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Localization and function of ET-1 and ET receptors in small arteries post-myocardial infarction: Upregulation of smooth muscle ET_B receptors that modulate contraction

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1 Endothelin-1 (ET-1) has been implicated as a mediator of increased vascular tone during development of heart failure post-myocardial infarction (MI). In the present study, expression and pharmacology of ET-1 and its receptors were studied in small mesenteric arteries from rats at 5 and 12 weeks after coronary artery ligation for induction of MI, or sham-operation.

2 In vessels from sham-operated and 5 week post-MI rats preproET-1mRNA, immunoreactive (ir) ET-1, ET_B receptor mRNA and irET_B receptor were confined to the endothelium, while ET_A receptor mRNA was distributed throughout the media. At 12 weeks post-MI, preproET-1 and irET_A receptor localization was similar but ET_B receptor mRNA and immunoreactivity were detectable in the media, as well as the endothelium.

3 The ET-1 concentration-response curve (CRC) was progressively shifted to the right in pressurized third generation mesenteric arteries from 5 and 12 week post-MI rats relative to sham-operated rats, with no change in the maximum. The ET_A receptor antagonist BQ-123 (10^{-6} M) induced a rightward shift of the ET-1 CRC in all vessels. Desensitization of ET_B receptors, by exposure to SRTX S6c (3×10^{-8} M), had no effect on the ET-1 CRC in vessels from 5 week post-MI or sham-op rats but induced a leftward shift in vessels from 12 week post-MI rats.

4 These results identify the endothelium as the primary site of ET-1 synthesis in small arteries and the ET_A receptor as mediating the effects of ET-1 in these vessels. However, ET_B receptor expression increases in vascular smooth muscle post-MI and is linked to mechanisms that inhibit the contractile response to ET-1.

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Abbreviations: ACh, acetylcholine; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BQ-123, (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]); CAL, coronary artery ligation; CHF, chronic heart failure; CRC, concentration-response curve; DAB, diaminobenzidine; DIG, digoxigenin; ET_A, endothelin type A receptor; ET_B, endothelin type B receptor; ET-1, endothelin-1; ir, immunoreactivity; KCl, potassium chloride; KH, Krebs-Henseleit solution; MI, myocardial infarction; NBT, nitro-blue tetrazolium chloride; PE, phenylephrine; SRTX S6c, sarafotoxin S6c

Introduction

Systemic vasoconstriction and reduced peripheral perfusion contribute to the vicious cycle that leads to the development of chronic heart failure (CHF) post myocardial infarction (MI). While augmented adrenergic activity and stimulation of the renin-angiotensin system have been implicated as mediators of increased vascular tone in this process, blockade of both systems is ineffective in normalizing peripheral resistance, or in preventing the high mortality associated with CHF (Dargie & McMurray, 1994). Plasma concentrations of the potent vasoconstrictor peptide endothelin-1 (ET-1) and its precursor big ET-1 are increased post MI and levels increase in proportion to the symptomatic and haemodynamic severity of CHF (Rodeheffer *et al*., 1992; Pacher *et al*., 1993; Wei *et al*., 1994). ET-1 has thus emerged as a potential mediator of the increased peripheral vascular resistance that contributes to progression of CHF post-MI.

The actions of ET-1 are mediated *via* ET-1 selective ET_A receptors and ET isopeptide unselective ET_B receptors (Gray & Webb, 1996). Antagonists of both receptor subtypes are now available and a number are in development for use in diseases where there is evidence of a pathophysiological role for ET-1 (Gray *et al*., 2000). In order to apply the most effective therapy it is important to characterize the receptors mediating the actions of ET-1 and to understand what the consequences of blockade of these receptors might be. In healthy blood vessels both ET_A and ET_B receptors are found on smooth muscle cells where they mediate the constrictor effects of ET-1, produced by vascular endothelial cells. Activation of ET_B receptors on endothelial cells results in the liberation of endothelium-derived relaxing factors that cause vasodilatation (D'Orleans-Juste *et al*., 1993). In most vessels studied the ET_A receptor is the principal subtype mediating smooth muscle contraction (Davenport *et al*.,

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1995), although the ET_B subtype can make a significant contribution (Haynes *et al*., 1995), particularly on the low pressure side of the circulation (Moreland *et al*., 1992; Gray *et al*., 1994; Strachan *et al*., 1995). In addition, smooth muscle ET_B receptors can influence ET_A receptor mediated contraction through intracellular cross-talk mechanisms (Fukuroda *et al*., 1994b; Clozel & Gray, 1995; Mickley *et al*., 1997a; Ozaki *et al*., 1997). A physiological role for ET-1 in the regulation of vascular tone in man has been demonstrated by infusion of ET_A receptor antagonists, either locally into the forearm where they increase blood flow, or systemically, where they reduce mean arterial blood pressure and peripheral vascular resistance (Haynes *et al*., 1995; 1996). Systemic infusion of a selective ET_B receptor antagonist results in peripheral vasoconstriction in experimental animals (Allcock *et al*., 1995) and in man (Strachan *et al*., 1999), demonstrating that liberation of relaxing factors is the principle role of ET_B receptor activation in healthy vasculature.

