 6), and/or elaboration of natriuretic factors such as nitric oxide (23). However, another possibility exists: CD-specific KO of ETA and/or ETB receptors may lead to compensatory changes in the remaining ET receptor. While such analysis has not been done, it is conceivable that such a scenario could exist if both ETA and ETB receptors affected a natriuretic response. One could envision that, since ETB receptors are normally much more abundant that ETA receptors in the CD, that ETB receptor KO could not be fully compensated for by increased ETA receptor expression, while ETA receptor KO could be compensated for by increased ETB receptor expression. Such analysis of ETA and ETB receptor expression is currently not possible to perform due to the limited amounts of protein and tissue (although mRNA could be assessed), hence an alternative means to examine this

THERE IS ABUNDANT EVIDENCE that collecting duct (CD) endothelin-1 (ET-1) is a physiologic regulator of arterial blood pressure (BP) and urinary Na excretion. The CD is the most abundant source of ET-1 in the body (15) and expresses the highest density of ET receptors of any renal cell type (16). In vitro ET-1 inhibits the epithelial Na channel (18) and Na-K-ATPase (25), suggesting that the peptide functions as an autocrine inhibitor of CD Na reabsorption. Further information about the CD ET system has been gleaned from cell-specific gene targeting studies. CD-specific knockout (KO) of ET-1 causes hypertension on a normal Na diet (~20 mmHg systolic BP elevation); a high Na diet is associated with a further elevation in systolic BP (~35–40 mmHg greater than controls) and impaired Na excretion (1). Notably, the salt-sensitive hypertension is partially corrected by amiloride, suggesting that the epithelial Na channel is involved. Total kidney and medullary ET-1 production is enhanced by Na loading (1). Taken together, these studies strongly suggest that CD-derived ET-1 could exert its full antihypertensive and natriuretic effects.

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question would be desirable. To address this, the current study examined BP and Na excretion in mice with CD-specific KO of both ETA and ETB receptors. By comparing these results to those obtained with CD-specific KO of ET-1 as well as CD-specific deficiency of either ET receptor alone, information on the combined effect of these receptors can be obtained.

**MATERIALS AND METHODS**

*Transgenic mice lines.* Mice with CD-specific disruption of both ETA and ETB receptor genes were generated in a manner similar to that previously described for each gene individually (1, 8, 9). The floxed ETB receptor mice have loxP sites flanking exons 3 and 4 of the ETB receptor gene, permitting selective deletion of essential coding regions on exposure to Cre recombinase (8). Similarly, floxed ETA receptor mice have loxP sites flanking exons 6–8 of the ETA receptor gene [these exons are critical to ETA receptor gene functional expression (9)]. Mice were bred to obtain homozygotes for both floxed ETA and ETB receptors (ETA/B); these were mated with aquaporin-2 (AQP2)-Cre mice with CD-specific expression of Cre recombinase. AQP2-Cre mice contain a transgene with 11 kb of the mouse AQP2 gene 5′ flanking region driving expression of Cre recombinase. The AQP2-Cre mice are identical to those fully characterized in previous publications by our group (1, 8, 9) and as used in collaboration with other investigators (11, 26). Female AQP2-Cre mice were mated with male floxed ETA/B receptor mice; female offspring heterozygous for both AQP2-Cre and floxed ETA/B receptors were bred with males homozygous for floxed ETA/B receptors. Animals homozygous for floxed ETA/B receptors and heterozygous for AQP2-Cre (CD ETA/B KO) were used in all studies. Sex-matched littermates that were homozygous for the floxed ETA/B receptor genes, but without Cre, were used as controls in all studies.

