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Detrimental Subtype-Specific Endothelin-1 (ET-1) Signaling in Myocardial Cells: the ET_A Mediated Proliferation and ET_B Receptor Down-Regulation

J. DŘÍMAL, M. MISLOVIČOVÁ, A. ISMAIL, F. MONČEK

Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovak Republic

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Summary

Many physiological and pathological processes in the cardiac tissue have been shown to be associated with a release of endothelin (ET) peptides and with induction of specific ET-receptors and G-protein-coupled ion channels. However, the exact mechanism regulating ET-receptors in the myocardium is controversial. The response to ET-1, the most important member of the ET family, is rapidly attenuated by down-regulation of ET-receptors. The internalization of ET-1 bound to two subclasses of specific receptors (ET_A and ET_B) that are abundant in the myocardium has been hypothesized to activate and/or inhibit a variety of intracellular signal transducing systems. The [^{125}I]ET-1, BQ-3020 and selective ET-antagonists were used to study the subtype-selective component of regulation of ET-1 receptors in myocardial membranes. We determined the characteristics of [^{125}I]ET-1 binding and [^{3}H]thymidine incorporation in whole cell saturation studies and measured Ca^{2+} channel induction and the total number of inactive Ca^{2+} channels in photoaffinity studies with [^{3}H]azidopine. Here we demonstrate four important components of the complex ET-1 response in human, porcine and rat myocardium, leading to aberrant responses of cells. After ET-1 induction, adaptive subtype-ET_B selective down-regulation predominated in human embryonic fibroblasts, in porcine membrane vesicles and in microsomal membranes of renal hypertensive rats, with preferential high affinity ET-1 binding to ET_A receptors and with the resultant ET_A mediated proliferative and mitogenic activation of human fibroblasts. The ET-1 induction was also accompanied by profound inactivation of Ca^{2+} channels in myocardial membranes.

Key words

Ligand binding • Endothelin-1 • ET_A-ET_B receptors • Photoaffinity labeling • Ca^{2+} channels • Human fibroblasts • Porcine myocardium • Microsomal membranes • Rat renal hypertension

Introduction

There is overwhelming evidence that endothelin-1 (ET-1) is a potent vasopressor and growth promoting peptide, which may play a role in cardiovascular pathology, e.g. in hypertension (Vanhoutte 1996), cardiac hypertrophy (Kanno *et al.* 1993), acute myocardial infarction and in congestive heart failure (Stewart *et al.* 1991). Activation of cardiac cells by ET-1 is presumably coupled to Ca^{2+} channels

Fax +420 2 293 894
E-mail physres@biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

(Bkaily *et al.* 1995). The resulting positive inotropic effect of ET-1 could be elicited in most mammalian species, however, it showed a wide range of species-dependent variations. It was mostly observed on the isolated heart (Ishikawa *et al.* 1988, Davenport *et al.* 1989, Hattori *et al.* 1993) but this was not in keeping with the very complex effects induced by ET-1 in cardiac cells (Masaki *et al.* 1992, Hilal-Dandan *et al.* 1997, Ozaki *et al.* 1997) and reported in recent studies on animal models (Pernow *et al.* 1997). The myocardial effects of ET-1 are presumably mediated by at least two types of G-protein-coupled ET-receptors (ET_A and ET_B) and both subtypes are expressed on the surface of the myocardial cell (Gu *et al.* 1989, Molenaar *et al.* 1993, Endoh *et al.* 1996), though in different ratios according to the species (Gu *et al.* 1992, Thibault *et al.* 1995, Ozaki *et al.* 1997, Wada *et al.* 1997). The ET_A-receptor was reported to launch most of the excitatory and proliferative response, whereas ET_B mediates predominantly inhibitory and/or excitatory responses (Russel *et al.* 1997, Davenport *et al.* 1995, Bacon *et al.* 1996, Dashwood *et al.* 1995, Clozel and Gray 1995, Opgaard *et al.* 1996, Sakai *et al.* 1996, Wada *et al.* 1997). The effects of ET-1 on the myocardium most probably also include quasi-irreversible ET-1 binding (Hilal-Dandan *et al.* 1997) with the observed negative effects of ET-1 on the size of myocardial infarction in animal models (Grover *et al.* 1993, Watanabe *et al.* 1995, Awane-Igata *et al.* 1997) with the release of ET-1 and with its own gene expression (see reviews Benatti *et al.* 1994, Gray and Webb 1996). The production of ET-1 was found to be markedly increased in patients with atherosclerotic coronary artery disease (Haug *et al.* 1996) and in acute myocardial infarction complicated with severe pump failure (Setsuta *et al.* 1995). It is still not clear, however, whether ET-1 is primarily responsible for initiating the majority of these effects in myocardial cells. The binding of ET-1 to two specific receptors ET_A and ET_B and the resultant internalization of bound ET-1 has been hypothesized to be activated by a signal from ET-receptors and regulatory G-proteins in cells. The participation of two distinct subtypes of ET-receptors in this regulation in myocardial cells as well as their specific role played in cardiovascular pathology are yet to be clarified.

The present experiments were, therefore, designed to study aberrant ET-1 signaling in myocardial cells. The end-stage of ET-1 receptor activation and the subtype-selective ET-1 receptor down-regulation were analyzed in human fibroblasts, in normal porcine left ventricular membrane vesicles and in microsomal myocardial membranes of rats with established chronic renal hypertension.

Methods

Human fibroblasts

Approved by the local Ethics Committee, human cardiac cells were isolated from fragments of primitive four chambered cardiac myotube (embryos of about 50 days of age) by enzymatic digestion in Dulbeco's modified Eagle's medium (DMEM) containing 0.1 % trypsin and 100 U/ml cellagen solution 2 (Worlington Biochem.). After sedimentation, the remaining tissue was digested for 15 min at 37 °C and this procedure was repeated three times. Then the isolated cells were pooled and centrifuged for 3 min at 2000 x g. The resulting pellet was resuspended in DMEM with 10 % fetal calf serum and seeded. After preplating, non-adherent cells were removed and fresh serum medium was added to the cells. The explants of adherent cells were used mostly between passages 5-13, usually at subconfluency, with a density of about 0.5-1.1 x 10^5 cells/cm^2. When examined by electron microscopy the cardiac cells were spindle-like in shape, their growth cycle and morphology remained as primary cultures of ventricular interstitial myofibroblasts with abundant collagen.

