Direct Evidence for Multiple Endothelin Receptors

Iris Schvartz, Olivia Ittoop, and Eli Hazum*

Division of Endocrinology, Glaxo Research Institute, 5 Moore Drive, Research Triangle Park, North Carolina 27709

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ABSTRACT: Competition binding experiments and peptide mapping techniques were employed in order to directly address the possible existence of endothelin (ET) receptor subtypes in the atria. Competition binding assays for ^{125}I-labeled ET-1 or ^{125}I-labeled ET-3 to bovine atrial membrane preparations suggest the existence of two ET receptor subtypes, one of which binds ET-1 and ET-3 with a similar affinity while the other shows preference for ET-3. However, cross-linking experiments of both peptides to this tissue resulted in the identification of a single 50-kDa protein. To identify directly the existence of multiple ET receptors, peptide mapping of cross-linked ^{125}I-labeled ET-1 or ^{125}I-labeled ET-3 receptors was conducted. Different peptide maps were obtained only under conditions that preferentially label one receptor subtype. These results indicate, for the first time, the existence of two ET receptor subtypes in the atria which differ from each other in both their binding characteristics and primary structure.

Endothelin- (ET-) 1 is a 21 amino acid peptide isolated from vascular endothelial cells and shown to be the most potent vasconstrictor known today (Yanagisawa et al., 1988). Numerous studies have demonstrated that in addition to its vasoconstrictive effects, ET possesses a wide variety of pharmacological activities in vascular and nonvascular tissues (Yanagisawa & Masaki, 1989; Giaid et al., 1989; Yoshizawa et al., 1990; MacCumber et al., 1989). Recently, the presence of the ET-1 gene as well as two other genes of ET, named ET-2 and ET-3, has been found in several mammalian species (Inoue et al., 1989). The amino acid sequences of the 21-residue peptides predicted by these genes were similar to, but distinct from, each other (Inoue et al., 1989). They all produced strong vasoconstrictive responses; however, their pharmacological activities were considerably different, suggesting the existence of endothelin receptor subtypes (Inoue et al., 1989). To directly address the question of whether different receptor subtypes exist, we conducted peptide mapping of the cross-linked ^{125}I-labeled ET-1 or ^{125}I-labeled ET-3 receptors from bovine atrial membrane preparations.

Experimental Procedures

Materials. ET-1, ET-3, and sarafotoxin-S6c (SRTX-c) were obtained from Peptides International, Inc. *Staphylococcus aureus* V8 protease and papain were purchased from Sigma Chemical Co. Disuccinimidyl suberate (DSS)[1] was purchased from Pierce Chemical Co. ^{125}I-ET-1 and ^{125}I-ET-3 (2200 Ci/mmol) were purchased from Amersham.

Membrane Preparations and Binding Assays. Atria were dissected from adult bovine hearts and brought on ice from a local slaughterhouse. The tissue was minced to small pieces and homogenized for 10 min (Polytron homogenizer) in 10 volumes (v/w) of ice-cold 20 mM Tris-HCl, pH 7.0, containing 5 mM EDTA (buffer A). The homogenate was centrifuged (1500*g* for 10 min at 4 °C), and the supernatant was collected and centrifuged again (38000*g* for 25 min at 4 °C). The pellet was resuspended in buffer A, homogenized again, and centrifuged at 38000*g* for an additional 25 min at 4 °C. The pellet was resuspended in 50 mL of buffer A, and protein was determined by using the Coomassie protein assay reagent. Binding assays were conducted exactly as previously described (Schvartz et al., 1990a) using ^{125}I-ET-1 or -ET-3 as tracers.

Cross-Linking. Cross-linking experiments were conducted essentially as described previously (Schvartz et al., 1990a), with several modifications. Briefly, the labeled peptide (800 000 cpm) was incubated with membrane preparations (800 μg of protein) in a total volume of 0.83 mL of 10 mM Hepes, pH 7.4 at 22 °C. After 100 min, the binding was terminated by centrifugation, and the pellet was resuspended in 10 mM Hepes. DSS, in a final concentration of 0.5 mM, was then added, and the reaction was continued for 20 min at 22 °C. Subsequently, 50 mM Tris-HCl, pH 7.4, was added for an additional 10 min at 4 °C; the pellet was washed twice, and the samples were then processed for gel electrophoresis as previously described (Schvartz et al., 1990a).

