

Endothelin–receptor interactions

Role of a putative sulfhydryl on the endothelin receptor

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The mechanism of action of endothelin–receptor interactions was studied, using radioligand binding assays and SDS-PAGE, to investigate the possibility of disulfide interchange. Electrophoretic analysis suggested involvement of disulfide bond(s) in the receptor–ligand complex. Treatment of Et receptors with sulfhydryl-specific alkylating reagents (NEM or others) resulted in decreased ability to bind [125 I]Et-1, [Dpr¹-Asp¹⁵]Et-1, an antagonist homologous to Et but with an amide link replacing one of the disulfides, bound to Et receptors reversibly, but binding of Et-1 was less reversible. Preincubation of receptors with Et-1, but not with [Dpr¹-Asp¹⁵]Et-1, protected receptors from alkylation with [14 C]NEM. The data suggest that the Et receptor has a sulfhydryl group at or near the Et binding site. A model is proposed in which the role of the putative sulfhydryl group is discussed.

Endothelin; Endothelin receptor; Antagonist; Mechanism

1. INTRODUCTION

Endothelin is a 21 amino acid vasoconstrictor peptide whose receptor interaction is, as yet, poorly characterized. Endothelin seems to function in a regulatory role as a paracrine hormone synthesized primarily by endothelial cells. It regulates vascular tone through binding to plasma membrane receptors on local target cells [1–4]. Receptor occupancy is reported to occur rapidly [2], with > 90% occupation of the available binding sites after a 30 s exposure to endothelin. This vasoconstrictor action of endothelin is a very long-lived one, as has been reported in a number of reviews [3,5–7]. There are many mechanisms by which a long-lived action could be effected, including the possibility that the ligand occupies the receptor for extended periods of time. Clozel et al. [8] reported that dissociation of the bound ligand is slow, with less than 10% dissociation occurring over a 48 h period. Waggoner et al. [9] have suggested that Scatchard analysis of endothelin binding to receptor is invalid, primarily because the binding is essentially irreversible. This irreversibility may be due to a covalent bond and this interaction may involve disulfide exchange. Hagiwara et al. [10] supported the possible involvement of disulfide exchange when they reported that the endothelin receptor is sensitive to the sulfhydryl

alkylating reagent, *p*-chloromercuriphenylsulfonic acid (PCMS). In addition, Wada et al. [11] reported that endothelin-1 binding is inhibited by the heavy metal cadmium, an observation which could also implicate sulfhydryl/disulfide involvement. A proposed mechanism which would account for these observations is that the endothelin receptor may have sulfhydryl group that can interact with at least one of the two disulfide bonds of endothelin, thus forming a covalent complex.

Recently, we described the design and synthesis of an endothelin-1 analog, [Dpr¹-Asp¹⁵]Et-1, in which the outer disulfide bond was replaced by an amide bond [1]. This molecule was shown to have antagonist activity (and no agonist activity) in a perfused lung vasoconstriction assay and in a bronchoconstriction assay [12]. It also was shown to bind to the endothelin receptor, although with a lower apparent affinity as compared to native endothelin [1]. If there is a sulfhydryl/disulfide interchange between the outer disulfide of endothelin and its receptor which is critical for endothelin activity, then this analog would be expected to bind more reversibly and to lack agonist activity. The purpose of this paper was to examine in greater detail the possibility that endothelin receptors may have critical sulfhydryl groups.

2. MATERIALS AND METHODS

2.1. Materials

Frozen Hartley guinea pig kidneys were purchased from Keystone Biologicals (Cleveland, OK). [125 I-Tyr¹³]endothelin-1 (specific activity

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of 2200 Ci/mmol) was obtained from New England Nuclear Research Products, DuPont Co. (Boston, MA) and unlabeled endothelin-1 was obtained from Peninsula Laboratories (Belmont, CA). *N*-Ethyl maleimide, iodoacetamide, and iodoacetic acid were obtained from Aldrich Chemicals (Milwaukee, WI). [Dpr¹-Asp¹⁵]Et-1 was synthesized as described [1]. [¹⁴C]*N*-Ethyl maleimide was obtained from Amersham (Arlington Heights, IL). [¹²⁵I]Samples were counted in a Auto-Gamma 5000 series counter (United Technologies Packard) with a counting efficiency for ¹²⁵I of 88%. [¹⁴C]Samples were counted in a liquid scintillation analyzer, 2000CA Tri-Carb (United Technologies Packard). Results were expressed as counts per minute (cpm).

