ETB Receptor in Renal Medulla Is Enhanced by Local Sodium During Low Salt Intake

ET_B Receptor in Renal Medulla Is Enhanced by Local Sodium During Low Salt Intake

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Abstract—Renal endothelin-1 participates in sodium and water handling, and its urinary excretion is increased in sodium-retentive states. We compared the cortical and medullary renal expression of prepro-endothelin-1, endothelin-converting enzyme-1, and endothelin type A and type B receptors in patients who underwent nephrectomy after normal (108 mmol/d NaCl; n=6) or low (20 mmol/d NaCl; n=6) sodium diet and investigated whether sodium exerts a direct role on endothelin receptor binding in vitro. With normal sodium diet prepro-endothelin-1 mRNA was 3-fold higher in renal medulla than in cortex ($P<0.01$), whereas endothelin-converting enzyme-1 mRNA was equally distributed. Endothelin-1 receptor density was 2-fold higher in renal medulla than in cortex ($P<0.05$). Type B was the main receptor subtype in both regions. In the renal cortex, low sodium diet caused a 194% increase in prepro-endothelin-1 mRNA ($P<0.05$), whereas endothelin-converting enzyme-1 type B and type A receptors remained unchanged. In contrast, in the renal medulla the increase in prepro-endothelin-1 mRNA (+30%, $P<0.05$) was associated with a selective increase in type B receptor for both mRNA expression (+37%, $P<0.05$) and binding density (+55%, $P<0.05$). Increasing in vitro sodium concentrations between 154 and 308 mmol/L significantly enhanced type B receptor density ($P<0.05$) and affinity ($P<0.05$). In conclusion, during low sodium diet, renal prepro-endothelin-1 synthesis increases mainly in the renal cortex (where no changes in receptors occur), whereas type B receptor is selectively enhanced in the renal medulla. The range of sodium concentrations that are physiologically present in vivo in the renal medulla selectively modulate type B receptor density and affinity. **(*Hypertension*. 2002;40:179-185.)**

Key Words: endothelin ■ receptors, endothelin ■ sodium ■ kidney ■ water-electrolyte balance ■ diet, sodium-restricted

Renal endothelin (ET)-1 is produced by vascular endothelium in the cortex, including glomerular capillaries, arterioles, and peritubular capillaries, and by tubular epithelial cells in the inner medulla.[1,2] These 2 compartments represent 2 distinct systems, both involved in volume homeostasis. ET-1 produced in the vasculature by endothelial cells is able to reduce blood flow at the renal cortex by acting on endothelin type A (ET_A) receptor subtype.[3] This vascular ET_A-mediated effect results in increased sodium reabsorption in both humans[3,4] and experimental animals.[5] Conversely, ET-1 produced by tubular epithelial cells inhibits arginine vasopressin (AVP)-stimulated osmotic water permeability in inner medullary collecting ducts[6–8] via endothelin type B (ET_B) receptor subtype,[5,9] thus increasing free water clearance.[2]

There is in vitro evidence that the increased osmolality increases preproET-1 (ppET-1) mRNA expression and ET-1 synthesis in epithelial tubular cells.[10,11] In vivo, an increased renal ET-1 production has been found in pathophysiological and clinical conditions characterized by increased medulla osmolarity, such as dehydrated physical exercise,[12] low sodium diet,[2] and heart failure.[13]

However, although cortical and medullary ET-1 systems participate differently in sodium and water handling, no studies exist comparing the activation of the ET-1 system in the 2 renal regions in the sodium-retentive states. Moreover, despite the fact that the use of ET-1 receptor antagonist has been proposed in heart failure,[14–16] no information is available as to whether the increased local ET-1 synthesis is associated with a consensual increase in receptor synthesis or whether it may cause downregulation of its specific receptors.

Therefore, the aims of this study are to compare the effects of low sodium diet on the expression of the various ET-1 system components (ppET-1, endothelin-converting enzyme [ECE]-1, ET_A, and ET_B) in the cortex and in renal medulla and to investigate whether sodium might play a direct role on endothelin receptor binding density and affinity.

