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Short communication

Endothelin ET_B receptors show different binding profiles in intact cells and cell membrane preparations

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Abstract

We examined the affinity of endothelin-1, endothelin-3 and four endothelin receptor ligands, BQ788 (*cis*-2,6-dimethylpiperidinocarbonyl-γ-methyl-Leu-D-Trp(1-CO_2CH_3-D-Nle-ONa), SB-209670 ((+)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid), IRL-1620 (succinyl-[Glu^9,$Ala^{11,15}$]endothelin-1(8-21)), and L-749329 (3′,4′-methylenedioxy-1-(2-propyl-4-carboxyphenoxy)-*N*-(4-isopropyl-phenylsulfonyl)-benzene acetamide), for endothelin ET_B receptors in human and rat heart cells. The affinities of these ligands showed good correlation between both types of living cells and between their membrane preparations ($r = 0.861$, $P < 0.001$), but less significant correlation between each of the living cells and its respective membrane preparation ($r = 0.569$, $0.02 < P < 0.05$). These results suggest that there is no species difference in the affinities of these ligands and that destruction of the intact cell membrane structure may lead to changes in binding properties of the endothelin ET_B receptor. © 1998 Elsevier Science B.V.

Keywords: Endothelin ET_B receptor; Endothelin-3; Girardi heart cell; Heart neonatal rat

1. Introduction

Endothelin has dual effects on blood vessels via two types of endothelin receptors, ET_A and ET_B (Sakurai et al., 1990). Endothelin ET_A receptors reside generally on smooth muscle cells and mediate vasoconstrictor responses, whereas the endothelial cells express endothelin ET_B receptors which mediate vasodilator effects via the endothelin-induced release of endothelium-derived relaxing factors (Yanagisawa and Masaki, 1989). However, endothelin ET_B receptors have also been shown to reside on vascular smooth muscle cells and mediate vasoconstriction (Clozel et al., 1992). Although only one gene has been cloned for endothelin ET_B receptors (Sakurai et al., 1990), the presence of endothelin ET_{B1} and ET_{B2} receptors is described by anatomical, functional, and pharmacological profiles (Warner et al., 1993; Sudjarwo et al., 1994). For example, endothelin ET_B receptors have been placed into two subclasses based on the sensitivity to PD 142893(Ac-(3,3-D-diphenylalanyl)-Leu-Asp-Ile-Ile-Trp trifluoroacetate) (Warner et al., 1993). Reynolds et al. (1995) suggested the possibility of subclassification due to species difference.

Here we tried to check for the existence of endothelin ET_B receptor subclasses by means of several receptor ligands. We detected neither subclass nor species difference, but were able to demonstrate that intact cells and membranes prepared from them showed different pharmacological profiles in the endothelin ET_B receptor binding.

2. Materials and methods

2.1. Materials

[^{125}I]Endothelin-3 was obtained from Amersham. Endothelin-1 and endothelin-3 were purchased from the Peptide Institute (Osaka, Japan). IRL-1620 (succinyl-[Glu^9,$Ala^{11,15}$]endothelin-1(8-21)) and BQ788 (*cis*-2,6-dimethylpiperidinocarbonyl-γ-methylleucyl-D-Trp (1-CO_2-CH_3-D-Nle-ONa) were from American Peptide (Sunnyvale, CA, USA). L-749329 (3′,4′-methylenedioxy-1-(2-propyl-4-carboxyphenoxy)-*N*-(4-isopropyl-phenylsulfonyl)-benzene acetamide) and SB-209670 ((+)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,

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4-methyl-enedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid) were kindly donated by Drs. Toshiro Konoike and Teruo Yamamori of our laboratories.

2.2. Cell cultures

Human Girardi heart cells were obtained from the American Type Culture Collection through Dainippon Seiyaku (Osaka, Japan). The cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (Gibco), and antibiotics in a 5% CO_2/95% air incubator at 37°C. Cultures of neonatal rat cardiac myocytes were performed as described by Jones et al. (1989).

2.3. [^{125}I]Endothelin-3 binding to cells

Binding studies with cells were performed as described previously (Mihara and Fujimoto, 1993). In brief, the cells were cultured in 24-well culture plates. After 3 to 5 days, the culture medium was aspirated, and the cells were washed twice with ice-cold HEPES (20 mM)-buffered Hanks' solution (pH 7.4). Each well was incubated with 8.3 pM [^{125}I]endothelin-3, unless otherwise noted, in 0.3 ml of HEPES-buffered Hanks' solution containing 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 250 μg/ml bacitracin and 10 μg/ml soybean trypsin inhibitor in the absence or presence of unlabelled agonists or antagonists. Binding was performed at 37°C for 60 min, unless otherwise stated. The incubation was terminated by rapid removal of the incubation medium and addition of 0.25 ml of ice-cold HEPES-buffered Hanks' solution. Free ligand was removed by washing the cells two times with ice-cold HEPES-buffered Hanks' solution. Cells were dissolved in 0.1 N NaOH and transferred to a test tube, then the radioactivity was counted. Nonspecific binding was determined in the presence of 10^{-7} M endothelin-3.