In cardiovascular pathophysiology there is increasing evidence for an alteration in the balance of receptors mediating the actions of ET-1 in blood vessels. While this can be explained in part through endothelial dysfunction which reduces the influence of endothelial ET_B receptor activation (Hasdai *et al*., 1997; Haynes & Webb, 1998; Kakoki *et al*., 1999), there is also evidence for changes in the expression and function of smooth muscle ET receptors. Downregulation of both ET_A and ET_B receptors occurs in hypertension (Schiffrin *et al*., 1995; Touyz *et al*., 1995) and ET_B receptor expression or function is reported to be increased in atherosclerosis (Dagassan *et al*., 1996), in spontanously hypertensive rats (Batra *et al*., 1993), in diabetes (Sullivan *et al*., 1997) and in response to increased flow (Barber *et al*., 1996). In CHF, there is functional evidence to support vascular ET_B receptor upregulation in patients (Love *et al*., 1996) and in an animal model (Cannan *et al*., 1996). ET_A receptor expression is reported to be decreased post-MI in mesenteric arteries (Fu *et al*., 1993). These effects might be associated with a phenotypic change of vascular smooth muscle cells in disease (Adner *et al*., 1998; Eguchi *et al*., 1994; Owe-Young *et al*., 1999), or they might occur under the influence of angiotensin II (Kanno *et al*., 1993) or ET-1 itself (Hamilton *et al*., 1994; Hirata *et al*., 1988). Circulating levels of ET-1 and its big ET-1 precursor are increased in a range of cardiovascular diseases, due to increased synthesis or reduced clearance (Haynes & Webb, 1998). In the vasculature, smooth muscle cells that are normally the target cell for ET-1 can generate ET-1 in pathology e.g. in coronary artery disease, in hypertension, or in tissue culture (Yu & Davenport, 1995; Schiffrin *et al*., 1997; Dashwood *et al*., 1998; Rossi *et al*., 1999).

The sites of ET-1 synthesis and the receptors mediating its effects in peripheral blood vessels during progression of CHF post-MI are not fully understood. The principal aims of the present study were therefore to characterize the receptors mediating the actions of ET-1 in resistance vessels from rats which had undergone coronary artery ligation and to correlate receptor function with distribution using *in situ* hybridization and immunocytochemistry. Furthermore, as receptor expression can be influenced by local ET-1 concentration prepro ET-1 mRNA and immunoreactive ET-1 were localized in the blood vessel wall and plasma levels of ET-1 and big ET-1 were measured by radioimmunoassay. Some of these data have been presented to the British Pharmacological Society (Mickley *et al*., 1997b) and to the International Society for Heart Research (Mickley *et al*., 1998).

Methods

Coronary artery ligation surgery

Adult, male Wistar rats, 250–300 g (Charles River), were subjected to ligation of the proximal portion of the left, main coronary artery according to the method of Pfeffer *et al*. (1979). Briefly, rats were anaesthetized by injection of sodium pentobarbital (Sagatal, 60 mg kg^{-1} i.p.), intubated with a plastic cannula and mechanically ventilated with oxygen enriched room air by use of a small rodent ventilator (Harvard Apparatus, U.K.) at a rate of 60 cycles min^{-1} and a tidal volume of 1 ml 100 g^{-1} body weight. A left thoracotomy was performed between the third and fourth ribs and the pericardium opened. The heart was gently but rapidly exteriorized and a 5/0 silk suture (Ethicon Ltd, U.K.) was passed around the left, main coronary artery. Sham-operated rats were subjected to the same protocol, except the suture was pulled through under the artery. The heart was subsequently returned to its position in the thorax and the rib cage closed with a 3/0 suture. A plastic catheter connected to a 1 ml syringe was placed in the chest before sewing and was used to remove air from the chest after closure. The animals were allowed to recover from anaesthesia, and were given buprenorphine (0.3 mg kg^{-1}) subcutaneously for analgesia. Ethical approval was granted by the Home Office.

Plasma endothelin levels

At 5 or 12 weeks post ligation or sham operation, rats were anaesthetized (Sagatal, 60 mg kg^{-1} i.p.) and blood was removed from the dorsal aorta into ice-cold tubes containing 5% EDTA for the measurement of plasma ET-1 and big ET-1 by radioimmunoassay, as previously described (Newby *et al*., 1998).

