



UNIVERSITÀ  
DEGLI STUDI  
FIRENZE

## FLORE

# Repository istituzionale dell'Università degli Studi di Firenze

### **Characterization of endothelin-1 receptor subtypes in isolated human cardiomyocytes.**

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

Characterization of endothelin-1 receptor subtypes in isolated human cardiomyocytes / P.A. MODESTI; VANNI S; PANICCIA R; BANDINELLI B; BERTOLOZZI I; POLIDORI G; SANI G; NERI SERNERI GG. - In: JOURNAL OF CARDIOVASCULAR PHARMACOLOGY. - ISSN 0160-2446. - STAMPA. - 34:(1999), pp. 333-339. [10.1097/00005344-199909000-00003]

*Availability:*

The webpage <https://hdl.handle.net/2158/213800> of the repository was last updated on

*Published version:*

DOI: 10.1097/00005344-199909000-00003

*Terms of use:*

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

*Publisher copyright claim:*

La data sopra indicata si riferisce all'ultimo aggiornamento della scheda del Repository FloRe - The above-mentioned date refers to the last update of the record in the Institutional Repository FloRe

(Article begins on next page)

## Characterization of Endothelin-1 Receptor Subtypes in Isolated Human Cardiomyocytes

Pietro Amedeo Modesti, Simone Vanni, Rita Paniccia, Brunella Bandinelli, Iacopo Bertolozzi, Gianluca Polidori, \*Guido Sani, and Gian Gastone Neri Serneri

*Clinica Medica e Cardiologia, University of Florence, Florence, and \*Department of Cardiosurgery, University of Cagliari, Cagliari, Italy*

**Summary:** On cardiac membranes and isolated cardiomyocytes from the human heart, cell-type distribution and functional activities of endothelin-1 (ET-1) receptor subtypes were investigated by using binding methods and messenger RNA (mRNA) in situ hybridization. The ET-receptor antagonist BMS-182874 selectively and competitively inhibits ET<sub>A</sub> receptors both on isolated myocytes and ventricular membranes with ~1,300 times greater affinity for ET<sub>A</sub> than ET<sub>B</sub> subtypes. The [<sup>125</sup>I]-ET-1 specific binding revealed  $42.851 \pm 2.546$  receptors/myocyte with a prevalent proportion of ET<sub>A</sub>-receptor subtypes on both myocytes (84 ± 2%) and ventricular membranes (66 ±

3%). In situ hybridization studies revealed that mRNA for ET<sub>A</sub> receptors was expressed on both myocytes and nonmyocyte cells, whereas mRNA for ET<sub>B</sub> receptors was almost exclusively expressed on fibroblasts and endothelial cells. Specific binding of [<sup>125</sup>I]-ET-1 to both myocytes and ventricular membranes in the presence of specific ET<sub>A</sub> (BMS-182874) and ET<sub>B</sub> (BQ-788)-receptor antagonists showed a displacement of [<sup>125</sup>I]-ET-1 by unlabeled ET-1, which were significantly faster from ET<sub>B</sub> than from ET<sub>A</sub>. This suggests a clearance function of ventricular ET<sub>B</sub> receptors. **Key Words:** Endothelin—Myocyte—Receptors—Growth factors—Receptor antagonists.

Endothelin-1 (ET-1) is a peptide that provides numerous biologic actions including potent and long-lasting vasoconstriction (1), and potent positive inotropic effect both in vivo (2) and on isolated cardiomyocytes (3,4). Cardiomyocytes express messenger RNA (mRNA) for pre-proET-1 (5,6), a precursor of ET-1, which in turn acts in the heart as an autocrine factor able to induce hypertrophy of myocytes (7) and proliferation of fibroblasts (8). Recent studies demonstrated that ET-1 is involved in pressure overload-induced hypertrophy (9,10). Cardiac activities of ET-1 are mediated by two ET-1-receptor subtypes, ET<sub>A</sub> and ET<sub>B</sub>, both represented in human myocardium (11) with an average proportion in the left ventricle of 60:40 (ET<sub>A</sub>/ET<sub>B</sub>) (12). Myocytes almost exclusively express the ET<sub>A</sub>-receptor subtype (>90%), whereas fibroblasts express both receptor subtypes (12-14). Due to the potential pathophysiologic and clinical importance of an increased ET-1 formation (15), a number of both nonselective and selective ET-1-receptor antagonists have been synthesized (16,17). Experimental studies have shown that short- or long-term administration of nonselective or selective ET-1-receptor antagonists can result in a favorable effect (18). BMS-182874 [5-(dimethylamino)-N-(3,4-dimethyl-5-isoxazolyl)-1-naphthalene sulfonamide] is an orally active, low-

molecular-weight, nonpeptidic, ET<sub>A</sub> receptor-selective antagonist (19). Although BMS-182874 has been found to be effective in a number of experimental settings (19-21), its capability to inhibit cardiac and human ET<sub>A</sub> receptors has been assessed only in cardiac rat membranes and in rat and Chinese hamster ovary cell lines transfected with the human complementary DNAs (cDNAs) for ET<sub>A</sub> and ET<sub>B</sub> receptors (21,22). No study has been performed on human myocytes. Discrepancies have been reported in the binding affinities for other ET-1-receptor antagonists (BQ 3020) among human, rat, and pig hearts (23). In addition, recombinant rat and human ET<sub>B</sub> receptors have shown different affinities when competing against several peptidic and nonpeptidic antagonists (24). Thus the extrapolation of data derived from animal studies to human subjects may not be correct (23). Moreover, the use of myocardial homogenates that contain both myocytes and a variety of other cell types does not allow to distinguishing the functional characteristics of ET<sub>A</sub> and ET<sub>B</sub> receptors expressed on myocytes (prevalently ET<sub>A</sub>) from those of the receptors expressed on myocardial interstitial cells (both ET<sub>A</sub> and ET<sub>B</sub>). This differentiation may be important, as several experimental studies have raised the possibility that ET<sub>A</sub> receptors mediate myocardial and coronary activities, whereas ET<sub>B</sub> recep-

