Autoradiographic distribution in rat tissues of binding sites for endothelin: a neuropeptide?

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Koseki, Chizuko, Masashi Imai, Yukio Hirata, Masashi Yanagisawa, and Tomoh Masaki. Autoradiographic distribution in rat tissues of binding sites for endothelin: a neuropeptide? Am. J. Physiol. 256 (Regulatory Integrative Comp. Physiol. 25): H858–H866, 1989.—Endothelin (ET) is a potent and long-acting vasoconstrictor peptide consisting of 21 amino acids and recently isolated from a medium of cultured porcine endothelial cells. To determine the possible sites of ET action, we have conducted autoradiography and receptor binding assays with 125l-labeled ET in rat tissues. The displaceable binding sites of the ligand were widely distributed, not only in the arteries and heart but also in various other organs, e.g., brain, kidney, lung, adrenal gland, and intestine. The systemically injected ET did not cross the blood-brain barrier, whereas the ligand, applied in vitro, was mainly located in the hypothalamic and thalamic areas, lateral ventricular region, subfornical organ, globus pallidus, and caudate putamen. Both membrane preparations from the brain stem including diencephalon and from the heart ventricle had similar, specific, and high-affinity binding sites for 125l-ET. We suggest that ET is involved in the regulation of a large variety of organ functions and may also act as a neuropeptide.

METHODS

Preparation of iodinated ET. The iodinated synthetic ET (porcine sequence) was prepared by the lactoperoxidase method (6). The iodinated ET was shown to retain the same biological potency as that of unlabeled ET using the vasoconstriction assay on the rat pulmonary artery. The resulting radiolabeled ET had a specific activity of 400–500 Ci/mmol.

Autoradiography of tissue labeled in vitro. The procedure was performed as previously described (11). Briefly, male Sprague-Dawley rats weighing ~230g were anesthetized with pentobarbital sodium. Ice-cold phosphate-buffered saline (PBS) was infused into the abdominal aorta. The tissues were immediately frozen in dry ice-n-hexane. Thin cryostat sections (10μm) were mounted on poly-D-lysine-coated glass slides and incubated with 1.25 × 10−9 M 125I-ET in the presence or absence of 2.5 × 10−7 M unlabeled ET for 10 min at 25°C. The incubation solution contained 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl) buffer (pH 7.4), 10 mM MgCl2·6H2O, 1 mM phenylmethylsulfonyl fluoride, 0.4 mg/ml soybean trypsin inhibitor, 1 mM tetrasodium EDTA, 100 U/ml aprotinin, and 2 mg/ml bovine serum albumin. The washed sections were mounted on X-ray film (Cronex no. 4, Dupont, Wilmington, DE) and exposed for 48 h under intensifying conditions (Cronex Lightning-Plus, Dupont). For microscopic observations of the binding sites, some slides were dipped into nuclear tracking emulsion (NTB-2, Eastman Kodak, Rochester, NY) and developed after 5–7 days. To estimate the binding amount of ET in each tissue section, the autoradiography was done by using plastic cover slips (Lux, Miles Laboratories, Naperville, IL). The area outlined by the tissue was cut out of the cover slip. The radioactivity in the tissue section was counted, and the tissues were then dissolved in 1 mg/ml sodium dodecyl sulfate to measure the amount of protein by the method of Lowry et al. (13).

Binding assay in microsomal fraction. The microsomal fraction was prepared as follows. The heart ventricles and brain stems including the diencephalon from two rats were homogenized in 10 vol (wt/vol) of 0.25 M sucrose including 1 mM EDTA (Phycotron setting no.
5, 2 × 30 s), and the homogenate was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was centrifuged (100,000 g for 1 h), and the resulting pellet was resuspended in the same incubation solution for autoradiography tissue labeled in vitro (see above) and kept in the deep freezer until use. A 20-μl aliquot of the microsomal fraction, containing ~130 μg protein in 0.25 M sucrose including 1 mM EDTA, was incubated at 25°C with various concentrations of 125I-ET in 20 μl of the incubation solution. The specific binding was calculated by subtracting the amount of nonspecific binding measured in the presence of 2.5 × 10⁻⁷ M unlabeled ET from the total amount bound. The amount of nonspecific binding was <30% of the total amount bound. Data were represented by the means of triplicate determinations.