Tissue preparation and measurement of infarct size

Following exsanguination, the mesenteric bed was rinsed and sections removed into either cold Krebs-Henseleit (KH) solution for functional studies or 10% neutral buffered formalin for fixation. After fixation the tissues were processed in ethanol and embedded in paraffin wax for immunohistochemistry and *in situ* hybridization. The hearts and lungs were removed, rinsed in saline to remove any residual blood and weighed. Hearts were bisected then fixed and processed as above. Infarct size was measured in paraffin sections as described previously (Mulder *et al*., 1998). Sections were placed under a CCD video camera module (Sony, U.K.) attached to a microscope with a $\times 20$ lens. The endocardial and epicardial circumferences of the infarcted tissue and of the LV were determined with image analysis software (Imaging Associates, Thame, U.K.). Infarct size was calculated as a percentage of [endocardial + epicardial circumference of the infarcted LV (mm)] / [endocardial + epicardial circumference of the whole LV (mm)].

In situ *hybridization*

The method for detecting preproET-1 and ET receptor mRNA expression *in situ* has been described previously (McEwan *et al*., 1998). In brief, cDNA fragments subcloned into pCR 2 vector (Stratagene) were linearised with *Hind*III, *Kpn*I and *Eco*RI restriction enzymes. cDNA templates for preproET-1, ET_A receptor and ET_B receptor were purified by phenol-chloroform extraction. Complementary mRNA anti-sense

probes were transcribed in the presence of digoxigenin (DIG)-labelled uracil (Boehringer Mannheim GmBH, Germany) and T7 and SP6 RNA polymerases. Sense probes were transcribed using T3 polymerase. Consecutive 3 μm paraffin sections were incubated with either sense or antisense DIG-labelled probes for preproET-1, ET_A receptor or ET_B receptor mRNA overnight. Following treatment with and a subsequent series of stringent washes in standard sodium citrate/formamide, sections were then treated with an alkaline phosphatase conjugated anti-DIG antibody and nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate. Prepro ET-1, ET_A receptor and ET_B receptor mRNA were detected as dark blue dots within the cytoplasm of cells in tissue sections. Transverse sections of aorta were used as positive controls. Negative controls were treated with RNAase prior to incubation with antisense or sense probes. Non-specific binding of the anti-DIG antibody was blocked using undiluted sheep serum for 1 h at 24°C.

Immunocytochemistry

Immunoreactive ET-1 and ET_B receptor were detected within parallel vessel sections using anti-ET-1 (Biodesign) and anti ET_B monoclonal antibodies (Calbiochem), followed by an alkaline phosphatase-conjugated goat anti-mouse IgG. ET-1 and ET_B receptor were detected using biotinylated secondary antibody (Dako) and a streptavidin-biotin-peroxidase kit (Dako) followed by incubation with diaminobenzidene (DAB). Positive signals appeared brown. Negative controls were treated with an antibody of the same immunoglobulin class not directed against the ET-1 epitope, or with DAB alone.

Perfusion myograph studies

Third order branches of the mesenteric artery (lumen diameters shown in Table 2) were excised and mounted between two fine glass cannulae ($\sim 100-150$ μm tip diameter) in a perfusion myograph (Living Systems Instrumentation, Burlington, VT, U.S.A) as previously described (Mickley *et al*., 1997a). Briefly, an intraluminal pressure of 60 mmHg was maintained by a peristaltic pump connected to a pressure servo unit. The arteries were superfused with warmed (37°C) and gassed (95% O_2 and 5% CO_2) KH solution (mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 5.5; BDH-Merck, Dorset, U.K.) containing indomethacin (10^{-4} M) at a flow rate of 5 ml min^{-1}. Lumen diameter and wall thickness were measured by video dimension analyser (Living Systems Instrumentation, Burlington, VT, U.S.A.) calibrated against a stage micrometer (resolution = 1 μm). After an equilibration period of 60 min the vessels were exposed twice to modified KH solution containing 60 mM KCl (equimolar replacement of NaCl by KCl) to produce maximum constriction. In all experiments included in this study the endothelium was removed by passage of an air bubble through the lumen of the mounted vessel (Falloon *et al*., 1993). Removal of the endothelium was confirmed by loss of acetylcholine (ACh 10^{-6} M) induced relaxation in vessels preconstricted with the adrenoceptor agonist phenylephrine (PE, 10^{-5} M).

