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Cardioprotective effect of an endothelin receptor antagonist during ischaemia/reperfusion in the severely atherosclerotic mouse heart

[1]Adrian T. Gonon, [1]Alexander Bulhak, [2]Anders Bröijersén & *,[1]John Pernow

[1]Department of Cardiology, Karolinska University Hospital, S-17176 Stockholm, Sweden and [2]Center for Metabolism and Endocrinology, Karolinska University Hospital, S-17176 Stockholm, Sweden

1 Endothelin (ET) receptor antagonists are cardioprotective during myocardial ischaemia and reperfusion through a nitric oxide (NO)-dependent mechanism. The aim of the present study was to investigate whether the ET receptor antagonist, bosentan, is cardioprotective in atherosclerotic mice.

2 Buffer-perfused hearts from apolipoprotein E/LDL receptor double knockout (KO) and wild-type (WT) mice were subjected to global ischaemia and reperfusion.

3 Following reperfusion, the recovery of rate–pressure product (RPP; left ventricular developed pressure (LVDP) × heart rate) was equally impaired in WT and KO mice given vehicle (34 ± 8 and $29\pm9\%$, respectively). The ET_A/ET_B receptor antagonist bosentan ($10\,\mu mol\,l^{-1}$) improved recoveries to $57\pm10\%$ in WT and to $68\pm10\%$ in KO mice ($P<0.01$). Similar effects were observed for the recovery of left ventricular end-diastolic pressure (LVEDP), developed pressure and dP/dt.

4 Bosentan improved the recovery of coronary flow in both KO and WT mice. Recovery of coronary flow was significantly higher in the KO mice given bosentan ($135\pm15\%$) than in the WT group ($111\pm12\%$; $P<0.01$). ET-1 ($1\,nmol\,l^{-1}$) impaired recovery of coronary flow in both WT and KO mice though this effect was more pronounced in the KO mice ($P<0.01$).

5 Coronary outflow of NO during reperfusion was enhanced in both KO and WT mice following bosentan administration.

6 The ET_A/ET_B receptor antagonist bosentan protects the atherosclerotic mouse heart from ischaemia/reperfusion injury. The observation that ET receptor blockade and stimulation have a greater effect on coronary flow in atherosclerotic hearts indicates an increased activation of the ET system in atherosclerotic coronary arteries.
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Abbreviations: ET, endothelin-1; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; KO, knockout; NO, nitric oxide; RPP, rate–pressure product; WT, wild type

Introduction

Atherosclerosis is the predominant underlying cause of coronary heart disease. It has become evident that the vascular endothelium, *via* the production of different vasodilator and vasoconstrictor substances (Lüscher & Noll, 1995), is an important regulator of cardiovascular function and that endothelial dysfunction plays an important role in the pathogenesis of atherosclerosis (Ross, 1999; Libby, 2000). Nitric oxide (NO) is constitutively produced in endothelial cells by endothelial NO synthase and exerts important protective effects by reducing vascular tone, inhibiting leucocyte and platelet activation and inactivating superoxide (Lüscher & Noll, 1995). Another endothelium-derived substance is the potent vasoconstrictor endothelin-1 (ET-1) (Yanagisawa *et al*., 1988). ET-1 mediates vasoconstriction by binding to the ET_A and ET_B receptors located on smooth muscle cells. ET-1 may also cause release of NO *via* stimulation of ET_B receptors on endothelial cells resulting in vasodilatation. NO not only counteracts the vasoconstrictor effect of ET-1 (Kourembanas *et al*., 1991) but also inhibits the production and release of ET-1 from endothelial cells (Boulanger & Lüscher, 1990). In atherosclerotic arteries, the production of ET is enhanced and the vasoconstrictor response to exogenous ET-1 is more pronounced (Jiang *et al*., 2000). Pharmacological blockade of ET receptors results in improved endothelium-dependent vasodilatation in atherosclerotic mice (Barton *et al*., 1998) and in patients with atherosclerosis (Bohm *et al*., 2002b).