*Genotyping.* Tail DNA was prepared by standard methods and PCR amplified for the AQP2-Cre transgene using oligonucleotide primers AQP2CreF (5′-CTCTCTCAGGAACGTGTTGCG-3′) and AQP2CreR (5′-GCGAACATCTTCAGGTTCTGCGG-3′) which amplify the 671-bp junction between the mouse AQP2 promoter and the Cre gene (8). The ETA receptor gene was amplified using ETAF1 5′-CCCATGCTTGAACAAACCAG-3′ and ETAR1 5′-GATGACACCAACACAGCAAGAAGACAG-3′. These primers span the loxP site upstream of exon 6. The wild-type allele product is 314 bp and floxed allele is 354 bp. ETA receptor gene recombination was detected using ETAF2 5′-CCTGCTCTTGGTGTG-3′ and ETAR2 5′-CGTTCCTCGGTGTGTTGTTG-3′; these yield a product size of 112 bp in unrecombined and no product in recombined in product recombinant DNA (9). The ETB receptor gene was amplified using ETBF1 5′-TCAGTGTGAATGAGACACAGAC-3′ (located in intron 2, 5′ to the first loxP site), ETBF1 5′-AGAGGATCATGCTTGGAC-3′ (located within intron 2 in the sequence that was deleted when constructing the 5′ loxP site), and ETBR2 5′-AGCCATAAAGGCTACGAC-3′ (located in intron 4, 3′ to the second loxP site). Products of primers ETF1 and ETBR2 give a product only in wild-type animals (865 bp), while ETBF1 and ETBR2 give products of 1,092 bp in unrecombined DNA and 186 bp after recombination (8). PCR products were visualized after electrophoresis through 1.5% agarose.

*Chronic telemetry and metabolic balance studies.* All mice were studied around 3 mo of age. All mice had catheters inserted into the right carotid artery, tunneled subcutaneously, and the attached radio-transmitter was localized to the back. Continuous recording of BP and pulse was performed using telemetry (Data Sciences International, Arden Hills, MN). Two days after the surgery, mice were placed into Hatteras metabolic cages (Hatteras Instruments, Cary, NC) and acclimated for 3 days. Mice were fed 9 ml of a gelled diet that contained no food or water and given 1.5 ml normal saline intraperitoneally (ip). Subsequently, urine was collected every 2 h for the next 6 h. Urine was analyzed for volume, Na, and K. To control for the volume load, separate mice were also given 2 ml water ip and urine osmolality and volume were determined every 2 h for the next 6 h.

*Acute Na loading.* After fasting for 12 h, mice were placed into small metabolic cages that contained no food or water and given 1.5 ml normal saline intraperitoneally (ip). Subsequently, urine was collected every 2 h for the next 6 h. Urine was analyzed for volume, Na, and K.

**ETA and ETB receptor gene recombination in nephron segments.** Kidney sections were incubated in 1 mg/ml collagenase and 0.1% DNase at 37°C for 1 h. Proximal tubule, thick ascending limb, and cortical, outer medullary, and inner medullary collecting ducts were microdissected, and DNA was isolated. Samples were amplified using real-time PCR (Smart Cycler, Cepheid, Sunnyvale, CA). The same primers as used for genotyping were employed.

*Electrolyte and hormone analysis.* Plasma and urine were analyzed for Na and K concentration (Easy Vet analyzer, Medica, Bedford, MA) and creatinine (Jaffe colorimetry, Sigma, St. Louis, MO). PRC was measured as previously described (8, 9) using an indirect radioimmunoassay (Phoenix Pharmaceuticals, Burlingame, CA). Aldosterone levels were determined by radioimmunoassay, after HCl hydrolysis and ethyl acetate extraction (Coat-a-Count, Diagnostic Products, Los Angeles, CA).

*Real-time PCR of CD.* Inner medullary CD (IMCD) were acutely isolated in a manner similar to that previously described (9). Briefly, inner medullas were minced in Kreb’s buffer with 1 mg/ml collagenase (Type IV, Worthington, Lakewood, NJ) and 0.1 mg/ml DNase (Sigma) and incubated at 37°C for 30–45 min. RNA was isolated, reverse transcribed, and real-time PCR was performed using a Smart Cycler (Cepheid). The primer sequences for ETA receptor mRNA were EdnrA F2 5′-TCTGACCACCTGGTCTGTGCTG-3′ and EdnrA R2 5′-CGTTCGATGTTGAGAGAC-3′ and GAPDH F 5′-TGGCCTTGGTG-3′ and GAPDH R 5′-CTGCTGTAAGGTG-3′ which yield a product size of 112 bp. The primers for GAPDH were GAPDH F 5′-TGGCCTTGGTG-3′ and GAPDH R 5′-CTGCTGTAAGGTG-3′ which yield a product size of 110 bp. The ratio of ETRA receptor:GAPDH mRNA was calculated for each sample.

