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ET_A receptor-mediated Ca^{2+} signaling in thin descending limbs of Henle's loop: Impairment in genetic hypertension

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ET_A-mediated Ca^{2+} signaling in thin descending limbs of Henle's loop: Impairment in genetic hypertension.

Background. Endothelins (ET) have diuretic and natriuretic actions via ET_B receptors that are found in most renal tubular segments, although the thin limbs have not been studied. Data also suggest that dysfunction of the renal ET system may be important in the pathogenesis of hypertension. The present study was aimed at determining the presence and nature of ET receptors in the thin limbs of Henle's loop and their ability to activate a Ca^{2+}-dependent signaling pathway, as well as whether ET-induced Ca^{2+} signals are altered in hypertension.

Methods. Reverse transcription-polymerase chain reaction (RT-PCR) and Fura 2 fluorescence measurements of $[Ca^{2+}]_i$ were made to characterize ET receptors in descending thin limbs (DTL) of Sprague-Dawley rats, spontaneously hypertensive (SH) rats, and control Wistar-Kyoto (WKY) rats, and the three selected strains of Lyon rats with low-normal (LL), normal (LN), and high (LH) blood pressure.

Results. In SD rats, ET induced Ca^{2+} signals in DTL of long-looped nephrons, but not in DTL of short loops, or in ascending thin limbs. Ca^{2+} increases were abolished by BQ123, an antagonist of the ET_A receptor, but not by BQ788, an antagonist of the ET_B subtype. Endothelin-3 and sarafotoxin 6c, two ET_B receptor agonists, were both inactive. RT-PCR showed the presence of both ET_A and ET_B receptor mRNA. Ca^{2+} signals measured in DTL of WKY LL and LN rats were similar to those in Sprague-Dawley rats, but were significantly diminished (LH) or abolished (SH) in hypertensive rats.

Conclusion. A functional ET_A receptor activating a Ca^{2+}-dependent pathway is expressed in DTL. This ET_A-induced calcium signaling is impaired in two strains of genetically hypertensive rats.

Key words: endothelin, ET_A receptor-mediated Ca^{2+} signaling, ET_A and ET_B receptor mRNA, type II descending thin limb, genetic hypertension.

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Since the original discovery of endothelins (ET) as potent vasoconstrictors, there is now increasing evidence to show that they also have significant diuretic and natriuretic actions [1]. In addition, they appear to work as local (rather than circulating) hormones, inhibiting salt and water reabsorption through a paracrine and/or autocrine action on renal tubular cells [2]. Although it is still uncertain whether the renal ET system is involved in the regulation of sodium and water balance under normal conditions [3], data suggest that it might play a primary role in some pathologic settings. Thus, in rats treated with salt and deoxycorticosterone acetate (DOCA), urinary excretion of ET-1 (which reflects renal synthesis) increases in parallel with urinary excretion of sodium and water [4], as do renal tissue levels of ET-1, ET-converting enzyme activity and the number of ET_B receptors in renal medullary tissue [4, 5]. Conversely, pharmacologic blockade of the ET_B receptor [6], the subtype usually expressed by renal epithelium [2], as well as targeted [7] or partial [8] deletion of the ET_B receptor gene, have been shown to cause salt retention and hypertension. Moreover, decreased urinary ET-1 excretion has been found in salt-sensitive hypertensive subjects [9], and decreased synthesis of ET-1 in renal papilla has also been demonstrated in different strains of spontaneously hypertensive (SH) rats [10–12]. Taken together, these findings support the concept that locally synthesized ET-1 and medullary ET_B receptors may serve to aid control of arterial blood pressure by promoting salt and water excretion; therefore, disorders of the renal ET-1 system could be important in the mechanisms underlying hypertension and salt sensitivity.

Because the inner medullary collecting duct (IMCD) is the main site of ET-1 synthesis [13], and it is also the nephron segment that expresses the highest number of ET_B receptors [14], it is often considered to be the major site of action of this peptide. However, ET-1 synthesis

and its effects are not restricted to the IMCD. Evidence for ET-1 mRNA [13], as well as ET_B receptor mRNA [14] and binding sites [15], has been found in most nephron segments. ET-1 has also been shown to inhibit water and/or sodium reabsorption in the proximal tubule [16], the thick ascending limb of Henle's loop [17, 18], the cortical portion of the collecting duct [19], and in the IMCD [20]. This suggests that the diuretic and natriuretic effects of ET-1 found in vivo may originate from ET-1 actions at different sites along the nephron.

Despite the wide expression of ET receptors, no published studies have reported whether or not the thin limbs of Henle's loop are also functional targets for ETs. Preliminary experiments from our laboratory have shown that basolateral ET-1 increases cytosolic calcium concentration ($[Ca^{2+}]_i$) in the descending thin limb (DTL), indicating the presence of functional receptors in this segment. The DTL plays an important role in the regulation of sodium and water excretion by the kidney. First, it participates in establishing the corticomedullary osmotic gradient. Second, it is an active site of prostaglandin E_2 synthesis [21–23], a prostanoid that may induce natriuresis by inhibiting salt reabsorption in the neighboring thick ascending limb. Therefore, the aim of the present study was to use a functional and molecular approach to (*1*) characterize the ET receptors expressed in DTL; and (*2*) examine whether ET receptors and ET's effect on $[Ca^{2+}]_i$ would be altered in DTL and collecting ducts of hypertensive rats.

METHODS

Animals

In a first set of experiments, ET-1 effects have been analyzed in normal adult male Sprague-Dawley rats (Harlan, Gannat, France). In a second set of experiments, its effects have been studied in two different models of genetic hypertension: the SH rats/Kyoto model, derived from the Wistar strain (group 1), and the Lyon model, derived from the Sprague-Dawley strain (group 2). In group 1, experiments were carried out on male prehypertensive (3 to 5 weeks old) [24], and hypertensive (8 to 10 weeks old) rats, and on age-matched Wistar-Kyoto (WKY) controls. In group 2, male rats of the three simultaneously selected Lyon strains [25] with high, normal, and low-normal blood pressure were studied (all aged 10 to 12 weeks). In some animals, indirect measurements of systolic blood pressure were made by the tail-cuff method 2 days before study.

All animals were fed a standard laboratory diet (UAR, Epinay, France; 2.5 g Na/kg) with free access to water until the day of study.