Endothelial dysfunction develops rapidly during reperfusion following myocardial ischaemia. This dysfunction includes both loss of NO-mediated vasodilatation and enhanced production and release of ET-1. Administration of selective ET_A and mixed ET_A/ET_B receptor antagonists during ischaemia and reperfusion results in reduction of infarct size, attenuation of neutrophil accumulation and improvement of myocardial contractile and endothelial function (Wang *et al*., 1995c; Brunner *et al*., 1997; Gonon *et al*., 1998; 2000; Gourine *et al*., 2001). It has been suggested that the cardioprotective effect afforded by ET receptor antagonists in healthy animal models is coupled to maintained bioavailability of NO. This suggestion is based on the finding that inhibition of NO synthase completely abrogated the ET receptor antagonist-

*Author for correspondence; E-mail: john.pernow@medks.ki.se
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induced protection (Gonon *et al*., 1998; 2000). However, it may be speculated that a cardioprotective effect of ET receptor blockade may not be evident in coronary atherosclerosis, which is the major underlying cause of acute myocardial infarction. Therefore, the aim of this study was to investigate whether the dual ET_A/ET_B receptor antagonist, bosentan, administered during ischaemia protects against ischaemia/reperfusion injury in an animal model with coronary atherosclerosis. This was investigated in knockout (KO) mice lacking the gene encoding apolipoprotein E and low-density lipoprotein (LDL) receptor and fed a cholesterol-rich diet. It has previously been demonstrated that this model develops an atherosclerosis that resembles human atherosclerotic lesions and endothelial dysfunction (Plump *et al*., 1992; Zhang *et al*., 1992; Nakashima *et al*., 1994; Jiang *et al*., 2000).

Methods

All investigations were approved by the Regional Ethics Committee for Animal Research and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH) publication No 85-23, revised 1985.

Animal preparation

Apolipoprotein E and LDL receptor double KO mice (Bomholt, Denmark) were fed a fat-rich diet (21% fat and 0.15% cholesterol; R683, AnalyCen, Lidköping, Sweden) for 7 months from the age of 6–8 weeks. Age-matched wild-type mice (WT; C57Bl/6) were fed only normal rodent food (BKU international fixed formula). The fat-rich diet leads to severe atherosclerosis in the KO mice (Figure 1) with increased cardiovascular mortality after 7 months (Caligiuri *et al*., 1999).

Only healthy mice without skin injuries and no neurological deficit were accepted for the study (Tokuno *et al*., 2002). WT and KO mice were heparinised and anesthetised with a mixture of fluanisonum, fentaylum and midazolam (2.5, 0.08 and 1.25 mg kg^{-1}, respectively, i.m.). The hearts were excised, the ascending aorta was cannulated and retrogradely perfused with noncirculating modified Krebs–Henseleit solution (in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.2, glucose 11.1 and pyruvate 2.0) at a constant pressure of 100 cmH_2O. The perfusate was bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. The trunk of the pulmonary artery was cut to drain the coronary effluent. To assess contractile function, a plastic balloon connected to a pressure transducer was inserted into the left ventricular cavity *via* the left atrium. Left ventricular end-diastolic pressure (LVEDP) was set at 4–8 mmHg by inflating the balloon with physiological saline. Left ventricular pressure and the electronically differentiated derivative, dP/dt, were continuously recorded by computer with PharmLab V3.0 (AstraZeneca R&D, Mölndal, Sweden). A Transonic flow probe (Transonic, Ithaca, NY, U.S.A.) connected to a Transonic flow meter (T208) was placed in the circuit proximal to the aortic cannula for continuous measurement of coronary flow. Heart rate was determined with pressure registration. A side arm connected to a mixing chamber was used for the administration of drugs at the onset of ischaemia.

Experimental protocol

Following stabilization for 35 min, hearts beating at a rate of more than 275 beats min^{-1} and with a left ventricular-developed pressure (LVDP) of more than 70 mmHg were randomised to receive 1 ml of vehicle ($n = 7$ in the WT and the KO groups), bosentan (10 μmol l^{-1} final concentration; $n = 7$ in