Ligand binding

The ligand binding experiments were performed on human fibroblasts (HF) maintained in DMEM supplemented with 10 % fetal calf serum, streptomycin/penicillin at 37 °C in 5 % of CO_2 in air, in enriched humidified atmosphere. Grown, confluent cultures of HF were used in the saturation studies. Whole-cell binding assays (measurements in triplicate) were performed at room temperature in HEPES-buffered physiological salt solution (buffer-1, in mmol/l): NaCl 135, KCl 4.2, $CaCl_2$ 1.25, $MgCl_2$ 1.0, KH_2PO_4 0.44, NaH_2PO_4 0.34, $NaHCO_3$ 2.6, HEPES 20.0, glucose 5.5, containing protease inhibitors (5 μg/ml pepstatin A, and (in mmol/l): 0.1 PMSF and 0.01 phosphoramidon, pH 7.4). HF were incubated for 120 min with increasing concentrations of [^{125}I]ET-1 (from 0.5 to 4.0 nmol/l) and nonspecific binding was determined in the presence 10^{-6} mol/l of unlabeled ET-1 or, in other experiments, with the sum of specific ET-antagonists in the same final concentration (ET_A selective tripeptide PD151242, non-selective pentapeptide PD142893 and the ET_B selective BQ-788).

[^{3}H]thymidine incorporation

The mitogenic potential of ET-1 was measured in HF by incorporation of [^{3}H]thymidine (0.5-1 x 10^5 cells/well). The HF (at approximately 80 % confluence) were at first growth-arrested by incubation (24 h at 37 °C) in DMEM with only 0.5 % FBS and then washed twice with the HEPES buffer-1 containing protease inhibitors, and finally stimulated with increasing concentrations of ET-1 (0.01-1.0 nmol/l) After exposure to the ligands, the cells were washed, incubated in 10 % trichloracetic acid (at 4 °C), dissolved in 0.1 mol/l of Tris (hydroxymethyl)-aminomethane, dried on Wathman filters and counted on a scintillation spectrometer (LKB Wallace, Turku, Finland).

Photoaffinity labeling experiments

Aliquots of HF cells (1.2 x 10^5 cells) were incubated with a 1,4 dihydropyridine-type photoaffinity probe (±)-[^{3}H]azidopine in twelve gradually increasing concentrations (0.5-12.0 nmol/l of ligand) in the presence or absence of unlabeled 1,4-dihydropyridine PN200-110 (1.0 μmol/l) in the dark. The mixtures were irradiated for 3 min with UV-light (40 W Phillips UV lamp at a distance of 50 mm) at the beginning of incubation. At the end of the 60 min incubation period, the cells were separated by rapid filtration on Wathman GF/C glass fiber filters, washed three times with 2.5 ml of cold assay buffer, the filters were collected in 10 ml scintillation cocktails (Bray) and counted on a 2500 TR Liquid Scintillation Analyzer (Tricarb, Packard) at 65 % efficiency.

Membranes from porcine myocardium

Viable porcine hearts (450-600 g) were obtained at a local slaughterhouse. Samples of the left ventricular myocardium (3.5 x 3.0 cm in diameter) were dissected out with a special drill, conductive coronary vessels, adhering tissues and fat were removed, and endocardial free wall segments were immersed in 12 volumes of ice cold buffer-2 (250 mmol/l sucrose, 2.5 mmol/l $MgCl_2$, 10 mmol/l Tris, saturated with 5 % of CO_2 in O_2, pH 7.5) and transported in Dewar vessels to the laboratory. The myocardial samples were homogenized and subfractionated as described by Ferry *et al.* (1985) and as previously used in our laboratory to obtain microsomal membranes suitable for ligand binding studies (Dřímal 1991). Aliquots of microsomal membrane proteins, an approximately 90-100 μg sample of protein in buffer-2 containing (in mmol/l): Tris HCl 50.0, $MgCl_2$ 2.5, PMSF 0.2, EDTA 0.1, EGTA 0.1 and in (μmol/l): pepstatin 1.0, leupeptin 1.0 were incubated in Eppendorf tubes (at room temperature). Protein concentrations in membrane preparations were assayed by the method of Bradford (1976).

Renal hypertensive rats

Male Wistar rats (n = 48, weighing from 190 to 220 g) were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Both kidneys were exposed under aseptic conditions by the classical Goldblatt-procedure in modification by Grollman (1975). Briefly, one kidney was removed and a „figure-of-eight" ligature and a silver clip were positioned on the contralateral kidney. Four weeks after the operation, after establishment of stabile chronic hypertension with arterial blood pressure higher than 25±1.5 kPa, the rats were again anesthetized, the thoracic cavity was opened, the heart was excised, immersed in an ice-cold buffer-1 and a segment of the free left ventricular wall was quickly removed. Myocardial segments (100 μm) were sliced, incubated in HEPES buffer-1 and used for ligand binding studies.

Chemicals:

[^{3}H]azidopine, specific activity 50 Ci/mmol (Amersham), [^{125}I]BQ-3020, (3-[^{125}I]endothelin-1, specific activity 2000 Ci/mmol, [^{125}I]PD151242 (both Amersham), [^{3}H]thymidine (DuPont), adenosine-3',5'-cyclic-monophosphate, P-1-(2-nitrophenyl)-ethyl ester, (caged cAMP, Calbiochem), synthetic human endothelin-1 (Sigma), BQ-788 (R.B.I), Serum for cell cultures and other cell culture reagents were from GIBCO (Grand Island, NY, USA). PN200-110 (Drug Research Institute, Modra, Slovakia)