Methods

Subjects Investigated and Experimental Protocol

Twelve patients affected by polar tumor and listed for elective nephrectomy were investigated. No subject was a smoker or had taken any drug for at least 4 weeks. Patients with hypertension, ischemic heart disease, heart failure, renal failure, abnormal liver

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TABLE 1. Characteristics of Subjects Investigated

	Normal Sodium Intake (n=6)	Low Sodium Intake (n=6)
Age, y	53±3	55±7
Gender, M/F	4/2	3/3
Systolic arterial pressure, mm Hg	125±7	128±8
Diastolic arterial pressure, mm Hg	82±3	83±3
Plasma glucose, mg/dL	80±6	81±7
Plasma sodium, mEq/L	139±4	137±5
Plasma creatinine, mg/dL	0.97±0.25	0.93±0.32
Creatinine clearance, mL/min	93±7	94±9
Sodium clearance, mL/min	0.6±0.04	0.09±0.03*
Plasma renin activity, ng Ang I/mL per hour	1.58±0.32	4.34±0.24*

Ang I indicates angiotensin I.
*$P<0.05$ vs normal sodium intake.

function tests or diabetes were excluded. The study was approved by the local review committee of the University of Florence, and all subjects gave their informed, written consent.

During the week preceding surgery, patients were randomized to receive a 7-day period of the same basic diet, with either normal (108 mmol/d NaCl; n=6) or low (20 mmol/d NaCl; n=6) sodium intake. Urine and plasma samples for the determination of sodium, creatinine, and plasma renin activity (PRA) were taken daily during the last 3 days of each diet regimen (Table 1).

Transmural kidney specimens, containing both cortex and medulla, were cut from the pole opposite the tumor and fixed in 4% paraformaldehyde for in situ hybridization studies. In addition, separated specimens of whole medulla (containing both inner and outer medulla) and cortex were dissected immediately after nephrectomy and frozen in liquid nitrogen for binding and RT-PCR studies.

Receptor Binding Studies

Cell membranes were obtained from homogenated tissue as previously described.[17] Competition and kinetic studies were performed in the presence of increasing concentrations of NaCl or NaH_2PO_4 (0, 77, 154, 231, and 308 mmol/L). Experiments were carried out in triplicate, and separate curves for renal cortex and medulla were obtained in each patient.

Equilibrium Binding Studies

Cell membranes (250 μg/mL) were incubated with ^{125}I-ET-1 (100 pmol/L, 2000 Ci/mmol) (Amersham) and increasing concentrations of unlabeled ET-1 (0 to 1 μmol/L) or selective ET_A (BQ123, 0 to 100 μmol/L) and ET_B (BQ788, 0 to 100 μmol/L) antagonists for 120 minutes at 22°C in a final volume of 0.2 mL.[17] The content was then rapidly filtered through glass fiber filters (Whatman GF/C). Binding data were analyzed using a nonlinear fitting computer program (LIGAND).[18]

Kinetic Analysis

The kinetics of association of ^{125}I-ET-1 (100 pmol/L) to cell membranes (250 μg/mL) were evaluated as previously described.[19]

RT-PCR Analysis

Levels of ppET-1, ECE-1, ET_A, and ET_B transcripts were quantified with reverse transcription-polymerase chain reaction (RT-PCR) using specific primers (Table 2) with GAPDH as the internal standard as previously described.[17] The efficiency of amplification of each primer pair was calculated beforehand from the slope of the semilogarithmic relationship between cycles of amplification (26 to 44) and amplification products (23% for GAPDH, 23% for ppET-1, 22% for ECE-1, and 22% for both ET_A and ET_B receptors) (Figure 1). All RT-PCR studies were performed in triplicate in all subjects investigated.

In Situ Hybridization Studies

In situ hybridization studies were performed as previously described[2] using specific cDNA probes for ET_A and ET_B receptor subtypes (ET_A, American Type Culture Collection, ATCC 105194, and ET_B, ATCC 1250426) and for GAPDH (ATCC 57090).