Received August 17, 1998; revision accepted March 1, 1999.  
Address correspondence and reprint requests to Dr. P. Amedeo

Modesti at Clinica Medica I, University of Florence, Viale Morgagni 85, 50134 Florence, Italy. E-mail: pa.modesti@dfc.unifi.it

tors act in the local clearance of ET-1 (25–27). This possibility suggests a different kinetics of [ $^{125}$ I]-ET-1 binding to ET<sub>A</sub>- and ET<sub>B</sub>-receptor subtypes. Therefore this study was planned to investigate the ET-1-binding inhibition of BMS-182874 in both isolated ventricular myocytes and cardiac membranes from the human heart and to analyze the kinetics of [ $^{125}$ I]-ET-1 binding to ET<sub>A</sub> and ET<sub>B</sub> receptors on both isolated human myocytes and human cardiac membranes.

## METHODS

### Tissue procurement

The characteristics of ET-1 binding and the inhibitory properties of BMS-182874 were investigated on isolated heart membranes and cardiomyocytes obtained from four nonfailing organ donors with no history of cardiac disease who were not taking any drugs. These donors were initially considered for cardiac transplantation but subsequently were deemed unsuitable for transplantation either because of age or size incompatibility with the recipient.

### Membrane isolation and cell separation

**Membrane isolation.** Three to five grams of left ventricular cardiac free wall were homogenized in an ice-cold buffer (20 mM NaHCO<sub>3</sub>, 0.1 mM phenyl-methyl-sulphonyl-fluoride, pH 7.4), and centrifuged at 1,500 g for 15 min at 4°C. Supernatant was then centrifuged at 48,000 g for 15 min at 4°C. The pellet was resuspended in ice-cold buffer (50 mM Tris-HCl, 1 mM EDTA, 0.1 mM phenyl-methyl-sulphonyl-fluoride, pH 7.4) and recentrifuged at 48,000 g for 15 min. This procedure was repeated once. Protein concentration was assessed according to Bradford (28). 5'-Nucleotidase assay (Sigma Chemicals, St. Louis, MO, U.S.A.) in the final fraction showed an enrichment of at least fourfold compared with those of the 1,500 g supernatant.

**Cardiomyocyte isolation.** The selected coronary artery was cannulated and perfused with a calcium-free buffer (minimal essential medium, MEM) Eagle Joklik (Sigma Chemicals) with 21 mM HEPES, 4.4 mM NaHCO<sub>3</sub>, 1.5 mM KH<sub>2</sub>CO<sub>3</sub>, 1.7 mM MgCl<sub>2</sub>, 11.7 mM glucose, 2 mM L-glutamine, 21 U/ml insulin (pH 7.2; HEPES-MEM buffer) gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 32°C for 10 min (blood washout). Then collagenase perfusion was carried out at 32°C with HEPES-MEM buffer gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> and Worthington-type II collagenase, 100 U/ml (20 ml/min). The collagenase-perfused tissue was then minced and shaken in a resuspension buffer (HEPES-MEM buffer supplemented with bovine serum albumin 0.5%, 0.3 mM CaCl<sub>2</sub>, 10 mM taurine) and Worthington type II collagenase, 100 U/ml, for 30 min at 37°C. After centrifugation for 4 min at 35 g, myocytes were enriched by centrifuging the resuspended pellet through Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). Cell purity (>99% cardiomyocytes) was assessed by using anti-human myosin monoclonal antibodies (M8421; Sigma Chemicals).

### Binding studies

**Kinetic analysis.** The kinetics of association of [ $^{125}$ I]-ET-1 (100 pM, 2,000 Ci/mmol, Amersham, Buckinghamshire, U.K.) to cardiac membranes (300 µg/ml) or isolated cardiomyocytes (10<sup>5</sup> cells/ml) was evaluated at selected times (30 s to 240 min), at 22°C. Nonspecific binding was obtained by adding unlabeled 1 µM ET-1 2 h before the addition of [ $^{125}$ I]-ET-1. The content was then rapidly filtered through Whatman GF/C filters (What-

man International Ltd., Maidstone, U.K.) presoaked with polyethylene glycol (PEG, 6.6%). The kinetics of dissociation was evaluated by adding unlabeled ET-1 (1 µM, final concentration (fc)) to the reaction mixture after 120 min of incubation. The kinetics of [ $^{125}$ I]-ET-1 binding to ET<sub>A</sub> or ET<sub>B</sub> subtypes was analyzed by using cardiac membranes or cardiomyocytes preincubated for 4 h with selective ET<sub>B</sub> (BQ-788, 10 nM) or ET<sub>A</sub> (BMS-182874, 1 µM) antagonists, respectively. Kinetic constants ( $K_{on}$ ,  $K_{off}$ , and  $K_d$ ) were calculated according to Weiland and Molinoff (29).

**Equilibrium studies and identification of endothelin-receptor subtypes.** In equilibrium binding studies, cell membranes (300 µg/ml) or isolated cardiomyocytes (10<sup>5</sup> cells/ml) were incubated with [ $^{125}$ I]-ET-1, 100 pM, and increasing concentrations of displacer, unlabeled ET-1 (0–1 µM) or BMS-182874 (0–1 mM) and BQ-788 (0–100 µM), for 120 min at 22°C in a final volume of 0.2 ml in the same experimental conditions as described earlier. Data were analyzed according to Scatchard (30) and Cheng and Prusoff (31). Competition binding data were analyzed by iterative curve fitting to a one- or two-site binding model by using a nonlinear-fitting computer program (LIGAND) (32) to obtain the final estimation of  $K_d$  for ET-1,  $K_i$  for BMS-182874 and BQ-788, and the receptor density ( $B_{max}$ ) values. To assess whether BMS-182874 is a competitive antagonist of ET<sub>A</sub> receptors, [ $^{125}$ I]-ET-1 binding experiments were performed in the presence of three fixed concentrations of BMS-182874 (0, 50, and 100 nM).