Results

Characterization of [^{125}I]ET-1 binding to HF

The binding of [^{125}I]ET-1 to whole cell preparations of HF was shown to be saturable and of high affinity. The analysis of the saturation curves by computer-assisted linear regression revealed one class of high affinity sites (n_H = 1.12±0.06) with K_D = 0.45±0.05 nmol/l (for radioligand concentrations ranging from 0.5 to 4.2 nmol/l) with the mean maximal binding capacity of 436±24 fmol/mg of protein. To determine the binding specificity of the non-selective ET agonist [^{125}I]ET-1, experiments were also performed after preincubation of human fibroblasts (HF) with the ET_A selective antagonist PD151242 and with the ET_B selective BQ-788 and non-selective PD142893. Figure 1 shows examples of [^{125}I]ET-1 specific binding in saturation experiments

with control, quiescent HF and in another experiments with HF preincubated with three different ET-antagonists. The specific binding remained unchanged after preincubation with PD151242 (B_{max} = 118±11 %), while it was significantly increased after preincubation with the ET_B selective antagonist BQ-788 (159±12 %, $p<0.05$) at reduced affinity. The characteristics of binding were also determined by competition analysis. The calculated values of K_D were close to the values determined in saturation experiments. In another group of experiments, HF were stimulated by preincubation with the low concentration of ET-1 (50.0 pmol/l for 60 min). The stimulation of HF with ET-1 in these experiments significantly reduced the total number of sites to 77±8 % ($p < 0.05$).

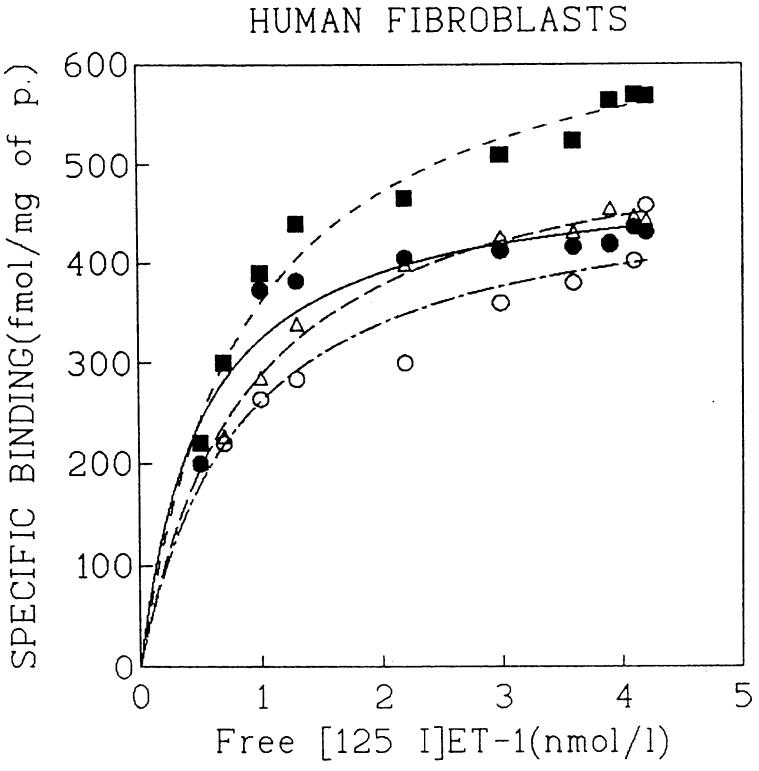

Fig. 1. *The comparison of the saturation binding isotherms obtained in experiments with the helical-type natural human peptide, synthetic (3-[^{125}I]iodotyrosyl) endothelin-1 (ET-1) in human embryonic fibroblasts (HF). Free radioligand concentrations (nmol/l) are on the abscissa and specific binding is on the ordinate (in fmol/mg of protein). Explanation and symbols: Control, growth-arrested, native HF (filled circles), cells preincubated with linear tripeptide PD151242, the ET_A-selective antagonist (triangles), HF preincubated with the non-selective pentapeptide PD142893 the ET-antagonist (open circles) and with ET_B - selective tripeptide BQ-788 (filled squares).*

Mitogenic potential of ET-1

The mitogenic effect of ET-1 was assessed by [^{3}H]thymidine incorporation during DNA synthesis in subconfluent whole cell preparations of HF. In a previous study on human vascular smooth muscle cells we reported significant induction of proliferation with ET-1 concentration of 0.1 nmol/l (Dřímal *et al.* 1998). We therefore, examined the proliferative response in HF also with this concentration. Additional experiments were performed to determine whether stimulation of DNA synthesis by ET-1 in HF was dose-dependent. The concentration-response curve for the ET-1 inductive effect in HF is shown in Figure 2. ET-1 dependent [^{3}H]thymidine incorporation doubled with 0.1 nmol/l of ET-1 and was maximal at 1.0 nmol/l of ET-1. Preincubation of the ET_A selective antagonist PD151242 in 10 nanomolar and higher concentrations significantly attenuated [^{3}H]thymidine incorporation into cells. The ET_B selective BQ-788 was ineffective in these experiments.

Effect of ET-1 on surface membrane Ca^{2+} channels in HF

To investigate whether the modulating effect of ET-1 plays any causal role in disorders involving membrane-bound Ca^{2+} channels in HF we also extended our studies to the analysis of specific photoaffinity-labeling of Ca^{2+} channels with 1,4-dihydropyridine [^{3}H]azidopine, a probe specific for inactivated Ca^{2+} channels. The specific photoincorporation of

(±)-[^{3}H]azidopine on surface membrane sites of L-type Ca^{2+} channels was found to be saturable and exhibited a high affinity. The photoaffinity ligand [^{3}H]azidopine specifically photolabeled a saturable population of high affinity binding sites on, with K_D = 0.82±0.05 nmol/l and density B_{max} = 746±89 fmol/mg of protein. The Hill coefficient in this group of experiments was close to unity (n_H = 0.92±0.05). Table 1 summarizes the results obtained with ET-1 preincubated cells. The specific photolabeling increased significantly in cells preincubated with 0.1-1.0 nmol/l of ET-1. This increase was concentration-dependent and was maximal after preincubation with 1.0 nmol/l of ET-1. Preincubation of adenosine-3',5'-cyclic monophosphate, P-1-(2-nitrophenyl)-ethyl ester, a membrane permeant photolabile derivative of cAMP (caged cAMP which is selectively released by a short pulse of light exactly at the time of photolabeling of Ca^{2+} channels) significantly attenuated the observed increase in specific photolabeling induced by ET-1 in these experiments. The powerful L-type Ca^{2+} channel blocker and K^+ channel activator S(+)-niguldipine slightly but significantly reduced the increased specific photolabeling induced by ET-1 preincubation. Similarly, did the protein-kinase inhibitor staurosporine that significantly reduced the photolabeling induced by ET-1.

