Mechanisms of the biphasic responses to endothelin-3 in dog coronary arteries

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1 Endothelin-3 (ET-3) elicited relaxations at low concentrations (up to 10^{-8} M) and contractions at higher concentrations in dog isolated coronary arteries precontracted with prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$). The relaxation by ET-3 was not affected by endothelium denudation nor treatment with N^G-nitro-L-arginine, but was abolished or reversed to a contraction by treatment with indomethacin and markedly suppressed by tranilcypromine, a PGI $_2$ synthetase inhibitor, or diphloretin phosphate, a prostaglandin receptor antagonist. ET-1 produced only concentration-dependent contractions.

2 BQ-123, a new selective ET $_A$ receptor antagonist, caused relaxation of the strips contracted with ET-3 in a dose-dependent manner and prevented the ET-3-induced contraction but did not affect the contraction produced by PGF $_{2\alpha}$. The relaxation caused by ET-3 was enhanced by treatment with BQ-123.

3 It is concluded that the relaxations elicited by ET-3 in dog coronary arteries are mediated via liberation of PGI $_2$ by activation of non-ET $_A$ receptors, located in subendothelial tissues, possibly smooth muscle cells, whereas the peptide-induced contractions are mediated via ET $_A$ receptors.

Keywords: Endothelin; ET $_A$ receptor; vasorelaxation; dog coronary artery; endothelium; PGI $_2$

Introduction

Endothelin (ET) was first described as an endothelium-derived, vasoconstrictor peptide (Yanagisawa *et al.*, 1988), but has also been demonstrated to have a vasodilator action. ET-1 or ET-3 has been reported to cause only contraction (Yanagisawa *et al.*, 1988; Fukuroda *et al.*, 1992) or relaxation at low concentrations (Fukuroda *et al.*, 1992; Ushio-Fukai *et al.*, 1992) in porcine coronary or pulmonary arteries. Intravenous bolus injections of ET-1 or ET-3 cause a transient, dose-dependent depressor response followed by a sustained pressor response in anaesthetized rats (Wright & Fozard, 1988; Gardiner *et al.*, 1989; Inoue *et al.*, 1989) and cats (Minkes *et al.*, 1989; Lippton *et al.*, 1988). ET-3 possesses greater vasodilator activity than ET-1 (Inoue *et al.*, 1989; Minkes *et al.*, 1989). The ET-induced vasodilatation has been considered to be mediated by endothelium-derived relaxing factor(s) (EDRF), since the depressor responses to ET are abolished by pretreatment with N^G-monomethyl-L-arginine (Fukuda *et al.*, 1990), a nitric oxide synthase inhibitor, or endothelium denudation (Warner *et al.*, 1989; Fukuda *et al.*, 1991; Ushio-Fukai *et al.*, 1992), and markedly inhibited by treatment with methylene blue or oxyhaemoglobin (Warner *et al.*, 1989) in rat isolated perfused mesenteric arteries. On the other hand, in *in vivo* experiments in dogs, N^G-nitro-L-arginine in concentrations sufficient to inhibit nitric oxide synthase partially inhibits but does not abolish the ET-induced depressor response, and the remaining response is abolished by additional treatment with cyclo-oxygenase inhibitors, suggesting the involvement of vasodilator prostaglandins (Yamashita *et al.*, 1991). An ability of endothelin to release prostacyclin (PGI $_2$) has been demonstrated in guinea-pig and rat isolated lungs (De Nucci *et al.*, 1988).

Recently, two different ET receptors have been cloned and termed ET $_A$ and ET $_B$ (Arai *et al.*, 1990; Sakurai *et al.*, 1990). BQ-123 has been introduced as a selective antagonist for the ET $_A$ receptor subtype (Ihara *et al.*, 1992). The present study was, therefore, undertaken to examine the mechanism of

ET-induced relaxations in dog isolated coronary arteries, with reference to endothelial function and receptor subtypes.

