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Role of endogenous endothelin in myocardial and coronary endothelial injury after ischaemia and reperfusion in rats: studies with bosentan, a mixed ET_A-ET_B antagonist

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1 Previous studies suggested that endothelin-1 (ET-1) may play a role in myocardial ischaemia and reperfusion. This study was designed to test the effect of a new nonpeptide antagonist of endothelin ET_A and ET_B receptors, bosentan, on myocardial infarct size, ventricular arrhythmias, and coronary endothelial dysfunction after ischaemia and reperfusion.

2 Anaesthetized male Wistar rats were subjected to 20 min ischaemia (left coronary artery occlusion) followed by 1 h (for the evaluation of coronary endothelial dysfunction) or 2 h (for the evaluation of infarct size) reperfusion, or 5 min ischaemia followed by 15 min reperfusion (for the evaluation of reperfusion arrhythmias). Vascular studies were performed on 1.5–2 mm coronary segments (internal diameter 250–300 μm) removed distal to the site of occlusion and mounted in wire myographs for isometric tension recording. Area at risk and infarct size were determined by Indian ink injection and triphenyl tetrazolium staining, using computerized analysis of enlarged sections after colour video acquisition.

3 Bosentan, administered at a dose which virtually abolished the pressor response to big ET-1 (3 mg kg^{-1}, i.v. before ischaemia) did not affect heart rate, arterial pressure or the rate pressure product before ischaemia, during ischaemia and during reperfusion. Bosentan did not affect the incidence of reperfusion-induced ventricular fibrillation (controls: 86%, $n = 14$; bosentan: 93%, $n = 15$), and did not modify infarct size (% of area at risk: controls: 63 ± 4, $n = 10$; bosentan: 60 ± 6, $n = 8$). Ischaemia followed by reperfusion markedly reduced the endothelium-dependent relaxations to acetylcholine (maximal response: sham: $59 \pm 4\%$, $n = 9$; ischaemia–reperfusion: $26 \pm 6\%$, $n = 8$; $P < 0.01$), characteristic of reperfusion-induced endothelial dysfunction, and this dysfunction was not prevented by bosentan (maximal response to acetylcholine: $25 \pm 5\%$, $n = 9$; $P < 0.01$ vs sham; P = NS vs ischaemia/reperfusion).

4 These experiments suggest that endogenous endothelin does not contribute to myocyte or coronary endothelial injury in this rat model of ischaemia and reperfusion.

Keywords: reperfusion; endothelin; bosentan; infarct size; coronary circulation; arrhythmias; endothelium

Introduction

Vascular endothelial cells synthesize various vasoactive substances, including endothelin-1 (ET-1), a 21 amino acid vasoconstrictor peptide. Several experiments have suggested that ET-1 is released in basal conditions (Ando *et al.*, 1989; Suzuki *et al.*, 1989), and that this release is increased in various pathophysiological conditions, including myocardial ischaemia or infarction (Miyauchi *et al.*, 1989; 1992; Yasuda *et al.*, 1990; Stewart *et al.*, 1991; Tsuji *et al.*, 1991; Watanabe *et al.*, 1991a; Vemulapalli *et al.*, 1992; Lechleitner *et al.*, 1993; Qiu *et al.*, 1993; Donckier *et al.*, 1994). ET-1 increases cardiac contractility (Shah *et al.*, 1989; Shomisch Moravec *et al.*, 1989) and induces coronary constriction (Tippins *et al.*, 1989), leading to myocardial ischaemia (Ezra *et al.*, 1989; Larkin *et al.*, 1989; Hori *et al.*, 1991) or arrhythmias (Yorikane & Koike, 1990; Salvati *et al.*, 1991). Furthermore, ischaemia can induce an externalization of ET-1 receptors in rat cardiac membranes (Liu *et al.*, 1990), and the response of rat isolated hearts to endothelin is increased during ischaemia and reperfusion (Nebauer *et al.*, 1990; 1991). Thus, based on those data, it is tempting to speculate that endogenous release of ET-1 may aggravate myocardial ischaemia, either through coronary constriction or through direct myocardial effects. This hypothesis has been difficult to test directly, due to the absence of specific antagonists of endothelin receptors. Recent studies, however, have suggested that monoclonal antibodies directed against ET-1, or phosphoramidon (an inhibitor of endothelin converting enzyme) could reduce ischaemic damage in experimental models of myocardial ischaemia and reperfusion in rats (Watanabe *et al.*, 1991a; Grover *et al.*, 1992).

