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Pharmacological characterization of cardiovascular responses induced by endothelin-1 in the perfused rat heart

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Abstract

The effects of the endothelin receptor antagonist TAK-044 (*cyclo*[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl]disodium salt) and BQ-123 (*cyclo*[D-Asp-Pro-D-Val-Leu-D-Trp]) were studied in the rat heart to characterize the receptor subtypes responsible for the cardiovascular actions of endothelin-1. Endothelin-1 induced a transient decrease and subsequent increase in perfusion pressure in perfused rat hearts, and increased left ventricular developed pressure. TAK-044 diminished these endothelin-1-induced responses (100 pmol/heart) with IC_{50} values of 140, 57 and 1.3 nM, respectively. BQ-123 (1–30 μM) partially inhibited the endothelin-1-induced hypertension (30–40%) in the rat heart, and failed to inhibit the hypotension. The positive inotropic effect of endothelin-1 was abolished by BQ-123. Neither indomethacin (10 μM) nor N^{ω}-nitro-L-arginine methyl ester (100 μM) attenuated the endothelin-1-induced hypotension. TAK-044 and BQ-123 attenuated the positive inotropic effect of endothelin-1 in rat papillary muscles. In rat cardiac membrane fractions, TAK-044 and BQ-123 inhibited [^{125}I]endothelin-1 binding to endothelin ET_A receptors with IC_{50} values of 0.39 ± 0.6 and 36 ± 9 nM, respectively, whereas only TAK-044 potently blocked the endothelin ET_B receptor subtype (IC_{50} value: 370 ± 180 nM). These results suggest that endothelin-1 modulates cardiovascular functions in the rat heart by activating both endothelin ET_A and endothelin ET_B receptors, all of which are sensitive to TAK-044.

Keywords: Endothelin-1; Heart, perfused, rat; TAK-044; BQ-123; Endothelin ET_A receptor; Endothelin ET_B receptor

1. Introduction

Endothelin-1 is a potent vasoconstrictive peptide isolated from the supernatant of cultured porcine aortic endothelial cells (Yanagisawa et al., 1988). Elevation of plasma endothelin-1 levels has been reported in patients with myocardial infarction and vasospasmic angina (Miyauchi et al., 1989; Matsuyama et al., 1991; Toyo-Oka et al., 1991), suggesting that endogenous endothelin-1 is related to the pathogenesis of ischemic heart disease. In fact, we have demonstrated that a monoclonal antibody against endothelin-1 and an endothelin receptor antagonist limit the extent of myocardial infarction in a rat ischemia-reperfusion model (Watanabe et al., 1991, 1995). However, it is unclear how endogenous endothelin-1 worsens myocardial infarction in the rat heart.

The effects of endothelin-1 on rat cardiovascular function are thought to be produced via activation of endothelin-1 receptors referred to as endothelin ET_A receptors (highly selective for endothelin-1 over endothelin-3) and endothelin ET_B receptors (non-selective for endothelin-1 and endothelin-3), since both receptor subtypes are present in the heart (Arai et al., 1990; Sakurai et al., 1990). However, there have been few studies linking the activation of endothelin ET_A and/or endothelin ET_B receptor subtypes to cardiovascular function in the rat. For example, there has been no confirmatory study evaluating endothelin ET_B-mediated coronary vasoconstriction using endothelin ET_B or endothelin ET_A/ET_B receptor antagonists. In rat ventricular muscle, Ishikawa et al. (1991) demonstrated that endothelin-1 but not endothelin-3 increases developed tension in adult rat papillary mus-

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cles. However, the detail of the receptor that is responsible for endothelin-1-induced increases in cardiac contractility has not been provided.

It has been reported that inhibition of endothelium-derived relaxing factor (EDRF) enhances the vasoconstrictive response of endothelin-1 in the rat heart, suggesting that endothelin-1 induces vasodilation by releasing EDRF (Wang et al., 1994). In contrast, Baydoun et al. (1990) have stated that the vasodilative action of endothelin-1 is independent of EDRF in the perfused rat heart. Therefore, it is a matter of controversy whether EDRF plays an important role in the vasodilation induced by endothelin-1 in the rat heart as it has been shown in other vascular beds (De Nucci et al., 1988; Ohlstein et al., 1990).

TAK-044 (*cyclo*[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl]disodium salt) is a new endothelin receptor antagonist which inhibits both 'constrictive' and 'vasodilative' endothelin ET_B receptors as well as endothelin ET_A receptors (Ikeda et al., 1994; Kikuchi et al., 1994; Kusumoto et al., 1994). Since TAK-044 reduces myocardial infarct size in an experimental rat model (Watanabe et al., 1995), it allows determination of endothelin-1-induced responses which may lead to extension of myocardial infarction induced by ischemia-reperfusion. In addition, TAK-044 is useful as a tool for pharmacological characterization of receptor subtypes in the coronary vasculature and myocardial cells of rats through which endothelin-1 modulates cardiac function. In the present study, we assessed the inhibitory effect of TAK-044 on endothelin-1-induced responses in rat heart preparations. The aims of this study were to determine (1) which receptor subtype is responsible for the endothelin-1-induced responses in rat coronary vessels and cardiac muscle, (2) whether endothelin-1-stimulated release of vasoactive substance can modulate cardiovascular tone and (3) which responses induced by endothelin-1 in these tissues is inhibited by TAK-044.