Cumulative agonist concentration-response curves (CRC) were obtained by addition of ET-1 ($10^{-13}-3\times10^{-8}$ M) or SRTX S6c ($10^{-12}-3\times10^{-8}$ M) to a 30 ml KH reperfusion circuit as previously described (Mickley *et al*., 1997a). Responses were recorded 5 min after addition of each agonist concentration, which was sufficient time for an equilibrium response. All of the following studies were carried out in random order and only one concentration response curve to ET-1 was performed per tissue. None of the drug treatments resulted in complete occlusion of the vessel lumen within the concentration range studied. To study the role of the ET_A receptor, BQ-123 (10^{-6} M) was added to the reperfusion circuit 30 min before commencement of the ET-1 CRC. To study the role of the ET_B receptor vessels were exposed to a supramaximal concentration of SRTX S6c (3×10^{-8} M) for 30 min, twice (with a wash out period of 10 min between each exposure), prior to commencement of the ET-1 CRC to desensitize ET_B receptors (Mickley *et al*., 1997a). Some vessels underwent both desensitization and exposure to BQ-123.

Peptides and reagents

ET-1 and SRTX S6c (Novabiochem, Nottingham, U.K.) were reconstituted in 50 : 50 methanol:distilled water. BQ-123 (cyclo [D - Trp - D - Asp - L - Pro - D - Val - 1 - L-Leu], Neosystems, France) was reconstituted in 0.9% saline, aliquoted and stored frozen at −20°C until use. All peptide agonists and antagonists were diluted in KH solution containing 0.1% bovine serum albumin (BSA: Sigma, Poole, U.K.). In all antagonist experiments the ET-1 concentrations were diluted in 0.1% BSA KH solution containing the appropriate antagonist. ACh (chloride salt, Sigma, Poole, U.K.) and PE (hydrochloride salt; Fisons, U.K.) were prepared in saline at stock concentration of 10^{-2} M, aliquoted, and stored at −20°C until use when diluted in KH solution.

Data analysis

Plasma peptide concentrations, tissue weights and infarct sizes data were analysed by one-way ANOVA and significance was established using Bonferroni's test. Unpaired observations were assessed by Student's *t*-test. For functional studies responses to receptor agonists were calculated as a percentage of maximum constriction obtained with the second exposure to 60 mM KCl Krebs solution and are expressed as mean ± s.e.mean. Where a maximum response to the agonist was obtained, the negative log of the concentration causing half-maximal contraction (pD_2) was calculated by linear regression

Table 1 Infarct size and the effects of myocardial infarction (MI) on lung and heart weights and on plasma concentrations of ET-1 and big ET-1

	Heart weight (g kg^{-1} BW)	*Lung weight* (g kg^{-1} BW)	*Infarct size* (% LV free wall)	*ET-1* (pg ml^{-1})	*Big ET-1* (pg ml^{-1})
5 weeks sham	0.64 ± 0.05	0.81 ± 0.08	No infarct	2.8 ± 1.0	23.4 ± 9.2
5 weeks post MI	*0.82 ± 0.06	1.14 ± 0.14	44 ± 8	5.4 ± 2.0	26.6 ± 9.4
12 weeks sham	0.79 ± 0.07	0.95 ± 0.11	No infarct	5.0 ± 1.6	24.7 ± 8.8
12 weeks post MI	*1.00 ± 0.02	1.39 ± 0.04	49 ± 11	5.6 ± 2.6	41.6 ± 14.7

Parameters were measured using rats at 5 or 12 weeks post myocardial infarction (MI) or sham-operation (sham), $n = 8$ per group. Infarct size is expressed as a percentage of the left ventricular (LV) free wall. Heart and lung weights are expressed as a proportion of body weight (BW). Values are the mean ± s.e.mean, $*P < 0.05$ compared to age-matched sham-op.

analysis and compared using unpaired *t*-test. The concentration-response curves were compared by one-way ANOVA followed by Fisher's least significant difference test. A P value of <0.05 was considered significant.

Results

Survival, infarct size and plasma ET-1/big ET-1

The survival rate for rats that underwent coronary artery ligation for induction of myocardial infarction (MI) was approximately 75%, all deaths occurred within 24 h of surgery. Infarct sizes at 5 and 12 weeks post MI were $44 \pm 8\%$ ($n=8$) and $49 \pm 11\%$ ($n=8$) of the left ventricular free wall, $n=8$. Despite significant thinning and scar formation in the infarcted region (not shown), heart weight was significantly increased post-MI at both time points (Table 1). Plasma ET-1 and big ET-1 concentrations were not significantly greater post-MI than sham controls at either of the time points studied (Table 1).