### In situ hybridization studies

The cDNA probes for ET<sub>A</sub>- and ET<sub>B</sub>-receptor subtypes were prepared from the phage clones of human endothelin receptors (ET<sub>A</sub>: American Type Culture Collection, Rockville, MD, U.S.A.: ATCC 105194 and ET<sub>B</sub>: ATCC 1250426). In situ hybridization studies were performed as previously described (33). Positive controls were obtained by using a cDNA probe for GAPDH (ATCC no. 57090). Myocytes were stained by using a specific anti-human myosin antibody (M8421; Sigma) and a secondary fluorescein-conjugated antibody (F4143; Sigma).

The specificity of the in situ hybridization signals was searched by testing the sections with hybridization mixture (a) without the probe, and (b) after incubation with RNAase A (1 Kunitz unit/L) for 1 h at 37°C before hybridization.

### Statistical analysis

Each single experiment was performed in triplicate. If not otherwise indicated, all data given in the text are expressed as mean ± S.D.

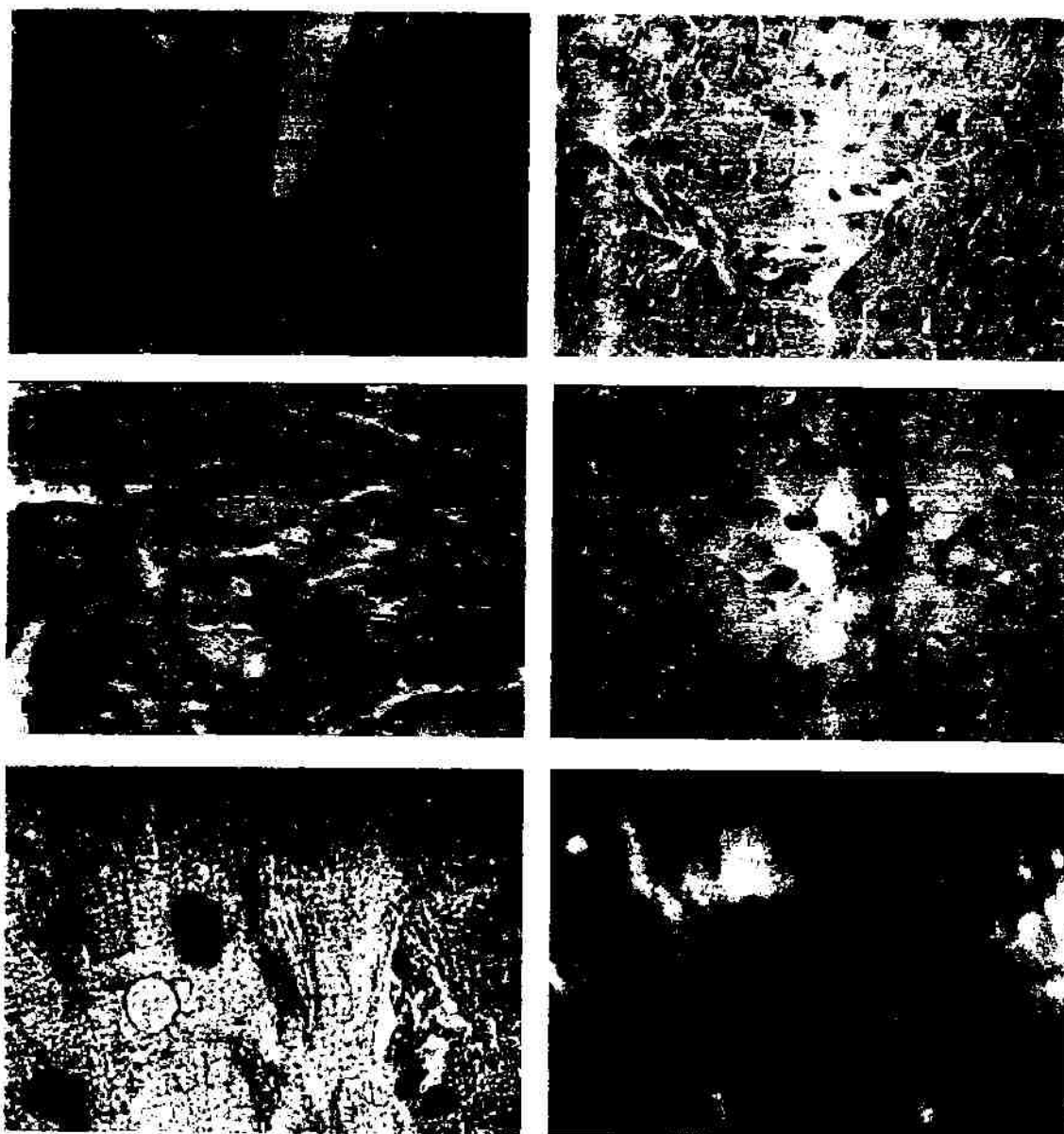
## RESULTS

### In situ hybridization studies

Negative and positive controls showed that the hybridization in left ventricular tissue was specific for mRNA and that the mRNA was intact (Fig. 1B and A). mRNA for ET<sub>A</sub> receptors in human left ventricle was expressed in both the myocytes and nonmyocyte cells (Fig. 1C). In contrast, mRNA for ET<sub>B</sub> receptors was almost exclusively expressed in nonmyocyte cells (fibroblasts and endothelial cells) but not in myocytes (Fig. 1D–F).