2.2. Methods

2.2.1. Radioligand binding assays

Guinea pig kidney apical membrane preparation. The method of Booth et al. [13] was utilized for preparation of brush-border membranes. All procedures were done in an ice bath. Membrane fragments were prepared from whole kidneys of Hartley guinea pigs of either sex. After 20 frozen kidneys had been allowed to warm at room temperature for approximately 10 min, the cortical tissue was carefully dissected. The cortex was then homogenized in 200 ml of 10 mM mannitol, 2 mM Tris-HCl, pH 7.1, at 4°C, in a Kenwood blender (model A 956A) run at full-speed for 2 min. The total volume of the homogenate was 400 ml. The homogenate was centrifuged on a Beckman Model TJ-6 centrifuge for 2 min at 200 × *g* (585 rpm).

After discarding the pellet, solid MgCl₂·6H₂O was added to the homogenate to give a concentration of 10 mM. This solution was centrifuged again for 12 min at 1500 × *g* (2525 rpm). The pellet was discarded and the supernatant was centrifuged on a Sorvall RC-5B Refrigerated Superspeed Centrifuge with a Sorvall SS-34 Rotor for 12 min at a *K* factor of 949 (13,000 rpm). The supernatant was discarded and the pellet was re-suspended in 6 ml of buffer. 100 μl aliquots were placed in polypropylene centrifuge tubes and stored frozen (−70°C) until needed. When the membranes were used in experiments these aliquots were diluted to 1 ml with Hanks buffered saline (pH 7.4) containing 0.5 mg/ml bovine serum albumin.

Effect of endothelin or [Dpr¹-Asp¹⁵]Et-1 on [¹²⁵I]endothelin-1 binding [¹²⁵I]Endothelin-1 binding assays were performed in polypropylene centrifuge tubes. Incubation medium consisted of Hanks buffered saline (pH 7.4) containing 0.5 mg/ml bovine serum albumin, 40,000 cpm [¹²⁵I]endothelin-1, 10 μl of graded concentrations of unlabeled ligand (endothelin or antagonist), and 5 μg membrane in a total volume of 0.2 ml. The tubes were incubated at room temperature for 1 h. Incubations were terminated by filtration using Whatman GF/C filters presoaked with Hanks/albumin buffer. Filtration was performed on a Millipore filtration apparatus. Filters were rinsed three times with 3 ml of Hanks balanced salt solution. The filters were then placed in polystyrene culture tubes and counted on the gamma-counter. Non-specific binding was defined as the counts remaining in the presence of 0.2 μM endothelin-1 and was approximately 19% of the total bound counts. Specific binding was defined as total binding minus non-specific binding. Each point represents *n* = 3, error bars are SD.

Effect of endothelin or [Dpr¹-Asp¹⁵]Et-1 on [¹⁴C]*N*-ethyl maleimide binding. [¹⁴C]*N*-Ethyl maleimide binding assays were performed in polypropylene centrifuge tubes. Membranes (10 μg) were first treated with graded concentrations of endothelin or antagonist for 1 h, then 100,000 cpm [¹⁴C]NEM were added in a medium consisting of Hanks/albumin buffer, total volume of 0.2 ml. The tubes were incubated at room temperature for 1 h. Incubations were terminated by spinning on a Beckman Microfuge at 12,000 rpm for 15 min. Supernatant was removed and discarded and pellet was resuspended in 1 ml of buffer. This solution was spun at 12,000 rpm for 15 min. The supernatant was again removed and discarded and the pellet was resuspended in 100 μl of 1 N NaOH. Each tube was rinsed into the liquid scintillation vials containing 5 ml of scintillation fluid.