Two receptor subtypes, ET_A and ET_B mediate the biological effects of endothelin (Arai *et al.*, 1990; Sakurai *et al.*, 1990). Although it was initially assumed that ET_A receptors were present on vascular smooth muscle and induced vasoconstriction, whereas ET_B receptors were present on vascular endothelium and mediated the vasodilator effect of endothelin (Sakurai *et al.*, 1992), recent experiments showed that ET_B receptors are in fact also present on smooth muscle cells and contribute to the vasoconstrictor effect of endothelin; indeed, in rats (McMurdo *et al.*, 1993; Bird *et al.*, 1993), as well as in isolated arteries (Seo *et al.*, 1994) the ET_A antagonists BQ-123 and FR-139317 are unable to antagonize fully the vasoconstrictor effect of endothelin-1, suggesting that part of this response could be due to stimulation of smooth muscle cell ET_B receptors. Thus, the physiological and pathophysiological effects of endogenous endothelin can be evaluated best through inhibition of both ET_A and ET_B-mediated responses.

Bosentan (Ro 47-0203) is a novel, non peptide, orally active ET-1 antagonist (Clozel *et al.*, 1994) derived from Ro 46-2005, a previously described ET-1 antagonist (Clozel *et al.*, 1993a,b). *In vitro*, bosentan binds competitively to both human ET_A and ET_B receptors, and inhibits the ET_A and ET_B-mediated responses of isolated arteries (Clozel *et al.*, 1994). *In vivo*, this compound inhibits the pressor and depres-

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sor responses induced by intravenous administration of ET-1, big ET-1, or sarafotoxin S6C, also suggestive of antagonism at both ET_A and ET_B receptors (Clozel *et al.*, 1994). Thus, this compound represents a new tool to investigate the role of endogenous endothelin in physiological and pathophysiological situations. Indeed, preliminary data suggest that bosentan decreases arterial pressure in various experimental models of hypertension (Clozel *et al.*, 1993c) and reverses cerebral vasospasm in subarachnoid haemorrhage, a situation associated with a marked increase in endothelin release (Roux *et al.*, 1993).

Thus, the purpose of the present study was to evaluate the effect of bosentan in a rat model of myocardial ischaemia followed by reperfusion. Specifically, the effect of bosentan was investigated on three major aspects of ischaemia/reperfusion injury: infarct size, reperfusion-induced arrhythmias and reperfusion-induced coronary endothelial dysfunction.

Methods

General animal preparation

The experimental protocol was similar to that described previously (Richard *et al.*, 1993; 1994). The study was performed in 96 male Wistar rats (Charles River, Saint Aubin les Elbeuf, France), weighing between 300 and 400 g, which were anaesthetized with sodium pentobarbitone, 40 mg kg^{-1} intraperitoneally. A midline incision was made in the neck and a tracheotomy performed. The rats were mechanically ventilated with room air supplemented with low flow oxygen using small rodent ventilators (Apelex, Massy, France or MDI, Mobile, AL, U.S.A.), at a rate of 60 cycles min^{-1} and a tidal volume of 10 ml kg^{-1} body weight. The respiratory rate and tidal volume were adjusted in order to maintain arterial blood gases within a normal range. Body temperature was maintained at 37°C with a thermostated heating blanket connected to a rectal thermometer. The right jugular vein was cannulated for injection of drugs and of Indian ink for the delineation of area at risk (see below). The left carotid artery was cannulated, and a small Millar Mikrotip catheter (Model SPR407, Millar, Houston, TX, U.S.A.) was inserted in the artery in order to measure arterial blood pressure. An electrocardiogram was also obtained with standard limb electrodes. A left thoracotomy was performed, and the heart exposed. A 7/0 polypropylene suture was passed around the proximal left coronary artery and the ends were passed through a small plastic tube to form a snare. The artery was occluded by pulling the snare, which was kept in place by means of a haemostatic clamp. Myocardial ischaemia was confirmed by visual cyanosis. Reperfusion was induced by releasing the snare.

Effect of bosentan on the pressor response to big endothelin in ganglion-blocked rats

Experiments were performed in 13 rats (7 controls, 6 bosentan). Rats were anaesthetized, ventilated and instrumented as described above, but the chest was not opened. The animals were allowed to rest for 15 min after completion of the surgical preparation, after which they received the ganglion blocking agent, chlorisondamine (2.5 mg kg^{-1}, i.v.). Fifteen min after chlorisondamine, rats received bosentan (Ro 47-0203, 3 mg kg^{-1}) or saline, given as an 0.2 ml i.v. bolus. Fifteen min after bosentan, the animals received increasing doses of big endothelin (0.01–1 nmol kg^{-1}). ECG and heart rate were monitored continuously on a Gould Windowgraph recorder (Gould, Ballainvilliers, France). Pressor effects of big endothelin were assessed 15 min after administration of each dose.