2. Materials and methods

2.1. Perfused rat heart preparations

Male Wistar rats (10 weeks old, CLEA, Japan) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Heparin (1000 IU/kg, Shimizu Co., Japan) was injected via the left femoral vein, and the heart was quickly removed. After removing adipose and connective tissue at 4°C, the heart was transferred to a heated organ chamber (37°C) and was perfused with oxygenated (95% O_2 and 5% CO_2) modified Krebs solution (in mM: NaCl 113, KCl 4.6, $CaCl_2$ 1.2, NaH_2PO4 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 22, glucose 10; pH 7.4) at a constant rate of 12 ml/min via a cannula inserted into the root of the aorta. Perfusion pressure was monitored with a pressure transducer (DX-312, Viggo-Spectramed, USA), and a balloon was placed inside the left ventricle to measure left ventricular developed pressure. The volume of the balloon was adjusted to maintain diastolic pressure below 5 mm Hg. The basal left ventricular developed pressure was around 80 mm Hg, which was similar to that observed in other studies (Liu et al., 1993; Hara and Abiko, 1995; Maulik et al., 1993). Heart rate was counted from the changes of left ventricular developed pressure wave using a tachometer (NEC Sanei, Japan). After a 30-min equilibration period, endothelin-1 was administered by bolus injection in the presence or absence of inhibitors. Since the vasoconstriction and inotropic effect induced by a higher dose of endothelin-1 (> 10 pmol/heart) did not return to the basal level even after 30 min, we injected endothelin-1 into each heart only one time. Infusion of the drugs was started 10 min before injection of endothelin-1, and they were continuously administered throughout the experiments. Effluent perfusate samples were collected with a syringe before and after injection of endothelin-1. Thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ output were measured using enzyme immunoassay kits (Amersham Japan).

2.2. Isolated papillary muscle preparations

10-week-old male Wistar rats were anesthetized, and the heart was removed. The left papillary muscle was cut and mounted between two platinum bipolar electrodes in a small organ bath filled with oxygenated (95% O_2-5% CO_2) modified Krebs solution (37°C, pH 7.4). The preparation was stimulated with a 1 Hz pulse (5 V, 3 ms). Resting tension of 0.5–1 g was applied where the developed tension exhibited sub-maximum responses. After a 1-h stabilization period, isoproterenol (1–10 μM, Wako Pure Chemical, Japan) was added to the bath to obtain the maximal increase in developed tension and followed by washout with the solution. 1 h after washing out isoproterenol, TAK-044 or BQ-123 was added 5 min prior to application of endothelin-1 at 10 nM. All data were normalized using maximal responses to isoproterenol.

2.3. Binding assay in rat ventricle

The heart was removed from anesthetized Wistar rats (10 weeks old). Ventricular membrane fractions were prepared according to the method previously reported (Glosmann and Ferry, 1985). Briefly, rat ventricle was homogenized in 20 mM $NaHCO_3$ containing 0.1 mM phenylmethyl sulfonyl fluoride and centrifuged at $1500 \times g$ for 15 min. The supernatant was then centrifuged at $45\,000 \times g$ for 15 min. The pellet was

homogenized with 50 mM Tris-HCl (pH 7.4) and 0.1 mM phenylmethyl sulfonyl fluoride. The same protocol was repeated twice, and the final suspension was stored at −80°C. All procedures were performed at 4°C. The protein concentration was determined according to the Lowry method (Lowry et al., 1951).

In the binding assay, [^{125}I]endothelin-1 (60 pM, Amersham Japan) was incubated with 20 μg of protein in medium (300 μl) containing 50 mM Tris-HCl (pH 7.2) and 0.2% bovine serum albumin (Amersham, UK) at 37°C for 90 min. The medium was filtered through a glass-fiber filter (GF/B, Whatman, UK), rinsing with a 45-fold volume of ice-cold buffer (12.5 ml) to stop the reaction. Radioactivity was measured with a gamma counter. Specific binding was determined as the difference between the counts in the presence and absence of 0.4 μM cold endothelin-1.

2.4. Chemicals

Endothelin-1 and endothelin-3 were purchased from Peptide Institute (Japan). N^{ω}-Nitro-L-arginine methyl ester hydrochloride and indomethacin were purchased from Sigma Chemical (USA). TAK-044 (*cyclo*[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl]-disodium salt) and BQ-123 (*cyclo*(D-Asp-Pro-D-Val-Leu-D-Trp)) were synthesized by Takeda Chemical Industries, and dissolved in distilled water before the experiments.