Effect of sulfhydryl alkylating reagents on [¹²⁵I]endothelin-1 binding. Binding to *N*-ethyl maleimide treated membranes (or membranes

treated with other sulfhydryl alkylating agents) was performed as in the [¹²⁵I]endothelin-1 binding studies except that membranes were preincubated with the indicated concentrations of alkylating agent (dissolved in buffer) at room temperature for 30 min (except as otherwise noted). The pH of the buffer was maintained at 7.4 with NaOH when preparing the iodoacetic acid solution. In separate experiments, the order of addition of *N*-ethyl maleimide, membrane and [¹²⁵I]endothelin-1 was varied. In one case, membranes were first preincubated with NEM for 1 h. In a second case, membranes were first preincubated with [¹²⁵I]endothelin-1 for 1 h followed by NEM for 1 h. In a third case, [¹²⁵I]endothelin-1 was preincubated with NEM for 1 h followed by incubation with membrane for 1 h. All incubations were at room temperature.

2.2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the endothelin-receptor complex

A 100 μl aliquot of the guinea pig kidney apical membrane preparation (described previously) was diluted to 1 ml in Hanks buffered saline (pH 7.4) containing 0.5 mg/ml bovine serum albumin. An endothelin dose-response binding experiment was carried out in duplicate, using 10 μl of membrane dilution, 10 μl of graded endothelin-1 concentrations, 50 μl of [¹²⁵I]endothelin-1 (15 pM), and brought to a total volume of 0.2 ml with Hanks buffer. The tubes were allowed to incubate at room temperature for 1 h. Two sample buffer solutions were prepared, one containing 2-mercaptoethanol ('reducing buffer') and one without 2-mercaptoethanol ('non-reducing buffer'). The non-reducing buffer contained 4.7 ml of distilled water, 1.0 ml of 0.5 M Tris-Cl, pH 6.8, 1.0 ml of glycerol, 1.0 ml of 10% (w/v) SDS, 0.2 ml of 0.05% Bromophenol blue, with the addition of 0.1 ml of 2-mercaptoethanol in the reducing buffer.

After the binding experiments were completed, the reaction mixtures were centrifuged at 10,000 × *g* for 10 min at 4°C. The pellets were rinsed and centrifuged two more times with 1 ml of the Hanks/albumin buffer. 50 μl of non-reducing buffer was added to one sample from each duplicate while the other sample had 50 μl reducing buffer added. These pellets were solubilized in the buffer and allowed to incubate at 37°C for 1 h, then subjected to electrophoresis. The gel was then subjected to autoradiography using the X-Omat AR film for 7 days at −70°C

3. RESULTS

3.1. Radioligand binding assays

3.1.1. Effect of endothelin-1 or [Dpr¹-Asp¹⁵]Et-1 on [¹²⁵I]endothelin-1 binding

Fig. 1A indicates that [Dpr¹-Asp¹⁵]Et-1 effectively competed with [¹²⁵I]endothelin-1 for binding to guinea pig kidney membrane preparation, although with a potency of about two orders of magnitude less than did unlabeled endothelin-1. A time course study was done to test the reversibility of the endothelin-receptor interaction (Fig. 1B). The control series exhibited essentially full binding of [¹²⁵I]endothelin-1 within minutes of addition of the radioligand. Preincubation of receptor (in guinea pig kidney membrane) with unlabeled endothelin (10^{−9} M, close to its IC₅₀) for 1 h effectively prevented the binding of [¹²⁵I]endothelin-1. The structural analog [Dpr¹-Asp¹⁵]Et-1 (at a dose well above its IC₅₀, 10^{−6} M) substantially blocked [¹²⁵I]endothelin-1 binding initially, but over the course of several minutes, [¹²⁵I]endothelin-1 binding increased to control levels. This suggested a largely irreversible interaction between endo-