Methods

Mongrel dogs of either sex, weighing 8 to 15 kg, were anaesthetized with intravenous injections of sodium pentobarbitone (30 mg kg $^{-1}$) and killed by bleeding from the carotid arteries. The heart was rapidly removed. Descending and circumflex branches of the left coronary artery (0.6 to 0.8 mm outside diameter) were isolated and cut into helical strips approximately 20 mm long, with special care being taken to avoid endothelial damage. The tissue was fixed vertically between two hooks in a muscle bath containing modified Ringer-Locke solution, which was maintained at $37 \pm 0.3^\circ\text{C}$ and aerated with a mixture of 95% O $_2$ and 5% CO $_2$. The hook anchoring the upper end of the strip was connected to the level of a force-displacement transducer (Nihon-Kohden Kogyo Co., Tokyo, Japan). The resting tension was adjusted to 1.5 g, which is optimal for inducing the maximal contraction. The composition of the bathing medium was (mM): NaCl 120, KCl 5.4, CaCl $_2$ 2.2, MgCl $_2$ 1.0, NaHCO $_3$ 25.0 and dextrose 5.6. The pH of the solution was 7.35 to 7.42. The strips were allowed to equilibrate for 60 to 90 min in the bathing medium during which time the solution was replaced three times every 10 to 15 min.

Isometric contractions and relaxations were displayed on an ink-writing oscillograph (Nihon-Kohden Kogyo Co.). A contractile response to 30 mM K $^+$ was first obtained, and the artery strips repeatedly washed with the fresh medium and reequilibrated. The strips were partially contracted with prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$, 10^{-7} to 10^{-6} M), the contraction being in the range of 20 and 35% of the contraction induced by 30 mM K $^+$. Concentration-response curves for endothelin-3 (ET-3), BQ-123 and beraprost, a PGI $_2$ analogue (Toda, 1988), were obtained by adding the agents directly to the bathing medium in cumulative concentrations. The artery strips were treated for approximately 20 min with antagonists, before the concentration-response curves for agonists

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were obtained. Because of a lack of reproducibility, the first concentration-response curves in the absence (control) and presence of pharmacological antagonists were compared in 2–4 strips obtained from the same dogs. At the end of each series of experiments, papaverine (10^{-4} M) was applied to attain the maximal relaxation. Contractions and relaxations induced by the test drugs are presented as percentage values relative to those caused by 30 mM K^+ and 10^{-4} M papaverine, respectively, unless otherwise mentioned. The endothelium of artery strips was removed by gently rubbing the intimal surface with a cotton pellet; unrubbed strips from the same dogs were used for comparison. Successful removal of the endothelium was determined by abolition of relaxations induced by 10^{-7} M acetylcholine and was confirmed histologically by the silver staining procedure.

The results shown in the text and figures are expressed as the mean $\pm$ s.e.mean. Statistical analyses were made using Student's paired and unpaired *t* test and Tukey's method after one-way analysis of variance. Drugs used were acetylcholine chloride (Daiichi Seiyaku Co., Tokyo, Japan), tranylcypromine, indomethacin (Sigma, St. Louis, MO, U.S.A.), prostaglandin $F_{2\alpha}$ (Ono Pharmaceutical Co., Osaka, Japan), endothelin (ET)-1 and 3, N^G -nitro-L-arginine (Peptide Institute Inc., Minoh, Japan), diphloretin phosphate (Leo, Helsingborg, Sweden), beraprost (sodium ($\pm$)-4[1R,2R, 3aS,8bS]-1,2,3a,8b-tetrahydro-2-hydroxy-1-[(3S,4RS)-3-hydroxy-4-methyl-oct-6-yne-(E)-1-enyl] -5cylcopenta [b] benzofuranyl] butyrate; Toray industries, Inc., Tokyo, Japan) and papaverine hydrochloride (Dainippon Pharmaceutical Co., Osaka, Japan). BQ-123 (cyclo-(D-Asp-L-Pro-D-Val-L-Leu-D-Trp-) sodium) was synthesized in the Tsukuba Research Institute of the Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan).