Peptide Mapping. Peptide mapping was conducted as described previously (Schvartz et al., 1990a). Briefly, bovine atrial membranes were cross-linked with either ^{125}I-labeled ET-1 or ^{125}I-labeled ET-3 and subsequently solubilized (1 h at 22 °C) in 50 mM Tris-HCl, pH 6.8, containing 10% SDS, 10% glycerol, and 5% dithiothreitol. The samples were then run on an SDS–polyacrylamide slab gel (10%) for 18 h at 12 mA. Second-dimension electrophoresis was performed by the method of Bordier and Crettol-Jarvinen (1979). Briefly, the lanes of interest from the first dimension were excised from the gel, trimmed to the 55 000 area, and incubated in 100 mL of 125 mM Tris-HCl, pH 6.8, containing 0.1% SDS for 40 min at room temperature. Thereafter, the bands were placed horizontally in a second gel containing 15% acrylamide. To fix the gel lanes, a 1% agarose solution in 125 mM Tris-HCl, pH 6.8, containing 0.1% SDS, was added to the level of the lanes. After the agarose had solidified, 0.25 mL of 50 mM Tris-HCl, pH 6.8, containing 2% SDS and 10% glycerol was added with the indicated amount of protease. Immediately thereafter, the electrophoresis was carried out at room temperature (18 h at 4 mA and an additional 4 h at 20 mA). The gels were dried and processed for autoradiography as described previously (Schvartz et al., 1990a).

Results and Discussion

Competition binding experiments for ^{125}I-labeled ET-1 or ^{125}I-labeled ET-3 to bovine cerebellum membrane preparations by unlabeled ET-1, ET-3, or SRTX-c (Takasaki et al., 1988)

* To whom correspondence should be addressed.

[1] Abbreviations: SDS, sodium dodecyl sulfate; DSS, disuccinimidyl suberate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Table I: IC_{50} Values (nM) of ET Analogues in Bovine Atria and Cerebellum[a]

	cerebellum		atria	
	^{125}I-ET-1	^{125}I-ET-3	^{125}I-ET-1	^{125}I-ET-3
ET-1	3 ± 2	4 ± 2	1 ± 0.1	0.5 ± 0.3
ET-3	4 ± 2	4 ± 2	2 ± 1	0.05 ± 0.01
SRTX-c	2 ± 0.5	7 ± 2	1000 ± 50	10 ± 3

[a] Membrane preparations (150 μg of protein) were incubated with either ^{125}I-labeled ET-1 or ^{125}I-labeled ET-3 in the presence of increasing concentrations of ET-1, ET-3, or SRTX-c, and the binding was measured as described previously (Schvartz et al., 1990a). IC_{50} is the concentration of peptide by which the specific binding of the labeled peptide is inhibited by 50%.

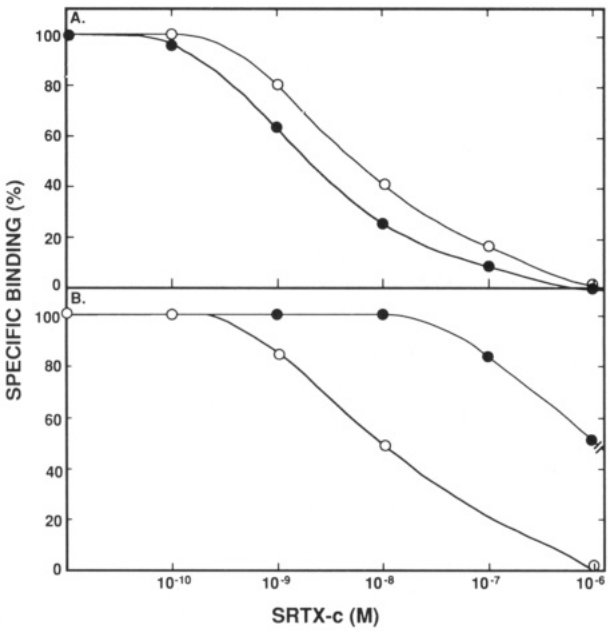

FIGURE 1: Dose-dependent inhibition of ^{125}I-ET-1 and ^{125}I-ET-3 binding to either cerebellum or atrial membranes by unlabeled SRTX-c. Cerebellum (A) or atrial (B) membranes (150 μg of protein) were incubated with either ^{125}I-ET-1 (●) or ^{125}I-ET-3 (○) (20 000 cpm) in the presence of increasing concentrations of unlabeled SRTX-c. After 90 min at 22 °C, the binding was measured as described under Experimental Procedures. Maximum specific binding was 15 fmol/mg of protein. Complete inhibiton of ^{125}I-ET-1 binding to atrial membranes was achieved by 10^{-4} M SRTX-c.