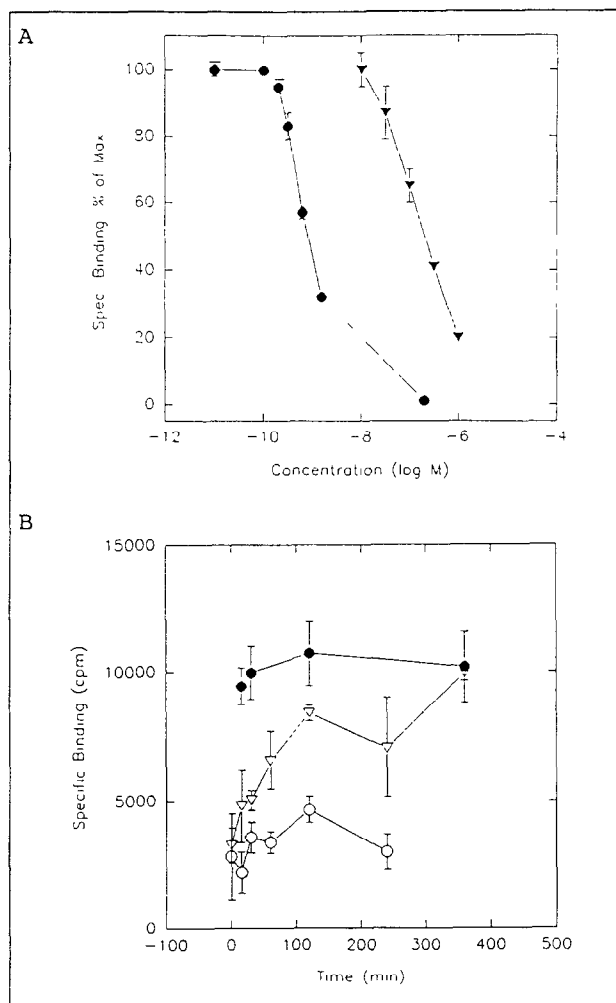


Fig. 1. Receptor-ligand interactions. Fig. 1A shows the results of a competitive radioligand-receptor assay. [¹²⁵I]endothelin-1 (40×10^3 cpm, specific activity 2200 Ci/mmol) and guinea pig kidney apical membranes ($10 \mu\text{g}$) were incubated with the indicated amounts of endothelin-1 (circles) or [Dpr¹-Asp¹⁵]Et-1 (triangles) in a total volume of 0.2 ml for 1 h at room temperature. Data are the mean \pm S.D. of triplicate determinations within one assay; the assay was repeated 3 times with two different receptor preparations. Fig. 1B is a time course of binding of [¹²⁵I]endothelin-1 after preincubating receptor with 1×10^{-9} M endothelin-1 (open circles), 1×10^{-6} M [Dpr¹-Asp¹⁵]Et-1 (triangles), or buffer (filled circles).

thelin and its receptor in comparison to a reversible interaction between [Dpr¹-Asp¹⁵]Et-1 and the endothelin receptor.

3.1.2. Effect of endothelin-1 or [Dpr¹-Asp¹⁵]Et-1 on [¹⁴C]N-ethyl maleimide binding

In order to demonstrate further the reversible nature of binding for the analog [Dpr¹-Asp¹⁵]Et-1 in comparison to the irreversibility of endothelin-1 binding, receptors were pretreated for 1 h with various concentrations of unlabeled endothelin or unlabeled [Dpr¹-Asp¹⁵]Et-1. Subsequently, [¹⁴C]NEM was added and allowed to incubate for 1 h. Increasing concentrations of endothelin

allowed for decreased uptake of [¹⁴C]NEM, whereas the analog at doses as high as 10^{-6} M was unable to prevent uptake of the labeled sulfhydryl alkylating agent (Fig. 2). Endothelin, at any concentration tested, could not prevent totally the uptake of [¹⁴C]NEM, suggesting that sulfhydryl groups other than endothelin-receptors were being alkylated.

3.1.3. Effect of sulfhydryl alkylating reagents on [¹²⁵I]endothelin-1 binding

Fig. 3A shows the results of alkylation with *N*-ethyl maleimide when the receptor was pretreated with increasing concentrations of NEM at room temperature for 30 min, then allowed to bind [¹²⁵I]endothelin-1 for 1 h (solid line), the results was a diminished capacity to bind endothelin. The dashed line shows the effects on binding when the receptor was pretreated with [¹²⁵I]endothelin-1, then subjected to the indicated doses of NEM. No loss of endothelin binding was noted. The dotted line is a control in which endothelin was preincubated with the indicated doses of NEM and then the mixture was added to the receptor. In Fig. 3B, receptor was pretreated with various sulfhydryl alkylating reagents (1×10^{-2} M) at room temperature for 30 min, then allowed to bind [¹²⁵I]endothelin-1 for 1 h. NEM exhibited the greatest inhibition of [¹²⁵I]endothelin-1 binding. This may have resulted from a decrease in the number of viable receptors available. In contrast, iodoacetamide (IAM) had little effect on binding of endothelin. These