Table 1. Effects of ET-1 on the surface membrane Ca^{2+} channels in human fibroblasts. DHP-sensitive, inactivated Ca^{2+} channels were covalently photolabeled with arylazide [^{3}H]azidopine.

Drug (nmol/l)	n	Number of sites (%)	n_H
ET-1 (0.1)	5	+146±30*	0.97±0.12
ET-1 (0.5)	5	+252±66*	1.05±0.12
ET-1 (1.0)	6	+468±81*	1.18±0.12
Caged cAMP (100.0) + ET-1 (1.0)	5	+115±29#	0.98±0.10
S(+)niguldipine (10.0) + ET-1 (1.0)	5	+330±82#	1.77±0.80#
PTX (100.0) + ET-1 (1.0)	5	+114±85#	1.43±0.20#
Staurosporine (10.0) + ET-1 (1.0)	4	+197±92#	1.45±0.15#

*Values are mean ± S.E.M. Number of sites specifically photolabeled on control cells was B_{max} = 764 ± 89 fmol/mg of protein and K_D = 0. 82 ± 0.05 nmol/l. The membrane permeant derivative of cAMP (caged cAMP) was selectively released from its cage by irradiation of cells exactly at the moment of covalent photolabeling of Ca^{2+} channels with [^{3}H]azidopine. * Significant change when compared to unstimulated cells; # significant change when compared to control response. n_H Hill coefficient, n = number of experiments.*

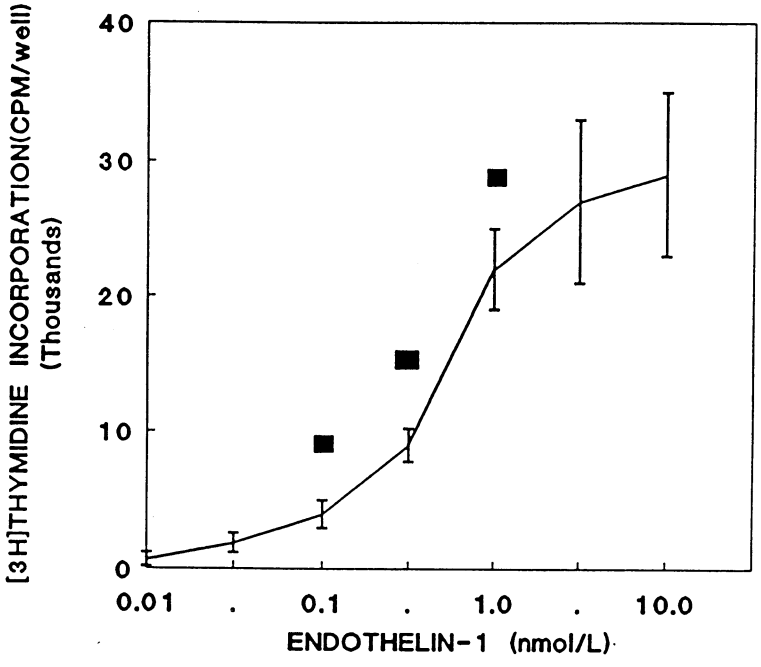

Fig. 2. *The effect of ET-1 preincubation on [^{3}H]thymidine incorporation in subconfluent HF. Concentration of ET-1 in nmol/l. Incorporation of [^{3}H]thymidine (x 10^3 cpm) on the ordinate. Results are mean ± S.E.M. (n = 6). * Significant increase in [^{3}H]thymidine incorporation after preincubation of ET-1.*

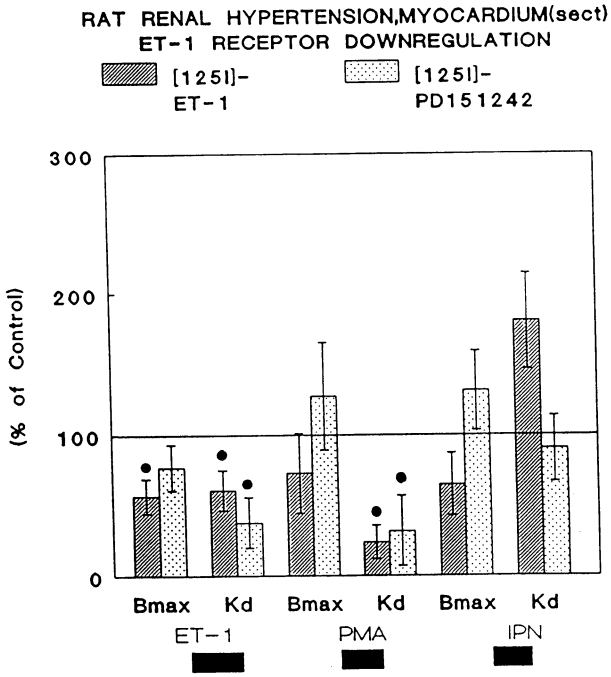

Fig. 3. *Down-regulation of ET-1 receptors in porcine endocardial membrane vesicles after preincubation of 0.1 nmol/l of non-selective natural peptide ET-1 or its truncated N-acetyl-[Ala^{11,15}]- (6-21) derivative, the ET_B selective agonist, BQ-3020 (1.0 nmol/l). Preincubation of ET-1 induced significant down-regulation of specific ET-1 binding sites (pmol/mg of protein) with a slight, but significant increase in affinity (here manifested by reduction in the numeric values of K_D). The inhibition of [^{125}I]ET-1 binding induced by BQ-3020 was accomplished mostly by reduced affinity . Protein kinase blocker staurosporine and Gi-protein uncoupler pertussis-toxin (PT-Toxin) significantly attenuated the reduction in affinity induced by BQ-3020.*