2.5. Data analysis

All values are shown as means ± S.E.M. The concentration at which compounds reduced the effect of endothelin-1 to half of the maximal response (IC_{50}) was determined in each preparation with a non-linear least square's program using one- or two-site analysis with LBS program (Ikeda et al., 1991). The doses producing the half-maximal responses to endothelin-1 (ED_{50}) and the IC_{50} in perfused heart experiments were calculated from mean values for each dose. The paired *t*-test or one-way ANOVA with Dunnett's test was used for statistical analysis. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cardiovascular effects of endothelin-1 in the perfused rat heart

As shown in Fig. 1, bolus injection of endothelin-1, 3–1000 pmol/heart, evoked a transient dose-dependent fall in perfusion pressure (< 1 min) followed by a sustained increase in perfusion pressure (> 30 min). Endothelin-1 also increased left ventricular developed pressure in a dose-dependent manner. Although heart rate tended to increase, it did not change significantly (Fig. 1d). The basal values of these parameters did not differ significantly at the various dosages (87 ± 4-102 ± 7 mm Hg, 71 ± 2–84 ± 7 mm Hg and 231 ± 7–244 ± 7 beats/min for perfusion pressure, left ventricular developed pressure and heart rate, respectively, $n = 3–9$, $P > 0.05$). It should note that the highest dose of endothelin-1 induced ventricular fibrillation/tachycardia in one of the four hearts and increased end diastolic developed pressure in all hearts examined.

3.2. Inhibitory effects of endothelin antagonists in the perfused rat heart

TAK-044 (1 nM–10 μM) concentration dependently inhibited cardiovascular responses induced by 100 pmol/heart of endothelin-1 at which endothelin-1 showed a submaximal response (70–80% of the maximal response) for each of the cardiac parameters (Fig. 2), without changing the time to peak response of endothelin-1. TAK-044 inhibited the endothelin-1-induced increase in perfusion pressure with an IC_{50} value of 57 nM, and markedly attenuated the increase in perfusion pressure by 82% at 10 μM (Fig. 2a). On the other hand, BQ-123 (1 and 30 μM) caused partial reduction of the pressor response to endothelin-1 by 30–40% (Fig. 2a) at the concentration which sufficiently inhibited [^{125}I]endothelin-1 binding to endothelin ET_A receptors (Ihara et al., 1992). TAK-044 attenuated the endothelin-1-induced decrease in perfusion pressure with an IC_{50} value of 140 nM, and abolished it over 1 μM (Fig. 2b). In contrast, BQ-123 (1 and 30 μM) did not alter it (Fig. 2b). TAK-044 inhibited the endothelin-1-induced increase in left ventricular developed pressure with an IC_{50} value of 1.3 nM and abolished the effect at 10 nM (Fig. 2c). In addition, BQ-123 at 1 μM completely inhibited the increase in left ventricular developed pressure (Fig. 2c). The basal values of the cardiovascular parameters did not differ at the various dosages of TAK-044 and BQ-123 (84 ± 5–93 ± 6 and 73 ± 6–83 ± 4 mm Hg for perfusion pressure and left ventricular developed pressure, respectively, $n = 6–8$, $P > 0.05$). Neither TAK-044 nor BQ-123 itself modified the above-mentioned basal values.

The effluent 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2 was measured. Endothelin-1 (100 pmol/heart) significantly augmented the release of 6-keto-prostaglandin $F_{1\alpha}$, a stable metabolite of prostacyclin, 22 times: 1.1 ± 0.1 pg/min before endothelin-1 and 24.6 ± 1.9 pg/min after the endothelin-1 (at the time of the maximal decrease in perfusion pressure, $n = 5$, $P < 0.01$, paired *t*-test). However, endothelin-1 did not

alter the efflux of thromboxane B_2, a thromboxane A_2 metabolite: 129.8 ± 22.6 pg/min before versus 145.4 ± 20.5 pg/min after treatment (at the time the maximal increase in perfusion pressure occurred, $n = 5$, $P > 0.05$, paired t-test). TAK-044 diminished 6-keto-prostaglandin $F_{1\alpha}$ release by endothelin-1 in a concentration-dependent manner with an IC_{50} value of 68 nM, and complete inhibition was obtained at 1 μM, which was consistent with the concentration dependency of TAK-044 observed in the inhibition of the endothelin-1-induced decrease in perfusion pressure. The cyclooxygenase inhibitor indomethacin (10 μM) decreased the basal release of 6-keto-prostaglandin $F_{1\alpha}$: 0.95 ± 0.23 and 0.38 ± 0.10 pg/min before and during infusion, respectively ($n = 6$, $P < 0.05$, paired t-test), and abolished endothelin-1-stimulated production of 6-keto-prostaglandin $F_{1\alpha}$: 0.32 ± 0.05 pg/min ($n = 6$) after endothelin-1. However, it did not significantly modify the transient decrease in perfusion pressure induced by endothelin-1 at 100 pmol/heart (Fig. 3). Basal perfusion pressure was reduced by indomethacin (10 μM): 110.0 ± 4.3 mm Hg versus 90.5 ± 5.7 mm Hg for the pre- versus post-treatment, respectively ($n = 6$, $P < 0.05$, paired t-test). Indomethacin did not alter the increases in perfusion pressure and left ventricular developed pressure induced by endothelin-1; the peak increases in perfusion pressure and left ventricular developed pressure were 31.2 ± 5.8 and 31.3 ± 8.5 mm Hg for indomethacin group ($n = 6$) and 27.9 ± 5.2 and 35.3 ± 4.4 mm Hg for vehicle group ($n = 9$), respectively. The NO synthetase inhibitor N^{ω}-nitro-L-arginine methyl ester (100 μM) itself increased perfusion pressure by 25.6 ± 2.5 mm Hg from the baseline value ($n = 6$). It displayed no effect on the depressor response of endothelin-1 at 100 pmol/heart; the peak value of vasodilation was 37.0 ± 2.0 mm Hg (Fig. 3).