### Competition binding studies at equilibrium

The [ $^{125}$ I]-ET-1 specific binding to heart membranes reached saturation at ~1 nM with a  $B_{max}$  of 183 ± 19



**FIG. 1.** In situ hybridization for GAPDH messenger RNA (mRNA) (A), ET<sub>A</sub> (C), and ET<sub>B</sub> (D, E) in left ventricular sections from human donors. A: Positive GAPDH mRNA signals in both myocytes and interstitial cells, and the same section after RNAase treatment (x400 magnification) (B). C: Positive ET<sub>A</sub> mRNA signals in both myocytes and nonmyocytes (x400). D: Positive ET<sub>B</sub> mRNA signals in interstitial cells at x400 (D) and x1,000 (E). In F, the same section stained for human myosin at fluorescent light (x1,000).

fmol/mg protein and a  $K_D$  of  $0.36 \pm 0.09$  nM (Table 1). Unlabeled ET-1 displaced the [<sup>125</sup>I]-ET-1 binding with a linear pattern of the Hill plot, indicating that ET-1 does not discriminate between receptor subtypes. Conversely, BMS-182874 (0–1 mM) inhibited the [<sup>125</sup>I]-ET-1 specific binding to isolated membranes with a biphasic pattern (Fig. 2; Table 1) and a 1260 times greater affinity for ET<sub>A</sub> than for the ET<sub>B</sub> subtype (Table 1). The calculated average proportions of ET<sub>A</sub> and ET<sub>B</sub> receptors were  $66 \pm 3\%$  and  $34 \pm 2\%$  (Table 1).

The selective ET<sub>B</sub> antagonist (BQ-788; 0–100 μM) showed also a biphasic pattern of inhibition of the [<sup>125</sup>I]-

ET-1 specific binding to isolated membranes confirming the presence of a balanced proportion of ET<sub>A</sub> ( $59 \pm 2\%$ ) and ET<sub>B</sub> receptors ( $41 \pm 2\%$ ) in isolated membranes (Table 1).

The [<sup>125</sup>I]-ET-1 specific binding to isolated cardiomyocytes showed the presence of  $42,851 \pm 2,546$  receptors/myocyte (Table 1). Unlabeled ET-1 displaced [<sup>125</sup>I]-ET-1 specific binding with a linear pattern.

BMS-182874 inhibited the [<sup>125</sup>I]-ET-1 specific binding to isolated cardiomyocytes with a nonlinear pattern of inhibition (Fig. 3; Table 1), indicating that both receptor subtypes are represented in cardiomyocytes, al-

**TABLE 1.** Competition studies of [<sup>125</sup>I]-ET-1 binding to human isolated heart membranes and cardiomyocytes by unlabeled ET-1 and selective ET<sub>A</sub> (BMS-182874) or ET<sub>B</sub> (BQ-788) receptor antagonists

	ET-1	BMS-182874	BQ-788
<b>Cardiac membranes</b>			
B <sub>max</sub> (fmol/mg)	183 ± 19	—	—
K <sub>D</sub> (nM)	0.36 ± 0.09	—	—
n <sub>H</sub>	0.90	0.44	0.43
ET <sub>A</sub> /ET <sub>B</sub> (%)	—	66 ± 3/34 ± 2	59 ± 2/41 ± 2
ET <sub>A</sub> B <sub>max</sub> (fmol/mg)	—	119 ± 10	126 ± 8
K <sub>i</sub>	—	57 ± 4.3 nM	266 ± 73 nM
ET <sub>B</sub> B <sub>max</sub> (fmol/mg)	—	64 ± 8	56 ± 9
K <sub>i</sub>	—	72 ± 12 μM	0.96 ± 0.16 nM
<b>Cardiomyocytes</b>			
B <sub>max</sub> (fmol/mg)	43 ± 7	—	—
K <sub>D</sub> (nM)	0.34 ± 0.05	—	—
n <sub>H</sub>	0.97	0.57	0.49
ET <sub>A</sub> /ET <sub>B</sub> (%)	—	84 ± 2/16 ± 2	86 ± 3/14 ± 2
ET <sub>A</sub> B <sub>max</sub> (fmol/mg)	—	35 ± 1	37 ± 2
K <sub>i</sub>	—	73 ± 4.3	318 ± 81 nM
ET <sub>B</sub> B <sub>max</sub> (fmol/mg)	—	7 ± 1	6 ± 1
K <sub>i</sub>	—	95 ± 32.1 μM	0.60 ± 0.37 nM

ET, endothelin; B<sub>max</sub>, maximal binding; K<sub>D</sub>, equilibrium dissociation constant; n<sub>H</sub>, Hill coefficient; K<sub>i</sub>, inhibitory constant.

though ET<sub>A</sub> is prevalent (84 ± 2%). Displacement studies of [<sup>125</sup>I]-ET-1 specific binding to cardiomyocytes by unlabeled ET-1, performed in the absence and in the presence of increasing concentrations of BMS-182874 (0, 50, and 100 nM), showed a progressive increase in the K<sub>D</sub> values of [<sup>125</sup>I]-ET-1 from 0.28 to 0.39 and 0.45 nM, respectively, whereas the B<sub>max</sub> remained unchanged (39, 42, and 43 fmol/mg protein, respectively; Fig. 4), indicating a competitive interaction between BMS-182874 and [<sup>125</sup>I]-ET-1 for binding to receptor sites.

