Endothelin A receptor-like immunoreactivity on the basal infoldings of rat renal tubules and collecting ducts*

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Summary. We investigated the distribution of endothelin A (ET_A) receptor-like immunoreactivity in the rat kidney using affinity-purified antibodies against amino acid residues 403-417 of the rat ET_A receptor modified by the multiple antigen peptide complex system. Western blot analysis using the affinity-purified anti-ET_A antibody detected bands of approximately 47.3 and 64.5 kDa in the rat kidney. By light microscopy, ET_A receptor-like immunoreactivity was seen in the basal side of the renal tubules and collecting ducts. The most intense immunoreactivity was present in the distal renal tubules and inner medullary collecting ducts. In addition to the basal infoldings, immunoreactive puncta were scattered in the epithelial cells of the renal tubules and collecting ducts. Specimens prepared using the pre-embedding method were examined by electron microscopy, and some immunopositive signals were seen on the basal infoldings of the renal tubules and collecting ducts. The lengths of immunopositive cytoplasmic membrane were far longer in the distal tubules and inner medullary collecting ducts than in the proximal tubules and outer medullary collecting

ducts. Immunopositive signals were also sometimes observed in the thick portion of Henle's loop, but never in the thin portion. We have not previously detected immunopositive signals on the renal vascular systems with the antibody used here. These results suggest that endothelin acts on the basal infoldings through the ET_A receptor, particularly in the distal tubules and inner medullary collecting ducts, although involvement of the ET_B receptor cannot be excluded.

Introduction

Endothelin (ET) has many physiological roles, including vasoconstriction, mitogenic effects, and the modification of pituitary hormone secretion (Simonson and Dunn, 1990; Naruse *et al.*, 1994). There are at least three distinct isoforms — ET-1, ET-2, and ET-3. Receptors for endothelins have been cloned and classified into three types, ET_A, ET_B, and ET_C. The ET_A receptor has a higher affinity for ET-1 than for ET-3, the ET_B receptor has an equal affinity for ET-1 and ET-3, and the ET_C receptor has a higher affinity for ET-3 (Masaki *et al.*, 1991; Karne *et al.*, 1993). Endothelin binding has been extensively studied in various organs using [¹²⁵I]ET combined with agonists and antagonists, the kidney being one of the most extensively studied organs for the distribution of endothelin and its receptors. Evidence of renal ET biosynthesis and binding suggests the possibility of a local role of ET-1 in the modulation of glomerular and tubular functions (see Rubanyi and Polokoff, 1994). Several lines of study indicate that endothelin in the kidney originates primarily in renal tubular cells (Kakui *et al.*, 2004) although there are quantitative and qualitative differences between nephron segments and between species. With

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regard to ET receptors, it has been shown that the ET_A receptor is the predominant type in the renal vasculature, mainly in smooth muscle cells, and that the ET_B receptor is located in renal tubules and collecting ducts in addition to endothelial cells lining the renal vasculature (Hori *et al.*, 1992; Terada *et al.*, 1992) and podocytes (Yamamoto *et al.*, 2002). However, recent findings on the distribution of the ET_A receptor are highly conflicting. For example, one immunohistochemical study reported the presence of the ET_A receptor in the renal tubules and collecting ducts (Wendel *et al.*, 2006), whereas another did not (Kuc and Dapenport, 2004). Moreover, an association of the ET_A receptor with collecting duct functions was suggested from observations made using mice with a knockout of the collecting duct-specific ET_A receptor (Ge *et al.*, 2005). Thus, there is a need to clarify the detailed distribution of the ET_A receptor in the kidney. The present report describes our findings on the distribution of the ET_A receptor in the rat kidney by light and electron immunomicroscopy with an affinity-purified antibody raised in our laboratory—against a partial peptide of the C-terminal portion of the rat ET_A receptor.

Materials and Methods

Experiments were performed under the authority of the Ethics Committee of Kanagawa Dental College, employing guidelines established by this committee.

