Evidence for receptor bound endothelin in renal but not in cardiac tissues from normal rats

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Abstract

Objectives: The stoichiometric binding model (Frelin C, Guedin D. Cardiovasc Res 1994;28:1613–1622) implies that most endothelin in tissues is bound onto receptors rather than in a free state. The objective of this study was to assay receptor bound endothelins in normal rat tissues.

Methods: We first defined acidic conditions that promoted a mild and reversible denaturation of ETA and ETB receptors and that allowed dissociation of bound [125I]endothelin-1. The action of an acid wash on [125I]endothelin-1 binding to cell or tissue homogenates was then investigated.

Results: Acid washing of homogenates prepared from rat brain capillary endothelial cells that express prepro endothelin-1 mRNAs unmasked receptor sites. Acid washing of cardiac, lung, brain or liver homogenates did not increase [125I]endothelin-1 binding. An acid wash of kidney homogenates increased 2.2 fold [125I]endothelin-1 binding. Experiments using BQ-123 further indicated that the acid treatment of renal homogenates mainly unmasked ETA receptors. Masked renal ET receptors were mainly localized in the medulla. Treatment of rats with phosphoramidon decreased the density of masked ET receptors in kidney homogenates.

Conclusion: As much as 50% of endothelin receptors in renal tissues are masked by endogenous endothelins. Most cardiac receptors are free of bound endothelins. These suggest that endothelins act as local rather than systemic mediators. © 1998 Elsevier Science B.V.

Keywords: Endothelin; BQ-123; Stoichiometric binding

1. Introduction

ET-1 is a potent vasoconstricotor peptide secreted by vascular endothelial cells. It belongs to a family of closely related peptides that comprise three mammalian isoforms (ET-1, ET-2 and ET-3) and snake venom toxins, the sarafotoxins. It is now well established that ETs are distributed in a large variety of organs and tissues. Their actions are mediated by their interaction with specific cell surface receptors of their target cells. Two receptors for ETs have been identified by molecular cloning techniques [1,2]. The ETA receptor mainly recognises ET-1 and ET-2. It is selectively antagonized by BQ-123. The ETB receptor binds with equal affinity to the different ET isoforms.

ETs are probably involved in the pathogenesis of some cardiovascular and renal diseases [3–7]. Evidence comes mainly from the fact that these conditions are associated to elevated circulating or urinary ET-1 levels [8]. It is, however, increasingly clear that circulating ET-1 levels are not representative of the real amount of ET present in tissues. For instance, plasma ET-1 levels (1–25 pM) [8] are too low to induce contractions in isolated vessel preparations. Conversely, mixed receptor antagonists which abolish peripheral vasoconstrictions in response to the infusion of ET-1, increase plasma immunoreactive ET-1 [9–11].

We previously suggested that a local action of ETs could be favoured by the fact that the $K_d$ value of ET-1 receptor complexes is much lower than the concentration
of free receptors in tissues, i.e. by conditions known as stoichiometric binding conditions [12]. Stoichiometric binding can account for many unusual features of ET actions such the fact that ET-1 + /− transgenic mice have a higher blood pressure than ET-1 + /+ mice [13], the presence of exceedingly low circulating levels of the peptides [8] and the fact that receptor antagonists act as clearance antagonists [9–11]. One essential feature of this model is that most ligand molecules cannot exist in a free state and must be bound onto receptors. Tissue ETs have been documented by cytochemical techniques. ETs have also been extracted from tissues under strong denaturing conditions. Although these techniques do indicate the presence of ETs in tissues, they provide no information about their form (free or bound) and about the receptors that are occupied.