PreproET-1 and ET-1 distribution

In situ hybridization in sections of third generation mesenteric arteries demonstrated that pre proET-1 mRNA is confined to the endothelial layer of the vessel wall (Figure 1a). The presence of signal in the adventitial layer of some vessels can

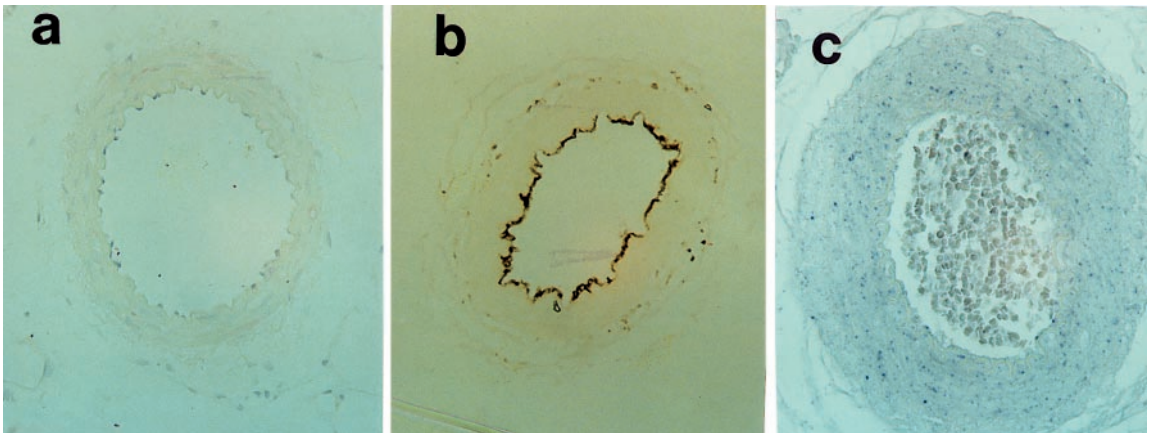

Figure 1 Representative mesenteric vessel sections from sham-operated rats showing localization of (a) pre pro ET-1 mRNA, and (b) immunoreactive ET-1 in the vascular endothelium, and (c) ET_A receptor mRNA (dark dots) in the smooth muscle media. Distribution was similar in vessels from sham-op and coronary artery ligation rats. Original magnification: $\times 240$. Figure is representative of at least seven sections.

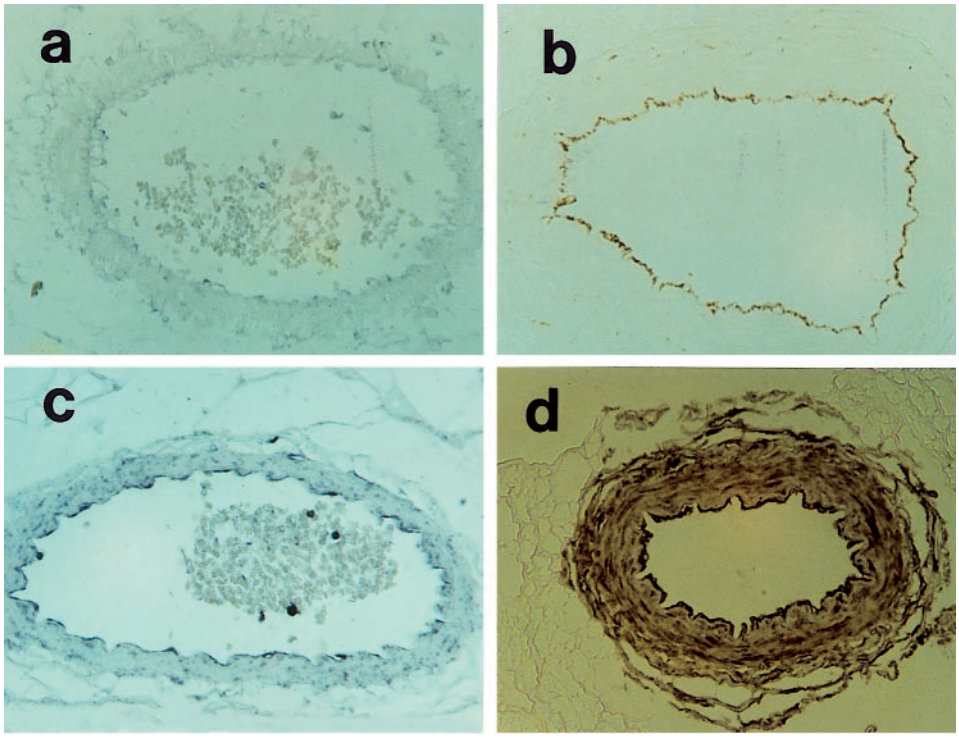

Figure 2 Representative mesenteric vessel sections showing localization of ET_B receptor mRNA (dark dots in a,c) and ET_B receptor protein (brown staining in b,d) in the endothelium of vessels from rats 12 weeks after sham-operation (a,b). Both are seen in medial smooth muscle in addition to the endothelium in vessels from rats 12 weeks after coronary artery ligation (c,d). Original magnification: $\times 240$. Figure is representative of at least six sections.