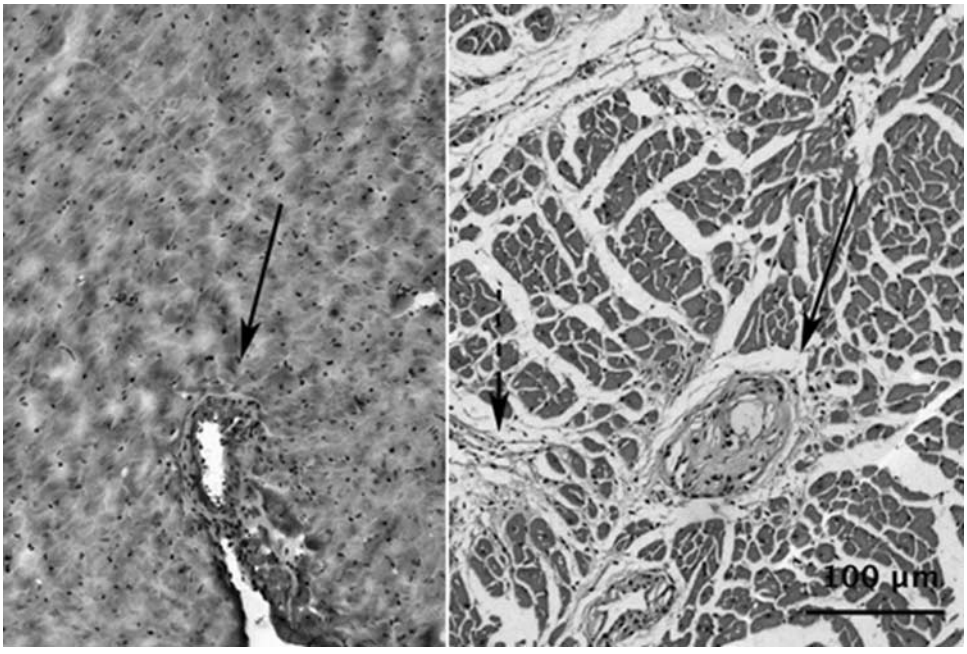

Figure 1 Haematoxylin–eosin stained section of myocardium from a WT mouse (left) and an apolipoprotein E/LDL receptor KO mouse (right). Coronary arteries (arrows) with atherosclerotic lesions with adjacent cardiac fibrosis (arrow with dashed line) are illustrated in the right panel.

the WT and $n=8$ in the KO groups) or ET-1 (1 nmol l^{-1} final concentration; $n=8$ in the WT and the KO groups). Warm global ischaemia was induced for 35 min by clamping the buffer perfusion followed by 30 min of reperfusion. The solutions containing vehicle, bosentan or ET-1 were injected into the aortic cannula within the first minute of ischaemia to ensure that the drugs were present in the heart during the entire ischaemic period. Haemodynamic parameters were determined preischaemia and then every 5 min during reperfusion. The coronary effluent of hearts treated with vehicle or bosentan was collected at preischaemia, then at 5, 10, 20 and 30 min during reperfusion and stored at $-80^{\circ}C$ until the analysis of NO.

NO analysis

NO concentrations in the rat cardiac effluent (250 μl) were determined using an NO analyser (NOA™280) from Sievers Instruments (Boulder, CO, U.S.A.), which measures NO based on a gas-phase chemiluminescence reaction between NO and ozone. NO in the samples was measured by reducing nitrite and nitrate (NO_X) with vanadium chloride in hydrochloric acid at 90°C to achieve high conversion efficiency. NO is therefore referred to as NO_X in the text. The inter- and intra-assay variations were 7.3 and 4.3%, respectively. The detection limit is 55 nmol l^{-1} in a 250 μl volume (14 pmol $sample^{-1}$).

Histopathological analysis

Hearts from two KO animals were fixed in 4% paraformaldehyde and embedded in paraffin. Cut sections were stained with haematoxylin and eosin for routine histological evaluation.

Materials

Sodium heparin was obtained from Lövens (Ballerup, Denmark), Dormicum (midazolam) from Hoffmann-LaRoche (Basel, Switzerland) and Hypnorm (fluanosonium + fentanylum) was from Janssen (Beerse, Belgium). All salts for the buffer were purchased from Sigma (Stockholm, Sweden). ET-1 was purchased from Alexis Corporation (Läufelfingen, Switzerland). Bosentan was kindly supplied by Dr Martine Clozel, Actelion, Switzerland.

Calculation and statistical analysis

LVDP is the difference between left ventricular systolic and end-diastolic pressures. Rate–pressure product (RPP) was calculated as the heart rate multiplied by the LVDP. The outflow of NO_X was calculated as the concentration of NO_X in the coronary effluent multiplied by the coronary flow. Recovery of myocardial performance and NO_X outflow are expressed as a percentage of the preischaemic value. All values are presented as the mean ± s.e.m. Comparison between the groups were made by one-way analysis of variance (ANOVA) followed by Fisher's PLSD test. The significance level was set at 0.05.