Results

In dog coronary arterial strips partially contracted with $PGF_{2\alpha}$, the addition of ET-3 in concentrations of 3×10^{-9} and 10^{-8} M produced a concentration-dependent relaxation. The relaxations at 3×10^{-9} M were inconsistent; slight relaxations were observed in three out of six strips but no relaxations in the remaining three, whereas those at 10^{-8} M were consistent. Therefore, the mechanism underlying relaxations was analysed at 10^{-8} M ET-3. Relaxations at 10^{-8} M slowly reversed to contraction to the level that was lower or higher than that prior to the addition of ET-3. The strips responded to the peptide in concentrations higher than 3×10^{-8} M with only contraction. After stabilization of the response to 3×10^{-8} M ET-3, indomethacin caused a marked contraction (Figure 1a). Removal of the endothelium did not influence these responses induced by ET-3 and indomethacin (Figure 1b). Concentration-response curves to ET-3 in the arterial strips with and without the endothelium are shown in Figure 2, in which the maximal level of relaxations and contractions is presented. The apparent median effective concentration (EC_{50}) of ET-3 for the relaxation in the endothelium-intact strips was $[4.2 \pm 0.7] \times 10^{-9}$ M ($n = 11$), and the maximal relaxation attained at 10^{-8} M was $25.1 \pm 5.5\%$ ($n = 11$) relative to that induced by 10^{-4} M papaverine.

The responses caused by ET-3 were not reproducible on repeated administration, even though the strips were repeatedly rinsed after the application of ET-3. Therefore, modification by antagonists of the ET-3-induced relaxation was tested on the first responses in different strips obtained from the same dogs; one was used as a control and the others were for treatment with antagonists. In endothelium-intact strips, treatment with N^G -nitro-L-arginine (L-NNA, 10^{-5} M), sufficient to suppress the EDRF-mediated relaxant response (Toda *et al.*, 1990), did not inhibit the ET-3-induced relaxation. The relaxant responses to 10^{-8} M ET-3 in the control and L-NNA-treated strips were $23.7 \pm 7.6\%$ and $21.8 \pm 5.3\%$ ($n = 6$). In the endothelium-denuded strips, treatment with indomethacin (10^{-6} M) reversed the relaxation

Figure 1 Modification by indomethacin (IM, 10^{-6} M) of the responses to endothelin-3 (ET-3) in dog coronary artery strips precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) with (a and c) and without (b) the endothelium. Concentrations of ET-3 from 10 to $7.5 = 10^{-10}$, 10^{-9} , 10^{-8} and 3×10^{-8} M, respectively. Horizontal lines just left of each tracing represent the level before addition of $PGF_{2\alpha}$ (10^{-7} to 10^{-6} M).

Figure 2 Concentration-response curves for endothelin-3 (ET-3) in dog coronary artery strips with (●) and without (○) the endothelium. The strips were partially contracted with prostaglandin $F_{2\alpha}$ (10^{-7} M to 10^{-6} M). Since ET-3 at 10^{-8} M caused a relaxation followed by a contraction, both responses to the peptide are plotted. Relaxations induced by 10^{-4} M papaverine and contractions by 30 mM K^+ were taken as 100% for relaxation and contraction, respectively; mean absolute values for the relaxation in the arteries with and without the endothelium were 270 ± 34 mg and 279 ± 39 mg ($n = 11$), respectively, and those for the contraction were 1722 ± 209 mg and 1752 ± 233 mg ($n = 11$), respectively. Points indicate means and bars indicate s.e.mean. Numbers in parentheses indicate the number of strips from separate dogs.

induced by 10^{-8} M ET-3 to a contraction. Typical tracings from strips with and without the endothelium are shown in Figures 1c and 3c, respectively. The ET-3-induced relaxation was markedly suppressed by tranylcypromine (3×10^{-4} M) or

Figure 3 Responses to endothelin-3 (ET-3), indomethacin (IM, 10⁻⁶ M) and BQ-123 (BQ, 10⁻⁷ M) in dog coronary arteries without endothelium precontracted with prostaglandin F_{2α} (PGF_{2α}). Concentrations of ET-3 from 10 to 7.5 = 10⁻¹⁰, 10⁻⁹, 10⁻⁸ and 3 × 10⁻⁸ M, respectively; concentrations of BQ-123 from 8 to 6 = 10⁻⁸, 10⁻⁷ and 10⁻⁶ M, respectively; PA = 10⁻⁴ M papaverine. Horizontal lines just left of each tracing represent the level before addition of PGF_{2α} (10⁻⁷ to 10⁻⁶ M).