Effect of bosentan on infarct size

Experiments were performed in 20 rats (10 controls and 10 bosentan). Rats were assigned to two groups, which were subjected to a 20 min coronary occlusion followed by 120 min reperfusion. Bosentan was given as an 0.2 ml i.v. bolus (3 mg kg^{-1}) 15 min before ischaemia. In these experiments, ECG and arterial pressure were monitored continuously on a Gould ES2000 recorder. The methods for quantitating infarct size were similar to those used in our previous studies (Richard *et al.*, 1993; 1994). At the end of the 120 min reperfusion period, the artery was briefly reoccluded, and 0.7 ml Indian ink was injected slowly into the jugular catheter, in order to delineate the area at risk of infarction. The heart was excised, the right ventricle and the atria were dissected away with small surgical scissors, and the remaining left ventricle was frozen in cold isopentane and kept in isopentane at − 10°C for 1 h. We have previously verified that this freezing procedure does not affect histochemical determination of necrosis as compared to fresh tissue (Richard *et al.*, 1993). The frozen ventricle was then sliced from apex to base into 7–8 sections. After thawing, the slices were immersed in 1% triphenyletrazolium chloride (TTC, Sigma Chimie) in pH 7.4 phosphate buffer for 20 min at 37°C, in order to delineate the infarcted tissue. The sections were then fixed in 10% phosphate buffered-formalin at room temperature for a minimum of 4 days. After fixation, each section was weighed and placed under a microscopic video camera (Microwatcher VS-30H, Mitsubishi Kasei Corporation, Tokyo, Japan) with a 20 fold enlargement lens. The camera was connected to an electronic colour digitalisation card (Matrox Illuminator 16) coupled to an AST computer. The digitized colour images were enlarged 5 fold (final enlargement 100 fold), and the resulting images were stored as bitmap files for later analysis. These stored images were later displayed on a 1024 × 768 pixel colour screen using a Windows-based image analysis software (Cyberview, Cervus Int., France), and the area (mm^2) of nonischaemic (Indian, ink stained), viable (TTC positive) and infarcted (TTC negative) tissue were determined on each section using the same image analysis software. From these measurements, infarct and area at risk weights were calculated knowing the individual weight of each section. The size of the area at risk was then expressed as a percentage of left ventricular weight, and infarct size was expressed as a percentage of the left ventricle and as a percentage of the area at risk.

Effect of bosentan on reperfusion-induced arrhythmias

Experiments were performed in 29 rats (14 controls and 15 bosentan). Animals were subjected to a 5 min coronary occlusion (corresponding to the duration of ischaemia for which the incidence of reperfusion arrhythmias is the highest) followed by 15 min reperfusion. We verified in pilot experiments that no severe arrhythmias occurred in this model after the first 15 min of reperfusion. Bosentan was given as a 0.2 ml i.v. bolus (3 mg kg^{-1}) 15 min before ischaemia. ECG and heart rate were monitored continuously on a Gould Windowgraph recorder. The occurrence of ventricular tachycardia and fibrillation (reversible and irreversible) during ischaemia and reperfusion was detected on the electrocardiogram and the blood pressure tracing; ventricular tachycardia was detectable on the ECG signal, whereas fibrillation was detectable both on the ECG and as a complete absence of arterial pressure.

Effect of bosentan on reperfusion-induced coronary endothelial dysfunction

Experiment were performed in 34 rats, which were assigned to three groups: group 1 rats (Sham, $n = 10$) were killed after a 100 min open-chest period without occlusion of the artery; group 2 rats (ischaemia/reperfusion, $n = 12$) were subjected

to a 20 min coronary occlusion followed by 60 min reperfusion; group 3 rats (bosentan, $n = 12$) were subjected to the same 20 min coronary occlusion/60 min reperfusion cycle as in group 2, but received bosentan given as a 0.2 ml i.v. bolus (3 mg kg^{-1}) 15 min before ischaemia.

Coronary endothelial dysfunction was assessed as described previously (Richard *et al.*, 1994). At the end of the experiment, the heart was removed and immediately placed in cold, oxygenated physiological saline (control solution) of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, EDTA 0.02 and glucose 11.1. The left coronary artery was carefully dissected free under a dissecting microscope. Segments of the artery (length 1.5–2 mm; internal diameter 250–300 μm) were taken distal to the occlusion site and mounted in a small vessel myograph for isometric tension recording (JP Trading, Aarhus, Denmark). For this purpose, the segments were threaded into two 40 μm stainless steel wires; the ends of the wires were then fastened to two stainless steel support blocks (Mulvany & Halpern, 1977). One block was mounted on a tension transducer and the other on a displacement device operated by a micrometer. Care was taken during the dissection procedure to avoid damage to the endothelium. During the mount process, the myograph chamber was filled with cold, oxygenated (95% O_2:5% CO_2, pH 7.4) control solution. Vessel length was measured with a calibrated lens placed in the dissection microscope. After equilibration, the vessel was progressively stretched using the micrometer; the internal circumference of the vessel was calculated from the micrometer reading and the corresponding force was read on the recorder. Wall tension was calculated for each level of stretch by dividing this reading by vessel length. From these measurements, the Laplace law was used to calculate the effective pressure (which corresponds to the pressure that would be necessary to extend the vessel to the measured internal circumference), as: effective pressure = wall tension × 2Π/internal circumference (Mulvany & Halpern, 1977).