Table 2 Lumen diameters of third generation mesenteric arteries at rest and in response to constrictor agents

	Resting diameter (μm)	*Post KCl* (60 mM) *diameter* (μm)	*Post PE* (10^{-5} M) *diameter* (μm)
5 weeks sham	292±10	58±3	54±2
5 weeks post MI	294±8	56±2	53±3
12 weeks sham	285±9	56±2	56±3
12 weeks post MI	305±12	60±3	62±3

Third generation arteries were dissected from the mesenteric beds of rats at 5 or 12 weeks post myocardial infarction (MI) or sham-operation (sham), $n=8$ per group. Vessel diameters are shown at rest and after exposure to potassium chloride (KCl, 50 mM) or phenylephrine (PE, 10^{-6} M) values are mean±s.e.mean, $n=8$.

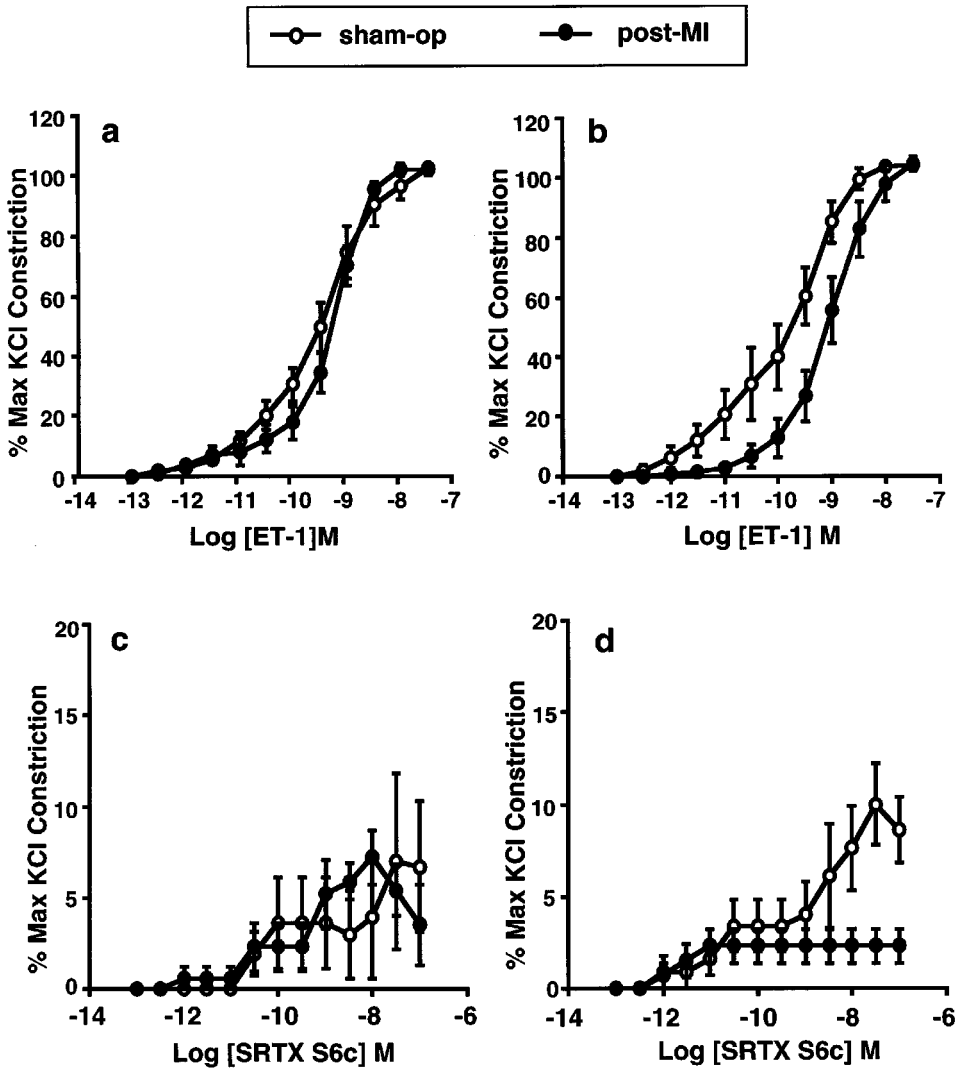

Figure 3 Cumulative concentration-response curves to ET-1 (a, b) and SRTX S6c (c, d) in small mesenteric arteries from rats 5 weeks (a, c) or 12 weeks (b, d) after coronary artery ligation (post-MI) or sham-operation (sham-op). Agonist responses are expressed as a percentage of the maximal contraction to KCl (60 mM) and are means±s.e.mean ($n=6-8$).

best be explained by synthesis in the endothelium of the vasa vasorum. Production of mature ET-1 in the endothelium of mesenteric arteries and in the vasa vasorum was confirmed by immunocytochemistry (Figure 1b). Immunoreactive ET-1 was seen in the medial smooth muscle layer of some vessels, most likely resulting from binding of ET-1 to receptors on smooth muscle cells.