The objective of this study was to devise a technique for assessing receptor bound ETs in tissues. It is based on the fact that ETs and their receptors form tight complexes that hardly dissociate at neutral pH. When binding experiments are performed on homogenate preparations, endogenous ETs that are already bound onto receptors, remain associated to the receptors and mask these sites to radiolabelled ligands. The masking of receptors by endogenous ET-1 is well recognized in cell culture systems. For instance, it has been reported that blocking the maturation of big ET-1 into ET-1 by phosphoramidon decreases the endogenous production of ET-1 by cultured cells and up regulates ET receptors [14]. In this paper we first defined conditions that induced a reversible denaturation of ET receptors and the dissociation of endogenous ETs from their receptors. We then applied these conditions to tissues from normal rats. The results indicated that the status of ET receptors was not identical in renal and cardiac tissues. Most receptors in cardiac tissues were available to exogenous ET-1. Only half of renal receptors was available to ET-1.

2. Methods

2.1. Materials

Biochemical reagents were from Sigma Chemical Co. unless otherwise indicated. ET-1 and BQ-123 were purchased from Neosystems (Strasbourg, France). [125I]ET-1 (2200 Ci/mmol) was obtained from Amersham Corp. or prepared using Chloramine T and purified by high pressure liquid chromatography [15]. A rat ETB receptor cDNA clone was kindly provided by Dr. M. Yanagisawa. A bovine ETA receptor cDNA clone was kindly provided by Dr. S. Nakanishi.

Earle’s salt solution was 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, buffered at pH 7.5 with 25 mM Hepes/Tris. In some experiments, the pH of the solution was brought to 2.5 with 25 mM glycine/HCl. Homogenization buffer was 250 mM sucrose, 1 mM EDTA buffered at pH 7.5 with 10 mM Tris/Cl. All solutions were supplemented with a cocktail of protease inhibitors (1 μM leupeptine, 0.1 mM bacitracin and 0.1 mM phenyl-methyl-sulphonyl fluoride).

2.2. Cell cultures

Stable transfectant CCI39 fibroblasts expressing bovine ETA receptors (ET/A/CCI39 cells) and rat ETB receptors (ET/B/CCI39 cells), prepared as previously described [16], were kindly provided by Dr. J. Pouyssegur. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Dutsch, Strasbourg, France), 100 units/ml of penicillin and 100 μg/ml of streptomycin. Rat brain capillary endothelial cells were grown as previously described [17].

To prepare cell homogenates, confluent monolayers were washed with serum free culture medium, scrapped with a rubber policeman into the homogenization buffer and homogenized with a Polytron (3 times 5 s at position 6). All operations were performed at 4°C. Homogenates were stored at −20°C.

2.3. Tissue preparation

Wistar rats (200 g from both sexes) were killed by cervical dislocation. Tissues were rapidly excised, and washed with ice cold Earle salt’s solution. Tissues were minced with scissors and homogenized with a Polytron (3 times 5 s at position 10) in homogenization buffer. All operations were performed at 4°C. Proteins were determined according to Bradford [18]. Homogenates were stored at −20°C.

In some experiments, rats were anesthetized with pentobarbital and a bolus injection of phosphoramidon (7 μmol/kg) was given in the tail vein. Control animals were injected with the same volume of saline. Animals were then returned to their home cage for 16 h and a second injection of phosphoramidon or of saline was given. Animals were killed 4 h later and renal homogenates were prepared as described above.

2.4. Acid wash

Homogenates (50 μg of proteins/ml) were diluted into 750 μl of Earle’s salt solution buffered at pH 2.5 with 25 mM glycine and left at room temperature for 90 min. The solution was then neutralized to pH 7.5 by addition of a 1 M Tris solution. Control preparations were maintained for 90 min at room temperature in Earle’s salt solution at pH 7.5. A mixture of glycine and Tris was then added to have identical buffer compositions in the two types of experiments. [125I]ET-1 binding was identical in freshly thawed homogenates and in homogenates that were left at room
temperature for 90 min. This indicated that the cocktail of protease inhibitor used prevented degradation of the receptors during the time course of our experiments.