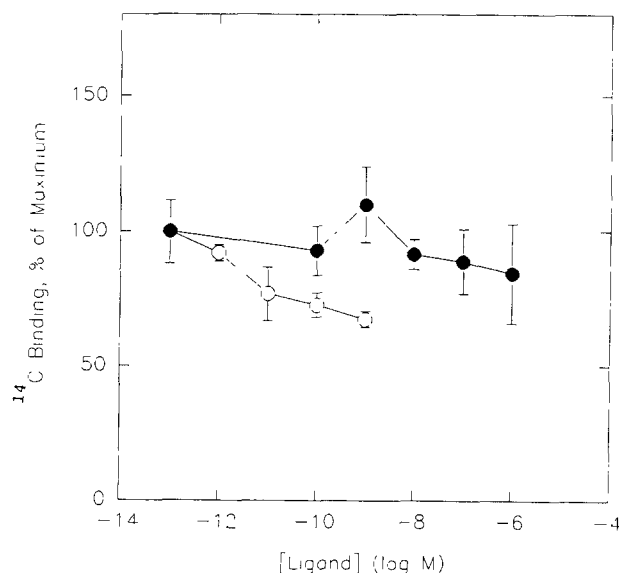


Fig. 2. Uptake of [¹⁴C]NEM by endothelin receptors. [¹⁴C]NEM binding to guinea pig kidney apical membranes pretreated with indicated doses of endothelin-1 (open circles) and [Dpr¹-Asp¹⁵]Et-1 (filled circles) indicates that increasing concentrations of endothelin, but not of [Dpr¹-Asp¹⁵]Et-1, were able to prevent the uptake of radiolabeled NEM. Values given as percent of maximal binding. Each data point represents the mean \pm S.D. of triplicate determinations from a representative experiment.

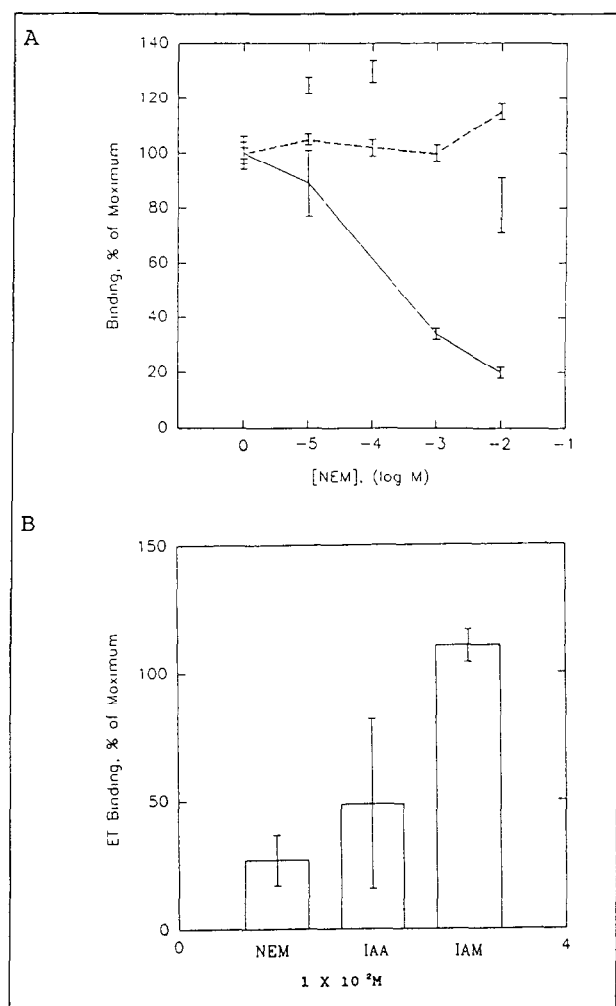


Fig. 3. Alkylation of receptor with *N*-ethyl maleimide (A) Order of addition: the solid line shows the effects on binding when receptor was preincubated with the indicated doses of NEM, then allowed to bind [¹²⁵I]endothelin-1. The dashed line shows the effects on binding when the receptor was pretreated with [¹²⁵I]endothelin-1, then subjected to the indicated doses of NEM. The dotted line is a control in which endothelin was preincubated with the indicated doses of NEM and then the mixture was added to the receptor. (B) Receptor was pretreated with 1 × 10⁻² M of the various sulfhydryl alkylating reagents at room temperature for 30 min, then washed and allowed to bind [¹²⁵I]endothelin-1 for 1 h. Values are given as percent of maximal binding. Maximal binding was defined separately for each treatment as binding in absence of alkylating agent (approx. 9000 cpm). Each data point represents the mean ± S.E.M. of triplicate determinations from a representative experiment.