ET-1 receptor mediated down-regulation in microsomal vesicles of porcine heart

Previously, we showed that porcine microsomal membrane vesicles retained some properties of the myocardial sarcolemma in whole cell preparations. Our partially purified microsomal membranes of porcine left ventricular endocardium contained reasonable amounts of both subtypes of ET-receptors as well as of myocardial, presumably L-type Ca^{2+} channel 1,4-dihydropyridine receptors. The specific [^{125}I]ET-1 binding to microsomal vesicular membranes in the absence of any test ligand was 688±25 fmol/mg of protein and K_D was 1.03±0.1 nmol/l. In order to identify the essential signaling for ET-1 receptor down-regulation, microsomal vesicles were preincubated with subnanomolar concentrations of the non-selective (ET_A/ET_B) agonist ET-1 and with the ET_B selective agonist BQ-3020 (Fig. 3). When 0.1 nmol/l of unlabeled ET-1 has been preincubated for 60 min with microsomal vesicles prior to [^{125}I]ET-1 addition, there was significant down-regulation of [^{125}I]ET-1 binding sites and a slight but significant increase in affinity. On the contrary, preincubation of the ET_B selective agonist BQ-3020 in low nanomolar concentrations significantly lowered the total number of ET-1 binding, mostly by reducing the affinity of [^{125}I]ET-1 for its receptors. Preincubation with pertussis toxin prior to ET-1 significantly attenuated the reduced affinity of receptors for [^{125}I] ET-1 induced by BQ-3020. Similarly, the protein kinase blocker staurosporine significantly attenuated the reduction in affinity induced by BQ-3020. The combined preincubation with two antagonists, the ET_A selective peptide PD151242 and the butenolide PD155080 prior to ET-1 testing (data not shown), significantly reduced the total number of [^{125}I]ET-1 binding sites (603±25 fmol/mg of protein) and its affinity (K_D =1.86±0.38 nmol/l).

Detrimental adaptation of ET-receptors in hypertensive myocardium

The basal values of systemic arterial blood pressure in the group of anesthetized renal hypertensive rats (RHR) were significantly increased (26.6 ± 1.9 kPa) and the vasodilatator response to acetylcholine was significantly reduced. Intravenous infusion of three vasopressors, i.e. norepinephrine, angiotensin and phenylephrine, in equipressor doses (ED_{25}) was accompanied by a significant initial depression of systemic arterial blood pressure, followed by an increase. Taking into account the potential importance of our findings concerning the subtype-ET_B selective receptor down-regulation in the compromised myocardium, we applied two ET-ligands, [^{125}I]ET-1 and [^{125}I]PD151242, on the left ventricular myocardium of RHR (Fig. 4). The density of ET_A receptors in the endocardium of RHR was found to be in the range of [^{125}I]ET-1 binding density. However, the affinity of ET-1 binding sites for [^{125}I]ET-1 was almost two times lower than that for ET_A selective ligands on ET_A receptors. In order to test the possibility that both subtypes of ET-receptors were modulated by independent, heterologous processes, we also analyzed the effects of two stimulants in the present study, namely

phorbol-myristate-acetate, a protein kinase activator, and isoproterenol, a specific beta-adrenergic stimulant. As is shown in Figure 5, preincubation of ET-1 induced significant down-regulation of ET-1 binding sites in the myocardium of RHR and significantly increased the affinity of ET_A receptors for their ligand. Phorbol-myristate-acetate (100 μmol/l) significantly enhanced the affinity and IPN (10 μmol/l) significantly attenuated the effect of ET-1 preincubation on binding.

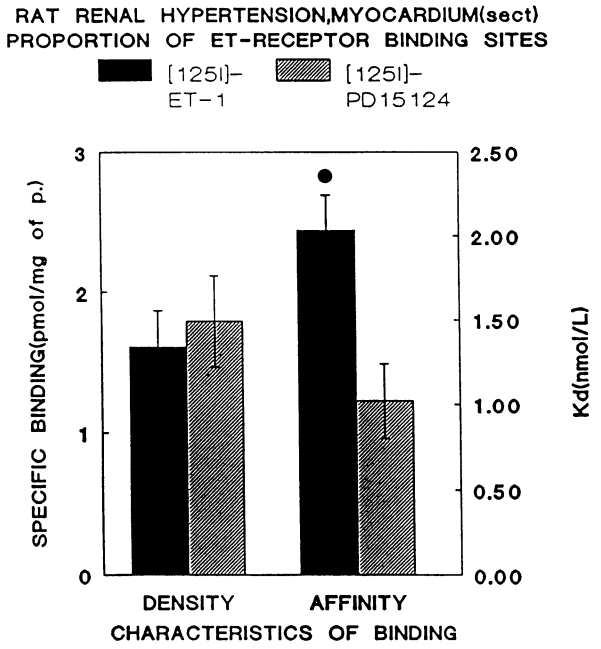

Fig. 4. *Characteristics of binding (B_{max} in pmol/mg of protein) and affinity (K_D in nmol/l) of non-selective agonist, human, synthetic [^{125}I]ET-1 and the ET_A selective antagonist [^{125}I]PD151242 in left ventricular myocardium of renal hypertensive rat heart (4 weeks after surgical induction of hypertension (solitary kidney, one clip method). Values are means ± S.E.M., n = 12. The dissimilarities in numeric values of K_D indicate preferential ET_A binding in renal hypertensive rat myocardium. Note the significant increase in K_D values with [^{125}I]PD151242.*

Discussion

The present study has shown that stimulation of human fibroblasts (HF) and of porcine endocardial membrane vesicles with synthetic, helical-type human and porcine endothelin-1 (ET-1) induced significant ET_B selective receptor down-regulation and reduced the total number of [^{125}I]ET-1 binding sites. This does not only represent an important adaptational mechanism. The dependence on the intensity and the proportion of ET_A and ET_B receptor subtypes also implies serious functional alterations leading to myocardial pathology, comparable to the findings in the endocardium of renal hypertensive rats. The study demonstrated that ET-1 had two receptors, ET_A and ET_B, to bind with and that both subtypes were present in various proportion on the surface membrane of human fibroblasts, in the endocardial membrane vesicles and in the myocardium of renal hypertensive rats. The higher human baseline plasma ET-1 levels in the range of pg/ml were reported previously in ischemic subjects (Ferri *et al.* 1997). According to this report, the subtype ET_B mediated down-regulation induced in the present study with subnanomolar concentrations of ET-1 that are clearly in the lower range of local human pathological concentrations of endothelin.