2.5. [125I]ET-1 dissociation experiments

Cellular homogenates (50 μg of protein/ml) were incubated in Earl’s salt solution (pH 7.5) supplemented with 12 pM [125I]ET-1 for 90 min at room temperature. The incubation medium was then acidified to pH 2.5 by the addition of concentrated HCl and the dissociation of [125I]ET-1 receptor complexes was followed by filtering aliquots of the incubation solution onto 0.22 μm Sartorius filters. Filters were washed 3 times with 5 ml of 0.1 M MgCl₂. Filters were then counted. Non-specific binding was determined in parallel experiments using 100 nM unlabelled ET-1. Triplicate assays were performed.

2.6. [125I]ET-1 binding experiments

Control and acid treated homogenates (after neutralization) were supplemented with 10–20 pM [125I]ET-1, 0.05% bovine serum albumin and competitor peptides. After 90 min of incubation at 20°C, aliquots of the incubation solutions were filtered under reduced pressure onto Sartorius filters. Filters were washed 3 times with 5 ml of 0.1 M MgCl₂ and counted. Non-specific binding was determined in parallel experiments using 100 nM unlabelled ET-1. Triplicate assays were performed. The properties of interaction of [125I]ET-1 with ETA receptors have been analyzed in great detail [16]. This study showed that because of time limited second order rate kinetics, apparent $K_d$ values measured in binding experiments are strongly dependent of the receptor concentration used in the assays. Care was taken in this study to perform binding assays at low protein concentrations (tissue homogenates: 67 μg/ml, cell homogenates: 10 μg/ml).

2.7. ET extraction and assay

Neutralized kidney extracts (4 animals in each experiment) were centrifuged at 120 000 g for 45 min. Supernatants were neutralized with Tris and passed through activated SepPak C18 cartridges (Waters). Columns were washed twice with 4 ml of 0.1% trifluoroacetic acid (TFA) and 0.05% triethylamine (TEA) in water and then with 4 ml of 20% acetonitrile, 0.1% TFA and 0.05% TEA. ETs were then eluted with 4 ml 50% acetonitrile, 0.1% TFA and 0.05% TEA. Fractions were lyophilized and the residue was dissolved into 0.1 ml of Earle’s salt solution supplemented with 0.05% bovine serum albumin. ET was then assessed by radioimmunoassay (Amersham kit), receptor assay or functional assays. The receptor assay was performed as previously described [17] and using membranes prepared from ETB receptor expressing fibroblasts. Functional assays were performed using rat aortic strips [19].

2.8. Data analysis

Binding isotherms were analyzed using the Ligand software to yield apparent $K_d$ values and maximum binding capacities. Means ± SEM and the number of animals used are indicated. Statistical significance of the differences was assessed using t tests. All animals used were housed and handled under protocols approved by the Institutional Animal Care and Use Committees.

3. Results

3.1. Acid induced dissociation of [125I]ET-1 receptor complexes

It is well known that complexes formed by ETs and their receptors are very stable and that an acidification promotes their dissociation [20]. We first defined conditions that promoted dissociation of preformed [125I]ET-1 receptor complexes. Experiments were performed using rat brain capillary endothelial cells which expressed ETA receptors [17,21] and CCl39 fibroblasts that were transfected with rat ETB receptors.

We first checked that ET-1 formed tight complexes with rat ETA and ETB receptors and that in 90 min dissociation experiments, less that 10% of [125I]ET-1 bound onto rat ETA or ETB receptors was released by 100 nM unlabelled ET-1. Fig. 1 shows that an acidification to pH 2.5 induced a rapid dissociation of preformed [125I]ET-1 ETA receptor complexes which was nearly complete after 20 min. Fig. 1 also shows that dissociation of [125I]ET-1 ETB receptor complexes proceeded much more slowly and that after a 90 min incubation at pH 2.5 only 70% of the complexes had dissociated. This result agrees with the
Fig. 2. Properties of acid washed ETA receptors. Control (○) and acid washed (●) homogenates were equilibrated with $^{[125]}$ ET-1 and the indicated concentrations of unlabelled ET-1. After 90 min of incubation, total binding was determined. Apparent $K_d$ values were 440 pM and 400 pM in control and acid washed homogenates respectively. Maximum binding capacities were 2.0 and 2.2 pmol/mg of protein in control and acid washed homogenates, respectively. Identical results were obtained in two other experiments.