2.4. [^{125}I]Endothelin-3 binding to membranes

Ventricles were isolated from neonatal (2- or 3-day-old) rats. Heart, liver, lung, kidney, cerebrum and cerebellum were excised from male Wister rats (5-week-old). Girardi heart cells cultured to confluence were scraped with a rubber policeman and washed in phosphate-buffered saline. Tissues or cultured cells were homogenized in 0.25 M sucrose/10 mM Tris–HCl buffer (pH 7.4). The homogenates were centrifuged at $1000 \times g$ for 10 min, and then the pellets were discarded. The supernatants were centrifuged at $100\,000 \times g$ for 30 min, and the resulting pellets were resuspended in the Tris buffer and stored at −80°C until use. Binding studies were performed as described previously (Fujimoto et al., 1992). In brief, membranes were incubated with 25 pM [^{125}I]endothelin-3, unless otherwise stated, in HEPES-buffered Hanks solution (0.1 ml), in borosilicated glass tubes, containing 0.1-mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 250 μg/ml bacitracin and 10 μg/ml soybean trypsin inhibitor (pH 7.4). Binding was performed for 60 min at 37°C, unless otherwise stated. The binding reaction was terminated by the addition of ice-cold 50 mM Tris–HCl (pH 7.4), following rapid filtration through a Whatmann GF/C glass fiber filter (pre-soaked in 1% polyethyleneimine) under reduced pressure. The filters were then quickly washed four more times with 2.5 ml of the buffer. Radioactivity retained on the filter was counted. Nonspecific binding was defined in the presence of 10^{-7} M endothelin-3.

3. Results

To detect only endothelin ET_B receptors, we used [^{125}I]endothelin-3 concentrations which were low enough to neglect its binding to coexisting endothelin ET_A receptors (Fujimoto et al., 1992). We first examined the potencies of IRL-1620, BQ788, SB-209670 and L-749329 for displacing [^{125}I]endothelin-3 binding in membrane prepara-

Table 1
Concentrations of endothelin receptor ligands needed to displace [^{125}I]endothelin-3 binding in living cells (C) and membrane preparations (M) by 50% (IC_{50})

Ligand	Human Girardi heart			Rat neonatal heart		
	IC_{50} (nM)		Ratio	IC_{50} (nM)		Ratio
	C	M	(M/C)	C	M	(M/C)
Endothelin-1	0.044	0.20	(4.5)	0.11	0.34	(3.1)
Endothelin-3	0.039	0.36	(9.2)	0.25	0.31	(1.2)
IRL-1620	2.3 ± 0.5	0.6 ± 0.2	(0.26)	1.8 ± 1.6	0.12 ± 0.03	(0.067)
BQ788	0.5 ± 0.1	3.5 ± 0.6	(7.0)	1.6 ± 0.9	5.9 ± 1.8	(3.7)
SB-209670	1.2 ± 0.2	3.24 ± 0.6	(2.7)	0.76 ± 0.09	1.3 ± 0.5	(1.7)
L-749329	3.3 ± 0.4	60 ± 9	(18)	1.1 ± 0.1	17 ± 2	(15)

Data indicated were means of two experiments or means ± S.D. of three experiments.

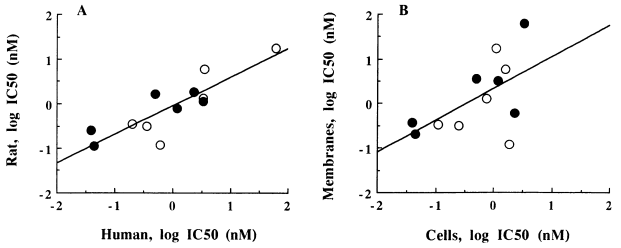

Fig. 1. Correlation of ligand affinities for human Girardi heart cells and rat neonatal heart cells (A) and living cells and their membrane preparations (B). In A, ●, living cells; ○, membrane preparations. In B, ●, human; ○, rat.

tions from rat heart, lung, liver, kidney, cerebellum and cerebrum (data not shown). The rank orders of the potencies were IRL-1620 > SB-209670 > BQ788 > L-749329 in all tissues.