3.3. The direct inotropic effects of endothelin-1 on cardiac tissue

Endothelin-1 at 10 nM increased the developed tension of the rat papillary muscle with a slow onset of

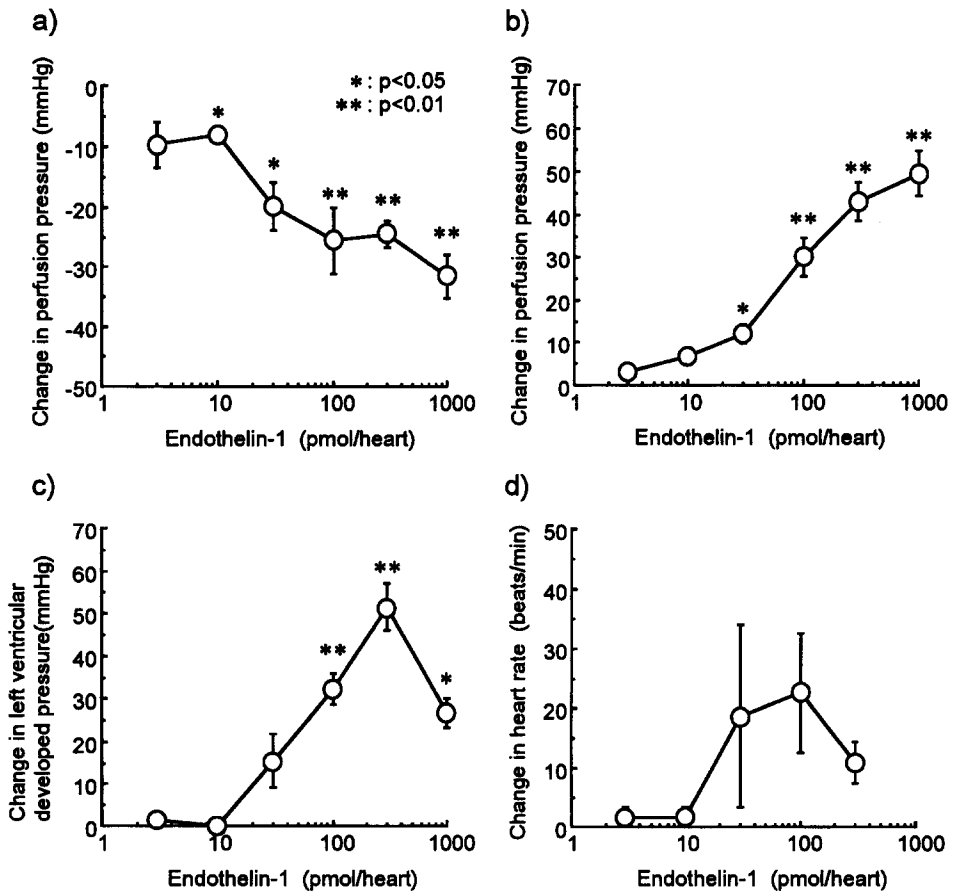

Fig. 1. Concentration-response curves for bolus injections of endothelin-1 in (a) decreasing perfusion pressure, (b) increasing perfusion pressure, (c) increasing left ventricular developed pressure and (d) heart rate in the perfused rat heart. Values shown are means ± S.E.M. $n = 3$–9. * $P < 0.05$, ** $P < 0.01$ versus the baseline value (paired t-test).

action, and took over 20 min to reach plateau level. It did not alter the resting tension of the muscle. Both TAK-044 and BQ-123 concentration dependently attenuated endothelin-1-induced increases in developed tension, and the highest concentrations of TAK-044 (100 nM) and BQ-123 (10 μM) inhibited them by about 80%. The peak values of the developed tension were 13.8 ± 2.0, 2.8 ± 1.7 and 2.7 ± 1.5% in the presence of vehicle (n = 7), TAK-044 (n = 5) and BQ-123 (n = 6), respectively (Fig. 4). Considering the concentration-response curves of TAK-044 and BQ-123, the inhibitory effect of TAK-044 was approximately 100 times more potent than that of BQ-123 (Fig. 4).