3.2. Acid treated ETA and ETB receptors renature after neutralization

Properties of acid washed ETA and ETB receptors were then analyzed. Figs. 2 and 3 compare ET-1 binding isotherms for ETA and ETB receptors in control homogenates and in homogenates that have been acid washed and neutralized. They show that the acid treatment did not modify the apparent affinity of ET-1 for its two receptors. It did not modify maximum ET-1 binding capacities. Taken together, these indicated that the conditions chosen were mild enough to allow a full renaturation of ETA and ETB receptors after neutralization.

3.3. An acid wash revealed new $^{[125]}$ ET-1 binding sites in homogenates prepared from capillary endothelial cells

The acid wash technique was then applied to cultured rat brain capillary endothelial cells. These cells were of interest as an autocrine action of ET-1 was suspected. Capillary endothelial cells express both ETA receptors and prepro ET-1 mRNAs. Fig. 4 shows that an acid wash of endothelial cell homogenates modified ET-1 binding isotherms. It increased the receptor density 2.4 fold from 1.1 to 2.6 pmol/mg of protein. It also induced a small increase in the apparent $K_d$ value of ET-1 receptor complexes from 120 pM to 200 pM. This increase probably reflected the increase in receptor density. Results obtained with endothelial cells were clearly different from those obtained with transfected fibroblasts (Figs. 2 and 3).

3.4. An acid wash unmasked new receptor sites in renal tissues

We then applied the acid wash technique to rat tissues. Tissue homogenates were prepared from different organs of normal rats. Half of the preparation from each animal was acid treated as described above. The other half served as a control. Following neutralization, receptors were titrated and the specific $^{[125]}$ ET-1 binding component was compared to that obtained in control homogenates that had
Table 1
Influence of an acid treatment on $^{[125]I}$ET-1 binding to homogenates from different rat tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>$2.18 \pm 0.14$ ($n=38$)</td>
</tr>
<tr>
<td>Heart</td>
<td>$1.28 \pm 0.23$ ($n=22$)</td>
</tr>
<tr>
<td>Brain</td>
<td>$1.14 \pm 0.02$ ($n=4$)</td>
</tr>
<tr>
<td>Lung</td>
<td>$1.01 \pm 0.04$ ($n=4$)</td>
</tr>
<tr>
<td>Liver</td>
<td>$0.96 \pm 0.13$ ($n=6$)</td>
</tr>
</tbody>
</table>

Homogenates were acid washed and neutralized as described in the Methods section. ET receptors were then titrated using 20 nM $^{[125]I}$ET-1. The specific binding component in acid treated tissues was expressed relative to the value obtained in preparations from the same animal that were not acid treated. Means ± SEM and the number of animals used are indicated.

not been acid treated. Table 1 shows that an acid treatment had no action on $^{[125]I}$ET-1 binding to brain, liver and lung homogenates. It increased $^{[125]I}$ET-1 binding to myocardial homogenates 1.3 fold. The difference was not statistically significant.

Acidification of whole kidney homogenates increased $^{[125]I}$ET-1 binding 2.18 ± 0.14 fold ($n=38$) (Table 1). We also noticed a large variability in the responses. No increase in $^{[125]I}$ET-1 binding was observed in two animals. In responsive animals, stimulation of $^{[125]I}$ET-1 binding by the acid wash ranged from 1.4 to 4.6 fold.