resulted in similar IC_{50} values for all these peptides (Table I and Figure 1A), suggesting that only one receptor subtype exists in bovine cerebellum. However, when these experiments were conducted using atrial membrane preparations, the binding of ^{125}I-ET-1 or ^{125}I-ET-3 was differentially inhibited by increasing concentrations of the unlabeled peptides (Table I and Figure 1B). The order of potency for inhibition of ^{125}I-ET-1 binding was ET-1 ≥ ET-3 ≫ SRTX-c, whereas that of ^{125}I-ET-3 was ET-3 ≫ ET-1 > SRTX-c. These findings suggest the existence of two ET receptor subtypes in the atria, one of which binds ET-1 and ET-3 with a similar affinity while the other shows preference for ET-3.

To identify directly the ET receptor subtypes in the atria, we cross-linked ^{125}I-labeled-ET-1 or ^{125}I-labeled ET-3 to atrial membrane preparations using the cross-linking reagent DSS. Cross-linking of either ^{125}I-labeled-ET-1 or ^{125}I-labeled ET-3 resulted in the identification of two bands with apparent molecular weights of 52K and 30K (Figure 2, lanes 1 and 3, respectively). The intensities of these two bands were markedly reduced when the cross-linking was conducted in the presence of excess unlabeled ET-1 or ET-3 (Figure 2, lanes 2 and 4, respectively), indicating that these bands represent specific binding proteins for both ET-1 and ET-3.

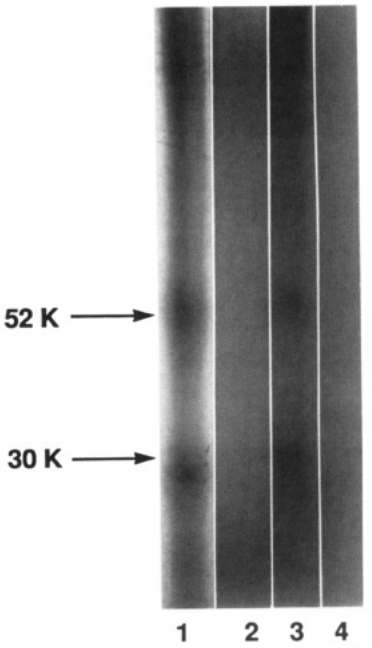

FIGURE 2: Autoradiogram of SDS–polyacrylamide gel electrophoresis of cross-linked ET receptors. Atrial membrane preparations were incubated with ^{125}I-ET-1 in the absence (lane 1) or presence of 0.1 μM ET-1 (lane 2), or with ^{125}I-ET-3 in the absence (lane 3) or presence of 0.1 μM ET-3 (lane 4) for 100 min at 22 °C. The cross-linking reaction was conducted as described under Experimental Procedures, and the samples were processed for gel electrophoresis.

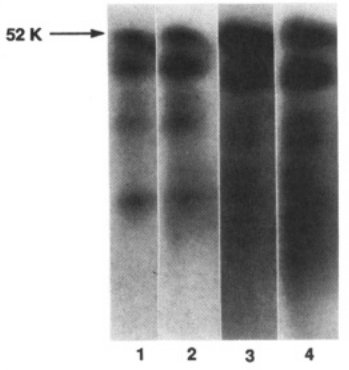

FIGURE 3: Peptide mapping of the 52-kDa binding protein cross-linked with either ^{125}I-labeled ET-1 or ^{125}I-labeled ET-3. The 52-kDa bands cross-linked with either ^{125}I-ET-1 (lanes 1 and 3) or -ET-3 (lanes 2 and 4) were excised from the gel, transferred to a 15% SDS slab gel, and digested with either 75 μg of papain (lanes 1 and 2) or 50 μg of *Staphylococcus aureus* V8 protease (lanes 3 and 4). For more details, see Experimental Procedures.

Recently, we demonstrated that the 30-kDa binding protein in bovine cerebellum membrane preparations is a proteolytic fragment of the native ET receptor (Schvartz et al., 1990a). Similar results were obtained with atrial membrane preparations (data not shown). These findings suggest that the apparent molecular weights of the ET receptor subtypes are similar, i.e., 50K when the molecular weight of ET is subtracted.