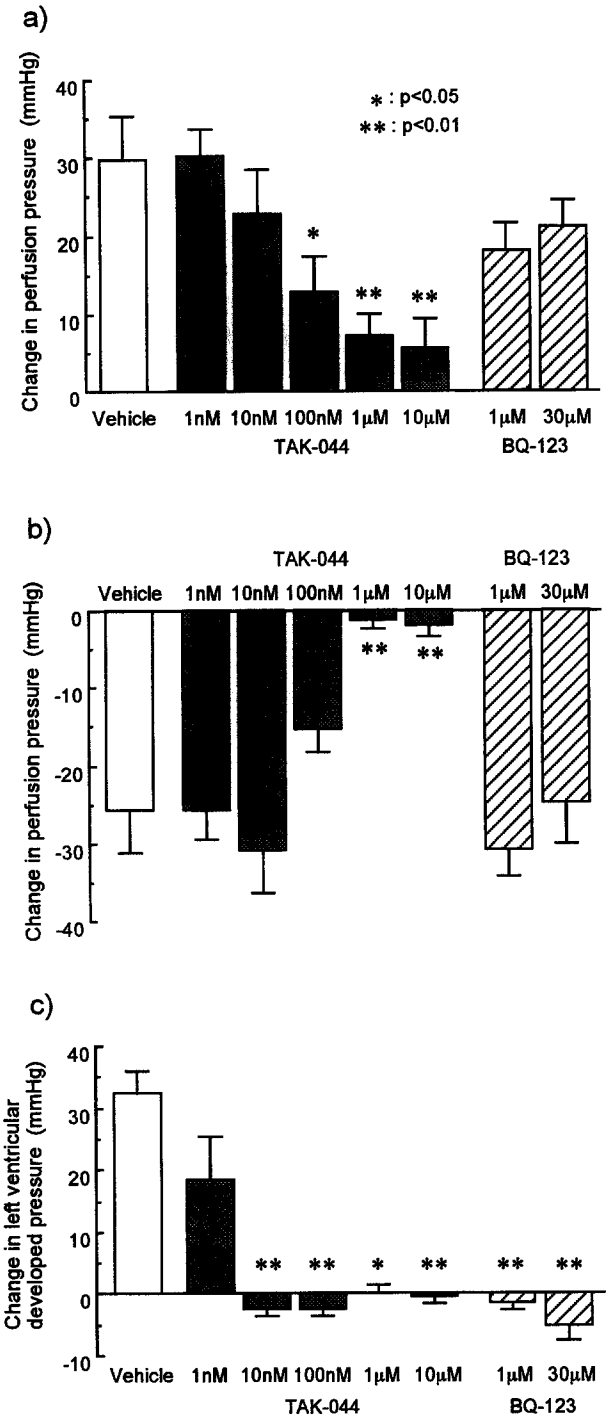

Fig. 2. Effects of vehicle, TAK-044 and BQ-123 on endothelin-1-induced (a) increase in perfusion pressure, (b) decrease in perfusion pressure and (c) increase in left ventricular developed pressure in perfused rat hearts. n = 6–8. Values shown are means ± S.E.M. * P < 0.05, ** P < 0.01 versus the vehicle group (one-way ANOVA and Dunnett's test).

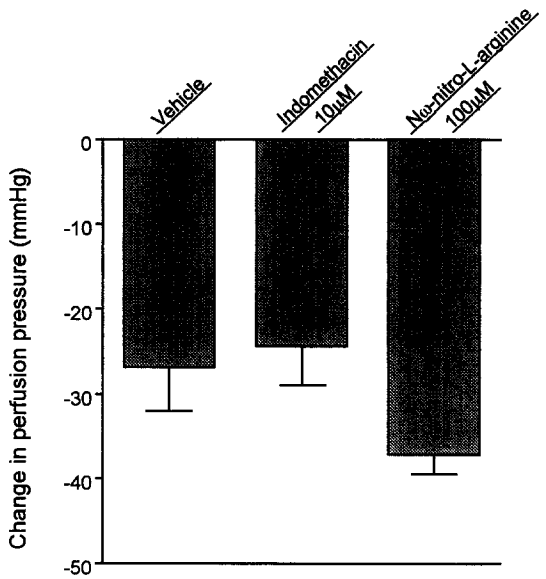

Fig. 3. Effects of vehicle, indomethacin and N^{ω}-nitro-L-arginine methyl ester on the endothelin-1-induced decrease in perfusion pressure in the perfused rat heart. n = 6–9. Values shown are means ± S.E.M.

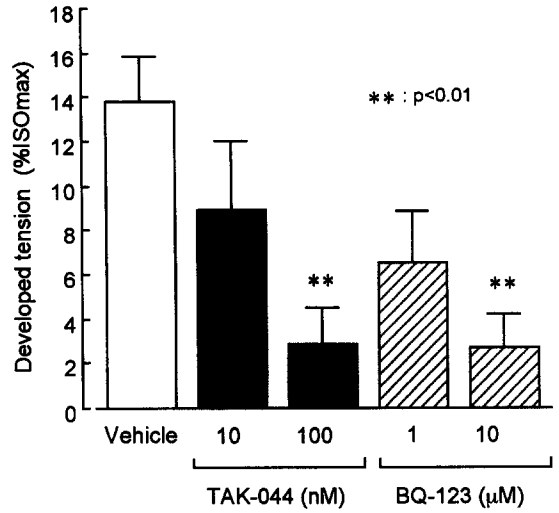

Fig. 4. Effect of vehicle, TAK-044 and BQ-123 on the endothelin-1-induced increase in developed tension in isolated rat papillary muscle. Values shown are means ± S.E.M. n = 5–7. ** P < 0.01 versus the vehicle group (one-way ANOVA and Dunnett's test).

Table 1
Calculated IC_{50} values of endothelin-1 ($n = 3$), endothelin-3 ($n = 5$), TAK-044 ($n = 4$) and BQ-123 ($n = 5$) for endothelin ET_A and endothelin ET_B receptors in the rat ventricular membrane preparation

	IC_{50} (nM)			
	ET_A receptor		ET_B receptor	
Endothelin-1		0.12 ± 0.02 (100%)		
Endothelin-3	11 ± 3	(54 ± 7%)	0.25 ± 0.09	(46 ± 7%)
TAK-044	0.39 ± 0.06	(53 ± 7%)	370 ± 180	(47 ± 7%)
BQ-123	36 ± 9	(60 ± 4%)	> 100000	(40 ± 4%)

Values shown are means ± S.E.M.