*Statistics and ethics.* Comparisons of single points on a single day between control and KO mice were analyzed by the unpaired Student’s t-test. Comparisons of multiple points (e.g., BP and Na excretion) were made using one-way ANOVA with the Bonferroni correction. P < 0.05 was taken as significant. Data are expressed as means ± SE.

All animal experiments were ethically approved by the University of Utah Institutional Animal Care and Use Committee.
RESULTS

Characterization of CD ETA/B KO mice. CD ETA/B KO mice developed normally until at least 5 mo of age and had no gross morphologic abnormalities. AQP2-Cre mice confer CD-specific KO, as determined by principal cell-specific Cre recombinase activity in mice heterozygous for AQP2-Cre and the ROSA26-YFP reporter, in situ hybridization, genomic PCR of microdissected CD, and genomic PCR for gene recombination in an organ panel of 15 different organs (1, 2, 8, 9). This was further confirmed by RT-PCR of ETA and B receptor mRNA as well as PCR of genomic DNA in microdissected CD. Cortical, medullary, and inner medullary CDs all showed ETA/B receptor gene recombination, while microdissected proximal tubules and thick ascending limbs had no recombination. In addition, CD from CD ETA/B KO mice had markedly reduced ETA/B receptor mRNA (20% of the levels expressed in control CD, n = 10 tubules). It should be noted that microdissected CD contain intercalated cells which may express ETA/B receptors and are not targeted by AQP2-Cre. Thus, CD ETA/B KO mice have principal cell-specific inactivation of ETA and ETB receptor genes.

Renal function and BP during normal Na intake. All mice were ration fed to match food and water intake (1, 8, 9). This was accomplished using a gelled diet containing all food and water as previously described. Under baseline conditions (0.3% Na intake), CD ETA/B KO mice had elevated systolic (18.4 ± 0.8 mmHg greater than controls, n = 10, P < 0.001) and diastolic BP (9.4 ± 2.1 mmHg greater than controls, n = 10, P < 0.005; Table 1 and Fig. 1). There were no differences in food or water intake, body weight, urine volume, urine K concentration, plasma Na or K concentration, urine Na or K excretion, or creatinine clearance (Table 1 and Fig. 2). PRC and urinary aldosterone excretion were similar between CD ETA/B KO and control mice (see Table 3).

Renal function and BP during a high Na diet. Following the 3 days of a normal Na diet, mice were placed on a high Na diet containing 6 ml gelled 1% Na diet and normal saline to drink. This diet caused a 15- to 20-fold increase in urinary Na excretion. CD ETA/B KO KO mice had further increases in systolic and diastolic BPs of −13 and 11 mmHg, respectively (Tables 1 and 2 and Fig. 1). BP was unchanged in control mice during a high Na diet. CD ETA/B KO mice also had enhanced Na retention that was first evident during the second of Na loading (Fig. 2). Importantly, Na intakes were similar between the two groups of mice over the course of the high Na diet.
Note that the data shown are cumulative Na retention, starting on the first day of a normal Na diet; this was done to help show significant differences in Na balance in the face of day-to-day variability. There were no differences in intake, plasma Na or K concentration, or creatinine clearance between CD ETA/B KO and control animals (Table 2 shows day 2 of the high Na diet). Urinary aldosterone excretion decreased to undetectable levels on a high Na diet in both groups (Table 3). PRC fell on a high Na diet in both CD ETA/B KO and control mice to a similar extent (Table 3).

**Effect of acute Na loading.** CD ETA/B KO mice had reduced urinary Na excretion within the first 2 h after an acute Na load (Fig. 3). Over the next 2-h period, CD ETB KO mice had similar Na excretion compared with controls. During the 4- to 6-h time period, CD ETA/B KO mice normalized Na excretion so that, after 6 h, they had similar Na excretion compared with controls. To control for a volume load effect, urine osmolality did not differ between CD ETA/B KO and control mice (n = 9 each group) after 2 h (648 ± 243 mosmol/kgH2O in CD ETA/B KO and 443 ± 220 mosmol/kgH2O in control) or after 6 h (644 ± 149 mosmol/kgH2O in CD ETA/B KO and 840 ± 135 mosmol/kgH2O in control). Similarly, urine volume was not different after 2 h (0.15 ± 0.04 ml in CD ETA/B KO and 0.20 ± 0.08 ml in control) or 6 h cumulatively (1.06 ± 0.12 ml in CD ETA/B KO and 0.98 ± 0.13 ml in control).