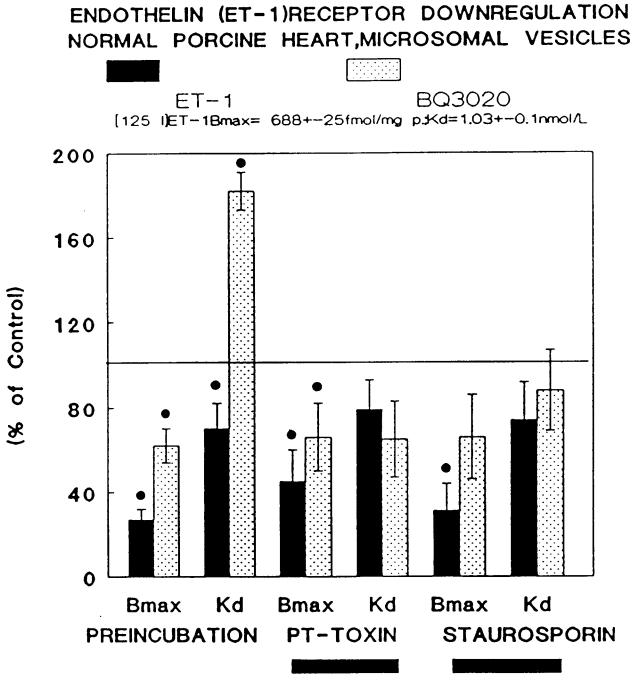

Fig. 5. *The changes in density of ET-1 and ET_A receptors induced by short-term stimulation (in nmol/l) with endothelin (ET-1, 1.0); phorbol-myristate-acetate (PMA, 100.0 μmo/l) and isoprenaline (IPN, 10.0 μmo/l) in sections of ventricular myocardium of renal hypertensive rats 4 weeks after induction of hypertension. Values are means ± S.E.M. Note the down-regulation of ET-1 binding with the significant increase in ET_A receptor affinity after ET-1 preincubation. We hypothesize that certain intramembrane signaling after PMA and IPN preincubation may affect the B_{max} and K_D of ET-1 and ET_A receptors. For further explanation see Methods and Results.*

Previously, we have shown that exogenous ET-1 induced selective ET_B receptor down-regulation in vascular smooth muscles. In the present report, we extended these studies to myocardial cells and have demonstrated that the potency of a selective ET_B agonist BQ-3020 (truncated structurally reduced analogue of ET-1, linear-type, Ala-derivative, which is devoid of the amino-terminal part) to induce the ET_B receptor down-regulation was significantly reduced. One possibility how to explain the differences between ET-1 and BQ-3020 induced down-regulation of ET-1 binding sites clearly demonstrated in our present study is that stimulation of ET_B receptors with ET-1 induced conformational changes selectively in ET_B receptors, reducing the affinity of ET-1 for this subtype. It is also possible that, in contrast to non-selective ET-1, the binding affinity of BQ-3020 to ET_A subtype is lower, most probably because of its truncated-peptide structure. The most frequent example of ET-receptor induction was reported under pathological circumstances with excessive endogenous concentrations of endogenous ET-1 (Ray *et al.* 1992). This may be an indication that acute myocardial disease states modify ET-receptor profiles to a far greater degree than previously suspected. The subtype ET_B mediated down-regulation in the present study was accompanied with adaptational ET_A predominance in renovascular hypertension. Furthermore, our results clearly indicate that the ET_A receptor predominance associated with ET_B selective down-regulation was accompanied by an increased affinity of ET_A binding sites for their ligands, including ET-1. This represents the potential detrimental factor that was present in all our experiments: in human fibroblasts, in porcine myocardial membranes and in the RHR endocardium.

The ET_B selective down-regulation induced in our study by exogenous ET-1 may be of pathophysiologic significance. In dependence on the intensity of ET_B down-regulation, it favored ET_A selective ligand binding with a predominance of enhanced vasoconstrictive and proliferative responses to ET-1.

Moreover, we found significant differences in the binding characteristics of 21 amino acid residues containing human, porcine ET-1 and of the BQ-3020, 6-21 amino-acid, [Ala ^{1,3,11,15}] ET-1 derivative, potent agonist at the ET_B sites. The different affinities of both ligands suggest that ET-1, as a representative of the ET-family of peptides, may have more than one binding site on the myocardial cell. Recently, the question of the multiplicity of conformations of G-protein-coupled receptors and their dependence on the ligands was interpreted as an allosteric phenomenon in agonist-receptor-G-protein interaction (Tuček 1977). After performing different ligand binding studies with peptide-type agonists, human [^{125}I]ET-1, BQ-3020 both in vascular smooth muscle and in myocardial cells, we are now convinced that the problem of R-R* dichotomy and allosteric phenomenology may also explain some effects induced by peptide-type ET ligands in myocardial cells. In our previous photoaffinity labeling studies, we repeatedly identified high- and low-affinity sites for DHPs in coronary vascular smooth muscle membranes (Dřímal 1991). In particular, saturable high-affinity sites covalently labeled with 1,4-dihydropyridine type of photolabel [^{3}H]azidopine are thought to correspond to 1,4-DHP binding sites on inactivated Ca^{2+} channels. The present results and interpretations of the stimulatory effect of ET-1 in cardiac microsomal membranes agree well with those reported in our previous studies (Dřímal 1994 Dřímal and Boháčik 1994). Using green monkey renal cells and human fibroblasts, it was shown that preincubation with endothelin-1 increased the total number of binding sites photolabeled on renal and vascular smooth muscle cells. However, the total number of sites specifically photolabeled on renal and vascular smooth muscle cells was lower in these experiments. The crucial point is whether the compromised mammalian heart is a source of elevated circulating endothelin-1 levels (Hasdai *et al.* 1996).