ET_A and ET_B receptor mRNA distribution

A high level of ET_A receptor expression in the medial smooth muscle of all vessels was demonstrated by *in situ* hybridization (Figure 1c). No obvious differences in expression of the ET_A receptor were detected between the different groups studied (results not shown). In contrast to the ET_A receptor, neither ET_B receptor mRNA expression nor significant immunoreactivity was detectable in the medial layer of arteries from sham-operated rats or from rats at 5 week post-MI (results not shown). ET_B receptor expression and immunoreactivity was confined to the endothelial and adventitial layers of these vessels (Figure 2a,b). However, in mesenteric arteries from rats 12 weeks post-MI there was clear evidence of ET_B receptor mRNA expression in the medial layer (Figure 2c). The presence of

ET_B receptor protein on smooth muscle cells was confirmed by immunoreactivity (Figure 2d).

Perfusion myography

The mean lumen diameter of the small mesenteric arteries was similar in all of the groups studied (Table 2). There was no difference in the maximal constriction obtained in response to KCl or PE in the arteries from either the 5 or 12 week post-MI rats compared to their respective sham-operated controls (Table 2).

ET-1 constricted all arteries in a concentration-dependent manner (Figure 3). There was no significant difference in the response to ET-1 of arteries taken from rats at 5 weeks post-MI relative to age matched sham-operated rats (pD_2 values 9.3 ± 0.2 and 9.2 ± 0.2 respectively, $n=8$ per group, Figure 3a) However, by 12 weeks post surgery the ET-1 CRC was significantly shifted to the right in arteries from post-MI rats relative to sham-operated controls (ANOVA $P<0.04$; pD_2 values 9.5 ± 0.2 and 8.9 ± 0.2 respectively, $P<0.05$, $n=8$ per group, Figure 3b), although there was no change in the maximum response.

In contrast to ET-1, contractile responses to the ET_B receptor agonist SRTX S6c were small and variable in vessels from sham-operated and post-MI rats at both time points studied (Figure 3c,d). At 12 weeks post-MI only one of the six arteries exposed to SRTX S6c responded to it by contracting.

ET_A receptor antagonism with BQ-123 caused a rightward shift of the ET-1 CRC in arteries from both 5- and 12-week sham-operated animals (Figure 4a,b). However, in arteries from rats post MI the effect of BQ-123 on the ET-1 CRC could be differentiated from that in the respective age-matched sham-operated controls. At 5 weeks post-MI BQ-123 inhibited responses to ET-1 above 10^{-9} M but had no significant effect