Isolation of nephron segments

The left kidney of sodium pentobarbital-anesthetized rats (Nembutal; 50 mg/kg body weight, intraperitoneally) was perfused via the renal artery with 5 mL HEPES-buffered saline solution (HBSS; containing 140 mmol/L NaCl, 5 mmol/L KCl, 0.8 mmol/L $MgSO_4$, 0.33 mmol/L Na_2HPO_4, 0.44 mmol/L NaH_2PO_4, 1.0 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mmol/L HEPES, and 5 mmol/L glucose, pH 7.4), followed by the same volume of a 0.16% collagenase solution (from *Clostridium histolyticum*; Serva, Boehringer Mannheim, Meylan, France). Thin corticomedullary slices were then incubated in 0.12% collagenase solution for 20 minutes at 30°C, carefully rinsed, and transferred into microdissection medium [HBSS to which 0.1% bovine serum albumin (BSA) had been added] for harvesting tubule segments at 4°C. All tubule samples were stored in a droplet of microdissection medium (2 μL) at 0 to 4°C until use.

The following structures were studied: DTL of superficial and juxtamedullary nephrons (type I and type II epithelium, respectively); outer medullary collecting ducts (OMCD) and IMCD, as well as thin ascending limbs (ATL) and glomeruli. DTL and OMCD were both isolated from the inner stripe of outer medulla. Type I DTL were identified anatomically by their attachment to the end of superficial nephron proximal straight tubules and functionally by the inability of 8-arginine vasopressin to generate calcium signals [26]. Type II DTL of deep nephrons were identified by their larger diameter and typical morphologic appearance under the stereomicroscope. ATL were microdissected in inner medulla. They were identified anatomically by their attachment to the end of medullary thick ascending limbs, and functionally by their ability to respond to 8-arginine vasopressin by generating calcium signals [26].

Expression of type A (ET_A) and type B (ET_B) endothelin-receptor mRNA

RNA was extracted from pools of 20 to 50 microdissected nephron segments as described previously [27] and summarized below. The pools of tubules were transferred with 10 μL microdissection solution into 400 μL denaturing solution [4 mol/L guanidium thiocyanate, 25 mmol/L sodium citrate (pH 7.0), 0.1 mol/L β-mercaptoethanol, and 0.5% sarcosyl) and 20 μg yeast tRNA used as carrier. After phenol/chloroform extraction and isopropyl alcohol precipitation, the final RNA pellet was dried under vacuum and dissolved in RNA dilution buffer (10 mmol/L Tris, pH 7.6, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 2 mmol/L dithiothreitol, and 40 U/mL ribonuclease inhibitor (Rnasin, Promega, Charbonnières, France). The yield of this RNA extraction procedure is ≥90% [27].

Expression of mRNA encoding ET_A and ET_B receptors was assessed by reverse transcription-polymerase chain reaction (RT-PCR), using the following specific primers [15]: ET_A-receptor sense (5′-GTGTTTAAGC TGTTGGCGGG-3′), and anti-sense (5′-CGAGGTCA

TGAGGCTTTTGG-3′), bases 405 to 424 and 1172 to 1191 from the initiating ATG codon, respectively, and ET_B-receptor sense (5′-AGCTGGTGCCCTTCATAC AGAAGGC-3′), and antisense (5′-TGCACACCTTTC CGCAAGCACG-3′), bases 521 to 545 and 1418 to 1439, respectively. RT was carried out for 2 hours at 37°C in a final volume of 10 μL in the presence of RNAs from 1 mm tubular length, random primers (PdN6, 5 μmol/L, Roche Diagnostics, Meylan, France), dNTPs (500 μmol/L) and Superscript™ II reverse transcriptase (100 U, Invitrogen, Ltd., Paisley, UK). After denaturation at 95°C for 30 seconds, PCR was carried out in the same tube in a final volume of 100 μL after addition of the sense and antisense primers (10 pmol), and Taq polymerase (1.25 U, Eurobio, Les Ulis, France). Samples were submitted to 30 to 35 cycles of three temperature steps: 94°C, 30 seconds; 60°C, 30 seconds; 72°C, 1 minute, except for the last cycle in which the elongation lasted 10 minutes. The DNA fragments were separated by electrophoresis on a 1.5% (wt/vol) agarose gel containing 10 μg/mL ethidium bromide, and visualized under ultraviolet illumination. For both ET_A and ET_B receptors, the nature of the PCR product (787 and 919 bp expected size, respectively) was confirmed by sequencing. In all experiments, possible contamination was checked by control RT-PCR reactions on samples in which reverse transcriptase had been excluded from the reverse transcription mixture (RTase −).

Measurement of intracellular free-calcium ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured using the method previously described [28]. Each tubular segment was loaded with Fura 2-AM (Molecular Probes, Interchim, Asnières, France; 10 μmol/L in microdissection medium) for 1 hour at room temperature and subsequently transferred to a superfusion chamber, where the ends of the segment were aspirated into two glass holding pipettes (to prevent possible perfusion of the luminal membrane). The segment was then superfused at a rate of 10 mL/min with HBSS for a 5-minute equilibration period. Fura 2 fluorescence was measured in a tubular portion of 20 to 30 cells using a standard photometric setup (model MSP 21, Zeiss, Jena, Germany) during superfusion with either HBSS alone or the same solution to which agonists had been added. All solutions were stored in individual reservoirs at room temperature until use; the temperature was raised to 37°C just prior to entry to the perfusion chamber.

Following subtraction of tubular autofluorescence from the fluorescence intensities of Fura 2 at 340 and 380 nm, $[Ca^{2+}]_i$ was calculated from the equation of Grinkievicz, Poenie, and Tsien [29], using a dissociation constant of Fura 2 for calcium of 224 nmol/L. The calibration parameters were determined from internal calibration using a solution containing 10 μmol/L ionomycin and both 0 Ca^{2+} and 1 mmol/L ethylene glycol bis(β-aminoethyl-ether)-N,N,N′,N′-tetraacetic acid (for the minimal ratio) or 3 mmol/L Ca^{2+} (for the maximal ratio).

Chemicals

ET-1, ET-3, sarafotoxin 6c, 8-arginine vasopressin, and norepinephrine (arterenol, bitartrate salt) were purchased from Sigma (Saint Quentin Fallavier, France). Other compounds were from Merck Eurolab (Fontenay-sous-Bois, France).

Statistics

Results are given as mean ± SE. Mean values from different groups were compared according to either unpaired Student *t* test, or, when comparing more than two groups together, according to ANOVA (analysis of variance) test with protected least significant difference (PLSD) Fisher test.

RESULTS

Functional evidence for ET receptors in type II DTLs

The representative trace, as well as calculated mean data in Figure 1 A and B, show that basolaterally superfused ET-1 (1 to 100 nmol/L) induced dose-dependent $[Ca^{2+}]_i$ increases in type II DTL The calcium response was sustained at 1 nmol/L, but transient at 100 nmol/L, returning spontaneously to basal levels under hormone stimulation. At 10 nmol/L, the pattern of the response was more variable, showing either moderate, slow, and sustained $[Ca^{2+}]_i$ increases ($\Delta[Ca^{2+}]_i \leq 200$ nmol/L; Fig. 1A), which resembled those observed in the OMCD (Fig. 1C); or high and sharp transients, such as those obtained at 100 nmol/L ET-1 ($\Delta[Ca^{2+}]_i \geq 200$ nmol/L; Fig. 1D, left). The effect of ET-1 persisted when Ca^{2+} was removed from superfusion medium (mean $\Delta[Ca^{2+}]_i$ in Ca^{2+}-free medium, 233 ± 40 nmol/L above basal, $N = 8$, Fig. 1D, right).