Antigen preparation and production of antisera

A peptide corresponding to amino acid residues 403–417 (a cytoplasmic domain) of the rat ET_A receptor, designated as the ET_A peptide, was synthesized (Accord Co. Ltd., Tokyo). This part of the peptide shares the minimum amount of homology with the rat ET_B receptor. A sequence comparison of the synthetic ET_A peptide revealed that it shares 66% homology with a phage resistance gene of *Lactococcus lactis lactis* (a Prokaryota) and a subtype of the ET_A receptor, ET_{AX} endothelin receptor cloned from the *Xenopus* heart (Kumar *et al.*, 1994). It also has 53% homology with the glycerol kinase of *Escherichia coli* and clathrin of *Saccharomyces cerevisiae* (a Fungi) based on data in the PRF-SEQDB database. The purity of the synthetic peptides was tested by high performance liquid chromatography (HPLC) analysis, which revealed a single peak. The peptide was modified by the multiple antigen peptide complex system (MAP) (Tam and Lu, 1989). Six guinea pigs were used for immunization, with one ml of pre-immune serum collected from each animal prior to the start of immunization. The

first immunization used an emulsion of Freund's complete adjuvant (Difco Labs., MI, USA) and MAP-modified ET_A peptide solution (2 mg/ml) at a ratio of 1:1. Each guinea pig was injected subcutaneously with 0.2, 0.2, 0.4, 0.4, 0.5 or 0.5 ml of the emulsion while the animal was under ether anesthesia. Booster injections of the same volume as the initial injection were given to each animal fortnightly, replacing the complete adjuvant with incomplete adjuvant (Difco Labs.). Three days after each booster, 1 ml of blood was collected and its serum was tested with sections of the rat kidney. When immunostaining was obtained at a dilution of 1:1,000, antisera were used for affinity purification.

Larsson's spot test

The specificity of the selected antisera was tested using the membrane blotting method (Larsson *et al.*, 1981). Various artificial protein samples were blotted on a nitrocellulose membrane (Pharmacia Biotech, Uppsala, Sweden): MAP-modified ET_A peptide; bovine serum albumin (BSA)-conjugated ET_A peptide with glutaraldehyde; MAP-modified ET_B peptide corresponding to amino-acid residues 425–439 of the rat ET_B receptor (Sakurai *et al.*, 1990); BSA-conjugated ET_B peptide with glutaraldehyde; and BSA-conjugated arginine-vasopressin (AVP; as a neuropeptide) with glutaraldehyde. The membrane was dried, blocked with 3% BSA in a 0.1 M sodium phosphate buffer (PB; pH 7.4), and stained with pre-immune serum (1:1,000) and antiserum (1:1,000), followed by incubation with biotinylated anti-guinea pig IgG (1:1,000; Vector Laboratories, Burlingame, CA, USA) and an avidin-biotin-horseradish peroxidase complex (ABC; Vector Laboratories) diluted to 1:1,000. The peroxidase complex was detected by incubation of the membrane with a 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.005% hydrogen peroxide and 0.6% ammonium nickel sulfate for 5–10 min.

Affinity purification of antibodies

ET_A peptide was conjugated to ECH Sepharose 4B and CNBr Sepharose 4B (Pharmacia Biotech) according to the manufacturer's instructions. After stabilization, each antiserum was added to peptide-conjugated ECH Sepharose 4B, or peptide-conjugated CNBr Sepharose 4B, and then the mixture was incubated overnight with gentle shaking at 4 °C. The mixture was packed into a column, and non-binding proteins were eluted with 0.1 M PB (pH 7.4) containing 0.5 M NaCl. The binding antibody was eluted with 0.1 M glycine-HCl buffer (pH 2.7) containing 0.5 M NaCl and the elution was neutralized with 0.1

N NaOH. The collected solution was dialyzed against distilled water and then freeze-dried using a VC-36N centrifugal concentrator (Taitec Corporation, Koshigaya).

Western blotting

Solubilization of endothelin receptors from the kidney was performed according to the method described by Kozuka *et al.* (1991). Briefly, two rat kidneys were placed on a metal plate on ice and 2 mm sagittal sections were cut by hand. The renal cortex was dissected out with the aid of a stereoscopic microscope. Fragments of the cortex (about 560 mg wet weight) were homogenized in 1 ml of a 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g/ml}$ pepstatin, and 10 $\mu\text{g/ml}$ leupeptin (solution A). The sample was then centrifuged at 10,000 $\times g$ for 1 hr at 4 °C. The pellet was suspended and washed twice with the same solution, and then further washed twice with 20 mM PB (pH 7.4) containing 130 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g/ml}$ pepstatin, and 10 $\mu\text{g/ml}$ leupeptin (solution B). The final pellet was added to 1 ml of solution B containing 0.25% 3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate and 0.4% digitonin and left for 12 h at 4 °C. The supernatant (designated here as the CHAPS fraction) was added to an equivalent volume of Laemmli's sample buffer (Laemmli, 1970). These samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE). After SDS-PAGE, separated proteins were transferred to Clear Blot Membrane-p (Atto Corporation, Tokyo) and probed with the affinity-purified antibody (5 $\mu\text{g/ml}$) in PB containing