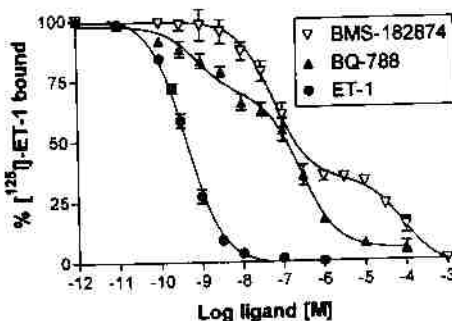
BQ-788 caused a lower inhibition of [<sup>125</sup>I]-ET-1 specific binding than BMS-182874 (Fig. 3; Table 1) indicating the presence of a low number (14 ± 2%) of ET<sub>B</sub> receptors on isolated cardiomyocytes.

#### Kinetic studies

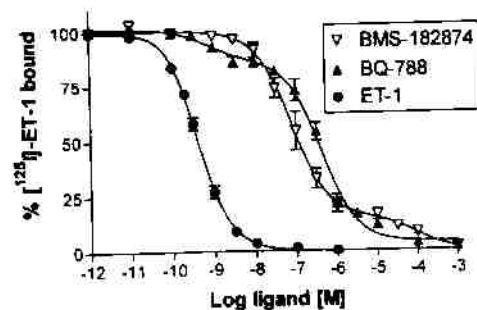
Specific binding of [<sup>125</sup>I]-ET-1 to heart membranes reached steady-state by 120 min (Fig. 5). The binding was only partially displaceable by unlabeled ET-1 (21% after 2 h) with a half-life of dissociation of 21 min (Table

2). When ET<sub>A</sub> receptors were blocked by preincubation with BMS-182874, specific binding of [<sup>125</sup>I]-ET-1 was 35% of maximal binding, and the [<sup>125</sup>I]-ET-1 displacement was more rapid with a half-life of dissociation of 13 min. In the presence of ET<sub>B</sub>-receptor blockade, specific binding was 65% of maximal binding and the half-life of dissociation increased to 28 min (Table 2), thus indicating that [<sup>125</sup>I]-ET-1 binds more tightly to ET<sub>A</sub> than to ET<sub>B</sub> receptors.

The [<sup>125</sup>I]-ET-1 specific binding to isolated cardiomyocytes reached a steady state after 120 min (Fig. 6) and was barely displaced by the addition of a large amount of unlabeled ET-1 (14% after 2 h with half-life of 20 min). The addition of BMS-182874 significantly decreased the [<sup>125</sup>I]-ET-1 specific binding to 15% of the maximal binding and caused a more rapid dissociation of [<sup>125</sup>I]-ET-1 (half-life of 15 min; Fig. 6; Table 2). On the contrary, when ET<sub>B</sub> receptors were blocked by preincubation with BQ-788 [<sup>125</sup>I]-ET-1, the specific binding was reduced to 80% of maximal binding, and dissociation of



**FIG. 2.** Displacement of [<sup>125</sup>I]-endothelin-1 specific binding by endothelin-1, BMS-182874, and BQ-788 from isolated cardiac membranes. Each point represents the mean ± SD.



**FIG. 3.** Displacement of [<sup>125</sup>I]-endothelin-1 specific binding by endothelin-1, BMS-182874, and BQ-788 from isolated cardiomyocytes. Each point represents the mean ± SD.

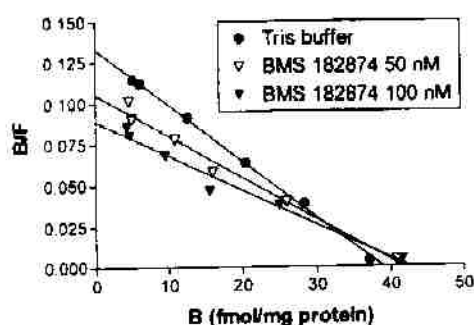


FIG. 4. Scatchard plot of [ $^{125}$ I]-endothelin-1 specific binding to isolated myocytes in the absence or presence of 50 or 100 nM BMS-182874. Each point represents the mean  $\pm$  SD.

[ $^{125}$ I]-ET-1 became slower, with a half-life of 36 min (Table 2).

## DISCUSSION

These results indicate that (a) BMS-182874 is able to antagonize [ $^{125}$ I]-ET-1 binding to ET<sub>A</sub> receptors on human cardiomyocytes; (b) in left ventricular human myocytes, ET<sub>A</sub> receptors are largely prevalent in respect to ET<sub>B</sub> subtypes; and (c) the two receptor subtypes are characterized by a different ET-1-binding tightness. BMS-182874 binds to ET<sub>A</sub> receptors on human myocytes with high selectivity because the absolute difference in the affinity for the two receptor subtypes is of three orders of magnitude (1,300 times), resulting in a clear differentiation between ET<sub>A</sub> and ET<sub>B</sub> receptors. The characteristics of [ $^{125}$ I]-ET-1 displacement in the absence and in the presence of different concentrations of BMS-182874 are consistent with a competitive interaction at the ET-1 binding site. The high selectivity of BMS-182874 for cardiac human myocyte ET<sub>A</sub> receptors makes BMS-182874 suitable for defining the presence and relative proportion of the two ET-1-receptor subtypes expressed on human heart myocytes. In human left ventricular myocytes, BMS-182874 reveals a 84:16 ET<sub>A</sub>/ET<sub>B</sub> receptor ratio. This high prevalence of ET<sub>A</sub> receptors on myocytes is confirmed also by *in situ* hybridization studies, which clearly showed a positive signal of mRNA of ET<sub>A</sub> but not of mRNA for ET<sub>B</sub> receptors. Also on human heart membranes, BMS-182874 binds to ET<sub>A</sub> receptors with high selectivity (~1,260 times greater for the ET<sub>A</sub> than the ET<sub>B</sub> subtype). The selectivity of BMS-182874 for human membrane ET<sub>A</sub> receptors is in the same order of magnitude as those reported for peptidic, nonorally active ET<sub>A</sub>-receptor antagonists such as BQ-123 (12) and FR-139317 (23). The affinity of BMS-182874 for human ET<sub>A</sub> subtypes is fourfold lower ( $K_i = 57$  nM) than that previously reported for rat heart membranes ( $K_i = 227$  nM) (21). Similar discrepancies regarding the affinity of ET<sub>A</sub>-receptor blocking agents between human and rat heart membranes have been reported by using BQ-123 (an ET<sub>A</sub>-selective antagonist)

(23,34). Minor discrepancies also were found for the affinity of the peptidic ET<sub>B</sub> antagonist used in our study (BQ-788), in comparison with the values previously reported in rats (35). These human-rat binding differences may be explained by the 7-9% and 12% species difference in the primary sequences of ET<sub>A</sub> and ET<sub>B</sub> receptors, respectively (34,36,37).