Statistical Analysis

Data are presented as mean±SD. Comparisons of a single observation between groups were made with ANOVA and 2-tailed *t* tests. All statistical analyses were performed with BMDP statistical software (BMDP Statistical Software, Inc).

Results

Renal ET-1 System

RT-PCR showed that the ppET-1/GAPDH ratio was 3-fold higher in renal medulla than in cortex (1.01±0.1 versus 0.31±0.24, respectively; $P<0.01$), whereas there was a comparable presence of ECE-1 mRNA in the 2 regions (ECE-1/GAPDH ratios of 0.85±0.45 and 0.78±0.44, respectively) (Figure 2A).

ET_B mRNA was the prevalent receptor transcript in both medulla (1.21±0.08 and 0.39±0.12 for ET_B/GAPDH and ET_A/GAPDH ratios, respectively) and renal cortex (0.95±0.33 and 0.45±0.14, respectively) (Figure 2A). In situ hybridization studies showed that mRNA for ET_B receptor was mainly expressed by tubular epithelial cells in renal medulla, whereas the ET_A subtype was almost exclusively

TABLE 2. GAPDH, ppET-1, ECE-1, ET_A, and ET_B Primers for RT-PCR

Primer	Sequence 5′—3′	cDNA Sizes (bp)	T Annealing (°C)	Cycles (n)
GAPDH	5′ TGAAGGTCGGAGTCAACGGA	987	58	32
	3′ CATGTGGGCCATGAGGTCCA			
ppET-1	5′ GTCAACACTCCCGAGCACGTT	304	60	32
	3′ CTGGTTTGTCTTAGGTGTTCCTC			
ECE-1	5′ TGCCATCTACAACATGATAG	572	52	32
	3′ GTCTTGACCCACTTCTTC			
ET_A	5′ TATCAATGTATTTAAGCTGCTGG	252	56	35
	3′ GGAATGGCCAGGATAAAGG			
ET_B	5′ TTGGAGCTGAGATGTGTAAGC	626	56	35
	3′ CCATAGTTGTACCGAAGTGAC			

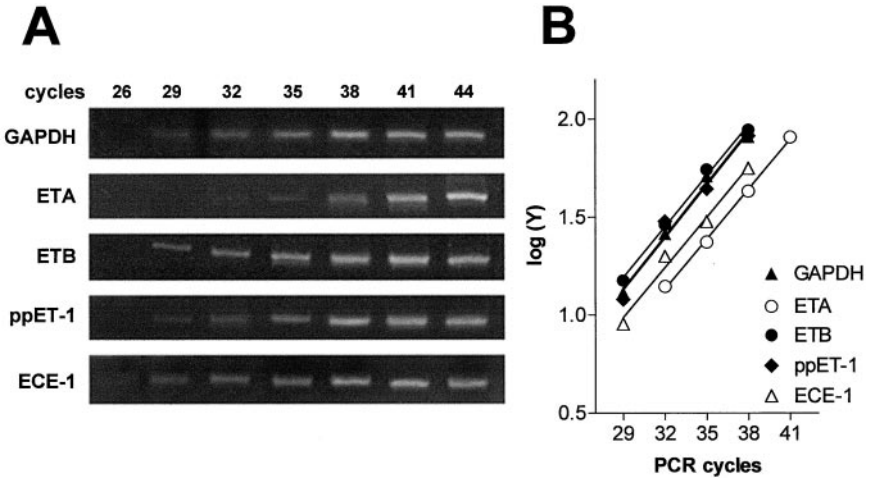

Figure 1. A, Amplification of GAPDH, endothelin type A (ET$_A$) and type B (ET$_B$) receptors, prepro-endothelin (ppET)-1, and endothelin-converting enzyme (ECE)-1 in human renal medulla at increasing numbers of cycles (26 to 44). B, Semilogarithmic representation of the relative extent of amplification (Y) of GAPDH, ET$_A$ and ET$_B$ receptors, ppET-1, and ECE-1.

localized in the endothelial cells of peritubular vessels (Figure 2B).