Arai *et al.* (1995) reported an elevated density of ET-1 receptors in the rat ventricular tissue after 8 days of pressure overload, suggesting that cardiac ET-1 is involved in the development of cardiac hypertrophy. The results of our present *in vitro* study, obtained on cultured human embryonic cells and on myocardial membrane vesicles from renovascular hypertensive rat hearts in the phase of stabilized hypertension, support the contention that ET-1 release and mitogenicity are also induced by exogenous supplementation of subnanomolar concentrations of ET-1. In response to injury of fibroblastic cells, ventricular myocardial cells most probably react *via* direct contact with other cells and *via* altered properties of membrane ET-1 receptors present on myocardial cells which may compromise myocardial function. Thus, excess of ET-1 with its ET_B selective down-regulation and with the prevalence of ET_A mediated mitogenic and proliferative response may act as promoter of hypertrophy in cardiovascular diseases. Ostensibly in contradiction with this view are the findings of our *in vivo* functional experiments on renal hypertensive rat models, where the basal values of systemic arterial blood pressure were significantly higher and ET-1 infusion induced less intensive increase in

systolic arterial blood pressure with a shorter duration of the effect. Careful hemodynamic analysis in these experiments provided an explanation for these results. Namely, there was always a significant reduction of cardiac output in RHR with increased total peripheral resistance and with increased diastolic left ventricular pressure, indicating incipient heart failure. In our model of renovascular hypertensive rats, both the constitutive or induced release of ET-1 may well cause functional and regulatory alterations in ET-receptors leading to heart failure.

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References

ARAI M, YOGUCHI A, ISO T, TAKAHASHI T, IMAI S, MURATA K, SUZUKI T: Endothelin-1 and its binding sites are upregulated in pressure overload cardiac hypertrophy. *Am J Physiol* **268:** H2084-H2091, 1995.

AWANE-IGATA Y, IKEDA S, WATANABE T: Inhibitory effects of TAK-044 on endothelin-1-induced vasoconstriction in various canine and porcine coronary arteries: a comparison with selective ET_A and ET_B receptor antagonists. *Br J Pharmacol* **120:** 516-522, 1997.

BACON CR, CARY NR, DAVENPORT AP: Endothelin peptide receptors in human atherosclerotic coronary artery and aorta. *Circ Res* **79:** 794-801, 1996.

BKAILY G, WANG S, BUI M, MÉNARD D: ET-1 stimulates Ca^{2+} currents in cardiac cells. *J Cardiovasc Pharmacol* **26** (Suppl 3): S293-S296, 1995.

BRADFORD MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254, 1976.

BENATTI L, FABBRINI MS, PATRONI C: Regulation of ET-1 biosynthesis. *Ann NY Acad Sci* **71**: 109-121, 1994.

CLOZEL M, GRAY G: Are there ET_B receptors mediating constriction and relaxation? *J Cardiovasc Pharmacol* **26** (Suppl 3): S262-S264, 1995.

DASHWOOD MR, TIMM M, KASKI JC: Regional variations in ET_A/ET_B binding sites in human coronary vasculature. *J Cardiovasc Pharmacol* **26** (Suppl 3): S351-S354, 1995.

DAVENPORT AP, NUNEZ DJ, HALL JA, KAUMANN AJ, BROWN MJ: Autoradiographical localization of binding sites for porcine [^{125}I]endothelin-1 in humans, pigs, and rats: functional relevance in humans. *J Cardiovasc Pharmacol* **13** (Suppl 5): S166-S170, 1989.

DAVENPORT AP, KUC RE, MAGUIRE JJ, HARLAND SP: ET-A receptors predominate in the human vasculature and mediate constriction. *J Cardiovasc Pharmacol* **26** (Suppl 3): S262-S267, 1995.

DŘÍMAL J: Regulation of 1, 4-dihydropyridine and beta-adrenergic receptor sites in coronary artery smooth muscle membranes. *Cell Signal* **3:** 225-232, 1991.

DŘÍMAL J. Identification of dihydropyridine binding sites on cultured monkey renal cells with a photoaffinity probe (–)-[^{3}H]azidopine. *Gen Physiol Biophys* **13:** 137-148, 1994 .

DŘÍMAL J, BOHÁČIK L: The effect of photogenerated site-directed free radicals on surface dihydropyridine binding sites identified on cultured monkey renal cells. *Meth Find Exp Clin Pharmacol* **16:** 397-404, 1994.

ENDOH M, NOROTA I, YANG HT, FUJITA S, TAKANASHI M: The positive inotropic effect and the hydrolysis of phosphoinositide induced by endothelin-3 in rabbit ventricular myocardium: inhibition by a selective antagonist of ET(A) receptors FR139317. *J Pharmacol Exp Ther* **277:** 61-70, 1996.

FERRI C, DE MARZIO P, DESIDERI G, BALDONCINI R, MORELLI S, SANTUCCI A: Plasma endothelin-1 levels during transient acute myocardial ischemia in men: effects of coronary revascularization. *Eur J Clin Invest* **27:** 526-532, 1997.

FERRY D.R, GOLL A, GLOSSMANN H: Photoaffinity labelling of the cardiac calcium channel. *Biochem J* **243:** 127-135, 1987.

GRAY GA, WEBB DJ: The endothelin system and its potential as therapeutic target in cardiovascular disease. *Pharmacol Ther* **72:** 109-148, 1996.

GROLLMAN A: Experimental induction of hypertension In: *Handbook of Experimental Pharmacology. Experimental Production of Diseases* Part 3. *Heart and Circulation*. Springer, New York, 1975, pp 71-96.

GROVER GJ, DZWONCZYK S, PARHAM CS: The endothelin-1 receptor antagonist BQ123 reduces infarct size in a canine model of coronary occlusion and reperfusion. *Cardiovasc Res* **27:** 1613-1618, 1993.