No response could ever be obtained in either type I DTL (Fig. 2A) or ATL (Fig. 2B), even though these segments did respond to extracellular adenosine triphosphate (ATP) and vasopressin (AVP) (Table 1), as anticipated from previous studies [26].

Pharmacologic characterization

To determine which ET receptor subtype is involved in the generation of calcium signals, we tested the effect of different agonists and antagonists specific for either the ET_A or the ET_B subtype on DTL. Because the OMCD is known to contain ET_B receptors [14, 15], it was also tested, as a control, in the same experiments. As shown in Figure 3 A and B, BQ788, a specific ET_B receptor antagonist, did not change $[Ca^{2+}]_i$ on its own and did not alter the effect of ET-1 in DTL (Fig. 3A). In contrast, it almost completely abolished the response to ET-1 in OMCD (Fig. 3B). Conversely, BQ123, a spe-

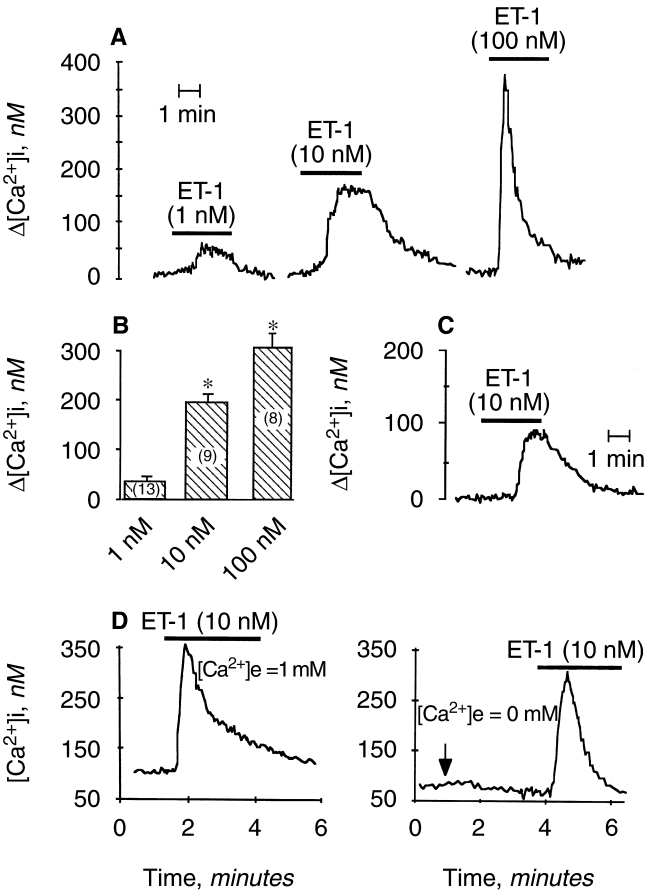

Fig. 1. Dose-dependent $[Ca^{2+}]_i$ increases induced by basolateral superfusion of endothelin 1 (ET-1) in thin descending limbs of long-looped nephrons (type II DTL). (*A*) Representative trace. (*B*) Mean increases (peak-basal) ± SE calculated for each ET-1 concentration from several tubules (number in parenthesis). Data obtained from four rats. (*C*) Control response obtained in the outer medullary collecting duct (OMCD). (*D*) Response of type II DTL in the presence (left) and absence (right) of calcium in extracellular medium.

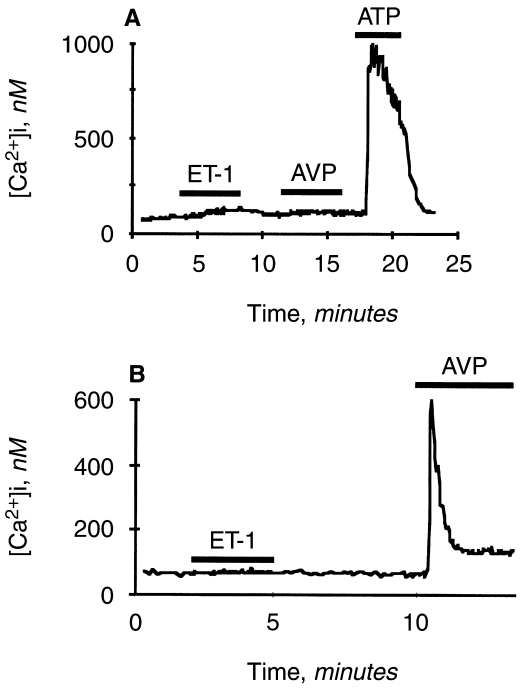

Fig. 2. Effect of basolaterally superfused endothelin 1 (ET-1) (10 nmol/L) on $[Ca^{2+}]_i$ in the descending thin limb of short loop nephrons (type I DTL) (*A*) and in the ascending thin limb (ATL) (*B*). ET-1 is devoid of effect in type I DTL and in ATL even though these segments respond to other activators of phospholipase C, as expected from previous studies [26]. Abbreviations are: AVP, 100 nmol/L 8-arginine vasopressin; ATP, 100 μmol/L adenosine triphosphate.

Table 1. Endothelin-1–induced Ca^{2+} signals in thin descending limbs (DTL) of the rat Henle's loop

	$\Delta[Ca^{2+}]_i$ *nmol/L*		
Segment	ET-1	AVP	ATP
DTL type I	12 ± 4 (9)	NT	771 ± 82 (8)
DTL type II	225 ± 19 (7)	1 ± 1 (7)	NT
ATL	3 ± 2 (4)	430 ± 153 (4)	NT

Values are mean $[Ca^{2+}]_i$ increases (peak-basal) ± SE induced by basolaterally superfused endothelin 1 (10 nmol/L ET-1) in thin descending (type I and type II DTL) and ascending limbs (ATL) from Sprague-Dawley rat kidneys (four rats; number of tubules in parentheses). Where necessary, 8-arginine vasopressin (AVP; 100 nmol/L) or adenosine-5′-triphosphate (ATP; 100 μmol/L) was tested as controls on the same tubules. NT is not tested.

cific ET_A receptor antagonist, blocked the ET-1 effect on DTL and was devoid of effect on OMCD (Fig. 3 C and D). In support of these observations, ET-3 and sarafotoxin 6c, two agonists specific for the ET_B receptor, were inactive on DTL (Fig. 4 A and C) and active on OMCD, where they generated calcium signals comparable in magnitude to those elicited by ET-1 (Fig. 4 B and D). Mean values for these experiments are reported in Table 2.