3.4. Binding assay in rat cardiac membrane fractions

Specific binding of [^{125}I]endothelin-1 in rat ventricular membrane fractions was displaced by cold endothelin-1 in a concentration-dependent manner with a single high affinity site (Hill slope: 1.02 ± 0.04, $n = 3$), and the IC_{50} value of endothelin-1 was calculated at 0.12 ± 0.02 nM ($n = 3$, Fig. 5 and Table 1). Endothelin-3, TAK-044 and BQ-123 also inhibited [^{125}I]endothelin-1 binding in membrane fractions (Fig. 5). However, the displacement profiles for these three ligands were different from that of endothelin-1. Endothelin-3 displaced bound [^{125}I]endothelin-1 more gradually than endothelin-1 (Fig. 5). In the case of TAK-044, it biphasically inhibited [^{125}I]endothelin-1 binding (Fig. 5). The first component of the displacement was observed between 0.03 and 30 nM, and inhibition reached the plateau phase. The second component occurred at over 0.1 μM and specific binding of [^{125}I]endothelin-1 was abolished at 10 μM. BQ-123 inhibited [^{125}I]endothelin-1 binding at concentrations as low as 10 nM, with a plateau phase between 300 nM and 1 μM, and a slight reduction was observed between 3 and 100 μM (Fig. 5). Even at the highest concentration of BQ-123 (100 μM), 28% of the total [^{125}I]endothelin-1 bound remained undisplaced. These findings clearly indicate that two or more receptor subtypes exist in rat ventricle preparations. Indeed, optimal curve fits for endothelin-3, TAK-044 and BQ-123 were obtained by two-site analysis. According to the results of computer analysis, endothelin-3 was found to have a high and low affinity site with IC_{50} values of 0.25 ± 0.09 nM (the ratios were 46 ± 7% of the total [^{125}I]endothelin-1 bound, $n = 5$) and 11 ± 3 nM (54 ± 7%, $n = 5$), respectively. Two different affinity sites for TAK-044 were also detected in rat cardiac membrane fractions; the IC_{50} values of these two sites were 0.39 ± 0.06 nM (53 ± 7%, $n = 4$) and 370 ± 180 nM (47 ± 7%, $n = 4$), respectively. BQ-123 possessed high affinity sites with an IC_{50} value of 36 ± 9 nM (60 ± 4%, $n = 5$), and it bound to another site over 100 μM (40 ± 4%, $n = 5$). A previous report on the affinities of endothelin-1, endothelin-3, BQ-123 and TAK-044 for endothelin receptor subtypes (Kikuchi et al., 1994) demonstrated that this membrane preparation derived from rat ventricle contains endothelin ET_A and endothelin ET_B receptors. As shown in Table 1, TAK-044 inhibited endothelin ET_A receptors 100 times more potently than BQ-123 did. TAK-044, unlike BQ-123, also markedly inhibited endothelin ET_B receptors, and it abolished the specific binding of [^{125}I]endothelin-1 at 10 μM.

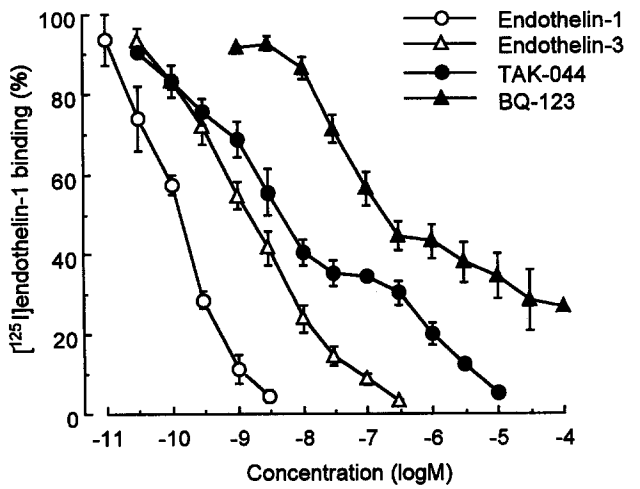

Fig. 5. Displacement of specific binding of [^{125}I]endothelin-1 by endothelin-1, endothelin-3, TAK-044 and BQ-123 in the rat ventricular membrane fraction. $n = 3$–5. Values shown are means ± S.E.M.

4. Discussion

In this paper, we have reported that endothelin-1 evoked transient vasodilation followed by sustained vasoconstriction, and that it also had a positive inotropic effect on the rat heart perfused with a constant flow. The endothelin ET_A/ET_B receptor antagonist TAK-044 concentration dependently attenuated all endothelin-1-induced responses. The selective endothelin ET_A receptor antagonist BQ-123, on the other hand,

abolished the endothelin-1-induced increase in cardiac contractility, but displayed partial or no inhibition against vascular events caused by endothelin-1.