With binding studies, ET-1 receptor density was 2-fold higher in renal medulla than in cortex (127±13 versus 59±9 fmol/mg protein, $P<0.05$) (Table 3). Again with binding studies, receptor population was almost exclusively represented by the ET$_B$ subtype in both districts (>80%) (Figure 3), with a 2.3-fold higher maximum binding (Bmax) in renal medulla than in renal cortex (Table 3). No differences in receptor affinity were observed between renal medulla and renal cortex.

Effect of Low Sodium Diet

In renal cortex, only mRNA for ppET-1 was markedly increased (+194% versus normal sodium diet) whereas mRNAs for ECE-1 and receptor subtypes remained unchanged (Figure 4). No changes in receptor binding were found in renal cortex (Table 3).

Conversely, in renal medulla, both ppET-1 and ET$_B$ genes were significantly enhanced (+30% and +37%, respectively; $P<0.05$ for both) without any changes in ECE-1 and ET$_A$ transcripts. Binding studies confirmed the selective increase in ET$_B$ receptor density in renal medulla during low sodium diet (+55% versus normal sodium diet) with no modifications in the ET$_A$ subtype (Table 3, Figure 5).

Effect of Sodium In Vitro

ET$_B$ receptor density and affinity showed a sodium-dependent increase in renal medullary membranes obtained from patients on normal sodium diet (Table 3, Figure 6). The increase was significant for NaCl concentrations greater than 154 mmol/L because, at 154 mmol/L, ET$_B$ receptor density was increased by 15% versus NaCl-free buffer (NS), whereas at 231 and 308 mmol/L increased by 28% ($P<0.05$ versus NaCl-free buffer) and 49% ($P<0.05$ versus both NaCl-free buffer and 154 mmol/L NaCl).

Likewise, the binding affinity remained almost unchanged versus NaCl-free buffer up to 154 mmol/L NaCl (1.5-fold, NS), and was 3.2-fold and 3.8-fold enhanced at 231 and 308 mmol/L NaCl, respectively ($P<0.05$ versus NaCl-free and 154 mmol/L NaCl for both) (Table 3).

In addition, the same effect was observed with membranes isolated from renal cortex. The relationship between the K×R product of ET-1 binding (a unitless measure of the amount of

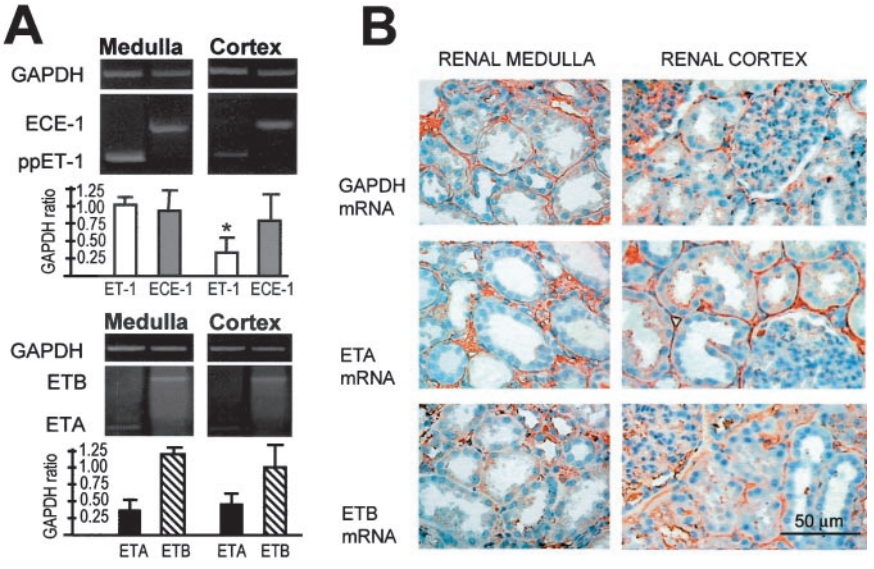

Figure 2. Expression of the components of the ET-1 system in human kidney. A, Representative RT-PCR of mRNA expression for ppET-1, ECE-1, and GAPDH (upper panel) and for ET$_A$ and ET$_B$ receptors and GAPDH (lower panel) in human kidney at normal sodium diet (n=6). Ratio to GAPDH mRNA expression is reported. *$P<0.01$ versus medulla. B, In situ hybridization studies for ET$_A$ and ET$_B$ receptor mRNAs in renal medulla and renal cortex. Prevalent ET$_B$ expression on tubular epithelial cells in renal medulla. Localization of ET$_A$ subtype in interstitial and endothelial cells of peritubular vessels.