Molecular characterization

Experiments have been carried out to investigate whether ET_A receptor mRNA is expressed in DTL, as suggested by functional data (Figs. 3 and 4). Figure 5 illustrates the results of one RT-PCR experiment (representative of four others) in which ET_A and ET_B receptor expression were studied in parallel on the same mRNA extracts from DTL, OMCD, IMCD, and glomerulus; OMCD was used because it is a good marker of the ET_B-receptor subtype (Figs. 3 and 4); IMCD and glomerulus were used because they have been reported to express both subtypes [14, 30, 31]. As shown by the results, clear signals for both ET_A and ET_B receptors were found in glomerulus and DTL. In contrast, OMCD and IMCD expressed exclusively mRNA for the ET_B receptor.

Altered response to ET-1 in SH rats

Several defects of the renal medullary ET system have been associated with hypertension [9–12]. Therefore, we examined whether calcium responses of DTL and OMCD

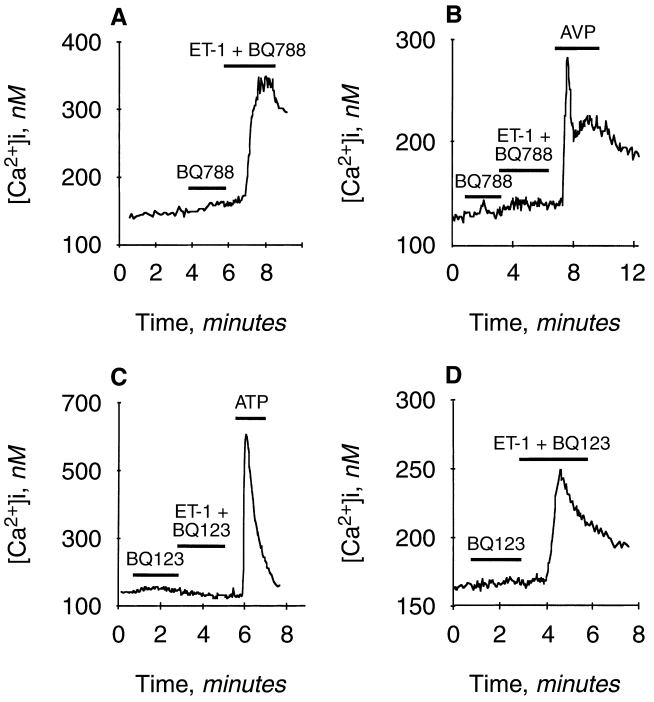

Fig. 3. Effect of specific antagonists of the ET_A and ET_B receptor subtypes on calcium responses to basolateral endothelin 1 (ET-1) in thin descending limbs (DTL) (*A* and *C*) of long-looped nephrons and outer medullary collecting ducts (OMCD) (*B* and *D*). (A and B) Effect of BQ788, a specific ET_B receptor antagonist. (C and D) Effect of BQ123, a specific ET_A receptor antagonist. BQ123 or BQ788 (10 nmol/L) were added to the bath 2 to 3 minutes before ET-1 (10 nmol/L). Where necessary, 8-arginine vasopressin (AVP; 100 nmol/L) and adenosine triphosphate (ATP; 100 μmol/L) were used as control of tubule viability in OMCD and DTL, respectively.

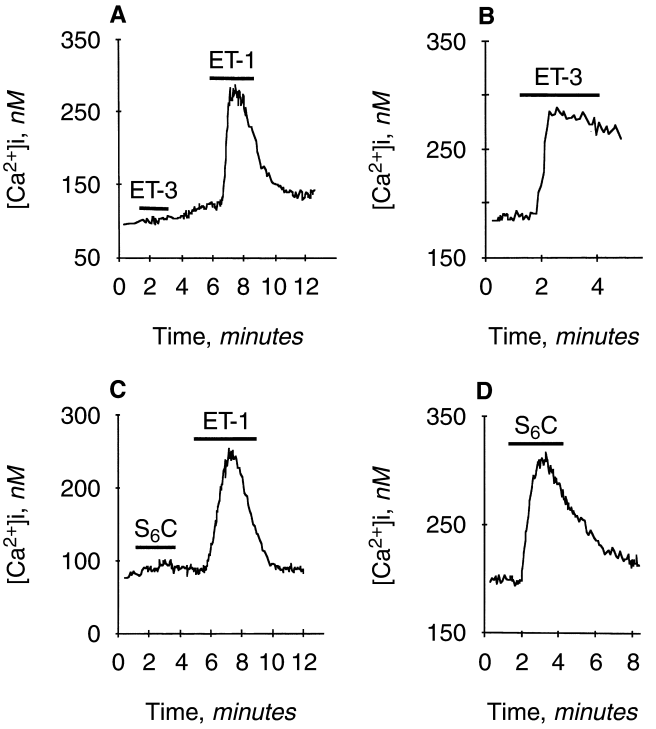

Fig. 4. Effect of endothelin 3 (ET-3) and sarafotoxin 6c (S_6C), two agonists specific for the ET_B receptor subtype, on $[Ca^{2+}]_i$ in type II thin descending limbs (DTL) (*A* and *C*) and outer medullary collecting ducts (OMCD) (*B* and *D*). ET-1, ET-3 and S_6C (all 10 nmol/L) were applied to the basolateral side of tubules.

to ET-1 would be altered in SH rats and Lyon hypertensive rats.

SH rats and their control WKY rats were studied at either 3 to 5 weeks old, before the onset of hypertension [24], or aged 8 to 10 weeks old, when hypertension is established. Mean arterial blood pressures in the older age group were 170 ± 6 and 127 ± 7 mm Hg ($N = 4$) in SH rats and WKY rats, respectively; $P \leq 0.001$.

Lyon rats from the three simultaneously selected strains referred to as low-normal, normal, and high blood pressure were studied at 10 to 12 weeks old. Mean arterial blood pressures were 120 ± 13, 131 ± 3, and 166 ± 8 mm Hg ($N = 4$), respectively. The mean arterial blood pressure of high blood pressure rats was significantly higher than that of both low-normal ($P \leq 0.005$) and normal rats ($P \leq 0.02$), whereas that of low-normal rats was not significantly different from normal rats. Therefore, as reported previously [25], Lyon rats with high blood pressure can be compared with two genetically distinct strains of control rats (low-normal and normal).

As illustrated by Figure 6, in 8- to 10-week-old SH rats, the calcium response to 10 nmol/L ET-1 was normal in OMCD, whereas it was blunted by about 90% in DTL. This decreased response of DTL could already be observed in 3- to 5-week-old animals. It was specific for ET-1, as the response to norepinephrine was similar to that of WKY rats in the same experiments. Finally, it did not result from a transcriptional defect, since ET_A and ET_B-receptor mRNAs were both expressed in the DTL of SH rats (Fig. 7).