**Comparison of BP between CD ETA/B KO mice and other CD ET system KO mice.** To evaluate the effect on BP resulting from the combined absence of CD ETA and ETB receptors, the systolic BP during normal and high Na diets in CD ET-1 KO, CD ETA KO, and CD ETB KO mice was examined (Fig. 4). The systolic BP of these latter three groups of mice was obtained by radiotelenometry and was as previously reported (1, 8, 9). CD ETA/B KO mice systolic BP was greater than that in CD ETB KO mice during normal or high Na intakes, suggesting that presence of the CD ETA receptor mitigates the degree of hypertension seen in CD ETB KO animals. However, CD ETA KO mice are not hypertensive. Notably, CD ETA/B KO systolic BP was similar to that in CD ET-1 KO on a normal Na diet, but was significantly less than CD ET-1 KO for the first several days of high Na intake, suggesting that CD-derived ET-1 effects on BP cannot be entirely explained by autocrine actions.

To help assess the possibility that ETA receptors may be altered in CD ETB KO mice, IMCD ETA receptor mRNA expression was quantified. Notably, ETA receptor mRNA was increased by 2.07 ± 0.24-fold (n = 3, P < 0.025) in ETB KO mice compared with controls when normalized for GAPDH.

## DISCUSSION

The current studies were undertaken to help define the role of CD ETA and ETB receptors in the regulation of systemic BP and urinary Na excretion. Based on previous studies wherein CD ETA KO did not affect BP or Na excretion, and CD ETB KO caused elevated BP and Na retention, it was expected that CD ETA/KO would have a similar phenotype as that seen in CD ETB KO animals. However, this proved not to be the case: combined KO of CD ETA and ETB receptors caused significantly greater hypertension than did KO of CD ETB receptors alone. Notably, CD ETA KO does not cause hypertension, yet deficiency of this receptor in the setting of CD ETB disruption appears to aggravate the hypertension—both during a normal Na diet and in response to high Na intake. As discussed earlier, quantitative assessment of ET receptor expression in CD is not technically possible; however, mRNA levels can be determined. We found that ETA receptor mRNA is significantly increased in CD ETB KO mice, supporting, although by no

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<th>Table 2. Metabolic balance data in control and CD ETA/B KO mice on day 2 of a high sodium diet</th>
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<td>Control</td>
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<td><strong>Systolic BP, mmHg</strong></td>
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<td><strong>Diastolic BP, mmHg</strong></td>
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<td><strong>Weight, g</strong></td>
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<td><strong>[Na] plasma, meq/l</strong></td>
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<td><strong>Cumulative Na retention, intake urinary excretion, meq, starting on day 1</strong></td>
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<td><strong>[K] plasma, meq/l</strong></td>
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<td><strong>Urinary K excretion, meq/day</strong></td>
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Data are means ± SE, n = 10 each data point. *P < 0.001 vs. control. †P < 0.05 vs. control.