The distension was stopped when effective pressure exceeded 100 mmHg. The relation between effective pressure and internal circumference was then fitted to an exponential curve, and the circumference of the vessel corresponding to a transmural pressure of 100 mmHg (IC_{100}) was calculated from the fitted curve. The vessel was then set to a circumference equal to $0.9 \times IC_{100}$, which corresponds to the circumference for which the active concentration is maximal (Mulvany & Halpern, 1977). After mounting, the vessels were allowed to equilibrate for 30 min, during which chamber temperature was progressively increased to 37°C.

After another 60 min equilibration period during which the vessels were washed, segments were exposed to increasing concentrations of 5-hydroxytryptamine (5-HT, 10^{-9}–10^{-5} M), after which concentration-response curves to acetylcholine (10^{-9}–10^{-5} M) or the NO donor SIN-1 (10^{-8}–10^{-4} M) were studied in each ring after precontraction by serotonin.

Drugs

Bosentan was a gift from F. Hoffmann-La Roche Ltd., Basel, Switzerland. SIN-1 (3-morphilinosydronimine) was a gift from Laboratories Hoechst, Paris, France. Chlorisondamine was a gift from Ciba-Geigy, Basel, Switzerland. Triphenyl tetrazolium, acetylcholine, 5-hydroxytryptamine, big endothelin-1 were all purchased from Sigma.

Data analysis

All results are expressed as mean ± s.e.mean. Area at risk and infarct size were compared by Student's unpaired t test. Comparisons of the incidence of arrhythmias were performed using a Pearson chi-square test. In all *in vitro* experiments, n refers to the number of animals from which the arteries were taken. Contractions to 5-HT are expressed as a percentage of maximal contraction, whereas relaxations to acetylcholine or SIN-1 are expressed as a percentage of the contraction to 5-HT. Results from *in vitro* studies were then compared using ANOVA followed when ANOVA was significant by a Tukey's test for multiple comparisons. A P value ≤ 0.05 was considered statistically significant.

Results

Pressor response to big ET-1

The effect of bosentan (3 mg kg^{-1}, i.v.) on the pressor response induced by increasing doses of big ET-1 (0.01–1 nmol kg^{-1}) in ganglion-blocked rats is shown in Figure 1. Bosentan was itself devoid of effect on blood pressure. In the absence of bosentan, big ET-1 induced a dose-dependent increase in blood pressure, the maximal increase in mean blood pressure being 68 ± 11% at the dose of 1 nmol kg^{-1}. The pressor response to big ET-1 was markedly reduced by bosentan (maximal increase in mean blood pressure: 13 ± 6%; $P < 0.01$ vs controls).

Haemodynamics during ischaemia and reperfusion

Table 1 shows the evolution with time of heart rate, systolic and diastolic blood pressure, and rate pressure product (an index of myocardial oxygen consumption) in the animals that entered the infarct size study and survived the entire protocol. Haemodynamic parameters were measured at baseline (i.e. before administration of bosentan), immediately before ischaemia (i.e. 15 min after administration of bosentan), at the end of the 20 min period of ischaemia, and after 120 min of reperfusion. In controls, neither ischaemia nor reperfusion affected heart rate, blood pressure or rate pressure product. Bosentan did not significantly affect the haemodynamic parameters measured at any time during the experiments, although it tended to decrease heart rate, blood pressure and the rate pressure product during reperfusion.

Area at risk and infarct size

Out of the 20 animals which entered the study (10 controls and 10 bosentan), one bosentan-treated rat died of ventricular fibrillation during ischaemia and one had to be excluded because of technical difficulties. Results on infarct size were thus obtained in 18 animals (10 controls and 8 bosentan). Figure 2 summarizes the area at risk and infarct size data for the two groups, and shows that the size of the area at risk, which is a major predictor of infarct size in models of

Table 1 Heart rate (HR, beats min^{-1}), systolic (SAP, mmHg) and diastolic (DAP, mmHg) arterial pressure, and rate pressure product (RPP 10^2 mmHg beats min^{-1}) measured at baseline, before ischaemia (i.e. 15 min after bosentan administration), at the end of the ischaemic period and after 120 min of reperfusion in control rats or in animals treated with bosentan (3 mg kg^{-1})

	Baseline		*Before ischaemia*		*End of ischaemia*		*End of reperfusion*	
	Control	*Bosentan*	*Control*	*Bosentan*	*Control*	*Bosentan*	*Control*	*Bosentan*
HR	421 ± 11	420 ± 11	389 ± 11	376 ± 13	399 ± 9	394 ± 16	393 ± 11	369 ± 13
SAP	124 ± 4	130 ± 11	120 ± 2	124 ± 11	116 ± 4	123 ± 10	122 ± 16	116 ± 8
DAP	102 ± 5	107 ± 7	96 ± 3	103 ± 8	96 ± 4	101 ± 7	97 ± 5	92 ± 7
RPP	597 ± 29	549 ± 54	536 ± 14	504 ± 59	535 ± 25	524 ± 62	556 ± 33	491 ± 41

regional ischaemia, was not significantly different in the two groups (controls: 51 ± 2%; bosentan 49 ± 6% of left ventricle). As compared to controls, bosentan did not affect infarct size, whether expressed as a percentage of left ventricle (controls: 32 ± 2; bosentan 31 ± 6%; Figure 2), or as a percentage of the area at risk (controls: 63 ± 4%; range 48–85%; bosentan 60 ± 6%; range 38–83%; Figure 2).