In the Lyon strain, the calcium responses (peak-basal)

Table 2. Pharmacologic characterization of the endothelin-receptor subtypes eliciting calcium signals in thin descending limb (DTL) and outer medullary collecting duct (OMCD)

Experimental condition	$\Delta[Ca^{2+}]_i$ *nmol/L*	
	DTL	OMCD
ET-1	128 ± 20 (18)	92 ± 16 (6)
ET-3	0 (10)	96 ± 12 (6)
S6c	0 (6)	93 ± 10 (4)
ET1 + BQ788	120 ± 19 (12)	8 ± 5 (9)
ET1 + BQ123	0 (7)	87 ± 6 (4)

Values are mean $[Ca^{2+}]_i$ increases (peak-basal) ± SE, calculated in each condition from several tubules (type II DTL and OMCD; number in parentheses) obtained from at least four rats. ET-1, ET-3, 10 nmol/L endothelin 1 and 3, respectively; S6c, 10 nmol/L sarafotoxin 6c; BQ788 (an ET_B-receptor antagonist) and BQ123 (an ET_A-receptor antagonist), each used at 10 nmol/L, were added to the superfusion medium 2 to 3 minutes before ET. Note that the response of DTL to ET-1 (with and without BQ) was of low magnitude as compared with those obtained in the other experimental series (see Fig. 1 and Table 1).

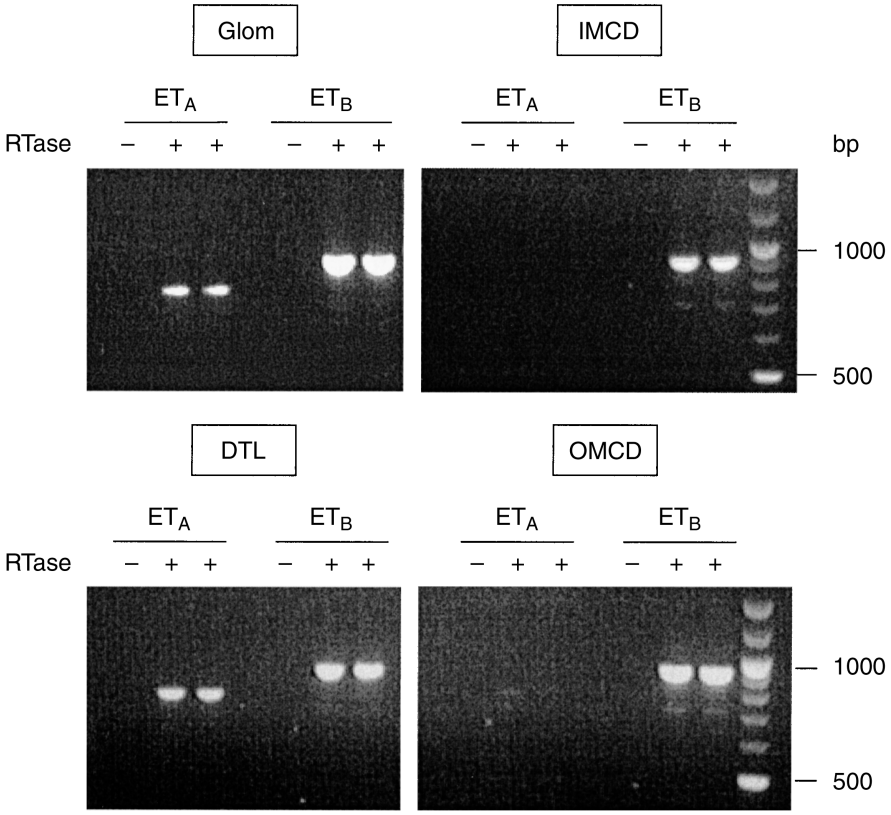

Fig. 5. Sample gels from one reverse transcription-polymerase chain reaction (RT-PCR) experiment (35 cycles). The relative expression of ET_A and ET_B receptor mRNA in glomerulus (Glom), type II thin descending limb (DTL), outer medullary collecting duct (OMCD) and inner (IMCD) medullary collecting duct. + and − are samples assayed in the presence and absence of reverse transcriptase, respectively. For each sample, 3 μL mRNA extract was used (the mRNA extracted from 1 glomerulus or 1 mm tubule length**).** ET_A and ET_B receptor mRNAs were looked for in the same extracts.

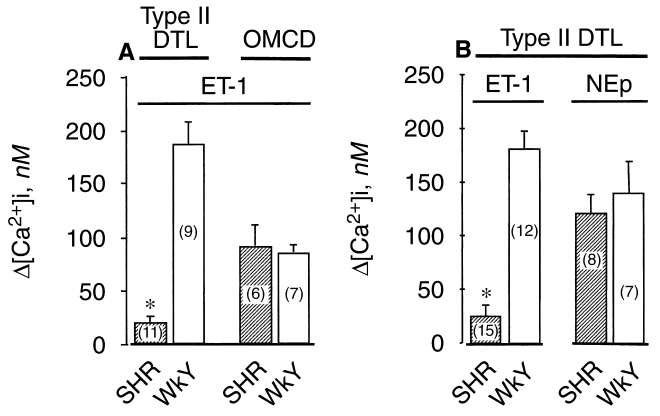

Fig. 6. Endothelin 1 (ET-1)–induced $[Ca^{2+}]_i$ increases (peak-basal) in thin descending limb (DTL) and outer medullary collecting duct (OMCD) of spontaneously hypertensive (SHR) and control Wistar-Kyoto (WkY) rats. (*A*) Data obtained in 8- to 10-week-old rats. (*B*) Data from 3- to 5-week-old (prehypertensive) rats. In each experiment, one SHR (▨) and one age-matched WkY rat (□) were studied in parallel. Values are means ± SE from several tubules (number in parentheses) obtained from four rats in each group. $*P \leq 0.001$ vs. WkY. ET-1 (10 nmol/L) or norepinephrine [1 μmol/L norepinehrine (NEp)] were added to the basolateral superfusion medium. Note that the response of DTL to norepinephrine was not altered in SHR.