After incubation, 200 μl of ice-cold incubation solution were added and then centrifuged at 100,000 g for 20 min at 4°C. The supernatant was rapidly aspirated, and the membrane-bound radioactivity was counted. In a binding inhibition assay, the following peptides or drugs were used: angiotensin II, α-rat atrial natriuretic peptide (α-rANP), bovine parathyroid hormone-(1–34), ω-conotoxin, apamin, verapamil, and nicardipine (Peptide Institute, Osaka, Japan).

**Autoradiography of tissue labeled in vivo.** 125I-ET (7.5 μCi) in saline was injected into the carotid artery. After 10 min, the tissues were rapidly perfused with ~100 ml of ice-cold PBS through the aorta and carotid artery and
RESULTS

Binding sites for $^{125}$I-ET in cardiovascular system. A significant amount of displaceable $^{125}$I-ET binding was observed in the smooth muscle layer of the pulmonary artery sections labeled in vitro [Figs. 1, A (arrow) and A', and 3A (double arrow)]. The comparable amount of ET binding was also found in the abdominal (Fig. 2, A-A') and thoracic aorta (data not shown). There was a diffuse and homogeneous binding of $^{125}$I-ET to the myocardium of the ventricles (Fig. 1, B and B') and the atrium. In the preparation of the emulsion-coated slides of cardiac sections, the grain density on the branches of the coronary artery was enriched much more than that on the surrounding cardiac muscle cells (Fig. 2, B-B').

In the kidney, we observed a high density of ET binding sites in the renal vein and artery, arcuate artery [Fig. 1C (arrows)], interlobular artery, vascular bundle in the outer medulla [Fig. 1C (double arrow)], glomerulus, and papilla, as seen in Fig. 1C. The papilla contained diffuse high-density distribution of grains (Fig. 1C). The detailed observations of binding sites at the microscopic level are indicated in Fig. 2, C-C'. The dense binding of ET in the interlobular artery was clearly observed, as seen in Fig. 2C (arrow). The afferent and/or efferent arterioles of the glomerulus in the emulsion-coated frozen sections also contained a high concentration of $^{125}$I-ET, as shown in Fig. 2C' (arrow).

Binding sites for $^{125}$I-ET in organs other than cardiovascular system. ET binding sites were also distributed in organs other than those of the cardiovascular system. In the lung, dense displaceable grains were found over the alveoli throughout the pulmonary tissue (Fig. 3, A and A'). The bronchi were also heavily labeled [Fig. 3A (arrow)]. In tracheal cross sections, binding sites were localized in the paries membranaceus of the tracheal muscle layer [Fig. 3B (arrow)]. As seen in Fig. 4, A-A' and B-B', the displaceable binding in the bronchi and trachea was localized not only on the muscle layer [Fig. 4, A and B (arrows)] but also considerably on the epithelial cell layer [Fig. 4, A and B (double arrows)]. Binding of $^{125}$I-ET was observed in the mucosal layer of the colon (Fig. 3, C and C'), intestine, and stomach (data not shown). In the spleen, the binding on the trabeculae was
FIG. 3. Autoradiograms of organs other than cardiovascular system labeled in vitro by $^{125}$I-endothelin. A: lung; B: spleen; C: trachea; D: colon; E: adrenal gland; F: eye. A'-C': examples of nonspecific binding. CB, ciliary body; Ir, iris; Ch, choroid; CEn, corneal endothelium. Bars, 1 mm.
clearly visualized in the autoradiogram of tissue labeled in vitro (Fig. 3, D and D'). In the adrenal gland, the medulla was heavily labeled. A less-dense displaceable binding was also seen in the cortex (Fig. 3, E and E'). The corneal endothelium, the iris, the ciliary body and processes, and the chorioida in the retina of the eyes (Fig. 3, F and F') also had a high density of $^{125}$I-ET binding sites. At the microscopic level, we confirmed these binding sites in the eye, as shown in Fig. 4, C-C' and D-D'$. When the ET binding was estimated in rat tissues, the brain, kidney, and lung were the tissues richest in ET binding sites, as shown in Table 1.