Fig. 1. Larsson's spot test (A and B) and Western blots of the CHAPS fraction of the rat kidney (C and D). Lane A was stained with anti-ET_A serum and lane B was stained with pre-immune serum. Lane C was stained with affinity-purified anti-ET_A antibody and lane D was processed from the secondary antibody. The spots indicated by arrowheads on the right side of lane B show MAP-modified ET_A peptide (AMAP), BSA-conjugated ET_A peptide (ABSA), BSA-conjugated arginine-vasopressin (VBSA), MAP modified ET_B peptide (BMAP) and BSA-conjugated ET_B peptide (BBSA), respectively. Small arrows on the left side of lane C indicate the positions of marker proteins at 74.4, 49.6, 37.2, 24.8, and 12.4 kDa (top to bottom, respectively). Large arrows on the right side of lane D indicate the immunoreactive bands that have migrated to approximately 64.5 kDa (higher) and 47.3 kDa (lower).

Fig. 2. Photomontage showing the overall distribution of ET_A receptor-like immunoreactivity in a coronal section of the rat kidney. Scale bar: 500 μm

Fig. 3. Light micrographs (**A** and **B**) showing ET_A-IR in the renal cortex, and electron micrographs (**C** and **D**) showing ET_A-IR in the proximal tubular cells. The boxed area in **A** indicates the magnified area shown in **B**. Arrowheads in **B** indicate ET_A-IR in the basal portion of renal tubules. Arrows in **B** indicate immunoreactive puncta. Arrows in **C** indicate DAB deposition on part of the cytoplasmic membrane of the basal infolding. Arrowhead in **D** indicates an immuno-positive membrane pit. CD: convoluted portion of the distal tubules, CP: convoluted portion of the proximal tubules, MD: macula densa, SD: straight portion of the distal tubules, SP: straight portion of the proximal tubules. Scale bars: 100 μ m (**A**), 25 μ m (**B**), 2 μ m (**C**), 500 nm (**D**)

Fig. 4. Light micrographs (**A** and **B**) showing ET_A-IR in the outer medulla and electron micrographs (**C** and **D**) showing ET_A-IR in the transitional area from the ascending thick portion of Henle's loop to the distal tubules. The boxed area in **A** indicates the magnified area shown in **B**. Arrowheads in **B** indicate ET_A-IR in the basal side of renal tubules. Arrows in **B** indicate immunoreactive puncta. Arrows in **C** indicate DAB deposition on part of the cytoplasmic membrane of basal infolding. Arrowheads in **C** indicate coagulation of DAB deposition within cytoplasm in a cell located in the thick portion of Henle's loop (Thick) and a cell located in the straight portion of the distal tubules (SD). The arrowhead in **D** indicates an immunopositive membrane pit. CO: collecting ducts, SD: straight portion of the distal tubules. Scale bars: 100 μ m (**A**), 25 μ m (**B**), 2 μ m (**C**) 500 nm (**D**)

0.9% saline (PBS) and 3% BSA. Probed membranes were then incubated with biotinylated anti-guinea pig IgG (1:1,000, Vector) and ABC complex (1:1,000) diluted in PBS containing 1% BSA and finally reacted with 0.02% DAB solution containing 0.005% hydrogen peroxide and 0.6% ammonium nickel sulfate for 5–10 min. Molecular marker proteins (cytochrome c, monomer to hexamer, Oriental East Co., Ltd., Osaka) were also electrophoresed simultaneously.