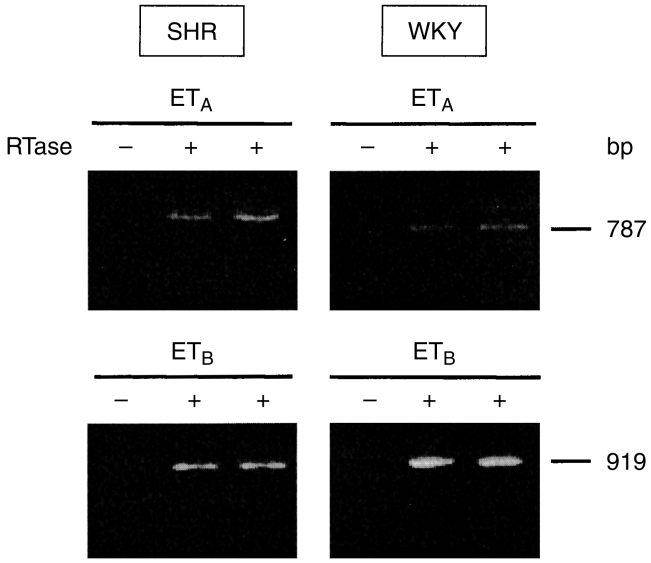

Fig. 7. Sample gels from one reverse transcription-polymerase chain reaction (RT-PCR) experiment (representative of two others). The expression of ET_A and ET_B-receptor mRNA in the thin descending limb (DTL) of the same spontaneously hypertensive rat (SHR) is shown. In each experiment, a Wistar-Kyoto (WKY) rat was also tested as positive control. + and − are samples assayed in the presence and absence of reverse transcriptase, respectively. For each sample, 3 μL mRNA extract were used (the mRNA extracted from 1 mm tubule length). For both SHR and WKY rats, three different extracts were prepared and tested in three separate experiments (30 cycles PCR each).

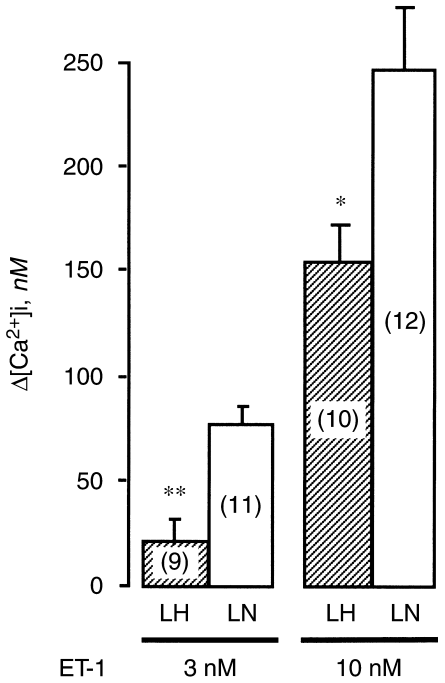

Fig. 8. Endothelin 1 (ET-1)–induced $[Ca^{2+}]_i$ increases (peak-basal) in thin descending limb (DTL) of the normotensive (LN; □) and hypertensive (LH; ▨) strains of Lyon rat. All rats were 10 to 12 weeks old. Values are means ± SE from several tubules (number in parentheses) obtained from five rats per group and per experimental condition. The effects of 3 and 10 nmol/L ET-1 are compared. $*P \leq 0.02$ vs. LN; $**P \leq 0.001$ vs. LN.

to 10 nmol/L ET-1 were LL, 236 ± 22 nmol/L ($N = 17$); LN, 219 ± 16 nmol/L ($N = 15$); LH, 146 ± 19 nmol/L ($N = 15$), respectively. The calcium rise in the LH strain was significantly blunted compared with the LL ($P < 0.002$) and LN ($P < 0.01$) strains. Figure 8 compares the calcium responses to 3 and 10 nmol/L ET-1 in the LN and LH strains. At 10 nmol/L, the impairment in LH rats was only moderate at ~33%, whereas at 3 nmol/L it was greater, almost double at ~66%, suggesting that the dose-response curve for Lyon rats with high blood pressure is shifted to the right.

DISCUSSION

This paper reports for the first time that (*1*) ET_A and ET_B receptor mRNA are expressed in the rat DTL of long-looped nephrons; (*2*) ET_A, but not ET_B, receptors activate a Ca^{2+}-dependent signaling pathway, and (*3*) ET-1–induced calcium signals are blunted in genetically hypertensive rats.

As already mentioned, numerous studies have been undertaken in the past to identify the sites of action of ET-1 along the nephron, its signaling mechanisms, and the nature of its biologic effects on renal tubular cells. However, none of these studies has investigated the thin limbs of Henle's loop. Our data show that in DTL of long-looped nephrons (Fig. 1), ET-1 elicits dose-dependent $[Ca^{2+}]_i$ increases that are similar in hormone sensitivity to those previously reported in the collecting duct [32]. As in the collecting duct, the calcium response persists in Ca^{2+}-free medium, suggesting that it is secondary to activation of a phospholipase C–dependent pathway [33–35].

On the basis of published data, it is generally assumed that the predominant ET receptor expressed in renal epithelium is the ET_B receptor. This conclusion is based on a large body of experimental evidence obtained from molecular and functional studies of microdissected nephron segments. Such experiments have shown that ET_B, but not ET_A, mRNA is expressed in the proximal tubule, the thick ascending limb and the collecting duct [14], and that ET_B receptors account fully for the inhibitory effects of ET-1 on sodium reabsorption in the thick ascending limb [17, 18], as well as on vasopressin-dependent cAMP accumulation [31] and water reabsorption [30] in the collecting duct. In proximal tubules, part of the ET-1 effect on phospholipid signaling might also be due to the presence of ET_C receptors in the basolateral membrane [36]. In contrast to these data, we have found that in the DTL of long-looped nephrons, both ET_A and ET_B mRNA are present (Fig. 5), and that ET-1–induced calcium signals are only linked to the ET_A-receptor subtype (Figs. 3 and 4). Indeed, the calcium response to ET-1 was totally blocked by an ET_A antagonist (Fig. 3), and ET_B agonists (ET-3 and sarafotoxin 6c) were devoid of effect in the DTL (Fig. 4). If mRNA is translated into functional ET_B receptor protein, these data indicate that the ET_B receptor subtype in the DTL is linked to a calcium-independent signaling pathway, as shown previously in our laboratory for the mouse and rat thick ascending limb [17].

Our data for OMCD (Fig. 5) are fully consistent with those published already, in that ET_B-receptor mRNA was clearly expressed, whereas ET_A-receptor mRNA was barely detectable in the same extracts [15]. In support of these data, we found that an ET_B, and not an ET_A, antagonist blocked the ET-1–induced calcium signals and that ET_B agonists elicited them (Figs. 3 and 4).

Up to now, the ET_A receptor subtype has been considered as a vascular receptor, because it is particularly abundant in vascular smooth muscle cells. In the kidney ET_A-receptor mRNA and/or binding sites have been found in renal vessels [37, 38], as might be expected, but also in intact glomeruli [14], cultured glomerular mesangial cells [39], inner medullary interstitial cells [38, 40], and (though to a lesser degree) in freshly prepared suspensions of IMCD cells [30, 31]. This latter finding is at variance with the data presented here (Fig. 5) and with that of others who also failed to detect the presence of mRNA specific for the ET_A-receptor in microdissected IMCD [14]. However, although the cell suspensions used to investigate ET receptors were highly

purified, it is possible that the trace of ET_A found in these preparations was due to some contamination with vascular and/or interstitial cells. Even if present in IMCD cells, ET_A receptors do not account for any known effect of ET-1 in this segment [30, 31]. In this respect, our study is the first to provide functional evidence for ET_A receptors in a renal tubular epithelial cell.