diphloretin phosphate (10⁻⁵ M). The quantitative data are summarized in Figure 4. Relaxations induced by beraprost were not significantly altered by treatment with tranilcypromine. The relaxant responses in the control and tranilcypromine-treated strips were 19.8 ± 6.8% and 31.3 ± 4.4%, respectively, at 10⁻⁸ M beraprost, and 55.5 ± 11.8% and 79.1 ± 2.4% (*n* = 5), respectively, at 10⁻⁷ M; the differences were not statistically significant.

ET-1 (10⁻⁹ to 10⁻⁸ M) elicited only concentration-dependent contractions in endothelium-intact and -denuded strips precontracted with PGF_{2α}. The ET-1-induced contractions at 10⁻⁹ and 3 × 10⁻⁹ M were 22.5 ± 10.1% and 51.0 ± 17.6%

Figure 4 Modification by tranilcypromine (TC, 3 × 10⁻⁴ M; a) and diphloretin phosphate (DPP, 10⁻⁵ M; b) of responses to endothelin-3 (ET-3, 10⁻⁸ M) in dog coronary artery strips without the endothelium. The strips were partially contracted with prostaglandin F_{2α} (10⁻⁷ to 10⁻⁶ M). Contractions induced by 30 mM K⁺ were taken as 100%; mean absolute values in control and TC-treated strips were 2242 ± 153 mg and 1895 ± 167 mg (*n* = 5), respectively, and those in control and DPP-treated strips were 1213 ± 187 mg and 1293 ± 354 mg (*n* = 3), respectively. Relaxations induced by 10⁻⁴ M papaverine were taken as 100%; mean absolute values in control and TC-treated strips were 220 ± 22 mg and 316 ± 77 mg (*n* = 5), respectively, and those in control and DPP-treated strips were 248 ± 29 and 223 ± 43 mg (*n* = 3), respectively. Significantly different from control (C), **P* < 0.01; ***P* < 0.001. Columns indicate means and bars represent s.e.mean.

relative to K⁺ (30 mM)-induced contraction, respectively, in the endothelium-intact strips, and 27.3 ± 7.4% and 54.0 ± 11.2%, respectively, in the endothelium-denuded strips.

The addition of BQ-123 (10⁻⁸ to 10⁻⁶ M) produced a dose-dependent relaxation in the denuded strips contracted by ET-3 in the absence and presence of indomethacin (Figure 3 a and c). Mean values of the relaxations at 10⁻⁸, 10⁻⁷ and 10⁻⁶ M BQ-123 were 69.0 ± 6.9%, 97.7 ± 1.6% and 99.4 ± 0.6% (*n* = 7), respectively, when the maximal contraction caused by 3 × 10⁻⁸ M ET-3 was taken as 100%. BQ-123 in these concentrations did not alter the tone of PGF_{2α}-contracted strips. Pretreatment with BQ-123 of the denuded strips suppressed the contractions caused by ET-3 (Figure 3b, Figure 5), but prolonged (Figure 3b) and potentiated the relaxations by the peptide (Figure 5).

Discussion

ET-3 elicited relaxation at low concentrations (up to 10⁻⁸ M) and contraction at higher concentrations in dog isolated coronary arteries precontracted with PGF_{2α}. Both responses were not endothelium-dependent, suggesting that endothelium-derived vasoactive substances such as EDRF (Furchgott & Zawadzki, 1980) or EDCF (De Mey & Vanhoutte, 1982; Toda *et al.*, 1988) are not involved. BQ-123 (Ihara *et al.*, 1992), a selective antagonist for ET_A receptors, significantly