The use of selective ET antagonists in time-course experiments allowed us to give an accurate estimate of binding characteristics of the two ET-receptor subtypes. Kinetic studies demonstrated that [ $^{125}$ I]-ET-1 binds more tightly to the ET<sub>A</sub> receptor than to the ET<sub>B</sub> subtype. The half-life for dissociation of ET-1 from cardiac membranes when ET<sub>A</sub> receptors were blocked was significantly shorter than that calculated in the presence of ET<sub>B</sub> inhibition. It seems unlikely that the different kinetics for ET<sub>B</sub> and ET<sub>A</sub> receptors may be due to the interference between nonpeptidic ET<sub>A</sub> (BMS-182874) and peptidic ET<sub>B</sub> (BQ-788) antagonists at receptor sites, because the concentrations of selective antagonists used in kinetic studies were far from inhibiting the other receptor class, as shown by competition studies.

Furthermore, mutational studies of both ET<sub>A</sub> and ET<sub>B</sub> receptors revealed that the sites required for antagonist binding and agonist-induced signal transduction are shared by both peptidic and nonpeptidic compounds (38-41). In particular, high-affinity binding of chemically distinct peptidic and nonpeptidic ET<sub>A</sub> antagonists is largely dependent on the residue at position 129, because high-affinity binding of BQ-123, SB-209670, bosentan, and BMS-182874 is not retained by Tyr-129 mutants (38,40,41). Conversely, binding of both peptidic and nonpeptidic ET<sub>B</sub>-receptor antagonists is dependent on Lys-182 on the ET<sub>B</sub> receptor sequence (39). Therefore no cross-interference seems to exist between both peptidic and nonpeptidic ET<sub>A</sub> and ET<sub>B</sub> antagonists.

The peculiar kinetic pattern of interaction of ET-1 at the two receptor subtypes observed in this study may account for the role played by ET<sub>B</sub> receptors in the cardiac clearance of ET-1, because the faster kinetics of ET<sub>B</sub> than ET<sub>A</sub> may cause a preferential ET-1 association or dissociation to or from ET<sub>B</sub> subtype receptors. Thus

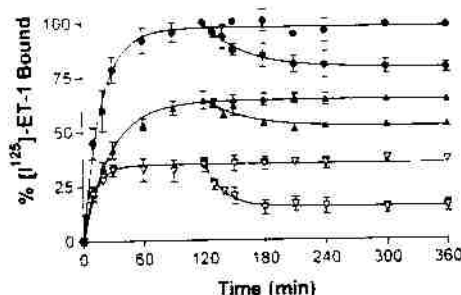


FIG. 5. Kinetic analysis of the [ $^{125}$ I]-endothelin-1 specific binding to isolated cardiac membranes in the absence (solid circles) and presence of BMS-182874, 1  $\mu$ M (open triangles), or BQ-788, 10 nM (solid triangles). Each point represents the mean  $\pm$  SD.

**TABLE 2.** Kinetic analysis of [<sup>125</sup>I]-ET-1 binding to human isolated heart membranes and cardiomyocytes in the absence and in the presence of selective ET receptor antagonists

	Buffer	BMS 182874	BQ-788
<b>Cardiac membranes</b>			
K <sub>obs</sub> (min)	0.046 ± 0.004	0.089 ± 0.016	0.036 ± 0.003
K <sub>-1</sub> (min)	0.030 ± 0.007	0.052 ± 0.013	0.026 ± 0.011
K <sub>1</sub> (nM/min)	0.163 ± 0.057	0.367 ± 0.09	0.104 ± 0.03
K <sub>D</sub> (nM)	0.183 ± 0.064	0.142 ± 0.025	0.25 ± 0.051
t <sub>1/2</sub> association (min)	14	8	19
t <sub>1/2</sub> dissociation (min)	21	13	28
<b>Cardiomyocytes</b>			
K <sub>obs</sub> (min)	0.051 ± 0.002	0.077 ± 0.021	0.028 ± 0.002
K <sub>-1</sub> (min)	0.034 ± 0.001	0.046 ± 0.023	0.019 ± 0.008
K <sub>1</sub> (nM/min)	0.159 ± 0.025	0.309 ± 0.082	0.089 ± 0.011
K <sub>D</sub> (nM)	0.217 ± 0.033	0.149 ± 0.086	0.215 ± 0.021
t <sub>1/2</sub> association (min)	14	9	25
t <sub>1/2</sub> dissociation (min)	20	15	36