*Authoradiography of tissue labeled in vivo.* The autoradiographic features of the tissues labeled in vivo (Fig. 5,
A-E) were essentially similar to those of the studies for
tissue labeled in vitro (Figs. 1 and 3) with exception of
the brain (see below). However, the relative ET labeling
densities were somewhat different. In the in vivo labeled
autoradiograms, the lung and kidney had the highest
gra
density, whereas the large vessels, renal arteries,
trachea, bronchus, and corneal endothelium of the eye
showed less dense binding of $^{125}$I-ET.

**Binding sites for $^{125}$I-ET in brain.** In the rat brain, the
$^{125}$I-ET injected into the aorta bound to the circumven-
tricular structures of the median eminence of the hypo-
thalamus (Fig. 6A) and subfornical organ (Fig. 6B) loc-
ated outside the blood-brain barrier and the choroid
plexus (Fig. 6, A and B). In marked contrast, the auto-
radiograms of the coronal section in the region of dience-
phal of tissue labeled in vitro clearly demonstrated
the high density of ET binding sites mainly in the
hypothalamic and thalamic areas, subfornical organ, lat-
eral ventricular region, globus pallidus, and caudate pu-

### TABLE 1. Quantity of ET binding in rat tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$^{125}$I-ET (1.25 x 10^{-8} M)</th>
<th>$^{125}$I-ET (1.25 x 10^{-8} M) + Unlabeled ET (2.5 x 10^{-7} M)</th>
<th>Displaceable Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart ventricle</td>
<td>0.99±0.06</td>
<td>0.41±0.06</td>
<td>0.58±0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.24±0.92</td>
<td>1.18±0.07</td>
<td>1.06±0.06</td>
</tr>
<tr>
<td>Lung</td>
<td>3.57±0.43</td>
<td>0.85±0.14</td>
<td>2.73±0.06</td>
</tr>
<tr>
<td>Colon</td>
<td>0.94±0.10</td>
<td>0.78±0.13</td>
<td>0.16±0.06</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.99±0.07</td>
<td>0.47±0.04</td>
<td>0.52±0.06</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.10±0.02</td>
<td>0.69±0.14</td>
<td>0.40±0.06</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.31±0.19</td>
<td>1.02±0.08</td>
<td>0.29±0.06</td>
</tr>
<tr>
<td>Liver</td>
<td>1.82±0.11</td>
<td>1.45±0.06</td>
<td>0.37±0.06</td>
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<tr>
<td>Skeletal muscle</td>
<td>0.57±0.03</td>
<td>0.51±0.04</td>
<td>0.05±0.06</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>2.25±0.19</td>
<td>1.41±0.16</td>
<td>0.84±0.06</td>
</tr>
<tr>
<td>Telencephalon</td>
<td>1.62±0.07</td>
<td>1.02±0.09</td>
<td>0.60±0.06</td>
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<tr>
<td>Mesencephalon</td>
<td>1.61±0.07</td>
<td>0.94±0.06</td>
<td>0.88±0.06</td>
</tr>
<tr>
<td>Metencephalon</td>
<td>3.97±0.56</td>
<td>1.12±0.03</td>
<td>2.86±0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE in pmol/mg protein; n = 3. ET, endothelin. Statistical differences were analyzed using the paired t test. * P < 0.05.
tamen excluding for the stria medullaris thalami (Fig. 6, C and C').