results imply that NEM and IAA bind to sulfhydryls on endothelin receptors and are successful in blocking binding of [¹²⁵I]endothelin-1. IAM likely binds to sulfhydryls on endothelin receptors but is not able to prevent [¹²⁵I]endothelin-1 binding.

3.2. SDS-PAGE of the endothelin-receptor complex

[¹²⁵I]Endothelin-1 was allowed to bind to receptor preparations, followed by dissolution in either non-re-

ducing or reducing buffer. Samples were then subjected to electrophoresis and Fig. 4 shows the autoradiogram. In the absence of reducing agents, a single band is observed at high molecular weight (≥ 100 kDa). However, upon reduction, the high molecular weight band disappears and there is probably a slight increase in the density of the band at 2500 Da (representing endothelin).

From the reported cloning of the ET_A receptor [14,15], and from the electrophoretic studies [16–18] in which endothelin-1 was covalently cross-linked to receptor and then treated with reducing buffer, it was expected that a band at approximately 50 kDa would be observed. In the autoradiograph, faint middle bands are present which are approximately 50 kDa, but the majority of the label is at higher molecular weight. Whether these bands truly represent endothelin receptors remains to be determined, but in this study, in which non-reducible cross-linking techniques were not used, it may be suggested that these bands represent receptor subunits that interact through disulfide bonds.

4. DISCUSSION

Several observations in the literature, when taken together, could suggest that endothelin receptors interact with their ligand through sulfhydryl/disulfide interchange. Among these observations are those of Waggoner et al. suggesting that endothelin binding is largely irreversible [9], of Hagiwara et al. suggesting that *p*-chloromercuriphenylsulfonic acid (PCMS) inactivates endothelin receptors [10], and of Wada et al. which indicates that the heavy metal cadmium inactivated ET receptors [11]. Further, we have shown that [Dpr¹-Asp¹⁵]Et-1, a structural analog of endothelin which has an amide bond replacing one of its disulfide bonds, is an endothelin antagonist. However, one of its analogs, a monocyclic derivative which lacks the amide bond is a weak agonist. In addition, the reported sequences of the ET_A and ET_B receptors demonstrate that there are 19 Cys in total, probably arranged as 4 on the cytoplasmic side, 7 in the membrane-spanning region, and 8 internal. One or more of these residues could possibly interact with a disulfide of endothelin. Therefore, we sought to test the possibility in a more direct fashion, employing standard alkylation studies, ligand binding studies, and electrophoretic analysis.

Alkylation of receptors with *N*-ethyl maleimide resulted in a loss of binding of endothelin, and this decrease was dependent on the concentration of NEM or the time of incubation with NEM. Conversely, incubation of receptor with endothelin, but not with [Dpr¹-Asp¹⁵]Et-1, decreased the uptake of [¹⁴C]NEM by receptor preparations. Total blockage was not observed in these crude membrane fractions, undoubtedly because sulfhydryls exist on protein other than the endothelin receptor. These data would suggest that the endothelin receptor may contain one or more sulfhydryl