Immunocytochemistry

Seven male Wistar rats (150–250g) were deeply anesthetized with pentobarbital sodium and perfused with 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB (pH 6.9). The kidneys were dissected out and post-fixed in the same fixative for 24 h at 4 °C, and 8 µm sections were then cut with an HM505E cryostat (MICROM, Walldorf, Germany). Immunocytochemical procedures for light and electron microscopies were carried out according to our routine methods (Yamamoto *et al.*, 1992). Briefly, sections were pretreated with 1% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity and with 3% BSA in PBS for 30 min to prevent non-specific protein binding to the sections. They were incubated with the affinity-purified antibody (10 µg/ml) diluted in PBS containing 1% BSA (PBS-BSA) for 2 days at 4 °C. After washing in PBS-BSA, the sections were incubated with biotinylated anti-guinea pig IgG (1:100) and then ABC (1:200) for 1 h each at room temperature. After washing again in PBS-BSA, the sections were incubated with 0.02% DAB solution containing 0.005% hydrogen peroxide for 5–10 min. Sections adjacent to those used for light microscopic immunocytochemistry were stained with hematoxylin and eosin to identify the segments of the urinary tubules and their locations. For electron microscopy, 20 µm sections were processed as described above. After incubation with DAB solution, they were fixed in 1% osmium tetroxide for 2 h at room temperature, dehydrated, and embedded in Quetol-812

(Nisshin EM Co., Ltd., Tokyo). Ultrathin sections were mounted on nickel grids, counterstained slightly with uranyl acetate and lead citrate, and examined with a JEM1220 electron microscope (JEOL, Tokyo). Antibody characteristics were further tested by Western blots of the CHAPS fraction of the rat kidney. Immunocytochemical controls were prepared by omitting the primary antibody or by incubating with the antibody preabsorbed with either the ET_A peptide (50 µg/ml) or the MAP-modified peptide (20 µg/ml).

Results

After five booster inoculations, we selected one antiserum based on the staining intensity of kidney sections and the result of the Larsson's spot tests. This antiserum recognized MAP-modified ET_A peptide, and ET_A peptide-conjugated with BSA (Fig. 1A). However, the antiserum labeled neither MAP-modified ET_B, ET_B peptide-conjugated with BSA, nor AVP-conjugated with BSA (Fig. 1B). The selected antiserum was affinity purified using synthetic ET_A peptide-conjugated ECH Sepharose 4B, or CNBr Sepharose 4B. Western blot analyses using the CHAPS fraction from rat kidney indicated that the affinity-purified anti-ET_A peptide antibody recognized two bands, a major band of approximately 47.3 kDa and a minor band of 64.5 kDa (Fig. 1C, D).

At the light microscopic level, ET_A receptor-like immunoreactivity (ET_A-IR) was seen in the renal tubules and collecting ducts but only in part of Henle's loop (data not shown). Under low magnification, the most intense immunoreactivity was seen in the outer medulla and secondarily in the cortex (Fig. 2). In the renal cortex, faint ET_A-IR was present in the proximal tubules, with intense staining in the distal tubules (Fig. 3A, B). In the proximal tubules, including the convoluted and straight portions, ET_A-IR tended to be restricted to the basal side of tubular cells. In addition, immunoreactive puncta were

Fig. 5. Light micrographs (**A** and **B**) showing ET_A-IR in the inner medulla, and electron micrographs showing ET_A-IR in the collecting duct cells (**C** and **D**), a thin portion of Henle's loop (**E**), and an endothelial cell of a blood capillary (**F**). The boxed area in **A** indicates the magnified area shown in **B**. Arrowheads in **B** indicate ET_A-IR in the basal portion of collecting ducts. Arrows in **C** and **D** indicate DAB deposition on part of the cytoplasmic membrane of the basal infolding. The arrowhead in **D** indicates an immuno-positive membrane pit. The cell lining a thin portion of Henle's loop (Thin) and an endothelial cell of a blood capillary (EC) seem to be immuno-negative. Some ribosomes in **F** show non-specific staining. CO: collecting ducts. Scale bars: 100 µm (**A**), 25 µm (**B**), 2 µm (**C**) and 500 nm (**D**)

Fig. 5. Legend on the opposite page.

sometimes scattered on the middle portion but were rare on the luminal side of tubular cells (Fig. 3B). By electron microscopic analysis, DAB deposition was seen on part of the cytoplasmic membrane comprising the basal infolding and also in part of the lateral cytoplasmic membrane adjacent to neighboring tubular cells (Fig. 3C, D). Immunopositive membrane pits were sometimes observed on parallel portions of the cytoplasmic membrane (Fig. 3D). In the distal tubules of the renal cortex, intense ET_A-IR was seen on the basal side of tubular cells, wider than in the proximal tubules, but the luminal side was generally free of ET_A-IR (Fig. 3B). In the macula densa, staining was not so evident, but a few immunoreactive puncta were seen in the basal side of the cells (Fig. 3B).