Data in Figure 2 and Table 1 show that ET-1 does not alter $[Ca^{2+}]_i$ in the DTL of short-looped nephrons. Because this type of DTL is difficult to isolate in the rat, we did not investigate whether it expresses mRNA for either the ET_A or the ET_B receptor. Therefore, we cannot say if it lacks ET receptors, or expresses ET receptors linked to calcium-independent pathways.

It is known that DTL from short and long-looped nephrons are morphologically and functionally different. The former are characterized by a flat type I epithelium that is permeable to urea, but almost impermeable to sodium; whereas the latter exhibit a tall type II epithelium that is impermeable to urea, but permeable to sodium [41]. The nature of any biologic effect exerted by ET-1 on this type II epithelium is still unknown. ET-1 might alter the passive movements of sodium and water that take place in this segment [41]. An alternative hypothesis is that ET-1 stimulates the calcium-dependent synthesis of an arachidonic acid metabolite [prostaglandin E_2, 20-hydroxyeicosatetraenoic acid (20-HETE), or others], as already demonstrated in different cell types for ET and other activators of phospholipase C [31, 42]. It is perhaps worth noting in this respect that type I DTL are located inside, or at the periphery of, vascular bundles and close to descending vasa recta, whereas type II DTL are located outside the bundles in close proximity to thick ascending limbs [43]. Thus, paracrine inhibition of sodium chloride reabsorption in the thick ascending limb as a result of ET-1–induced eicosanoid synthesis in type II DTL could be one of the mechanisms by which ET-1, via its ET_A receptors, exerts a diuretic and natriuretic effect [44].

The calcium responses to ET-1 in type II DTL have been studied in three different strains of normotensive rats: Sprague-Dawley rats from our usual supplier (Figs. 1 to 4), Wistar-Kyoto rats (Fig. 6), and Lyon normotensive rats (Fig. 8). In all three strains, the DTL response to ET-1 was of comparable magnitude. It was also similar in Lyon low-normal rats, a strain initially selected to generate genetically hypotensive animals [25], but which has a blood pressure comparable to that of normal rats. In contrast, the DTL response was significantly impaired in hypertensive rats, although the response of the OMCD was normal in these animals (Fig. 6). These results are unlikely to be due to genetic variability; rather they indicate a possible link between a defect in ET-1 signaling in DTL and hypertension. The fact that the defect in ET-1 action was already present in 3- to 5-week-old SH rats before the onset of hypertension (Fig. 6) suggests, but does not prove, that it could in some way be involved in the onset of hypertension. This possibility is at least consistent with the many published examples demonstrating that salt-dependent hypertension is associated with disturbances of the hormonal mechanisms regulating salt and water reabsorption, for example, disorders in medullary prostaglandin E_2 synthesis [45], prostaglandin E_2 action on the medullary thick ascending limb [46], as well as genetic defects of the renin-angiotensin-aldosterone and atrial natriuretic peptide systems [47].

At present, we do not know anything about the cellular events underlying the loss of ET-1 calcium signals in the DTL. RT-PCR analysis of mRNA shows that ET_A-receptor mRNA is expressed in DTL of both WKY rats and SH rats (Fig. 7), as in normal Sprague-Dawley rats (Fig. 5). Therefore, if the defect in the hypertensive strains concerns the ET_A receptor, it is not transcriptional in origin. It might also involve a protein located downstream in the chain of events leading to phospholipase C activation, including phospholipase C itself, or another protein coupling it to the ET_A receptor. The fact that in the same DTL the calcium response to norepinephrine was preserved, but that to ET-1 abolished (Fig. 6), is in keeping with the latter possibility.

CONCLUSION

Our study provides the first evidence for a functional ET_A receptor in a native renal tubular epithelial cell. This receptor is specifically located in the DTL of long-looped nephrons. It triggers mobilization of intracellular calcium upon binding to ET-1, probably as a result of phospholipase C activation. Finally, ET_A-dependent calcium signals in DTL are diminished, or abolished, in genetically hypertensive rats. Therefore, we speculate that this novel defect in the medullary ET system may have some role in disordered renal sodium and water handling, and thus contribute to the onset of hypertension.

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REFERENCES

1. Simonson MS: Endothelins: Multifunctional renal peptides. *Physiol Rev* 73:375–411, 1993
2. Nord EP: Renal actions of endothelins. *Kidney Int* 44:451–463, 1993
3. Kohan DE: Intrarenal endothelin-1 and hypertension. *Am J Kidney Dis* 36:Iiv–Ivi, 2000
4. Hsieh TJ, Lin SR, Lee YJ, *et al*: Increased renal medullary endo-