Reperfusion arrhythmias

Figure 3 shows the incidence of ventricular fibrillation upon reperfusion after a 5 min period of ischaemia in controls or bosentan-treated rats. In controls, reperfusion was associated with the occurrence of ventricular fibrillation in 86% of the animals; fibrillation was irreversible (i.e. no reversion to normal sinus rhythm during the first 15 min of reperfusion) in 21% of the animals. Bosentan did not affect the incidence of reperfusion-induced ventricular fibrillation (93 and 26% of total and irreversible fibrillation, respectively).

Coronary endothelial dysfunction

Out of the 34 rats that entered the study, 5 animals (2 controls and 3 bosentan) died of ventricular fibrillation. Experiments on isolated blood vessels were then performed in arteries taken from the 29 surviving animals (10 sham, 10 ischaemia/reperfusion controls and 9 ischaemia/reperfusion + bosentan). Responses of isolated arteries to increasing concentrations of 5-HT or SIN-1 are shown in Figure 4. As compared to sham-operated animals, neither ischaemia/reperfusion nor its treatment by bosentan affected the contractile responses to 5-HT (Figure 4). Similarly, the endothelium-independent relaxant response to the nitric oxide donor SIN-1 was not affected by ischaemia/reperfusion or by bosentan (Figure 4).

Responses of isolated coronary arteries to the endothelium-dependent vasodilator acetylcholine are shown in Figure 5. In arteries taken from sham-operated animals, acetylcholine induced concentration dependent relaxations which reached 59 ± 4% at the highest concentration (10^{-5} M). This response to acetylcholine was virtually abolished by L-arginine analogues such as N^G-nitro-L-arginine or N^G-nitro-L-arginine methyl ester (data not shown). The response to acetylcholine was markedly reduced after ischaemia and reperfusion (Figure 5; maximal response: 26 ± 6%; $P<0.01$ vs sham). As compared to sham-operated rats, the response to acetylcholine was significantly reduced by ischaemia/reperfuson at all concentrations from 3×10^{-7} M to 10^{-5} M. This ischaemia/reperfusion – induced impairment of endothelium-dependent response to acetylcholine was not affected by bosentan (maximal relaxation: 25 ± 5%; $n=9$; $P<0.01$ vs sham; P = NS vs ischaemia reperfusion).

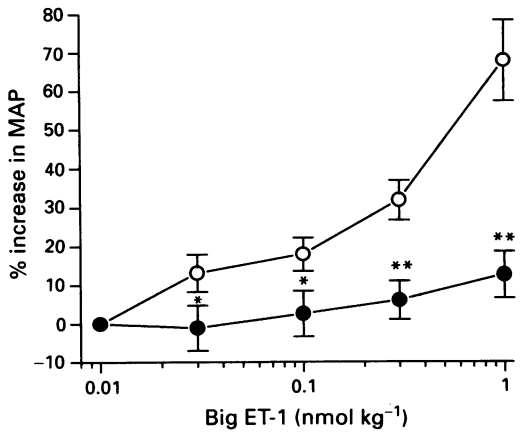

Figure 1 Percentage increase in mean arterial pressure (MAP) induced by increasing doses of big endothelin-1 in control rats (○) and in rats treated with bosentan (3 mg kg^{-1}, ●), in the presence of the ganglionic blocking agent chlorisondamine (2.5 mg kg^{-1}, i.v.). *$P<0.05$ and **$P<0.01$ vs controls.

Discussion

The present study shows that a mixed ET_A-ET_B endothelin antagonist, bosentan, administered at a dose which virtually abolished the pressor response to big ET-1, had no effect of infarct size, reperfusion arrhythmias or reperfusion-induced coronary endothelial dysfunction in a rat model of ischaemia/reperfusion. These results suggest that ET-1 probably does not contribute to the postischaemic myocardial or coronary endothelial injury in this model.

Effect of bosentan on the pressor response to big ET-1

In the present experiments, bosentan (3 mg kg^{-1}) markedly inhibited the pressor response to big ET-1, in agreement with previous results (Clozel *et al.*, 1994). *In vivo*, the major part of the pressor effect of big ET-1 is considered to be ET_A-mediated, but a small component is resistant to inhibition by the ET_A antagonist BQ-123 (McMurdo *et al.*, 1993) and could be the consequence of activation of the ET_B receptor present at the level of the smooth muscle. We chose to assess the effect of bosentan on the response to intravenous administration of big ET-1, rather than ET-1 itself on the basis that big ET-1 mimics better than ET-1 the physiological