TABLE 3. Effects of 7-Day Normal Sodium and Low Sodium Diets on ET-1 Receptors

	Normal Sodium Intake (108 mmol/d NaCl)		Low Sodium Intake (20 mmol/d NaCl)	
	Tris Buffer	Tris Buffer+NaCl[a]	Tris Buffer	Tris Buffer+NaCl[a]
Cortex				
Bmax total, fmol/mg	59±9	121±12*	61±11	130±16*
ET_A:ET_B	18:82	11:89	16:84	9:91
ET_A, fmol/mg	11±2	13±1	10±2	12±1
ET_B, fmol/mg	49±7	107±10*	51±9	118±14*
Kd ET-1, pmol/L	55±14	13±2*	47±6	15±5*
Kobs, min^{-1}	0.064±0.012	0.106±0.010*	0.065±0.015	0.103±0.018*
K-1, min^{-1}	0.0076±0.0019	0.0034±0.0005*	0.0066±0.0008	0.0038±0.0005*
Medulla				
Bmax total, fmol/mg	127±13†	182±18*†	184±26‡†	279±17*‡†
ET_A:ET_B	12:88	8:92	6:94	5:95
ET_A, fmol/mg	15±2	15±1	11±3	14±1
ET_B, fmol/mg	112±12†	167±17*†	173±24‡†	265±17*‡†
Kd ET-1, pmol/L	38±17	10±3*	32±11	12±4*
Kobs, min^{-1}	0.043±0.010	0.097±0.032*	0.060±0.007	0.095±0.023*
K-1, min^{-1}	0.0063±0.0019	0.0029±0.0008*	0.0060±0.0011	0.0033±0.0007*

Bmax indicates maximum binding; Kd ET-1, dissociation constant of ET-1; Kobs, kinetically derived observed association rate constant; K-1, kinetically derived dissociation rate constant.

[a]The effect of local sodium concentration on ET-1 binding is investigated in vitro in the presence of 308 mmol/L NaCl.

*$P<0.05$ vs Tris-buffer; †$P<0.05$ vs cortex; ‡$P<0.05$ vs normal sodium intake.

ligand-receptor binding) and sodium concentration in the incubation medium showed a sigmoid pattern with a maximum rate of increase for sodium concentrations ranging between 154 and 308 mmol/L, which corresponded to a 4-fold increase in ET-1 binding efficiency (Figure 7). The enhancement of binding efficiency was not attributable to chloride because similar results were obtained when NaH_2PO_4 was used instead of NaCl (Figure 7).

Discussion

The present study shows that during low sodium diet (1) the synthesis of renal ppET-1 is increased mainly in the cortex, where no changes in endothelin receptors occur, whereas (2) ET_B receptor density selectively increases in renal medulla, and, moreover, that (3) sodium in vitro directly enhance ET_B receptor density and affinity.

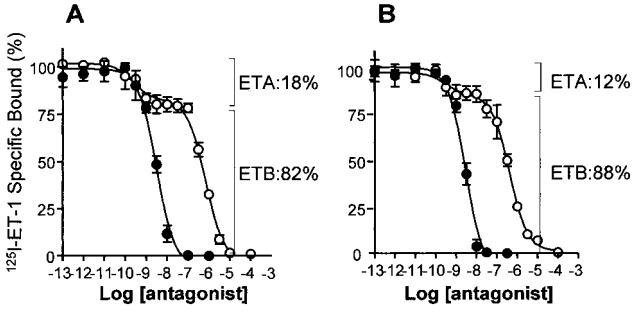

Figure 3. Competition experiments in human renal cortex (A) and medulla (B) of patients on normal sodium diet (n=6). ET-1 (filled circles), BQ123 (empty circles). Experiments are performed in triplicate.