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<th>Table 3. Plasma and urine hormone levels in control and CD ETA/B KO mice under varying normal and high Na intake</th>
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<td><strong>Aldosterone, ng/day</strong></td>
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<td><strong>PRC, pg Al μl⁻¹ h⁻¹</strong></td>
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Data are means ± SE, n = 5–6 each data point. Aldosterone, urinary aldosterone excretion; Al, angiotensin I; PRC, plasma renin concentration.
means proving, the notion that CD ETA receptors may play a role in mitigating the hypertension and Na retention in this model. It is not possible to interpret changes in response to in vivo administration of ETA- or ETB-selective antagonists since these agents directly affect systemic BP, renal blood flow, glomerular filtration rate, and numerous other parameters. Activation of ETB receptors inhibits Na and/or Cl reabsorptive pathways in vitro, including reduction of epithelial Na channel activity in distal nephron cells (18), inhibition of NaCl reabsorption in isolated perfused cortical CD (17), and reduction of chloride flux in isolated medullary thick ascending limb (20). In contrast, the role of ETA receptors in regulating renal tubule Na reabsorption remains uncertain. Initial data suggested that nephron ETA receptors may exert antinatriuretic effects in that high concentrations of ET-1 (10 nM) increased epithelial Na channel activity in A6 distal nephron cells via ETA receptor activation (18). However, in preliminary studies, infusion of ET-1 into the renal medulla of rats genetically deficient in ETB receptors causes a natriuresis, and this effect is blocked by ETA antagonism (19). How then can these data be reconciled? The answers are entirely speculative; however, several possibilities exist. One possibility, albeit clearly unproven, is that CD ETA receptors can be natriuretic, but that such effects are primarily manifest in the absence of CD ETB receptors. ET receptor isofoms have been reported to form homo- and heterodimers, which may differentially affect cellular trafficking, ET antagonist binding, and activation of signal transduction pathways (10, 12). If ETB receptors normally (at least in mice and rats) predominate in the CD, then ETB receptor homodimers and ETA/ETB receptor heterodimers may be the major receptor forms present. However, if ETB receptors are absent, then ETA receptor homodimers might form, creating a receptor structure that now could exert a natriuretic effect. However, proof of such differential dimerization in vivo is not currently possible. Another possibility is that ETA receptors, whether homo- or heterodimers, are natriuretic in vivo, but that because ETB receptors predominate in the CD, absence of CD ETA receptors does not normally have physiologic significance. Finally, it is conceivable that changes in ET receptor expression on neighboring intercalated cells might play a role; however, there have been no studies in this regard. Resolution of this question is rather problematic, but will need addressing since it is of substantial clinical significance. Antagonists selective for the ETA receptor cause fluid retention and edema formation in humans; this side effect caused halting of a diabetic nephropathy trial, may have limited the utility of ETA antagonists in congestive heart failure (3), and was also a significant issue in patients with metastatic prostatic cancer treated with ETA blockers (5).

The current study found that CD ETA/B KO mice increased BP in response to Na loading more slowly than did mice with CD ET-1 KO. Furthermore, there was no detectable effect on Na excretion on the first day of Na loading in CD ETA/B KO mice. These findings suggest that CD-derived ET-1 may exert primarily paracrine effects on the first day of Na loading, while such paracrine effects may continue to play a role, in addition to autocrine regulation of CD function, during subsequent days of Na loading. Such paracrine actions could include inhibition of thick ascending limb NaCl transport (20), dilation of medullary vasa recta (6), and/or elaboration of natriuretic factors (nitric oxide and prostaglandin E2) from medullary interstitial cells (27).

CD ETA/B KO mice had similar PRC and urinary aldosterone excretion as control animals, despite being hypertensive. Furthermore, Na loading suppressed PRC (aldosterone became immeasurable) to a similar degree in CD ETA/B KO and control mice, despite CD ETA/B KO mice manifesting salt-sensitive hypertension. Thus, in essence, CD ETA/B KO mice do not appear to have an appropriately suppressed renin-angiotensin-aldosterone axis. These findings are in contrast to those in CD ETB KO mice who have markedly suppressed PRC and urinary aldosterone excretion compared with normotensive controls (8). Hence, it is possible that ET-1 interaction with CD ETA receptors may lead, through as yet uncertain
mechanisms, to inhibition of renin release. Notably, CD ET-1 KO mice also have failure to suppress the renin-angiotensin-aldosterone axis (1). The initial theory was that CD-derived ET-1 could directly inhibit renin release, so CD ET-1 KO mice were missing this regulatory pathway (1). While the current study does not preclude this possibility, it does suggest that other mechanisms must be involved in potential CD ET-1 regulation of renin release.

In summary, the current study demonstrates that the CD ET system involves complex regulation of renal tubule Na transport and systemic BP. ET-1 could conceivably interact with both ETA and ETB receptors in the CD; if such a scenario exists, activation of either receptor could potentially exert a natriuretic effect. CD-derived ET-1 likely exerts part of its actions with neighboring cells. This system of BP regulation may exist, activation of either receptor could potentially exert a natriuretic effect. CD-derived ET-1 likely exerts part of its actions with neighboring cells. This system of BP regulation may exist.

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