ET, endothelin; K<sub>obs</sub>, observation constant; K<sub>-1</sub>, kinetic constant for dissociation; K<sub>1</sub>, kinetic constant for association; K<sub>D</sub>, equilibrium dissociation constant; t<sub>1/2</sub> association, half time of association; t<sub>1/2</sub> dissociation, half time of dissociation

this receptor subtype might act as a clearance receptor. Recent studies both in isolated rat hearts (27) and in humans (42) showed that infusion of ET<sub>B</sub> (BQ-788), but not of ET<sub>A</sub>-selective antagonists (PD-155080 or BQ-123), caused an increase in ET-1 plasma concentrations. These results indirectly support the hypothesis that ET<sub>B</sub> receptors act as clearance or buffer receptors for ET-1. The slower dissociation of ET-1 from ET<sub>A</sub> than from ET<sub>B</sub> receptor may have a functional consequence, because ET<sub>A</sub> is the receptor subtype that prevalently mediates the cellular effects of ET-1 (43,44). The in situ hybridization studies showed that mRNA for ET<sub>A</sub> receptor was expressed in both cardiomyocytes and interstitial cells, whereas mRNA for ET<sub>B</sub> receptor was expressed almost exclusively in the interstitial cells. As a consequence, in conditions of an increased ET-1 local concentration, as occurs in heart failure, the biologic effects of ET-1 especially target the myocytes, which are void of ET<sub>B</sub>-subtype receptors.

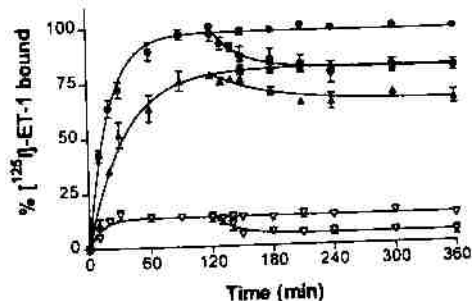
In conclusion, BMS-182874 antagonizes with high selectivity ET<sub>A</sub>-receptor subtypes on both human isolated myocytes and ventricular membranes. The different

binding tightness of ET-1 to ET<sub>A</sub> versus ET<sub>B</sub> ventricular receptor subtypes, revealed by specific antagonists, supports the hypothesis that ET<sub>B</sub> plays a main role in the cardiac local clearance of ET-1.

**Acknowledgment:** The financial support of Telethon-Italy (grant 864), and of the Ministero dell'Università e della Ricerca Scientifica (cofinanziamento MURST 1998) are gratefully acknowledged. We thank Dr. G.B. Leproux for providing BMS-182874 and Jane R. Wertheimer for the careful editing of the manuscript.

## REFERENCES

1. Yanagisawa M, Kurihara H, Kimura S, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;341:1-5.
2. Kitayoshi T, Watanabe T, Shimamoto N. Cardiovascular effects of endothelin in dogs: positive inotropic action in vivo. *Eur J Pharmacol* 1989;166:519-22.
3. Jones L, Rozick J, Tsutsui H, Cooper G IV. Endothelin stimulates multiple responses in isolated ventricular cardiac myocytes. *Am J Physiol* 1992;263:H1447-54.
4. Qiu Z, Wang J, Perreault CL, Meuse AJ, Grossman W, Morgan JP. Effects of endothelin on intracellular Ca<sup>2+</sup> and contractility in singular ventricular myocytes from the ferret and human. *Eur J Pharmacol* 1992;214:293-6.
5. Giaid A, Saleh D, Yanagisawa M, Clarke Forbes RD. Endothelin immunoreactivity and mRNA in the transplanted human heart. *Transplantation* 1995;59:1308-13.
6. Plimpton C, Ashby MJ, Kuc RE, O'Reilly G, Davenport AP. Expression of endothelin peptides and mRNA in the human heart. *Clin Sci* 1996;90:37-46.
7. Ito H, Hirata Y, Miroe M, et al. ET-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ Res* 1991;69:209-15.
8. Takuwa N, Takuwa Y, Yanagisawa M, Yamashita K, Masaki T. A novel vasoactive peptide stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblast. *J Biol Chem* 1989;264:7856-61.
9. Arai M, Yoguchi A, Iso T, et al. Endothelin-1 and its binding sites are upregulated in pressure overload cardiac hypertrophy. *Am J Physiol* 1995;268:H2084-91.
10. Yamazaki T, Komuro I, Kudoh S, et al. Endothelin-1 is involved



**FIG. 6.** Kinetic analysis of the [<sup>125</sup>I]-endothelin-1 specific binding to isolated cardiomyocytes in the absence (solid circles) and presence of BMS-182874, 1 μM (open triangles), or BQ-788, 1 nM (solid triangles). Each point represents the mean ± SD.