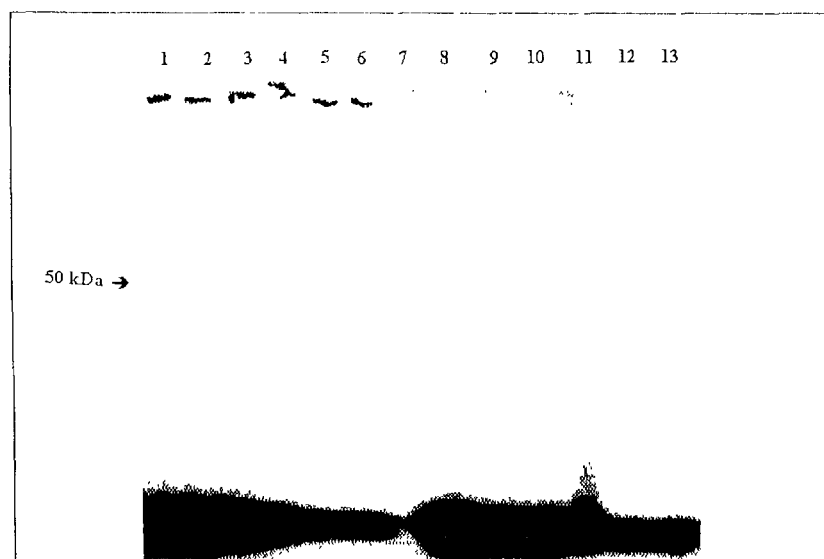


Fig. 4. Effect of sodium dodecyl sulfate-polyacrylamide gel electrophoresis on [125 I]endothelin-1–receptor interaction. [125 I]Endothelin-1 binding to guinea pig kidney apical membranes in the presence of graded concentrations of unlabeled endothelin-1 (1×10^{-10} to 1×10^{-6} M). Lanes 1 to 6 represent an endothelin-1 dose–response curve subjected to non-reducing conditions after [125 I]endothelin-1 binding was completed. Lanes 8 to 13 represent an endothelin-1 dose response curve subjected to reducing conditions after [125 I]endothelin-1 binding was completed. 5 μ l of molecular mass standard was placed in lane 7 for determination of the M_r .

groups which are sensitive to alkylation. That endothelin can block uptake of NEM suggests that a sulfhydryl may be near the ligand binding site, though further work would be needed to confirm this unequivocally.

Further observations concerning the alkylation studies are of interest. First, fairly high concentrations of NEM were required for maximal loss of endothelin binding, and this may suggest that the putative sulfhydryl is not easily accessible to a bulky group such as NEM. This led to the use of alternative alkylating agents and to the second observation. Iodoacetic acid (IAA) and iodoacetamide (IAM) were employed because they are smaller than NEM. However, only IAA blocked endothelin binding. Likely mechanisms for this observation include the possibility that IAM did not alkylate the receptor, which seems unlikely, or that the small size and lack of charge on IAM allowed endothelin to bind. That is to say, it may be that the larger size of NEM and the charge of IAA interfere with binding (Table I). No biological studies were employed with these toxic alkylating agents for obvious reasons, but it

may be that endothelin bound to IAM-alkylated receptors would be unable to cause the normal signal transduction.

Electrophoretic analysis using radiolabeled endothelin resulted in the observation of high molecular weight bands which may represent endothelin–receptor complexes. These bands were not detected in the presence of reducing agents, implicating disulfide interchange. If these bands do represent endothelin receptors, which have a molecular weight near 50 kDa [14,15], then they must have been aggregated in a manner that can be overcome by the addition of reducing agents. Other workers have not reported such an observation, but most have employed photoactively-crosslinked endothelin receptor complexes in the presence of reducing agents. These conditions would lead to identification of the 50 kDa polypeptide, but not to larger molecular weight complexes.

A model for the interaction of endothelin with its receptor can be proposed which is consistent with all the data and which offers new leads on the nature of the interaction and the nature of the antagonist, [Dpr¹-Asp¹⁵]Et-1. As shown in Fig. 5, the antagonist properties [1] of [Dpr¹-Asp¹⁵]Et-1 suggest that there are two distinct endothelin conformations. A 'receptor binding' conformation is depicted as a bicyclic structure, and this conformation may or may not be the solution structure of endothelin, but if it is not the solution structure, it is some conformation easily accessible to the molecule. A 'receptor activating' conformation is proposed to be a more open, monocyclic structure. It is proposed that these two structures can interconvert

Table I

Physical properties of sulfhydryl alkylating reagents and their effect on [125 I]endothelin-1–receptor binding

Reagent	Physical properties	Effect on endothelin binding
Iodoacetic acid	Small; charged	Decreases
Iodoacetamide	Small; uncharged	None
N-Ethylmaleimide	Large; uncharged	Decreases