In the outer medulla, intense ET_A-IR was seen in the straight portion of the distal tubules (Fig. 4A, B). Staining profiles in these tubules were similar to those seen in cortical distal tubules (Fig. 3A, B). At the electron microscopic level, DAB deposition was seen to some extent on the cytoplasmic membrane forming the basal infoldings of the distal tubules (Fig. 4C, D). Immunoreactive membrane pits were often seen on these immuno-positive membranes (Fig. 4D). A considerable part of Henle's loop seemed to be immunonegative for the ET_A antibody although some DAB deposition was observed within the cytoplasm (Fig. 4C). The collecting ducts that intermingled with intensely stained distal tubules contained a few immunoreactive puncta (Fig. 4B).

In the inner medulla, intense ET_A-IR was seen in the collecting ducts with staining profiles similar to those seen in the distal tubules (Fig. 5A, B). The collecting ducts in this area showed more intense staining than those in the outer medulla. In the collecting ducts, some DAB deposition was seen on the cytoplasmic membrane of the basal infoldings (Fig. 5C, D) and part of the lateral membrane (data not shown), similar to that seen in the proximal tubules. Other areas of the inner medulla consisting of blood vessels and a thin portion of Henle's loop seemed to be immuno-negative (Fig. 5E, F).

There were no differences in staining profiles between antibodies that had been affinity-purified with ECH Sepharose 4B and these with CNBr Sepharose 4B. The staining profiles produced by these antibodies were abolished by preabsorption with ET_A peptide or with MAP-modified ET_A peptide (data not shown). There was no immunostaining in the sections processed with pre-immune serum or the secondary antibody (not shown).

Discussion

The specificity of the antibody produced here was confirmed by the disappearance of the staining profiles following preabsorption with ET_A peptide or MAP-modified ET_A peptide. The anti-ET_A antiserum subjected to affinity purification recognized the MAP-modified ET_A peptide, and the peptide conjugated to BSA, but not the MAP-modified ET_B peptide or, by Larson's spot test, the peptide conjugated to BSA. Western blot analysis indicated that the anti-ET_A antibody detected two bands from the CHAPS fraction of the rat kidney. The lower band (47.3 kDa) was close to the deduced molecular weight from cloned cDNA of the human ET_A receptor (427 amino-acid residues with a relative *Mr* of 48,722 by Adachi *et al.*, 1991), bovine ET_A receptor (427 amino-acid residues with a relative *Mr* of 48,516 by Arai *et al.*, 1990), and rat ET_A receptor (426 amino-acid residues with no description about *Mr* by Lin *et al.*, 1991). The higher band (64.5 kDa) might be a glycosylated form. In fact, the presence of higher species (53 kDa by Watanabe *et al.*, 1989 and 73 kDa by Martin *et al.*, 1990) of the ET_A receptor, having a higher affinity for ET-1 than ET-3, has been documented by SDS-PAGE electrophoresis.

It is well known that ET induces natriuresis, acting locally in a paracrine and/or autocrine mode, by reducing Na⁺, K⁺-ATPase activity in the rat proximal tubule (Garvin and Sanders, 1991) and in the rabbit inner medullary collecting duct (IMCD) (Zeidel *et al.*, 1989). However, the identities of the ET receptors that mediate ET-induced inhibition of sodium transport are uncertain. The exception is in dogs, in which there is evidence that the ET_B receptor is involved (Brooks *et al.*, 1994). In our electron microscopic analysis, we found ET_A receptor-like immunoreactivity in the basal infoldings of tubular cells in the proximal and distal tubules as well as of inner medullary collecting ducts (IMCD) cells. These membranes are known to contain Na⁺, K⁺-ATPase. In agreement with our data, ET binding sites have also been demonstrated on basal infoldings of these cells by a radio-binding study (Yukimura *et al.*, 1996). Thus, the ET_A receptor may be involved in the ET-mediated reduction of Na⁺, K⁺-ATPase activity. We cannot, however, exclude the possible involvement of ET_B receptors in this ET function, because both ET_A and ET_B receptors were implicated by binding studies and reverse transcription-PCR (RT-PCR) in the rat IMCD (Kohan *et al.*, 1992). The proportion of ET_A to ET_B receptors was found by binding studies to be 2:3 in the basolateral membrane in the renal cortex (Edwards *et al.*, 1998). Furthermore, we also found ET_B receptor-like immunoreactivity in the rat proximal tubules and IMCD in a preliminary study (Yamamoto

and Uemura, 1998), although the stained areas were far smaller than in the glomeruli (Yamamoto *et al.*, 2002). Further studies are needed to clarify the subtype(s) of ET receptors involved in the inhibition of Na⁺, K⁺-ATPase activity.