Results

Histopathological analysis

Histological examination of hearts from KO mice revealed severe atherosclerotic lesions in the coronary arteries. All arteries had an increased wall-to-lumen ratio compared to WT hearts and some thrombi were apparent in the KO hearts. Fibrosis was observed adjacent to occluded coronary arteries. Representative sections of a KO heart and a WT heart are depicted in Figure 1.

Haemodynamics during ischaemia and reperfusion

Preischaemia, there were no differences in coronary flow, LVDP, LVEDP, heart rate or RPP between the vehicle groups of each mouse strain and their corresponding treated groups (Table 1), or between the two mouse strains.

Following ischaemia, recovery of myocardial function was generally poor in both WT and KO vehicle groups with no significant differences between the strains. At the end of the reperfusion period, LVDP was only 30 ± 9 and 34 ± 11% of the preischaemic value in the WT and KO groups, respectively, in those mice administered vehicle only. Administration of bosentan improved the recovery of LVDP to more than 60% in both the WT and KO groups ($P<0.001$, Figure 2a). ET-1 administration slightly depressed the recovery of LVDP to 18 ± 4% in the WT group, but ET-1 exerted no major effect on recovery of LVDP during the late phase of reperfusion in the KO group.

Table 1 Preischaemic values of left ventricular end-diastolic pressure (LVEDP), developed pressure (LVDP), rate–pressure product (beats $min^{-1} \times$ LVDP), coronary flow (CF) and effluent nitric oxide (NO_X) in wild-type (WT) and apolipoprotein E/LDL receptor knockout (KO) mice given vehicle, bosentan and endothelin-1 (ET-1)

	Vehicle WT (n = 7)	*Bosentan WT* (n = 7)	*ET-1 WT* (n = 8)	*Vehicle KO* (n = 7)	*Bosentan KO* (n = 8)	*ET-1 KO* (n = 8)
LVEDP (mmHg)	5.5 ± 0.3	5.6 ± 0.2	5.4 ± 0.4	5.8 ± 0.3	5.9 ± 0.3	5.9 ± 0.4
LVDP (mmHg)	87 ± 6	84 ± 5	103 ± 7	91 ± 8	91 ± 5	92 ± 5
RPP (mmHg × beats min^{-1})	26,966 ± 2646	29,357 ± 4454	33,127 ± 3050	36,467 ± 3517	28,434 ± 2765	33,618 ± 1173
CF (ml min^{-1})	2.5 ± 0.2	2.4 ± 0.2	2.6 ± 0.2	2.5 ± 0.2	2.0 ± 0.3	2.0 ± 0.2
NO_X (nmol min^{-1})	0.69 ± 0.21	0.72 ± 0.13		1.19 ± 0.22	0.94 ± 0.19	