Figure 5 Modification by BQ-123 (BQ) and indomethacin (IM) of contractile (a) and relaxant (b) responses to endothelin-3 (ET-3) in dog coronary arteries without the endothelium. The strips were partially contracted with prostaglandin F_{2α} (10⁻⁷ to 10⁻⁶ M). Since ET-3 at 10⁻⁸ M caused a relaxation followed by a contraction, both responses to the peptide are plotted. Contractions induced by 30 mM K⁺ and relaxations by 10⁻⁴ M papaverine were taken as 100% for contraction and relaxation, respectively; mean absolute values for the contraction in control (●), BQ (10⁻⁸ M)-treated (○), BQ (10⁻⁷ M)-treated (×) and IM (10⁻⁶ M)-treated (Δ) strips were 2344 ± 323 mg, 2190 ± 498 mg, 2230 ± 307 mg and 1924 ± 698 mg (*n* = 5), respectively; and those for the relaxation were 268 ± 49 mg, 294 ± 47 mg, 201 ± 12 mg and 308 ± 24 mg (*n* = 5), respectively. Points indicate means and bars represent s.e.mean. Significantly different from control, **P* < 0.05; ***P* < 0.01 (Tukey's method).

inhibited the ET-3-induced contractions in control and indomethacin-treated arteries and potentiated the relaxations in a concentration-dependent manner, whereas the PGF_{2α}-induced contraction was unaffected. Therefore, it appears that the contractions induced by ET-3 in dog coronary arteries are associated with ET_A receptors located in the smooth muscle as previously reported in porcine blood vessels (Fukuroda *et al.*, 1992), whereas the receptor subtype responsible for the relaxation induced by the peptide is distinct from ET_A. N^G-nitro-L-arginine failed to suppress the relaxations induced by ET-3 in endothelium-intact strips providing further evidence that the peptide-induced relaxation in dog coronary artery is unlikely to be mediated by endothelium-derived nitric oxide. On the other hand, indomethacin abolished the ET-3-induced relaxation in endothelium-intact and -denuded strips. Tranylcypromine, a PGI₂ synthetase inhibitor (Gryglewski *et al.*, 1976), markedly suppressed the relaxation caused by ET-3, but did not influence the response to beraprost, a PGI₂ analogue. Further, the peptide-induced relaxation was abolished by diphloretin phosphate, a prostaglandin receptor antagonist (Eakins, 1971), as is the response to PGI₂ (Akiba *et al.*, 1986). These results suggest that the relaxations elicited by ET-3 are mediated via liberation of PGI₂ by activation of non-ET_A receptors located in subendothelial tissues, possibly smooth muscle cells. Involvement of endogenous PGI₂ has been postulated in the relaxation induced by other peptides such as angiotensin II (Okamura *et al.*, 1990) and bradykinin (Toda *et al.*, 1987).

It is assumed that the ET receptor(s) responsible for

vasoconstriction is (are) distinct from those mediating vasorelaxation (Vane, 1990). In the present study, ET-1 did not produce significant relaxation. The difference between ET-1 and ET-3 may be explained by their different affinities for ET_A and the receptor(s) mediating relaxation. Potent vasoconstriction by ET-1 may mask the weak relaxation by the peptide. Recently, contraction-mediated by ET_B receptors has been demonstrated in porcine isolated arteries (Fukuroda *et al.*, 1992); however, the present results suggest that this does not occur in dog coronary arteries. Although numerous studies have demonstrated that ET-3-induced relaxations are endothelium-dependent (Warner *et al.*, 1989; Fukuda *et al.*, 1990; 1991), the present study has revealed an endothelium-independent, PGI₂-mediated mechanism for the ET-induced relaxation.

Endothelial cells synthesize and release vasoactive substances spontaneously or in response to physical and chemical stimuli and thus play an important role in the regulation of vascular tone. Although ET was introduced as a potent vasoconstrictor (Yanagisawa *et al.*, 1988), low concentrations of ET-3 elicited hypotension *in vivo* and vasorelaxation in certain isolated blood vessels. Chronic infusion of ET did not cause hypertension (Tanabe *et al.*, 1992). Therefore, ET in physiological concentrations may not produce vasoconstriction but rather vasodilatation. The present study suggests that ETs might produce vasodilatation mediated via ET receptors distinct from ET_A even when endothelial functions are damaged by vascular disorders such as atherosclerosis or severe hypertension.

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