Increased ppET-1 mRNA expression in the endothelial cells of the peritubular capillary network and in epithelial cells of the medullary collecting tubules during low sodium diet was previously reported by our group.[2] In the same study, the increased ppET-1 mRNA expression was associated with an increased renal ET-1 production and urinary ET-1 excretion linearly related to sodium retention.[2] The present study extends these results and offers new insights into the role of ET-1 in sodium retentive states in humans. The comparison between cortex and renal medulla of ppET-1 mRNA expression at RT-PCR shows that, during low sodium diet, the ppET-1 gene is enhanced to a greater extent in renal cortex, where the vascular component is highly represented, than in renal medulla. Moreover, mRNA studies performed in cortical tissue have revealed that the ET_A receptor, previously found in homogenates from renal cortex,[20,21] is mainly localized in the vasculature (in situ hybridization) and is not downregulated during low sodium diet (RT-PCR studies). Sodium reabsorption mainly occurs at the proximal tubule with an active process. In addition, when extracellular volume is contracted, as during low sodium diet, and postglomerular resistances are increased to maintain the glomerular filtration rate, the decreased hydrostatic pressure and the increased colloid osmotic pressure in peritubular capillaries further enhance the rate of sodium reabsorption at the proximal tubule. The wide range of increase in ppET-1 expression in renal cortex (+194%) might contribute to

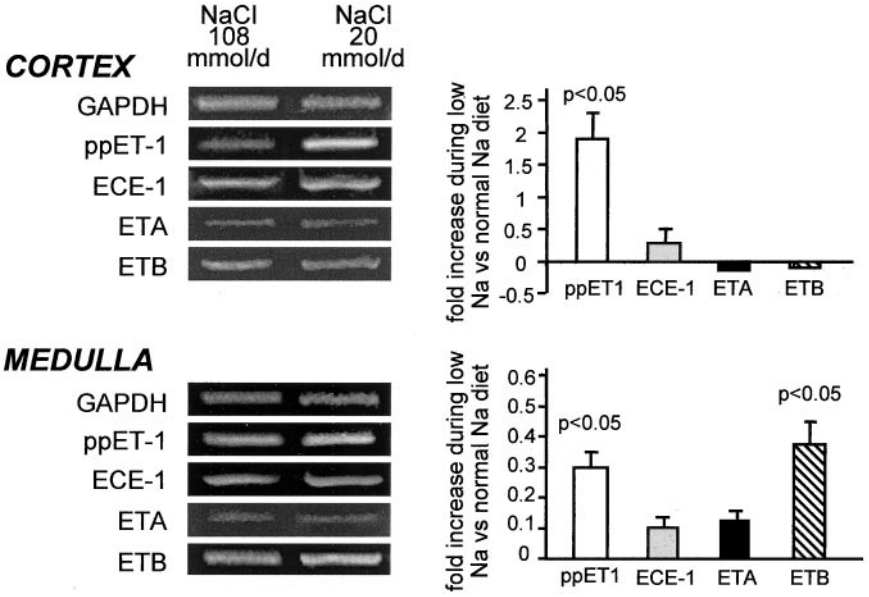

Figure 4. Representative mRNA expression of the components of the ET-1 system (ppET-1, ECE-1, and ET_A and ET_B receptors) after normal (108 mmol/d NaCl) and low sodium diet (20 mmol/d NaCl) in renal cortex (upper panels) and in renal medulla (lower panels). The right graphs represent the increase in mRNAs (expressed as ratio to GAPDH) during low sodium diet (n=6) compared with normal sodium diet (n=6). All RT-PCR studies were performed in triplicate in all subjects investigated.