Since the molecular weights of these receptors are similar while their binding characteristics are different, we conducted peptide mapping experiments to detect differences in their primary structures. As shown in Figure 3, identical peptide maps were generated by either papain (lanes 1 and 2) or *Staphylococcus aureus* V8 protease (lanes 3 and 4) from the atrial ET receptors when cross-linked with either ^{125}I-labeled ET-1 (lanes 1 and 3) or ^{125}I-labeled ET-3 (lanes 2 and 4). Since both ^{125}I-ET-1 and ^{125}I-ET-3 bind to the ET-1 and ET-3 receptor subtypes, these peptide maps probably represent a

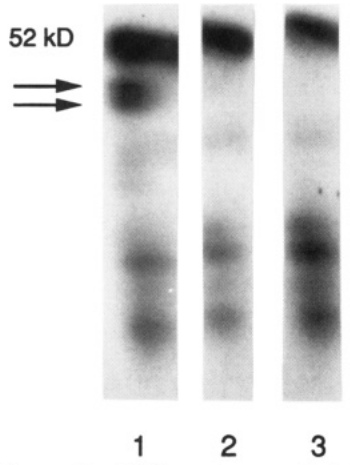

FIGURE 4: Peptide mapping of ET receptors cross-linked with ^{125}I-ET-1 in the presence of increasing concentrations of SRTX-c. ^{125}I-ET-1 was incubated with membrane preparations in the absence (lane 1) or presence of 10^{-8} (lane 2) or 10^{-6} M (lane 3) SRTX-c as described in the legend to Figure 1. The 52-kDa bands were excised, transferred to a 15% SDS slab gel, and digested with 50 μg of *Staphylococcus aureus* V8 protease as described under Experimental Procedures.

combination of the peptide fragments generated from each of the receptor subtypes. In order to preferentially label only one receptor subtype, we took advantage of the binding characteristics of these peptides. As shown in Figure 1B and Table I, the IC_{50} value of SRTX-c for ^{125}I-ET-3 binding to the atria was 10 nM, whereas that for ^{125}I-ET-1 was 1 μM. Thus, incubation of atrial membrane preparations with ^{125}I-ET-1 in the presence of high concentrations of SRTX-c, e.g., 10^{-8}–10^{-6} M, would inhibit the binding of the labeled peptide to the ET-3 receptor subtype without affecting the binding to the ET-1 receptor subtype.

Figure 4 shows the different peptide maps generated from ET receptors cross-linked with ^{125}I-ET-1 in the presence of increasing concentrations of SRTX-c. As shown in the autoradiogram, several peptide fragments were generated from ^{125}I-ET-1-labeled receptors when the binding was conducted in the absence of SRTX-c (lane 1). However, in the presence of SRTX-c, i.e., 10^{-8} or 10^{-6} M, the upper two peptide fragments disappeared completely (lanes 2 and 3, respectively). Since under these experimental conditions all ET-3 receptors are blocked (Table I), the peptide maps represent the ET-1 receptor subtype. These findings, in combination with the binding results, suggest the existence of two ET receptor subtypes in atrial membrane preparations.

The presence of ET receptors in vascular and nonvascular tissues has been well established (Yanagisawa & Masaki, 1989). However, the molecular properties of these receptors are still under intensive investigation (Ambar et al., 1990; Watanabe et al., 1989; Martin et al., 1989; Masuda et al., 1989; Miyazaki et al., 1990). In a recent study (Watanabe et al., 1989), it was demonstrated that chick cardiac membranes contain two distinct types of ET receptors, one of which is a 53-kDa protein possessing a higher affinity for ET-1 and ET-2 than for ET-3. The other consists of two major proteins of 34 and 46 kDa and has higher affinity for ET-3 than for ET-1 or ET-2. In our previous (Schvartz et al., 1990a, b) and unpublished studies, we have clearly demonstrated that both ET-1 and ET-3 bind to a single protein with an apparent molecular weight of 52K. Therefore, other proteins with lower apparent molecular weights probably represent proteolytic fragments of the receptor. These results and our present findings indicate that the distinct binding properties exhibited by ET-1 or ET-3 in the atria can be accounted for by the existence of ET receptor subtypes. These receptors have an identical apparent molecular weight of 50K but differ in their peptide maps, suggesting minor differences in their primary structure. Indeed, following our manuscript submission, two cDNA clones encoding for an ET-1-specific receptor subtype and a non-isopeptide-selective subtype have been identified and expressed (Arai et al., 1990; Sakurai et al., 1990). Thus, it appears that the cerebellum ET receptor which binds ET-1, ET-3, and SRTX-c with similar affinity (our present findings) is, probably, the nonselective subtype. However, the nature of the atrial receptors as well as ET-3 or SRTX-c receptors awaits further studies.

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