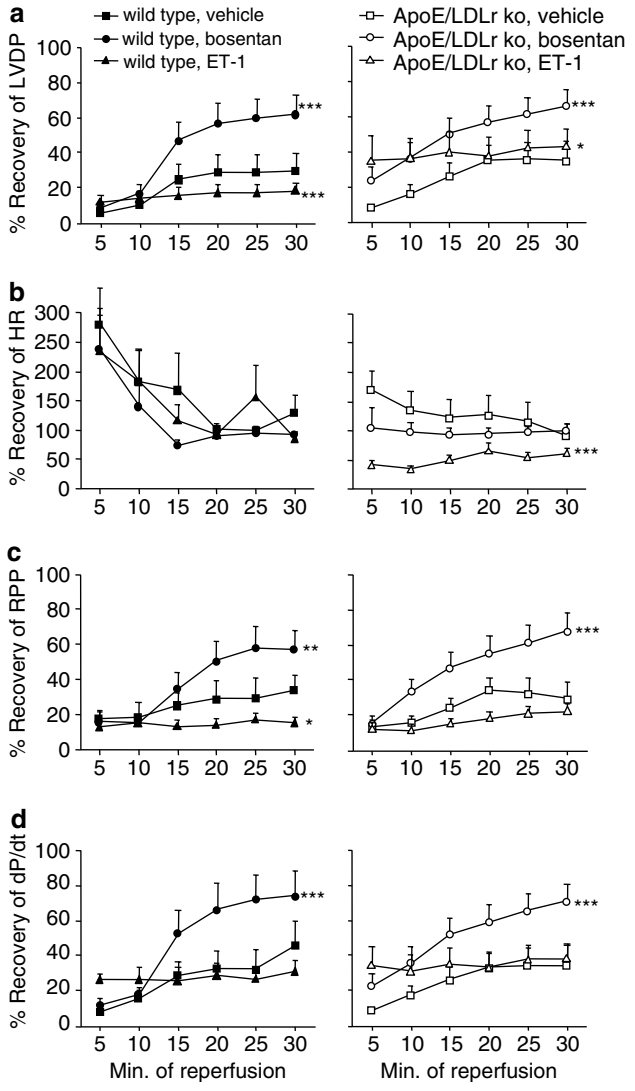

Figure 2 Percentage recovery of (a) left ventricular-developed pressure (LVDP), (b) heart rate (HR), (c) rate-pressure product (RPP) and (d) first derivative of left ventricular pressure (dP/dt) during reperfusion in mouse hearts given vehicle, the ET_A/ET_B receptor antagonist bosentan or endothelin-1 (ET-1) at the onset of ischaemia. The left panel presents data from WT mice and the right panel from apolipoprotein E/LDL receptor KO mice (ApoE/LDLr KO). Data are depicted as the mean ± s.e.m. Significant differences from the vehicle groups during the reperfusion period are illustrated; $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Heart rates during reperfusion were variable, especially during the first 15 min of reperfusion. There were no significant differences in heart rate recovery between vehicle- and bosentan-treated groups. In the KO animals, heart rate recovery was depressed by ET-1 (Figure 2b). Administration of bosentan produced a significantly improved recovery of RPP during reperfusion than was observed with vehicle administration (57 ± 10 and 68 ± 10% vs 34 ± 8 and 29 ± 9%, respectively; $P<0.001$; Figure 2c). Administration of ET-1 to the WT hearts depressed the recovery of RPP to 15 ± 3% ($P<0.05$ vs WT vehicle). Owing to a lower heart rate, there was a nonsignificant trend towards attenuation of the recovery of RPP by ET-1 in the KO hearts (Figure 2c). Bosentan also improved the recovery of the first derivative of left ventricular pressure (dP/dt; Figure 2d). LVEDP during reperfusion was significantly lower in the bosentan groups (46 ± 6 mmHg in the WT group and 41 ± 5 mmHg in the KO group at 30 min of reperfusion, respectively) than in the groups receiving vehicle (64 ± 5 mmHg in the WT group and 57 ± 8 mmHg in the KO group; $P<0.001$). Administration of ET-1 did not significantly effect LVEDP in either group. There were no significant differences in postischaemic recovery of myocardial function between WT and KO animals following administration of ET-1 or bosentan.

Administration of bosentan significantly enhanced the recovery of coronary flow during reperfusion in both the WT and the KO groups (Figure 3), while ET-1 reduced the recovery of coronary flow in both the WT and KO groups (Figure 3). The effects of both bosentan and ET-1 on coronary flow were significantly more pronounced in the KO mice than in the WT mice ($P<0.01$).

NO_X outflow

Although there was a trend towards a higher outflow of NO_X in the coronary effluent of the hearts from KO animals, there was no significant difference between KO and WT hearts prior to ischaemia (Table 1). During reperfusion, the outflow of NO_X was significantly attenuated in vehicle-treated WT and