It has been shown that ET inhibits AVP-dependent cAMP accumulation in the rat collecting duct, possibly by the inhibition of adenylate cyclase activity (Tomita *et al.*, 1990). Furthermore, ET inhibited AVP-stimulated water permeability in the rat collecting ducts (Oishi *et al.*, 1991; Tomita *et al.*, 1993). However, conflicting results have been obtained regarding the receptor subtype involved in the inhibition of AVP: Nadler and Zimpelmann (1992) suggested that this occurred via the ET_A receptor, whereas Edwards *et al.* (1993) suggested it was via the ET_B receptor. Moreover, the presence of ET_B receptor mRNA, but not ET_A receptor mRNA, has been shown by RT-PCR in the IMCD (Terada *et al.*, 1992) and inner and outer medullary collecting ducts (Chow *et al.*, 1995). Another study, however, found both ET_A and ET_B receptors in the IMCD (Kohan *et al.*, 1992). A recent immunohistochemical study showed the presence of the ET_A receptor in the distal tubules and cortical collecting ducts, but not in the outer medullary collecting ducts (Wendel *et al.*, 2006). In contrast, the present data showed ET_A receptor-like immunoreactivity in the outer medullary collecting ducts and IMCD. The collecting duct, especially the IMCD, is the major nephron site of ET-1 synthesis and binding. Moreover, a collecting duct-specific knockout of the ET_A receptor shows altered renal AVP responsiveness (Ge *et al.*, 2005). Taking all these results into account, both types of ET receptor may be present in the IMCD and involved in ET modulation of the AVP function. However, this issue requires further clarification. ET may stimulate ET receptors on the basal infoldings to influence, directly or indirectly, Na⁺, K⁺-ATPase, and AVP receptors.

It is well known that stimulation of the ET_A receptor leads to the vasoconstriction of preglomerular arteries and efferent arterioles (Lanese and Conger, 1993) and that stimulation of the ET_B receptor is able to cause both vasodilation—with the release of prostacyclin and nitric oxide—and vasoconstriction without such a release (Hyslop and de Nucci, 1992). Immunohistochemical studies have indicated the presence of the ET_B receptor over the glomerular and peritubular capillaries (Hagiwara *et al.*, 1993; Yamamoto and Uemura, 1998; Wendel *et al.*, 2006) and on podocytes (Yamamoto *et al.*, 2002). On the other hand, the present study did not detect ET_A-IR in the renal vascular system, but Wendel and co-workers found it in the renal vascular system, in addition to the collecting ducts (Wendel *et al.*, 2006). This discrepancy

may be a consequence of the antibodies utilized, because Western blot analysis indicated slight differences between the antibody used here and the antiserum used by Wendel and co-workers in the protein species recognized. An alternative explanation may lie in the fact that the antibody used here labeled only a subtype of the ET_A receptor. The presence of a subtype of the ET_A receptor, designated ET_{AX}, has been demonstrated in the *Xenopus* heart and oocyte follicle (Kumar *et al.*, 1993, 1994). The binding properties of the ET_{AX} receptor for ET-1, ET-3, and sarafotoxin 6c (a selective agonist for the ET_B receptor) were similar to those of the ET_A receptor, but the ET_{AX} receptor had a different binding property for BQ123 (a selective antagonist for the ET_A receptor) compared with the ET_A receptor. Although the existence of the ET_{AX} receptor in mammalian species has not yet been confirmed, the presence of a novel ET-3 binding site—which has a different binding profile from the traditional ET_A or ET_B receptor and highly binds a ET_A antagonist, A-127722—has been demonstrated in the inner medulla of ET_B receptor-deficient rats (Taylor *et al.*, 2003). Therefore, it is possible that the antibody used here recognized a subtype of mammalian ET receptors for which amino acid sequences have not yet been clarified.

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