Fig. 5 compares $^{[125]I}$ET-1 binding to control and acid washed homogenates prepared from renal cortex and medulla. It shows (i) that $^{[125]I}$ET-1 binding was larger in the medulla than in the cortex and (ii) that an acid wash revealed more $^{[125]I}$ET-1 binding sites in homogenates prepared from the medulla than in homogenates prepared from the cortex. Thus masked renal ET receptors are mainly localized in the renal medulla.

3.5. Properties of masked renal ET receptors

Fig. 6 presents typical dose–response curves for the inhibition by unlabelled ET-1 of the specific $^{[125]I}$ET-1 binding in control and acid washed homogenates from the same animal. It shows that an acid wash did not alter the apparent properties of interaction of ET-1 with its receptors. Renal tissues express ETA and ETB receptor subtypes [4]. To identify the receptors recognized by $^{[125]I}$ET-1 we used BQ-123, a selective antagonist of ETA receptors [23]. Fig. 7 shows that BQ-123 hardly prevented $^{[125]I}$ET-1 binding to control homogenates. It decreased $^{[125]I}$ET-1 binding to acid washed homogenates in a clear biphasic manner. Actions of nanomolar concentrations of BQ-123 probably corresponded to ETA receptors. Actions of sub micromolar concentrations of BQ-123 probably corresponded to ETB receptors. Taken together these results confirmed the presence of both ETA and ETB receptors in renal tissues. They further indicated that $^{[125]I}$ET-1 mainly titrated ETB receptors in untreated homogenates. $^{[125]I}$ET-1 titrated both ETA and ETB receptors in acid washed homogenates.

The relative proportions of ETA and ETB receptors in control and acid washed renal homogenates were evaluated.
using a standardized assay. Homogenates prepared from single animals were incubated with 10 nM $[^{125}\text{I}]$ET-1 in the absence or the presence of 100 nM BQ-123 or of 100 nM ET-1. Knowing that at 100 nM, BQ-123 completely prevented $[^{125}\text{I}]$ET-1 binding to capillary endothelial cells or to ETA receptor expressing fibroblasts and had no action on $[^{125}\text{I}]$ET-1 binding to ETB receptor expressing fibroblasts (data not shown), we considered that the BQ-123 sensitive $[^{125}\text{I}]$ET-1 binding component represented the contribution of ETA receptors to the measured signal. Conversely, the BQ-123 insensitive, specific $[^{125}\text{I}]$ET-1 binding component was considered to represent the contribution of ETB receptors. Data indicated that ETB receptors accounted for 74 ± 4% ($n = 18$) of the specific $[^{125}\text{I}]$ET-1 binding in normal homogenates. They accounted for only 63 ± 6% ($n = 18$) of the specific $[^{125}\text{I}]$ET-1 binding in acid washed homogenates. Although the difference suggested a selective unmasking of ETA receptors by the acid treatment, it was not statistically significant.

A more sensitive manner of analyzing the data is to calculate the stimulation by an acid wash of the BQ-123 sensitive and insensitive signals. Data indicated that an acid wash increased 2.21 ± 0.29 fold ($n = 18$) the BQ-123 sensitive $[^{125}\text{I}]$ET-1 binding, meaning that 54% of renal ETA receptors were masked. Conversely, an acid wash increased the BQ-123 insensitive $[^{125}\text{I}]$ET-1 binding only 1.39 ± 0.17 fold ($n = 18$) meaning that 28% of renal ETB receptors were masked. The difference was statistically significant ($p < 0.01$, $t$ test). It suggested a selective unmasking of ETA receptors by the acid treatment. It should be noted, however, that the acid wash procedure only released 70% of ET-1 bound to ETB receptors (Fig. 1) and that the proportion of masked ETB receptors may be slightly higher than 28%.