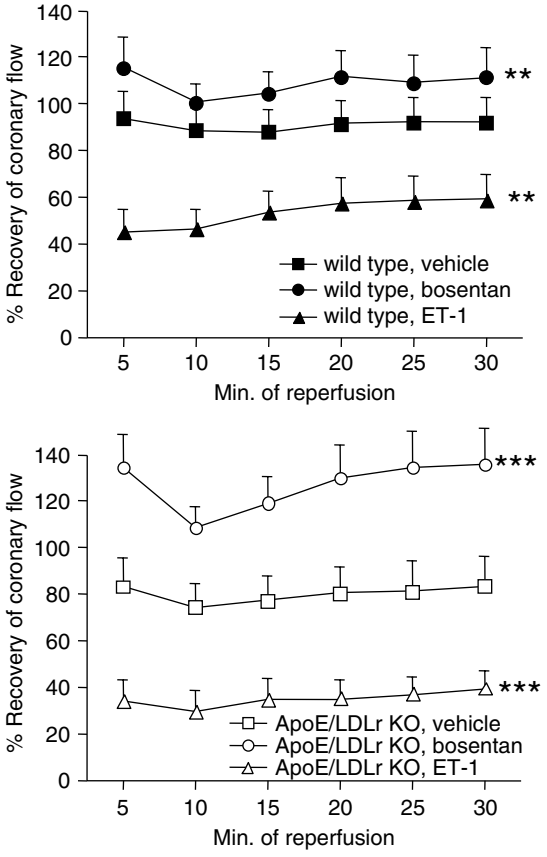

Figure 3 Recovery of coronary flow during reperfusion in WT mice and apolipoprotein E/LDL receptor KO mice (ApoE/LDLr KO) given vehicle, the ET_A/ET_B receptor antagonist bosentan or endothelin-1 (ET-1), respectively. Data (mean ± s.e.m.) are expressed as a percentage of the preischaemic values. Significant differences from vehicle groups are presented; $^{**}P<0.01$, $^{***}P<0.001$.

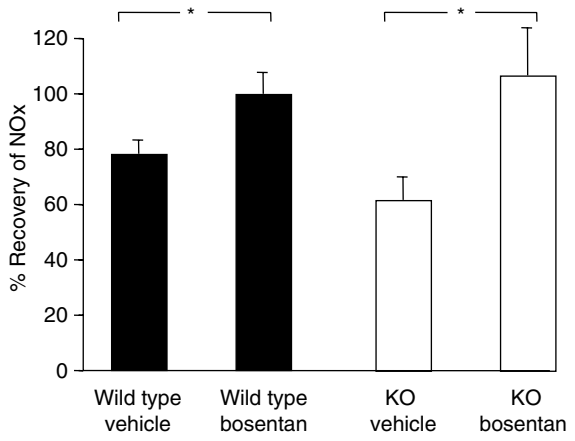

Figure 4 Outflow of nitric oxide (NO_X) during reperfusion in the cardiac effluent from WT and apolipoprotein E/LDL receptor KO mice (ApoE/LDLr KO) given vehicle and the ET_A/ET_B receptor antagonist bosentan expressed as a percentage of preischaemic values. Significant differences between the groups are depicted; $^*P<0.05$. Data are mean ± s.e.m.

KO mice (Figure 4). The recovery of the outflow of NO_X during reperfusion was significantly better preserved in the bosentan-treated groups ($P<0.05$; Figure 4).

Discussion

The main finding of the present study is that the dual ET_A/ET_B receptor antagonist bosentan protects against ischaemia and reperfusion injury in hearts with severe coronary atherosclerosis. This important finding adds to previous data from animal models with healthy arteries and demonstrates that pharmacological blockade of ET receptors gives protection against ischaemia/reperfusion injury in this model of coronary artery disease.

Previous studies have demonstrated that ET receptor antagonists reduce the extent of myocardial necrosis and improve myocardial function following ischaemia and reperfusion in different animal models with normal coronary arteries (Wang *et al*., 1995b; Gonon *et al*., 1998). Furthermore, recent results show that the cardioprotective effect of ET receptor antagonism following ischaemia and reperfusion is abrogated by inhibition of NO synthase (Gonon *et al*., 2000; Gourine *et al*., 2001), suggesting that it is dependent on production of NO (Wang *et al*., 1995a; Gonon *et al*., 2000; Gourine *et al*., 2001). It is therefore of pathophysiological and therapeutic importance to elucidate whether ET receptor antagonism protects the myocardium in a situation of severe atherosclerosis with endothelial dysfunction, as is the situation in patients with acute myocardial infarction. The apolipoprotein E/LDL receptor-deficient mouse is an atherosclerotic mouse model characterised by endothelial dysfunction due to impaired response to the endothelium-dependent vasodilatator acetylcholine and increased expression of ET-1 and ET receptors (Barton *et al*., 1998; Jiang *et al*., 2000; Kobayashi *et al*., 2000; d'Uscio *et al*., 2002). Therefore, this model is useful to investigate whether cardioprotection is achieved under severely atherosclerotic conditions. The dual ET_A/ET_B receptor antagonist bosentan significantly improved the recovery of LVDP, RPP and dP/dt and decreased LVEDP in the hearts of both WT mice and in the atherosclerotic mouse model following ischaemia and reperfusion when compared to hearts treated with vehicle only. These results clearly demonstrate that bosentan attenuated both systolic and diastolic myocardial dysfunction following ischaemia.