thelin-1 synthesis in prehypertensive DOCA and salt-treated rats. *Am J Physiol* 279:F12–F21, 2000
5. Pollock DM, Allcock GH, Krishnan A, *et al*: Up regulation of endothelin B receptors in kidneys of DOCA-salt hypertensive rats. *Am J Physiol* 278:F279–F286, 2000
6. Pollock DM, Pollock J: Evidence for endothelin involvement in the response to high salt. *Am J Physiol* 281:F144–F150, 2001
7. Ohuchi T, Kuwaki T, Ling GY, *et al*: Elevation of blood pressure by pharmacological disruption of the ET_B receptor in mice. *Am J Physiol* 276:R1071–R1077, 1999
8. Gariepy CE, Ohuchi T, Williams SC, *et al*: Salt-sensitive hypertension in endothelin-B receptor deficient rats. *J Clin Invest* 105:925–933, 2000
9. Hoffman A, Grossman E, Goldstein DS, *et al*: Urinary excretion rate of endothelin-1 in patients with essential hypertension and salt sensitivity. *Kidney Int* 45:556–560, 1994
10. Kitamura K, Tanaka T, Kato J, *et al*: Immunoreactive endothelin in rat kidney inner medulla: Marked decrease in spontaneously hypertensive rats. *Biochem Biophys Res Commun* 162:38–44, 1989
11. Hughes AK, Cline RC, Kohan DE: Alterations in renal endothelin-1 production in the spontaneously hypertensive rat. *Hypertension* 20:666–673, 1992
12. Vogel V, Backer A, Heller J, Kramer HJ: The renal endothelin system in the Prague hypertensive rat, a new model of spontaneous hypertension. *Clin Sci* 97:91–98, 1999
13. Ujiie K, Terada Y, Nonoguchi H, *et al*: Messenger RNA expression and synthesis of endothelin-1 along rat nephron segments. *J Clin Invest* 90:1043–1048, 1992
14. Takemoto F, Uchida S, Ogata E, Kurokawa K: Endothelin-1 and endothelin-3 binding to rat nephrons. *Am J Physiol* 264:F827–F832, 1993
15. Terada Y, Tomita K, Nonoguchi H, Marumo F: Different localization of two types of endothelin receptor mRNA in microdissected rat nephron segments using reverse transcription and polymerase chain reaction assay. *J Clin Invest* 90:107–112, 1992
16. Garcia NH, Garvin JL: Endothelin's biphasic effect on fluid absorption in the proximal straight tubule and its inhibitory cascade. *J Clin Invest* 93:2572–2577, 1994
17. De Jesus Ferreira MC, Bailly C: Luminal and basolateral endothelin inhibit chloride reabsorption in the mouse thick ascending limb via a Ca^{2+}-independent pathway. *J Physiol* 505:749–758, 1997
18. Plato CF, Pollock DM, Garvin JL: Endothelin inhibits thick ascending limb chloride flux via ET_B-mediated NO release. *Am J Physiol* 279:F326–F333, 2000
19. Tomita K, Nonoguchi H, Terada Y, Marumo F: Effects of ET-1 on water and chloride transport in cortical collecting ducts of the rat. *Am J Physiol* 264:F690–F696, 1993
20. Oishi R, Nonoguchi H, Tomita K, Marumo F: Endothelin-1 inhibits AVP-stimulated osmotic water permeability in rat inner medullary collecting duct. *Am J Physiol* 261:F951–F956, 1991
21. Imbert-Teboul M, Siaume S, Morel F: Sites of prostaglandin E_2 (PGE_2) synthesis along the rabbit nephron. *Mol Cell Endocrinol* 45:1–10, 1986
22. Bonvalet JP, Pradelles P, Farman F: Segmental synthesis and actions of prostaglandins along the nephron. *Am J Physiol* 253:F377–F387, 1987
23. Grupp CG, Begher M, Cohen D, *et al*: Isolation and characterization of the lower portion of the thin limb of Henle in primary culture. *Am J Physiol* 274:F775–F782, 1998
24. Cabassi A, Vinci S, Quartieri F, *et al*: Norepinephrine reuptake is impaired in skeletal muscle of hypertensive rats in vivo. *Hypertension* 37:698–702, 2001
25. Sassard J, Vincent M, Orea V, *et al*: Genetics of blood pressure and associated phenotypes in the Lyon rat. *Clin Exp Hypertens* 19:5–6, 1997
26. Bailey MA, Imbert-Teboul M, Turner C, *et al*: Axial distribution and characterisation of basolateral P2Y receptors along the rat renal tubule. *Kidney Int* 58:1893–1901, 2000
27. Elalouf JM, Buhler JM, Tessiot C, *et al*: Predominant expression of beta 1-adrenergic receptor in the thick ascending limb of rat kidney: Absolute mRNA quantitation by reverse transcription and polymerase chain reaction. *J Clin Invest* 91:264–272, 1993
28. Champigneulle A, Siga E, Vassent G, *et al*: A V_2-like vasopressin receptor mobilizes intracellular calcium in rat medullary collecting tubules. *Am J Physiol* 265:F35–F45, 1993
29. Grinkievicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450, 1985
30. Edwards RM, Stack EJ, Pullen M, Nambi P: Endothelin inhibits vasopressin action in rat inner medullary collecting duct via the ET_B receptor. *J Pharmacol Exp Therap* 267:1028–1033, 1993
31. Kohan DE, Padilla E, Hughes AK: Endothelin B receptor mediates ET-1 effects on cAMP and PGE_2 accumulation in rat IMCD. *Am J Physiol* 265:F670–F676, 1993
32. Naruse M, Uchida S, Ogata E, Kurokawa K: Endothelin increases cell calcium in mouse collecting tubule cells. *Am J Physiol* 261:F720–F725, 1991
33. Tomita K, Nonoguchi, Marumo F: Effects of endothelin on peptide-dependent cyclic adenosine monophosphate accumulation along the nephron segments of the rat. *J Clin Invest* 85:2014–2018, 1990
34. Nadler SP, Zimplemann JA, Hebert RL: Endothelin inhibits vasopressin-stimulated water permeability in rat terminal inner medullary collecting duct. *J Clin Invest* 90:1458–1466, 1992
35. Woodcock EA, Land S: Functional endothelin ET_B receptors on renal papillary tubules. *Eur J Pharmacol* 247:93–95, 1993
36. Knotek M, Jaksic O, Selmani R, *et al*: Different endothelin receptor subtypes are involved in phospholipid signaling in the proximal tubule of rat kidney. *Pflgers Arch Eur J Physiol* 432:165–173, 1996
37. Hori S, Komatsu Y, Shigemoto R, *et al*: Distinct tissue distribution and cellular localization of two messenger ribonucleic acids encoding different subtypes of rat endothelin receptors. *Endocrinology* 130:1885–1895, 1992
38. Chow LH, Subramanian S, Nuovo GJ, *et al*: Endothelin receptor mRNA expression identified by in situ RT-PCR. *Am J Physiol* 269:F449–F457, 1995
39. Hughes AK, Padilla E, Kutchera WA, *et al*: Endothelin-1 induction of cyclooxygenase 2 expression in rat mesangial cells. *Kidney Int* 47:53–61, 1995
40. Wilkes BM, Ruston AS, Mento P, *et al*: Characterization of endothelin-1 receptor in rat medullary interstitial cells. *Am J Physiol* 260:F579–F589, 1991
41. Imai M, Yoshitomi H: Heterogeneity of the descending thin limb of Henle's loop. *Kidney Int* 38:687–694, 1990
42. McGiff JC, Quilley J: 20-HETE and the kidney: Resolution of old problems and new beginnings. *Am J Physiol* 277:R607–R623, 1999
43. Lemley KV, Kriz W: Cycles and separations: The histotopography of the urinary concentrating process. *Kidney Int* 31:538–548, 1987
44. Gellai M, Jugus M, Fletcher T, *et al*: Reversal of postischemic acute renal failure with a selective $endothelin_A$ receptor antagonist in the rat. *J Clin Invest* 93:900–906, 1994
45. Craven PA, Studer RK, DeRubertis FR: Decreased cytosolic calcium and prostaglandin synthesis in prehypertensive rats. *Hypertension* 15:388–396, 1990
46. Good DW, Caflisch CR, George T: Prostaglandin E_2 regulation of ion transport is absent in medullary thick ascending limbs from SHR. *Am J Physiol* 269:F47–F54, 1995
47. Garber DL: The molecular basis of hypertension. *Annu Rev Biochem* 68:127–155, 1999