Finally relative densities of masked ETA and ETB receptors were estimated from the acid inducible BQ-123 sensitive and insensitive $[^{125}\text{I}]$ET-1 bindings. Results indicated masked receptors consisted of 46 ± 10% ETA receptors and 54 ± 10% ETB receptors ($n = 18$). In other words, endogenous ETs bind equally to ETA and ETB receptors but the density of ETA receptors being less than that of ETB receptors, a larger fraction of ETA receptors is occupied by endogenous ETs.

3.6. Influence of phosphoramidon on renal ET receptors

Phosphoramidon inhibits conversion of big ET-1 into ET-1 by endothelin converting enzyme [24] and can be used to decrease tissue ET. Rats were injected with phosphoramidon and renal ET receptors were titrated 20 h later. Fig. 8 shows that $[^{125}\text{I}]$ET-1 binding to untreated kidney homogenates prepared from control and phosphoramidon treated animals were similar. An acid wash increased $[^{125}\text{I}]$ET-1 binding in the two groups of animals but the increase ET-1 receptor density in phosphoramidon treated animals was lower than in controls. Thus phosphoramidon treatment decreased densities of both total and masked ET receptors in renal homogenates.

3.7. Extraction of endogenous ETs from renal tissues

Provided that masked receptors were receptors that had been freed of bound ETs, the amount of receptor bound ET was estimated to 300 fmol/mg of proteins, i.e. about 10 pmol per kidney. This value is close to the amount of ET-1 that can be extracted from kidney samples under strong denaturing conditions [25].

Different assays (RIA, receptor assays and functional assays) were used to assay free ET in renal extracts. The results indicated (i) undetectable ET levels in neutral kidney extracts and (ii) the presence of low amounts of ET in acid extracts (3–5 fmol/mg of protein). This amount was much lower than that expected from the density of masked sites (300 fmol/mg of protein). The main reason for this difference was that ET-1 released from the receptors was degraded at pH 2.5. This was checked by an experiment in which a kidney homogenate was incubated for 90 min at pH 2.5 in the presence of a known amount of exogenous ET-1. This amount was only partially recovered after neutralization of the extract and purification of ET-1. We further checked that aqueous solutions of ET-1 were stable at pH 2.5 and that degradation of the peptide by kidney homogenates was reduced at pH 7.5. One hypothesis for these results could be that lysosomal proteases were activated at pH 2.5 and degraded endogenous ETs. Because of these difficulties, it was not possible to relate the amount of masked ET receptors and the concentration of endogenous ET in tissues.
4. Discussion

Results presented in this paper indicate the presence in rat tissues of two forms of \(^{125}\text{I}\)ET-1 binding sites: (i) sites that are freely accessible to the radiolabelled ligand and (ii) binding sites that become accessible to the radiolabelled ligands only after an acid treatment of the homogenates. Masked sites were only observed in renal tissues (Table 1); they were mainly localized in renal medulla (Fig. 6) and may be of the ETA or ETB subtype (Fig. 7). Masked receptors were not observed in transfected fibroblast cell lines (Figs. 2 and 3). They were observed in capillary endothelial cells that expressed pre-pro ET-1 mRNAs (Fig. 4). Different hypotheses may account for the existence of masked receptors. First it could be that the acid treatment induced a conformational change of the receptors and unmasked inactive, but ET free binding sites. This hypothesis was unlikely for the acid treatment did not unmask binding sites in fibroblasts and had different actions in different rat tissues. Another hypothesis could be that ET-1 was stored by the cells, was released during homogenization and masked receptor sites after homogenization of the tissues. Although an intracellular storage of ET-1 by endothelial cells has been documented [26,27], this hypothesis is unlikely to account for our results in kidney membranes for no detectable ET could be recovered in neutral kidney extracts. Another hypothesis is that masked ET receptors were receptors that were occupied by endogenous ETs. This hypothesis is supported by the fact that phosphoramidon, an inhibitor of endothelin converting enzyme [24] decreased the density of masked renal receptors (Fig. 8). The fact that important amounts of ETs are associated to their receptors in renal tissues and cannot be extracted from the tissues under mild conditions provides direct support of our hypothesis that stoichiometric binding conditions determine local actions of ETs [12].