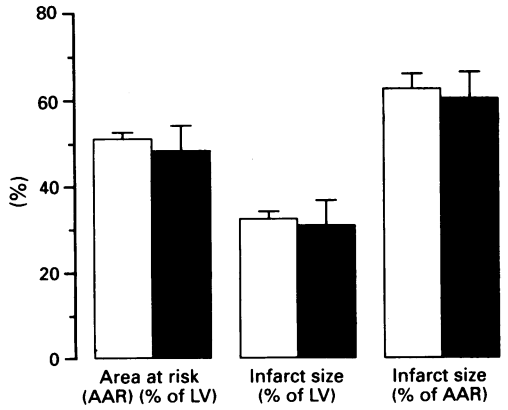

Figure 2 Area at risk (AAR), expressed as % of left ventricle (LV), and infarct size, expressed as % of left ventricle and % of the area at risk, in controls rats (open columns), or in animals treated by bosentan (3 mg kg^{-1}, solid columns).

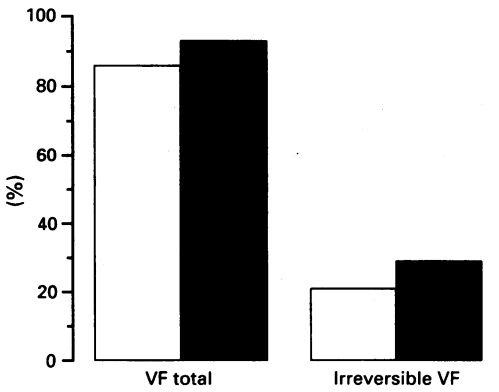

Figure 3 Incidence of total and irreversible ventricular fibrillation (VF) upon reperfusion after a 5 min period of ischaemia in control rats (open columns) or in rats treated by bosentan (3 mg kg^{-1}, solid columns).

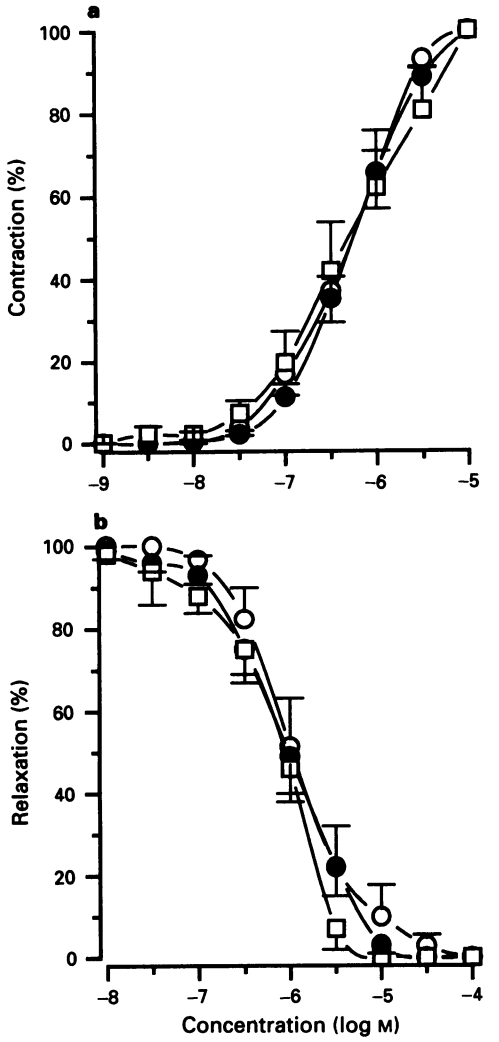

Figure 4 Contracting responses of rat coronary arteries to 5-hydroxytryptamine (5-HT) (a) and relaxant responses to the nitric oxide donor SIN-1 (in 5-HT-precontracted rings; b). Arteries were taken from sham-operated animals (○), or animals subjected to ischaemia followed by reperfusion in the absence (●) or in the presence of bosentan (□). Values for 5-HT are expressed as a percentage of maximal response, and values for SIN-1 are expressed as a percentage of the contraction to 5-HT.

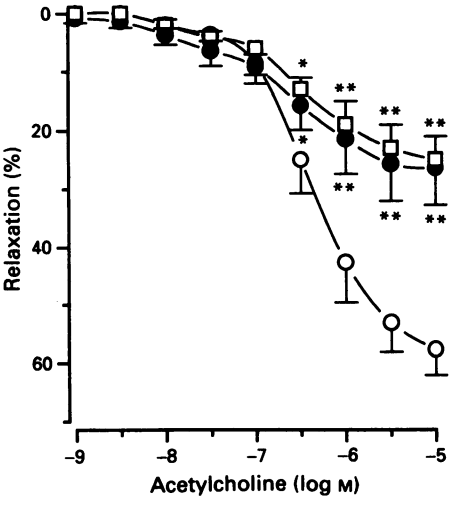

Figure 5 Relaxant responses of rat coronary arteries to acetylcholine. Arteries were taken from sham-operated animals (○), or animals subjected to ischaemia followed by reperfusion in the absence (●) or in the presence of bosentan (□). Values are expressed as a percentage of the contraction to 5-hydroxytryptamine. $*P<0.05$ and $**P<0.01$ vs sham.