GU XH, CASLEY DJ, NAYLER WG: Characterization of [^{125}I]endothelin-1 binding sites in rat cardiac membrane fragments. *J Cardiovasc Pharmacol* **13** (Suppl 5): S171-S173, 1989.

GU XH, OU RC, CASLEY DJ, DALY MJ, NAYLER WG: Effects of age on endothelin-1 binding sites in rat cardiac ventricular membranes. *J Cardiovasc Pharmacol* **19:** 764-769, 1992.

HASDAI D, EREZ E, GILL AI, RAANANI E, SCLAROVSKY S, BARAK Z, SULKES J, VIDNE BA: Is the heart a source for elevated circulating endothelin levels during aorta-coronary artery bypass grafting surgery in human beings? *J Thorac Cardiovasc Surg* **112:** 531-536, 1996.

HAUG C, VOISARD R, LENICH A, BAUR R, HOHER M, OSTERHUES H, HANNEKUM A, VOGEL U, MATTFELDT T, HOMBACH V, GRUNERT A: Increased endothelin release by cultured human smooth muscle cells from atherosclerotic coronary arteries. *Cardiovasc Res* **31:** 807-813, 1996.

HATTORI Y, NAKAYA H, NISHIHIRA J, KANNO M: A dual component positive inotropic effect of endothelin-1 in guinea pig left atria: a role of protein kinase C. *J Pharmacol Exp Ther* **266:** 1202-1212, 1993.

HILAL-DANDAN R, VILLEGAS S, GONZALES A, BRUNTON LL: The quasi-irreversible nature of endothelin binding and G-protein linked signaling in cardiac myocytes. *J Pharmacol Exp Ther* **281:** 267-273, 1997.

ISHIKAWA T, YANAGISAWA M, KIMURA S, GOTO K, MASAKI T: Positive inotropic action of novel vasoconstrictor peptide endothelin on guinea pig atria. *Am J Physiol* **255:** H970-H973, 1988.

KANNO K, HIRATA Y, TSUJINO M, IMAI T, SCHICHIRI M, ITO H, MARUMO F: Up-regulation of ET_B receptor subtype mRNA by angiotensin in rat cardiomyocytes. *Biochem Biophys Res Commun* **194:** 1282-1287, 1993.

MASAKI T, YANAGISAWA M, GOTO K: Physiology and pharmacology of endothelins. *Med Res Rev* **12:** 391-421, 1992.

MOLENAAR P, O'REILLY G, SHARKEY A, KUC RE, HARDING DP, PLUMPTON C, GRESHAW GA, DAVENPORT A.P: Characterization and localization of endothelin receptor subtypes in the human atrioventricular conducting system and myocardium. *Circ Res* **72:** 526-588, 1993.

OPGAARD OS, CANTERA L, ADNER M, EDVINSSON L: Endothelin-A and B receptors in human coronary arteries and veins. *Regul Pept* **63:** 149-156, 1996.

OZAKI S, OHWAKI K, IHARA M, ISHIKAWA K, YANO M: Coexpression studies with endothelin receptor subtypes indicate the existence of intracellular cross-talk between ET_A and ET_B receptors. *J Biochem* **121:** 440-447, 1997.

PERNOW J, AHLBORG G, LUNDBERG JM. KAIJSER L: Long-lasting coronary vasoconstrictor effects and myocardial uptake of endothelin-1 in humans. *Acta Physiol Scand* **159:** 147-153, 1997.

RAY SG, McMURRAY JJ, MORTON JJ, DARGIE HJ: Circulating endothelin in acute ischaemic syndrome. *Br Heart J* **67:** 83-386, 1992.

RUSSELL FD, SKEPPER JN, DAVENPORT AP: Detection of endothelin receptors in human coronary artery vascular smooth muscle cells but not endothelial cells using electron microscope autoradiography. *J Cardiovasc Pharmacol* **29:** 820-826, 1997.

SAKAI S, MIYAGUCHI T, SAKURAI T, KASUZA Y, IHARA M, YAMAGUCHI I, GOTO K. SUGISHITA Y: Endogenous endothelin-1 participates in the maintenance of cardiac function in rats with congestive heart failure. Marked increase in endothelin-1 production in failing heart. *Circulation* **93:** 1214-1222, 1996.

SETSUTA K, SEINO Y, TOMITA Z, NEJIMA J, TAKANO T, HAYAKAWA H: Origin and pathophysiological role of increased plasma endothelin-1 in patients with acute myocardial infarction. *Angiology* **46:** 557-565, 1995.

STEWART DJ, KUBAC G, COSTELLO KB, CERNACEK P: Increased plasma endothelin-1 in the early hours of acute myocardial infarction. *J Am Coll Cardiol* **18:** 38-43, 1991.

THIBAULT G, ARGUIN CH, GARCIA R: Cardiac endothelin-1 content and receptor subtype in spontaneously hypertensive rats. *J Mol Cell Cardiol* **27:** 2327-2336, 1995.

TUČEK S: Observations of allosteric phenomena on G-protein-coupled receptors point to problems in interpretation of agonist-receptor-G-protein interactions. *Trends Pharmacol Sci* **18:** 414-416, 1997.

VANHOUTTE PM: Endothelial dysfunction in hypertension. *J Hypertens* **14** (Suppl 5): S83-S93, 1996.

WADA A, TSUKAMOTO T, FUKAI D, OHNISHI M, MAEDA K, HISANAGA T, MAEDA Y, MATSUDA Y, KINOSHITA M: Comparison of the selective endothelin ET_A and ET_B receptor antagonists in congestive heart failure. *J Am Coll Cardiol* **30:** 1385-1392, 1997.

WATANABE T, AWANE Y, IKEDA S, FUJIWARA S, KUBO K, KIKUCH T, KUSAMOTO K, WAKIMASU M, FUJINO M: Pharmacology of nonselective ET_A and ET_B receptor antagonists in rats. *Br J Pharmacol* **114:** 949-954, 1995.

Reprint requests

J. Dřímal, M.D, D.Sc, Slovak Academy of Sciences, Institute of Experimental Pharmacology, Dúbravská cesta 9, 842 16 Bratislava, Fax: +421 07 375928, E-mail: exfadrim@savba.sk.