reducing cortical blood flow through ET_A receptors,[4,5,22] enhancing sodium and water reabsorption at the proximal tubule. The reduction in cortical blood flow also attenuates the flow in the vasa recta, so that the reduced washout of osmolytes in renal medulla increases intratubular sodium concentration and passive sodium reabsorption at the thin ascending limb of Henle's loop.[23,24]

Therefore, the high ppET-1 overexpression in the cortex at low sodium diet, without changes in vascular ET_A receptor, indicate that, at normal sodium diet, the ET_A receptor population exceeds the physiological requirement and that, during low sodium diet, ET-1 contributes to sodium reabsorption mainly via the ET_A -mediated reduction of cortical blood flow and, finally, that ET-1 does not downregulate the ET_A receptor.

Unlike in the cortex, in renal medulla the increased ppET-1 transcript (30%) is paralleled by selective ET_B receptor gene overexpression (37%) with selective increase in the number of ET_B binding sites (55%). The mechanisms responsible for the increased medullary de novo synthesis of ET_B receptors during low sodium diet remain to be investigated, and a possible role for medulla hyperosmolarity can only be postulated. In situ hybridization indicates that the ET_B receptor gene in the renal medulla is expressed by epithelial cells of distal tubules and collecting ducts, where previous autoradiographic studies had localized the receptor protein.[19] ET_B receptors in inner medullary collecting ducts mediate the ET-1 inhibitory effect on AVP-stimulated osmotic water permeability.[6,25,26] In addition, ET_B stimulation also inhibits sodium reabsorption along the thick ascending limb.[5,27] ET_B-selective overexpression in the renal medulla during low sodium diet may modulate ET_A-mediated sodium retention, at the same time contrasting AVP-mediated water reabsorption and possible hyponatremia in clinical conditions characterized by nonosmotic AVP secretion, such as heart failure.

During a low sodium diet, the efficiency of ET_B receptor binding in renal medulla also appears to be magnified, in addition to increased ET_B receptor synthesis. In vitro studies have indeed shown a direct effect of increased local sodium concentration on ET_B receptor density and affinity in medullary membranes. The maximum effect of sodium on ET_B receptors in vitro is evident between 154 and 308 mmol/L

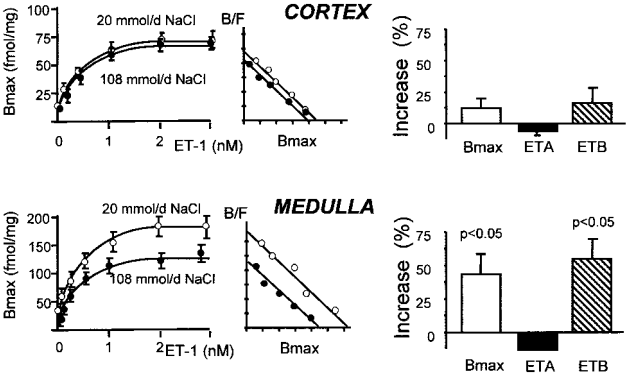

Figure 5. Binding of ET-1 at equilibrium to cortical (upper part) or medullary (lower part) membranes of kidneys obtained after normal (filled circles, n=6) or low sodium diet (empty circles, n=6). Experiments were performed in triplicate.

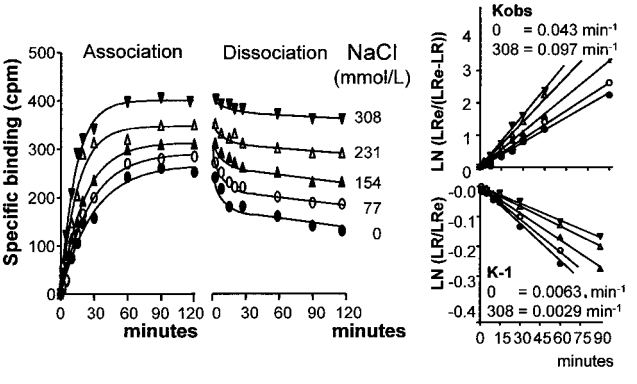

Figure 6. Effect of increasing sodium concentration on the association and dissociation phases of the ET-1 binding to membranes from renal medulla of patients on normal sodium diet (n=6). Experiments were performed in triplicate.