**Binding characteristics in microsomal fraction from heart ventricle and brain stem.** Based on the time course of binding, we found that the specific $^{125}$I-ET binding reached equilibrium within 5 min in the brain stem and 15 min in the heart ventricular preparation, as shown in Fig. 7. We therefore selected an incubation time of 10 min for the brain stem and 15 min for the heart. The receptor binding assay showed high-affinity ET binding sites in both of the microsomal fractions from cardiac ventricles and brain stem including diencephalon as shown in Fig. 8. The Scatchard analysis from these two binding curves showed a single class of binding sites for $^{125}$I-ET, respectively (Fig. 8, inset). The values of the apparent dissociation constant ($K_D$), binding maximum ($B_{max}$), and correlation coefficient ($r$) were shown in the legend for Fig. 8. The value of the apparent $K_D$ of the brain was comparable to that of the heart ventricle. As indicated in Fig. 9, the binding of $1.25 \times 10^{-9}$ M $^{125}$I-ET was competitively displaced by unlabeled ET in a dose-dependent manner, with the concentration producing 50% inhibition being $7.1 \times 10^{-9}$ M in the brain and $2.2 \times 10^{-8}$ M in the heart ventricle. It was not inhibited in both preparations by peptides including $10^{-6}$ M angiotensin II, $10^{-6}$ M $\alpha$-rANP, $10^{-6}$ M bovine parathyroid hormone, $5 \times 10^{-8}$ M $\omega$-conotoxin, and $5 \times 10^{-8}$ M apamin. It should be emphasized that the binding was unaffected by Ca$^{2+}$ channel blockers including $10^{-3}$ M verapamil and $10^{-3}$ M nicardipine.

**DISCUSSION**

This study demonstrated that the binding sites for ET were distributed not only in the arterial smooth muscle and heart but also widely in various other organs, including the brain, kidney, lung, adrenal gland, and intestine. In fact, the functional significance of ET was obtained in some of these organs. ET had a constrictive effect on the trachea, having the same order of half-maximal effective dose for the aorta (22). In the heart, ET diffusely bound to the myocardium of the ventricle and the atrium. The apparent $K_D$ of ET binding in the cardiac microsomal fraction was comparable to the ET-induced chronotropic and inotropic effects in isolated atria (7, 8, 24). Accordingly, these ET induced cardiac effects may have resulted from a direct action of ET on the myocardial cells. Although the vasoconstrictor effect induced by ET was attenuated by nicardipine (7, 8, 24), voltage-dependent Ca$^{2+}$ channel blockers did not compete with ET for the ET binding sites. This was also the case for
cultured vascular smooth muscle cells (6). Therefore the inhibitory effect of the Ca²⁺ antagonists may be noncompetitive in binding in nature.

Our results revealed that the kidney and lung were the tissues richest in ET binding sites (Table 1). According to Yanagisawa et al. (24), however, mRNA for the ET precursor could not be demonstrated for any of these organs in porcines. Since the distribution of the binding sites for ET is not identical to that of its production, it is possible that ET acts not only as an autacoid but also as a circulating hormone. It should be noted that the precursor could not be demonstrated for any of these tissues richest in ET binding sites (Table 1).

In the brain, the large amount of specific high-affinity binding sites for ET were localized in the microsomal fraction from the brain stem, including diencephalon. The brain also showed discrete distribution of binding sites for ET. This pattern of distribution was distinct from those of other biologically active substances such as ω-conotoxin (10), vasopressin (23), endorphin (4), neurotensin (17), and other substances (3, 5, 15, 18, 19, 26). Since the intra-aurically injected ET does not cross the blood-brain barrier, it is possible that ET or an analogous peptide(s) might be produced in the brain and might act as a neuropeptide. Furthermore, the circumventricular structures bound by systemic ET described above have been shown to contain binding sites for ANP (14, 20) and angiotensin II (20, 27). These peptides are involved in the regulation of vasopressin secretion (9), salt and water intake (2), and the systemic blood pressure (21). Recently, Yoshida et al. (25) demonstrated that intracerebroventricular administered ET caused an increase in blood pressure at a low concentration. Thus our results suggest that ET acts on the central nervous system as a neurotransmitter or neuromodulator to control these functions.

In conclusion, our observation that the ET binding sites are located in organs other than those of the cardiovascular system suggests that ET may be involved in the regulation of a wide variety of organ functions including those of the central nervous system.

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