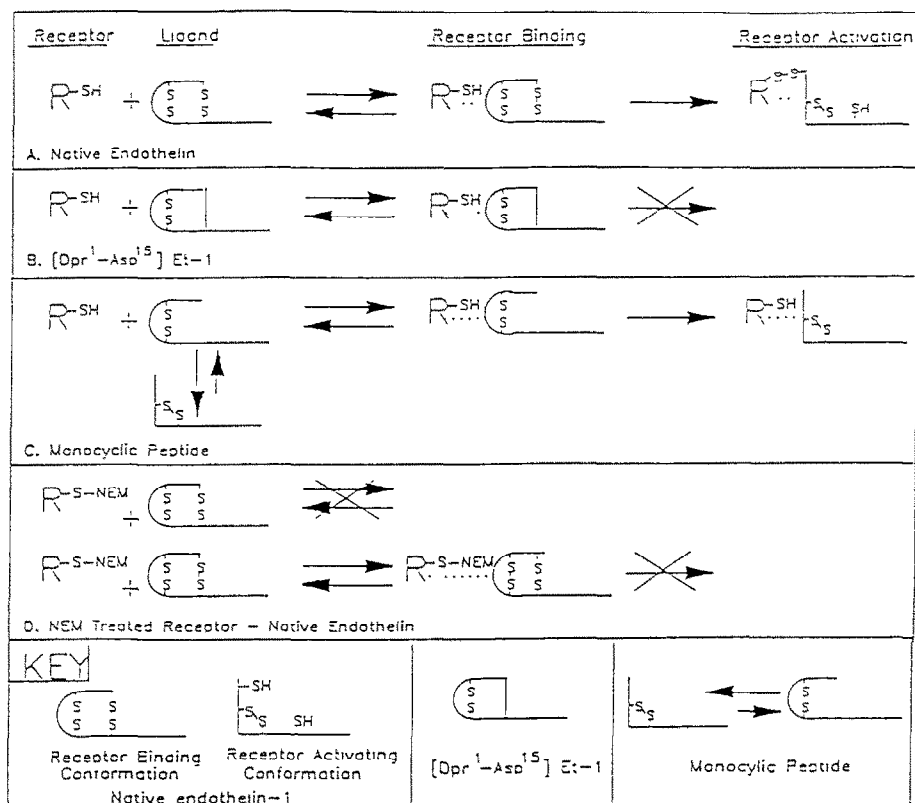


Fig. 5. Proposed model for endothelin-receptor interactions

by means of disulfide interchange with the receptor. $[Dpr^1-Asp^{15}]Et-1$ is depicted as being 'locked' in the receptor binding conformation, unable to achieve the receptor activating conformation because it has an amide bond instead of the outer disulfide bond of endothelin. Thus $[Dpr^1-Asp^{15}]Et-1$ can bind to receptor but not interact with the receptor through disulfide exchange in order to generate the flexibility necessary to achieve receptor activation. Other conformationally constrained antagonists [19,20] have been reported, and it may be that these molecules are similarly unable to achieve the flexibility required for receptor activation. As shown previously [1], a control peptide is devoid of an outer disulfide bond, and is depicted as an open, monocyclic structure. A small percentage of the monocyclic peptide molecules may approximate the bicyclic, receptor binding conformation even though there is no outer bridge. Once bound to the receptor, the monocyclic peptide is flexible enough to assume the receptor activating conformation, circumventing the disulfide exchange requirement. This is an important distinction. The model proposes that it is not a disulfide bonded complex between endothelin and receptor, per se, that is responsible for receptor activation, but that disulfide exchange is a necessary intermediate step for endothelin to achieve receptor activation. This may explain the weak agonist activity [1] of the monocyclic peptide and

similar monocyclic analogs reported in the literature [19]. When the receptor is first treated with NEM or other sulfhydryl alkylating reagents, the 'critical' free sulfhydryl at the ligand binding site becomes blocked, leading to a decrease in receptor binding through steric or charge effects. Although not tested in this work due to the expected toxicity of alkylating reagents, endothelin binding to modified receptors would be predicted by this model to be biologically inactive. This could best be tested using site-directed mutagenesis of the endothelin receptor.

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