- in mechanical stress-induced cardiomyocyte hypertrophy. *J Biol Chem* 1996;271:3221-8.
11. Hosoda K, Nakao K, Hiroshi-Arai, et al. Cloning and expression of human endothelin-1 receptor cDNA. *FEBS Lett* 1991;287:23-6.
  12. Molenaar P, O'Reilly G, Sharkey A, et al. Characterization and localisation of endothelin receptor subtypes in the human atrioventricular conducting system and myocardium. *Circ Res* 1993;72:526-38.
  13. Fareh J, Toujz RM, Shiffrin EL, Thibault G. Endothelin I and angiotensin II receptors in cells from rat hypertrophied heart. *Circ Res* 1996;78:302-11.
  14. Thomas PB, Liu ECK, Webb ML, Mukherjee R, Spinale FG. Evidence of the endothelin-1 autocrine loop in cardiac myocytes: relation to contractile function with congestive heart failure. *Am J Physiol* 1996;40:H2629-73.
  15. Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *J Pharmacol Exp Ther* 1994;46:325-415.
  16. Moreland S. Endothelin receptor antagonists: a brief review. *Can J Physiol Pharmacol* 1994;72:1469-71.
  17. Ohlstein EH, Elliott JD, Feuerstein GZ, Ruffolo RR Jr. Endothelin receptors: receptor classification, novel receptor antagonists, and potential therapeutic targets. *Med Res Rev* 1996;16:365-90.
  18. Yazaki Y, Yamazaki T. Reversing congestive heart failure with endothelin receptor antagonists. *Circulation* 1997;95:1752-4.
  19. Stein PD, Hunt JT, Floyd DM, et al. The discovery of sulfonamide endothelin antagonist and the development of the orally active ET<sub>A</sub> antagonist 5-(dimethylamino)-N-(3,4-dimethyl-5-isoxazolyl)-1-naphthalene sulfonamide. *J Med Chem* 1994;37:329-31.
  20. Thabes JS, Lefferts PL, Lu WX, Snaffer JR. An endothelin-A receptor antagonist (BMS-182874) attenuates endotoxin-induced lung dysfunction in chronically instrumented awake sheep. *Am J Respir Crit Care Med* 1994;149:A470.
  21. Webb ML, Bird JE, Liu ECK, et al. BMS-182874 is a selective, nonpeptide endothelin ET<sub>A</sub> receptor antagonist. *J Pharmacol Exp Ther* 1995;272:1124-34.
  22. Buchan KW, Alldus C, Christodoulou C, et al. Characterization of three non-peptide endothelin receptor ligands using human cloned ET<sub>A</sub> and ET<sub>B</sub> receptors. *Br J Pharmacol* 1994;112:1251-7.
  23. Peter MG, Davenport AP. Characterization of the endothelin receptor selective agonist, BQ3020 and antagonists BQ123, FR139317, BQ-788, 50235, Ro-462005 and bosentan in the heart. *Br J Pharmacol* 1996;117:455-62.
  24. Reynolds EE, Hwang O, Flynn MA, et al. Pharmacological differences between rat and human endothelin B receptors. *Biochem Biophys Res Commun* 1995;209:506-12.
  25. Fukunoda T, Fujikawa T, Ozaki S, Ishikawa K, Yano M, Nishikibe M. Clearance of circulating endothelin-1 by ET<sub>B</sub> receptors in rats. *Biochem Biophys Res Commun* 1994;199:1461-5.
  26. Wang QD, Li NS, Lundberg JM, Pernow J. Protective effects of non-peptide endothelin receptor antagonist bosentan on myocardial ischaemic and reperfusion injury in the pig. *Cardiovasc Res* 1995;29:805-12.
  27. Brunner F, Doherty AM. Role of ET<sub>B</sub> receptors in local clearance of endothelin-1 in rat heart: studies with the antagonists PD 155080 and BQ-788. *FEBS Lett* 1996;396:238-42.
  28. Bradford AM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
  29. Weiland GA, Molinoff PB. Quantitative analysis of drug-receptor interactions: determination of kinetic and equilibrium properties. *Life Sci* 1981;29:313-30.
  30. Scatchard G. The attractions of proteins for small molecules and ions. *Ann N Y Acad Sci* 1949;51:660-72.
  31. Cheng YC, Prusoff WH. Relation between the inhibition constant (K<sub>i</sub>) and the concentration of inhibitor which causes 50% inhibition (IC<sub>50</sub>) of an enzyme reaction. *Biochem Pharmacol* 1973;22:3099-109.
  32. Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* 1980;107:220-39.
  33. Modesti PA, Cecioni I, Migliorini A, et al. Increased renal formation is associated with sodium retention and increased free water clearance. *Am J Physiol* 1998;275:H1070-7.
  34. Elshourbagy NA, Korman DR, Wu HL, et al. Molecular characterization and regulation of human endothelin receptors. *J Biol Chem* 1993;268:3873-9.
  35. Russel FD, Davenport AP. Characterization of the binding of ET<sub>B</sub> selective ligands in human and rat heart. *Br J Pharmacol* 1996;119:631-6.
  36. Adachi M, Yang YY, Furuchi Y, Miyamoto C. Cloning and characterization of cDNA encoding human A-type endothelin receptor. *Biochem Biophys Res Commun* 1991;180:1265-72.
  37. Ogawa Y, Nakao K, Arai H, et al. Molecular cloning of a non-isopeptide-selective human endothelin receptor. *Biochem Biophys Res Commun* 1991;178:248-55.
  38. Lee JA, Elliott JD, Sutiphong JA, et al. Tyr-129 is important to the peptide ligand affinity and selectivity of human endothelin type A receptor. *Proc Natl Acad Sci U S A* 1994;91:7164-8.
  39. Lee JA, Brinkmann JA, Longton ED, et al. Lysine 182 of endothelin B receptor modulates agonist selectivity and antagonist affinity: evidence for the overlap of peptide and non-peptide ligand binding sites. *Biochemistry* 1994;33:14543-9.
  40. Rose PM, Krystek SR, Patel PS, et al. Aspartate mutation distinguishes ET<sub>A</sub> but not ET<sub>B</sub> receptor subtype-selective ligand binding while abolishing phospholipase C activation in both receptors. *FEBS Lett* 1995;361:243-9.
  41. Webb ML, Pate PS, Rose PM, et al. Mutational analysis of the endothelin type A receptor (ET<sub>A</sub>) interactions and model of the selective ET<sub>A</sub> antagonist BMS-182874 with the putative ET<sub>A</sub> receptor binding cavity. *Biochemistry* 1996;35:2548-56.
  42. Cowburn PJ, Cleland JGF, Mc Donagh TA, et al. Endothelin clearance in patients with chronic heart failure: implications for anti-endothelin therapy. *Circulation* 1998;98(suppl 1):3A.
  43. Cramer H, Muller-Esterl W, Schroeder C. Subtype-specific desensitization of human endothelin ET<sub>A</sub> and ET<sub>B</sub> receptors: reflects differential receptor phosphorylation. *Biochemistry* 1997;36:13325-32.
  44. Pönicke K, Volgelsang M, Henroth M, et al. Endothelin receptors in the failing and nonfailing human heart. *Circulation* 1998;97:744-51.