release of this peptide from endothelial cells (Clozel *et al.*, 1993b; 1994). Indeed, big ET-1 must be converted to ET-1 for activity (Gardiner *et al.*, 1991). This conversion, which is inhibited by phosphoramidon, seems to take place mainly in the tissues (Watanabe *et al.*, 1991b), and ET-1 is then preferentially released toward the vascular smooth muscle rather than toward the lumen (Wagner *et al.*, 1992). Indeed, the vasoconstrictor effects of big ET-1 can be observed at doses for which no increase in plasma ET-1 can be detected (Teerlink *et al.*, 1993). For this reason, i.v. administration of ET-1 probably leads to an overestimation of the endothelium-mediated depressor response to ET-1, which might not be a physiologically relevant phenomenon if ET-1 is preferentially released toward the vascular smooth muscle *in vivo*.

Effect of bosentan on infarct size

In the present study, we used a rat model of *in vivo* myocardial ischaemia and reperfusion similar to the one used in our previous studies (Richard *et al.*, 1993; 1994). Although the rat offers numerous advantages in terms of cost and reproducibility (especially because of the absence of native collaterals), the small size of the heart may be the cause of various potential difficulties and imprecision during infarct size quantification. In our experiments, necrosis was quantified by computerized image analysis of ventricular sections after a 100 fold enlargement, i.e. on >10 cm diameter images. Thus, despite the small size of the heart, we believe that our quantification of infarct size was highly accurate.

In the present experiments, we used a short (20 min) duration of ischaemia. Nevertheless, such ischaemia still resulted in the development of large myocardial infarcts, averaging 60% of the area at risk, and this is consistent with results from our previous studies (Richard *et al.*, 1993; 1994). It must be noted that this duration of ischaemia is shorter than that used in other infarct size studies performed in rats. However, despite this shorter duration of ischaemia, infarcts in our studies are of similar size or even larger than those obtained by other groups (e.g. Li *et al.*, 1992; Yellon *et al.*, 1992; Liu & Downey, 1992; 1993; Li & Kloner, 1993). The reasons for these differences in the rate of development of necrosis could be due in part to differences in the anaesthetics used or to differences in the strains of rats used (Wistar in the present study vs Sprague-Dawley in other studies). Finally, using the same experimental model, we have been previously able to detect significant limitation of infarct size with ischaemic preconditioning (Richard *et al.*, 1993; 1994), and with calcium antagonists such as verapamil (unpublished data). Thus, despite the development of large infarcts, this model appears adequate to evaluate the efficacy of potential anti-ischaemic drugs.

Our infarct size studies were performed after a short (2 h) period of reperfusion. However, it is unlikely that limiting reperfusion to 2 h affected the outcome of the study or the delineation of the infarcts based on tetrazolium staining. Indeed, in a previous study, we have shown that varying the duration of reperfusion from 1 to 6 h in the same experimental model did not affect the size of the infarcts measured by tetrazolium staining. This suggests that prolonging the reperfusion period does not contribute to the extension of infarct size in this model and that, despite the short duration of reperfusion used in the present experiments, our measurements of infarct size are valid.

In the present experiments, blockade of endothelin receptors with bosentan did not affect infarct size. It is unlikely that this lack of effect of bosentan is due to an insufficient

blockade of the endothelin-induced responses, since we showed that the same dose markedly reduced the pressor response induced by big ET-1 (Figure 1), and we verified in pilot experiments that this inhibitory effect of bosentan on the response to big ET-1 persists for at least 2 h. Furthermore, the half-life of bosentan in these conditions is known to be at least 3 h (M. Clozel, Roche, Basel, personal communication).

Our results are in contrast to those of two other studies in rats which reported a significant limitation of infarct size either with a monoclonal antibody against ET-1 (Watanabe *et al.*, 1991a), or with phosphoramidon (Grover *et al.*, 1992). It should be noted that, in both previous studies (unlike the present study), the area at risk was not quantified, and infarct size was expressed as a percentage of left ventricle. It is now clearly established that the size of the area at risk is a major determinant of infarct size in all models of regional ischaemia (e.g. Reimer *et al.*, 1985). Thus, a study on the effect of a treatment on infarct size should include a measurement of area at risk in order to express infarct size as a percentage of area at risk and eliminate individual variations of infarct size due to variations in the anatomical area at risk (Schaper, 1984). Furthermore, in both previous studies, the duration of reperfusion was 24 h. It has been shown previously that rat infarcts undergo rapid remodelling characteristic of infarct healing, such as wall thinning, increased lumen volume and decreased infarct size secondary to scar formation. Indeed, such an early healing is already markedly evident in 24 h old infarcts, since at this time the anatomical area at risk is reduced by about 30% as compared to early values (Hearse *et al.*, 1988). One consequence of this is that any drug that can influence the rate of early infarct remodelling may give an impression of infarct size limitation when assessed at 24 h (especially when area at risk is not quantified), when in reality no such event may have occurred (Hearse *et al.*, 1988). Thus, differences in the duration of reperfusion or absence of measurements of area at risk may explain the differences between our results and those of the two previously published studies performed in rats.