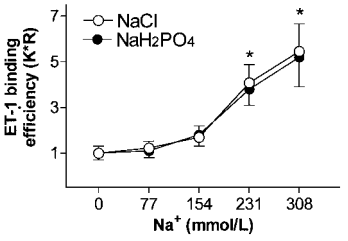

Figure 7. Effect of increasing NaCl and NaH_2PO_4 concentration in the incubation buffer on the ET-1 binding efficiency as expressed by the K×R product (affinity constant × receptor binding concentration). *$P<0.05$ versus NaCl-free buffer and versus NaCl 154 mmol/L.

(Figure 7), ie, within the range of sodium concentration in renal medulla.[28] This effect is observed in membranes obtained from patients at both low and normal sodium diet and is not due to chloride in itself because the same pattern of response is observed when sodium phosphate is used.

The adaptation of ET_B receptors to different sodium intake is not an isolated result. Type I angiotensin (Ang) II receptors in the kidney also exhibit physiological adaptation during chronic changes in sodium intake because sodium restriction increased renal Ang II receptor density and AT1 mRNA levels in the 2 main sites of sodium reabsorption, proximal tubules[29,30] and the medullary thick ascending limb of loop of Henle.[31] Conversely, a high salt diet was associated with a downregulation of AT1 receptors.[32] Therefore, during low sodium diet the upregulation of angiotensin II receptors cooperates with angiotensin II formation to enhance sodium retention. In the same condition, the endothelin-1 system seems to operate as a modulating system because it selectively activates the ET_B receptor subtype, which causes natriuresis and increases free water clearance.

Experiments performed in rats indicated that AT1 receptor upregulation during low sodium diet is Ang II–dependent because it was prevented by losartan treatment.[31] At variance with Ang II, endothelin-1 seems not responsible for receptor upregulation because, according to the present findings, ET_B receptor is not enhanced in the cortex where ET-1 is overexpressed to a greater extent than in medulla. Moreover, the in vivo increase in ET_B receptor binding efficiency during low sodium diet is reproduced in vitro by sodium concentrations expected to be present in renal medulla. Sodium was reported to interact at the level of the second transmembrane domain of the other 7 membrane domain receptors, such as the D2 receptor[33] and the bradykinin B2 receptor,[34] resulting in enhanced stability of the agonist-receptor complex and enhanced binding efficiency.[35] The present study does not clarify the responsible mechanism at the molecular level but, for the first time, indicates that only the type B of the 2 endothelin receptors dynamically adapts its binding efficiency in a range of sodium concentration that is physiological for renal medulla.

In conclusion, present findings indicate that the endothelin system in the kidney is regulated by sodium intake and that the local levels of sodium directly modulate ET_B receptor density and affinity, resulting in a different adaptations of the cortex and renal medulla to low sodium diet.

Perspectives

The enhanced ET-1 synthesis with increased efficiency of the ET_B receptor in human renal medulla during low sodium diet shows a dynamic modulation of the renal ET-1 system in a physiological condition of sodium retention. ET_B enhances free water clearance so that medullary ET_B receptor adaptation might indeed play a key role in counteracting cortical ET_A-mediated sodium retention and AVP-mediated water reabsorption in sodium retentive states.

These results might also bear implications for the proposed therapeutic use of endothelin receptor antagonist in heart failure. Notwithstanding the fact that acute nonselective ET-1 antagonism showed hemodynamic benefits in heart failure patients,[36,37] the ET_B blockade might be responsible for the early and sustained increase in body weight and frequency of edema caused by fluid retention, recently observed during chronic mixed ET_A/ET_B receptor blockade.[38] It is worth recalling that, in experimental heart failure, only the use of selective ET_A antagonists was shown to improve diuresis, whereas no effects were observed following the administration of nonselective ET-1 antagonists.[39,40] Therefore, the enhanced ET_B efficiency might play a relevant role, especially under conditions of marked sodium retention.

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