Next, we studied the affinities of these ligands and endothelin isopeptides for [^{125}I]endothelin-3 binding in two types of intact cells, human Girardi heart cells and rat primary neonatal heart cells. Both [^{125}I]endothelin-3 bindings in human Girardi and rat neonatal heart cells were displaced by endothelin-1 or endothelin-3 with similar potency (Table 1), indicating that the bindings are of the endothelin ET_B-type. Each membrane preparation was also characteristic of the endothelin ET_B-type. [^{125}I]Endothelin-3 binding in the membrane preparation from human Girardi heart cells was displaced by endothelin ET_B receptor ligands with the same rank order of endothelin-1 = endothelin-3 > IRL-1620 > SB-209670 > BQ788 > L-749329, which was obtained with rat neonatal heart membranes (Table 1). Unexpectedly, however, the rank orders obtained with human and rat living cells were different from those obtained with the corresponding membrane preparations. Fig. 1 shows the correlation on affinities of these ligands for human Girardi heart cells and rat neonatal heart cells from two points of view. There was good correlation between human and rat ($r = 0.861$, $P < 0.001$), but less significant correlation between the living cells and their respective membrane fractions ($r = 0.569$, $0.02 < P < 0.05$).

Temperature dependency of [^{125}I]endothelin-3 binding was studied using human Girardi heart cells and their membranes. Bindings of 8.3 pM [^{125}I]endothelin-3 to the cells were 2.77 ± 0.03 (100%), 1.50 ± 0.05 (54%) and 0.18 ± 0.02 fmol/well (6%) at 37, 25 and 4°C, respectively ($n = 3$), whereas bindings of 25 pM [^{125}I]endothelin-3 to the membranes were 8.55 ± 0.13 (100%), 6.98 ± 0.13 (82%) and 1.08 ± 0.21 fmol/tube (13%) at 37, 25 and 4°C, respectively ($n = 3$).

4. Discussion

The affinities of six ligands for endothelin ET_B receptors in living cells and their membrane preparations were examined using human Girardi heart cells and rat neonatal heart cells. The affinities for human and rat receptors showed a good correlation when compared between the living cells or between the membrane preparations, but there was less significant correlation between the living cells and their respective membrane preparations. For example, IRL-1620 showed higher affinity for membranes than for cells, while BQ788, SB-209670 and L-749329 showed lower affinities for membrane preparations than for cells. The difference in affinity varied with the antagonists (L-749329 > BQ788 > SB-209670). Affinities of peptide ligands or agonists were not necessarily higher for membranes because endothelin-1 and endothelin-3 showed lower affinity for the membrane fractions than for living cells (Table 1).

We found no difference between rat and human endothelin ET_B receptors, inconsistent with some previous studies (Tanaka et al., 1995; Reynolds et al., 1995; Yang et al., 1997). We cannot precisely explain the reason for this discrepancy at present, but one possibility is that it may depend upon antagonists used. Some antagonists, like RES-701-1 (Tanaka et al., 1995) and PD147452 (Reynolds et al., 1995), might be able to discriminate between rat and human endothelin ET_B receptor binding sites. Another possibility is that a sub-population of endothelin ET_B receptors we failed to detect under our experimental condition might give rise to species difference.

What causes the difference between bindings to living cells and to their membranes? Recent studies suggest that the conformation of receptors in cell membranes is very flexible and can change from one state to another (Kenakin, 1995). Not every receptor ligand binds to the same conformational state. Antagonists are believed to bind in a different way from agonists. Destruction of the intact cell membrane structure is thought to have a great effect on the proportion of various conformational states of the receptor. [^{125}I]endothelin-3 binding decreased as the temperature lowered, and the decrease occurred more markedly with living cells. The difference in temperature dependence between living cells and their membranes suggests that the disintegration of the membrane structure exerted a profound effect on the property of endothelin ET_B receptors. This might be related to the difference in affinity of receptor ligands between living cells and their membrane preparations.

Another explanation is due to the agonist-induced internalization of receptors in intact cells. However, this may not be the case because if the agonist IRL-1620 induces internalization of receptors, its affinity should be higher for living cells than for their membrane preparations. Furthermore, antagonists should show the same affinity for living cells and their membrane preparations since antagonists cannot induce receptor internalization.

In this study, we could not find any species or tissue difference, but found that the binding properties of the endothelin ET_B receptor changed on destruction of the

intact cell membrane structure. Thus, when discussing the existence of a subclass of endothelin ET_B receptors, the receptor preparation should be taken into consideration.

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