The most important observation of this paper is that the status of ET receptors is different in renal and cardiac tissues. While about 50% of renal ET receptors are occupied by endogenous ETs, most cardiac receptors are available to exogenous ET-1 (Table 1). This suggested that the kidney but not the heart is an important site of action of ETs in normal rats. This conclusion is consistent with results showing that ET receptor antagonists have no action on cardiac hemodynamics [28] but markedly altered renal function in normal rats [29–31]. The absence of receptor bound ETs in heart tissues also suggests that cardiac receptors are unlikely to be desensitized. This could mean a larger sensitivity of the heart to ETs. It should further be stressed that ET has two actions on receptors. First ET regulates expression of receptors [14,15]. This action determines the total density of sites (free and masked). Second, ET binds to receptors and masks receptors to exogenous ET-1. This action determines the density of masked sites. Therefore, a measure of the fraction of masked sites in a membrane preparation cannot be used as a measure of endogenous ETs.

This study further confirms the presence in renal tissues of both ETA and ETB receptors. As observed previously [4], ETB receptors are more abundant than ETA receptors. The two receptors have different cellular localization and probable functions. ETB receptors are localized in arterioles, glomeruli, proximal convoluted tubule, medullary thick ascending loop and collecting duct [32]. ETB receptors in glomerular endothelial cell couple to NO and prostaglandin syntheses, i.e. to vasorelaxing mechanisms [33]. ETB receptors in distal segments of tubules are natriuretic [31]. ETA receptors are localized in arterioles, glomeruli and proximal straight tubule [31]. They probably mediate vasoconstrictor [30,34] and antinatriuretic [29,31] actions of the peptide. Renal synthesis of ETs has been documented in glomerular endothelial cells, mesangial cells and epithelial cells of the proximal tubule, medullary thick ascending limb, medullary collecting duct and cortical collecting tubule [35]. Results presented in this paper further suggested that as much as 50% of renal ET receptors are occupied by endogenous ETs and that most of these receptors seem to be localized in the renal medulla. Endogenous ETs (about 300 fmol/nig of protein) bind equally to ETA and ETB receptors. Yet, the density of ETA receptors being less than that of ETB receptors, a larger fraction of ETA receptors than of ETB receptors is occupied by endogenous ETs. These could suggest a preferential activation of ETA receptors by endogenous ETs. This hypothesis is consistent with the observation that bosentan, a mixed receptor antagonist, has the same renal actions as BQ-123, an ETA receptor selective antagonist [30]. Further studies are required to define which kidney cells bind endogenous ETs.

Intravenously injected \(^{125}\text{I}\)ET-1 disappears from the circulation with a half life of 1–2 min. The label accumulates in lung, kidney and liver tissues [36,37]. Evidence using receptor antagonists have further suggested that ETB receptors play an important role in the clearance of ET-1 [9–11]. Results presented in this paper indicate the presence of receptor bound ETs in kidney but not in lung and liver tissues (Table 1). This does not mean that pulmonary ET-1 clearance is not receptor mediated. ETs bound to ETB clearance receptors may be rapidly internalized and degraded. Conversely, the existence of masked ET receptors in kidney tissues does not mean that these receptors are involved in the clearance of ET-1. Whether ETs bound to renal receptors have a local or systemic origin cannot be defined from our experiments.

In conclusion, this study indicates that an important fraction of renal ET receptors are not accessible to exogenous \(^{125}\text{I}\)ET-1. They become available to labelled ligands only after an acid wash of homogenates. All available evidence suggests that these masked sites are receptors that have bound endogenous ETs. The status of ET receptors is not identical in kidneys and hearts. The marked tissue...
distribution of masked receptors suggests that ETs should be considered as local mediators rather than as circulating hormones.

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