The constriction evoked by endothelin-1 in rat coronary resistance vessels is believed to be mediated by activation of both endothelin ET_A and endothelin ET_B receptor subtypes based on the following considerations. First, TAK-044 inhibited almost all the sub-maximal pressor effects of endothelin-1 over 1 μM (Fig. 2a), where it completely displaces the specific binding of [^{125}I]endothelin-1 to both endothelin ET_A and endothelin ET_B receptors in various preparations (Kikuchi et al., 1994; Watanabe et al., 1995; Table 1). Second, pretreatment with BQ-123 (1 and 30 μM) caused a partial attenuation of the increase in perfusion pressure induced by endothelin-1 at which it fully inhibited endothelin ET_A receptors (Ihara et al., 1992; Fig. 2). Finally, endothelin ET_B receptor-mediated coronary constriction is also observed in dog and pig coronary vessels (Teerlink et al., 1994; Watanabe et al., 1995). In contrast, Wang et al. (1994) claimed that the endothelin ET_B receptor antagonist IRL 1038 failed to inhibit endothelin-1-induced coronary vasoconstriction in the rat heart. However, it has recently been reported that IRL 1038 antagonizes only the endothelin ET_{B1} receptor subtype, which contributes to vasodilation by endothelin isomers, and not the endothelin ET_{B2} receptor subtype, which mediates the venous constriction and pressor responses of endothelin (Sudjarwo et al., 1993; Waner et al., 1993; Clozel et al., 1994). Since TAK-044 inhibits both 'vasodilative' and 'vasoconstrictive' endothelin ET_B receptor subtypes in anesthetized rats (Ikeda et al., 1994), it seems that the endothelin ET_{B2} receptor (the 'vasoconstrictive' endothelin ET_B receptor) is a major pathway of endothelin-1-induced constriction in rat coronary resistance vessels. Thus, the constriction of coronary arteries via activation of endothelin ET_B receptors seems to be an important pathway for endothelin-1 in the regulation of coronary blood flow in mammalian hearts, the same as shown in other organs (Clozel et al., 1992; Cristol et al., 1993). To confirm our conclusion, we should conduct an experiment with a specific endothelin ET_{B2} receptor antagonist, but none is yet available.

In contrast to our observation that BQ-123 does not significantly attenuate endothelin-1-induced vasoconstriction, Wang et al. (1994) reported that BQ-123 significantly inhibited the coronary vasoconstriction evoked by endothelin-1 in the Sprague-Dawley rat. Although it is difficult to explain why we did not detect a significant effect of BQ-123, one possible explanation is that the contribution of endothelin ET_A receptors to the endothelin-1-induced coronary constriction in our preparation (30–40%) was lower than in the preparation Wang et al. (1994) used (50–60%). Although the cause of the difference between the two studies remains to be determined, it may be attributable to the strain or age of the rats.

It has been reported that endothelin-1 constricts rat aorta not only directly but indirectly via the production of thromboxane A_2 (Reynolds and Mok, 1990; Taddei and Vanhoutte, 1993). However, no significant stimulation of thromboxane B_2 release was detected in response to an application of endothelin-1 in coronary resistance arteries, as shown in the present study. In addition, we have demonstrated that indomethacin causes no attenuation of endothelin-1-induced coronary vasoconstriction. Although thromboxane A_2 may be a modulator of basal coronary tone in rats, because of the reduction of basal perfusion pressure accompanied by a decrease in thromboxane B_2 release after application of indomethacin, it does not produce any additional effects on the endothelin-1-induced coronary constriction in rats.

The IC_{50} value of TAK-044 for the depressor effect of endothelin-1 in perfused rat heart (coronary vasodilative effect of endothelin-1) was 140 nM, which is consistent with that for endothelin ET_B receptors (Kikuchi et al., 1994; Watanabe et al., 1995). BQ-123, on the other hand, did not affect the depressor action of endothelin-1 (Fig. 2b). These results suggest that the endothelin-1-induced reduction of perfusion pressure was mediated by vasoactive substances produced via stimulation of endothelin ET_B receptors in the perfused rat heart, and not by those increased via activation of endothelin ET_A receptors, such as the atrial natriuretic peptide released from rat atrial cells (Thibault et al., 1994). Endothelin-1-induced vasodilation has been reported not to be inhibited by oxyhemoglobin, and no stimulation of prostacyclin release was observed in response to application of endothelin-1 to the rat heart (Baydoun et al., 1990). Our findings in this study support the former observation, since N^{ω}-nitro-L-arginine methyl ester did not attenuate endothelin-1-induced vasodilation, indicating that EDRF plays no or only a minor role in the effects of endothelin-1. On the other hand, we observed that endothelin-1 increased 6-keto-prostaglandin $F_{1\alpha}$ release. The discrepancy regarding the effect on prostacyclin production in the two studies seems to depend on the dosage of endothelin-1. The former study used 10 pmol/heart of endothelin-1, a dose 10 times lower than we used. However, the augmented prostacyclin release by endothelin-1 does not seem to fundamentally contribute to its vasodilative effects in the rat heart, since the depressor action of endothelin-1 was not modulated by indomethacin at 10 μM where it completely abolishes the 6-keto-prostaglandin $F_{1\alpha}$ release augmented by endothelin-1. One possible explanation is that the amount of prostacyclin released may be far too small to dilate coronary vessels. Alternatively, inhibition of prostacyclin efflux can not counteract endothelin-1-induced