Bosentan also enhanced the recovery of coronary flow. Conversely, administration of ET-1 markedly attenuated the recovery of coronary flow. It is interesting to note that the effects of bosentan and ET-1 on coronary flow were significantly greater in KO animals than in WT animals. Thus, ET-1 seems to play a more prominent role during ischaemia and reperfusion in the regulation of coronary vascular tone in atherosclerotic mice than in WT mice. Accordingly, Caligiuri *et al*. (1999) demonstrated that the ET_A receptor mediates stress-induced myocardial infarction in apolipoprotein E/LDL receptor KO mice but not in WT mice. These observations suggest that ET receptor blockade evokes more pronounced vascular effects in atherosclerotic arteries than in normal arteries, which concords with findings in patients with atherosclerosis (Bohm *et al*., 2002a).

Bosentan is a mixed ET_A/ET_B receptor antagonist with approximately 20 times higher affinity for the ET_A receptor (Clozel *et al*., 1994). It is difficult to establish with certainty whether the present cardioprotective effect of bosentan is mediated *via* the ET_A receptor or dual receptor blockade. It is, however, interesting that the vasoconstrictor ET_B receptor is upregulated in atherosclerotic patients (Bohm *et al*., 2002a) as well as in experimental hypercholesterolemia (Hasdai *et al*., 1997), which may contribute to the enhanced effect of ET-1 and bosentan in the KO animals.

Previous observations support the suggestion that ET receptor blockade enhances NO bioavailability in atherosclerosis. Chronic treatment of apolipoprotein E-deficient mice with a selective ET_A receptor antagonist restored NO-mediated endothelial function and reduced elevated tissue ET-1 levels (Barton *et al*., 1998; d'Uscio *et al*., 2002). More importantly, it has been shown that selective ET_A receptor antagonism improves endothelium-dependent vasodilatation in patients with atherosclerosis (Bohm *et al*., 2002b). In the present study, the outflow of NO_X in the cardiac effluent was better preserved following administration of bosentan than in the presence of vehicle alone. This indicates that ET receptor antagonism maintains the release of NO_X, which may contribute to the cardioprotective effect of ET receptor blockade. A recent study demonstrated that the protection achieved with ET receptor blockade is associated with increased expression of endothelial NO synthase (Gonon *et al*., 2004), which may explain the enhanced recovery of NO_X in the presence of bosentan.

The preischaemic concentration of NO_X in the cardiac effluent did not differ between the WT group and KO group. This appears to be in contrast to previous reports demonstrating impaired endothelium-dependent vasorelaxation and reduced concentration of NO derived from the aorta of the atherosclerotic mouse (Barton *et al*., 1998; Jiang *et al*., 2000; Scalia *et al*., 2001). However, in the isolated whole heart preparation, it cannot be determined whether the released NO_X is derived from the coronary vessels or the myocardium. Thus, a limitation of the present model using the whole heart preparation is that it does not allow selective determination of NO_X release from the coronary vasculature. Furthermore, it cannot be determined which isoforms of NO synthase

(endothelial and inducible NO synthase) generated the NO_X detected in the coronary effluent. It has been demonstrated that there is expression of inducible NO synthase in the aortic root of the apolipoprotein E KO mouse, which contributes to progression of the atherosclerotic lesions (Detmers *et al*., 2000). Another study limitation is that it was performed on isolated buffer-perfused hearts to monitor myocardial function and coronary flow. The protective effect of ET receptor blockade in atherosclerotic animal models needs to be confirmed in other models *in vivo*.

In conclusion, the dual ET_A/ET_B receptor antagonist, bosentan, protects against ischaemia and reperfusion injury as demonstrated by improved myocardial function and coronary flow in an isolated heart model of coronary atherosclerosis. These results suggest that ET receptor blockade may be a feasible treatment for limiting ischaemia and reperfusion injury in conditions with severe atherosclerosis.

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