Another difference between our study and that of Grover *et al.* (1992) relates to the drug used to inhibit the endothelin effects. We used a specific inhibitor of endothelin receptors which does not show any binding inhibitory activity against various eicosanoids, ions or peptides (Clozel *et al.*, 1994). In contrast, the study of Grover *et al.* (1992) was performed using phosphoramidon, a metalloprotease inhibitor that is not specific for endothelin converting enzyme but may inhibit other metalloproteases such as neutral endopeptidase EC.3.4.24.11 (Turner, 1987). Thus, it is possible that the positive results obtained by Grover *et al.* (1992) could be the consequence of nonspecific effects of phosphoramidon, independent of its inhibitory effect on endothelin converting enzyme. Indeed, recent experiments showed that a specific antagonist of ET_A receptors, FR 139317, also did not affect infarct size in a rabbit model of ischaemia–reperfusion (McMurdo *et al.*, 1994). Thus, our results confirm those of McMurdo *et al.* and extend those to ET_B receptors. Taken together, our results and those of McMurdo *et al.* (1994) suggest that endogenous endothelin does not contribute to the extension of infarct size after myocardial ischaemia and reperfusion.

One possible explanation of this lack of effect would be that endothelin is not released in quantities sufficient to induce deleterious effects during ischaemia and reperfusion, or to express its pro-arrhythmic effects. Although we have not measured plasma levels of ET-1 in our model, a previous study showed a fourfold increase in plasma ET-1 10 min into reperfusion after a 1 h episode of *in vivo* ischaemia in rats (Watanabe *et al.*, 1991a). In dogs, a short episode of coronary occlusion (10 min) induces an even less marked increase in plasma ET-1 (from 1.3 to 2.0 pM; Donckier *et al.*, 1994). Although the pro-ischaemic effects of such an increase in plasma endothelin cannot be easily assessed, it is possible that such moderate increases in plasma ET-1 are not sufficient to induce significant myocardial or vascular effects.

Effect of bosentan on coronary endothelial dysfunction

In our experiments, ischaemia followed by reperfusion did not affect the coronary response to 5-HT or to the nitric oxide donor SIN-1, but markedly reduced the response to acetylcholine, confirming our previous results in the same experimental model (Richard *et al.*, 1994). Such an endothelial dysfunction also confirms previous results obtained in other species (Vanbenthuysen *et al.*, 1987; Dauber *et al.*, 1990; Pearson *et al.*, 1990a; Tsao *et al.*, 1990). Moreover, we showed previously that a similar alteration was not observed in arteries obtained from hearts subjected to ischaemia without reperfusion (Richard *et al.*, 1994), suggesting that this coronary endothelial dysfunction is a manifestation of reperfusion injury.

One limitation of the study is that only one endothelium-dependent vasodilator was used (acetylcholine). We attempted to study other potential endothelium-dependent vasodilators, i.e. adenosine di-phosphate, α_2-adrenoceptor agonists, histamine and bradykinin, and found in pilot studies that none of them induced significant endothelium-dependent relaxations. In addition, we found previously that receptor-independent, endothelium-dependent relaxations to the calcium ionophore, A23187, were rather weak in this preparation (Richard *et al.*, 1994). Furthermore, substance P and 5-HT, which induce endothelium-dependent relaxations in other species, also do not relax rat coronary arteries (Nyborg & Mikkelsen, 1990; Prieto *et al.*, 1991). Thus, to our knowledge, acetylcholine is the only endothelium-dependent relaxing agent active on rat isolated coronary arteries. Because of this limitation, we could not determine whether the impairment observed in the present experiment reflects a true defect in nitric oxide synthase activity or a specific impairment of the transduction pathway linking muscarinic receptors to NO-synthase. Such a selective impairment has already been shown to occur in other pathological situations such as hypercholesterolaemia or atherosclerosis, which selectively affect Gi protein-mediated transduction pathways (Flavahan, 1992), and in canine experiments involving chronic reperfusion (Pearson *et al.*, 1990b). In any case, the absence of any effect of bosentan on the response to acetylcholine suggests that this compound was unable to prevent coronary endothelial dysfunction, and thus that endogenous endothelin probably does not contribute to reperfusion-induced coronary endothelial dysfunction in this model.

In conclusion, our results obtained in rats suggest that administration of the mixed ET_A and ET_B endothelin antagonist, bosentan, at a dose which virtually abolished the pressor response to big ET-1, did not have any significant effect of infarct size, reperfusion arrhythmias, and reperfusion-induced coronary endothelial dysfunction. These results suggest that endogenous endothelin probably does not contribute to myocyte or coronary endothelial injury in this model of ischaemia with reperfusion.

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