effects, because endothelin-1 produced other vasorelaxant substances. Recently, it was claimed that the vasodilation induced by endothelin-1 in the rat heart was completely inhibited under high extracellular K^+ condition (Sakuma et al., 1993). This suggests that endothelium-derived hyperpolarizing factor may play an important role in the vasodilation induced by endothelin-1 in the rat heart. In contrast, the opposite observation has been reported, i.e., that the NO synthetase inhibitor N^G-nitro-L-arginine enhances endothelin-1-induced coronary flow reduction in the rat heart perfused with a constant pressure (Wang et al., 1994). However, under those experimental conditions, coronary flow is reduced by application of endothelin-1 even in the absence of N^G-nitro-L-arginine. Since EDRF is released under hypoxic conditions and dilates vessels (Pohl and Busse, 1989), contribution of EDRF to endothelin-1-induced modulation of vascular tone may be indirect and secondary to the reduction of coronary flow by endothelin-1.

In isolated rat ventricular muscle, endothelin-1 has often been reported to have a positive inotropic effect (Takanashi and Endoh, 1991; Moravec et al., 1989; Li et al., 1991 and Fig. 4), weaker than that of the β-adrenoceptor agonist isoproterenol and the Ca^{2+} channel activator BAY K 8644, and similar to that of the α_1-adrenoceptor agonist phenylephrine and the phosphodiesterase type III inhibitor milrinone (Ruch et al., 1992; Katano and Endoh, 1992; Li et al., 1991). In the Langendorff perfused rat hearts, we also showed that endothelin-1 increases cardiac contractility (Fig. 1). In contrast, some investigators have failed to find any positive inotropic effect of endothelin-1 in rat heart perfused at a constant pressure (Neubauer et al., 1990; Wang et al., 1994). The lack of a positive inotropic effect of endothelin-1 in their studies is probably attributable to the marked reduction of coronary flow which leads to an ischemic condition of the heart. This suspicion is supported by the observation of Firth et al. (1990) that low doses of endothelin-1 increase the cardiac output of the rat heart without modulating coronary flow, whereas high doses decrease cardiac output and are associated with a large fall in coronary flow. Therefore, in our study, the positive inotropic effect of endothelin-1 appears to be isolated from the secondary counteraction produced by reduced coronary flow, since coronary flow was kept constant by our experimental method.

The positive inotropic effect of endothelin-1 in the rat heart is considered to represent a direct action on cardiac myocytes, not an indirect effect via the production of the cardiotonic substance prostacyclin (Pavlovic et al., 1992), since we demonstrated that indomethacin does not modify the effects of endothelin-1. The effect of endothelin-1 is probably evoked by activating endothelin ET_A receptors based on the following considerations. First, BQ-123 markedly inhibited endothelin-1-induced positive inotropic effects in perfused hearts and isolated papillary muscles (Fig. 2 and 4). Second, the concentration ranges of TAK-044 and BQ-123 in the prevention of endothelin-1-induced positive inotropic effects in isolated rat papillary muscle are consistent with those obtained for the blockage of endothelin ET_A receptors (Fig. 4 and Table 1).

It has been reported that endothelin ET_A receptors in rat cardiac cells, but not endothelin ET_B receptors, mediate various responses, such as phosphoinositide hydrolysis, adenylate cyclase inhibition and protein synthesis (Ito et al., 1993; Hilal-Dandan et al., 1994). Thus, the cardiac effects of endothelin-1, including its positive inotropic action, seem to mainly occur via endothelin ET_A receptors. In this study, however, we have reported that rat heart membrane preparations also contain a rather large amount of endothelin ET_B receptors (about 40% of total [^{125}I]endothelin-1 binding). The endothelin ET_B receptors observed in this preparation may represent contamination by coronary smooth muscle or endothelium, since it is difficult to separate these cells from ventricular myocytes. Alternatively, endothelin ET_B receptors may exist on the surface of cardiac cells, since there is evidence that the endothelin ET_B receptor and its mRNA are expressed in human and rat ventricular myocytes (Hori et al., 1992; Molenaar et al., 1993). Further study is necessary to determine the role of the endothelin ET_B receptor in ventricular myocytes.

It has been demonstrated that increases in endogenous endothelin-1 extend myocardial infarction in the rat model, since a monoclonal antibody against endothelin-1, endothelin-converting enzyme inhibitor and TAK-044 reduced infarct size (Watanabe et al., 1991, 1995; Grover et al., 1992). In this study, we have shown that TAK-044 attenuates the constriction of coronary vessels and the increase in cardiac contractility by endothelin-1 in the rat heart. This may explain the cardioprotective effects of TAK-044 in the rat ischemia-reperfusion model, since endothelin-1-induced coronary vasoconstriction and increased cardiac contractility results in decreased oxygen supply and increased oxygen demand in the rat heart, detrimental factors in the progression of ischemic damage to the heart. However, further study, especially in regard to energy metabolism, is necessary to clarify the precise mechanism of endothelin-1-induced extension of myocardial infarction in the rat heart.

In conclusion, endothelin-1 evoked several responses such as coronary vasoconstriction and vasodilation and displayed a positive inotropic effect in the rat heart by activating endothelin ET_A and endothelin ET_B receptors, and TAK-044 attenuated the changes in these cardiovascular parameters induced by endothelin-1 by blocking these receptor subtypes.

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