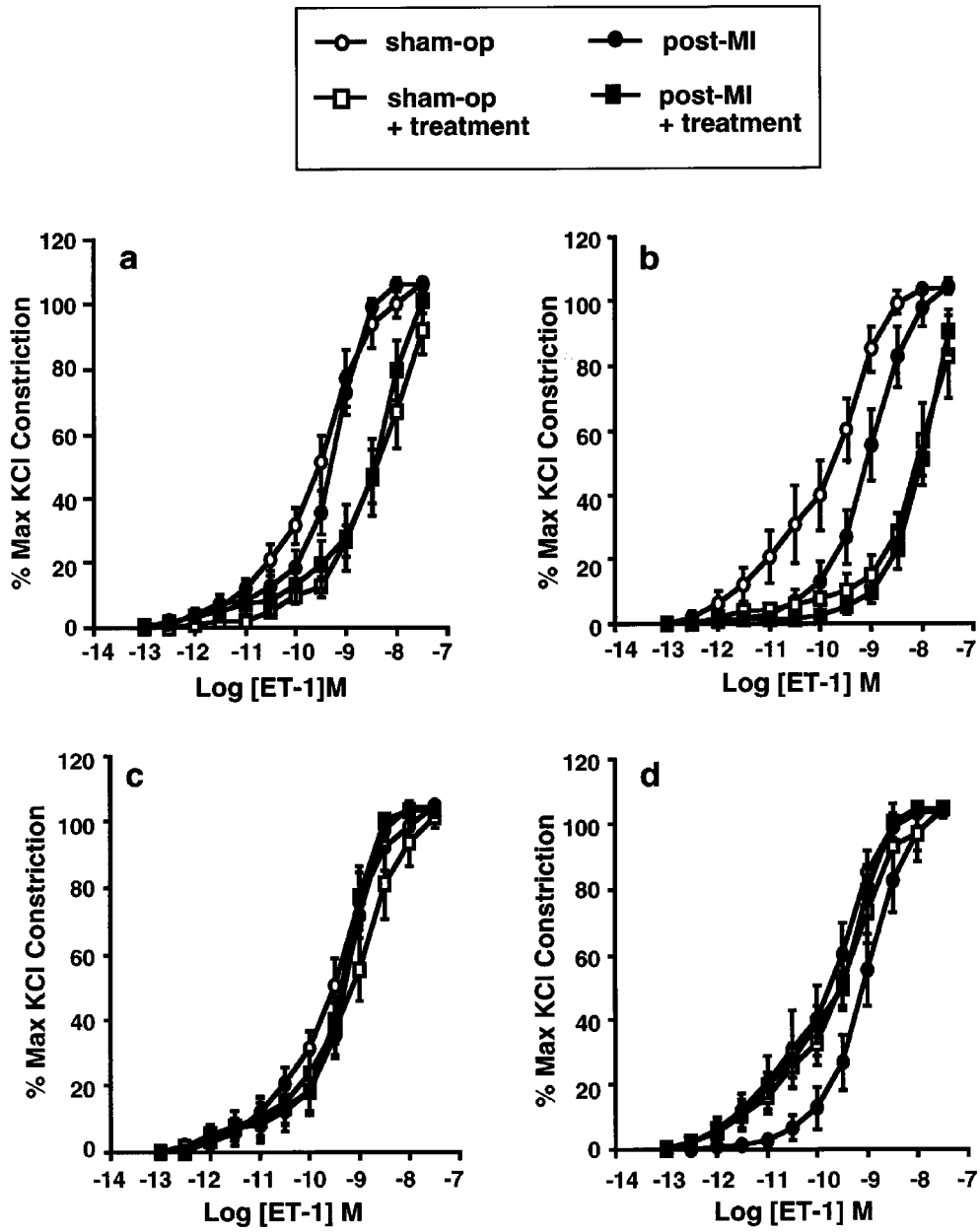

Figure 4 The effect of ET_A receptor antagonism by BQ-123 (10^{-6} M, a,b) or ET_B receptor desensitization by exposure to SRTX S6c (3×10^{-8} M, c,d) on cumulative concentration-response curves to ET-1 in small mesenteric arteries from rats 5 weeks (a,c) or 12 weeks (b,d) after coronary artery ligation (post-MI) or sham-operation (sham-op). Agonist responses are expressed as a percentage of the maximal contraction to KCl (60 mM) and are means $\pm$ s.e.mean ($n=6-8$).

on responses elicited by doses of ET-1 between 10^{-13} and 3×10^{-10} M (Figure 4a). At 12 weeks post MI the rightward shift was parallel but the magnitude of the shift was reduced relative to that in the sham-operated control arteries (Figure 4b). The pD_2 values could not be calculated for the studies involving BQ-123 as maximum responses were not reached in any group.

Desensitization of ET_B receptors following exposure to a supramaximal concentration of SRTX S6c (LaDouceur *et al*., 1993; Mickley *et al*., 1997a) was confirmed by loss of response to a second exposure to SRTX S6c (not shown). Desensitization of the ET_B receptor had no effect on responses to ET-1 in arteries from sham-operated or 5-week post-MI rats (Figure 4c,d). In contrast, the ET-1 CRC in arteries from 12-week post-MI rats was shifted to the left following ET_B receptor desensitisation so that it became superimposable on the ET-1 CRC obtained in arteries from sham-operated animals (Figure 4d, pD_2 values of 9.4 ± 0.2 and 9.4 ± 0.3 respectively, $n = 6$ per group).

Combined removal of ET_A and ET_B receptor influence was achieved by exposure to a supramaximal concentration of SRTX S6c in the presence of BQ-123. In comparison to ET_A receptor blockade alone, addition of ET_B receptor desensitization resulted in an increased rightward shift of responses to low concentrations of ET-1 at 5 weeks post sham-operation or MI (Figure 5a). In vessels from 5-week post-MI rats the threshold concentration for ET-1 increased from 3×10^{-13} M with BQ-123 alone to 3×10^{-10} M with additional exposure to SRTX S6c (Figure 5a). Responses to concentrations of ET-1 above 10^{-8} M were similar whether BQ-123 was administered alone or in combination with ET_B receptor desensitization. This differential effect of BQ-123 or in combination with SRTX S6c on the ET-1 CRC was not present in arteries from rats 12 weeks post-MI.

Discussion

Although it is known that plasma levels of ET-1 increase during the progression of CHF following MI (Tomoda, 1993; Omland *et al*., 1994), little is known of the receptors mediating the vascular effects of ET-1 in these conditions. The present study demonstrates that vascular responsiveness to ET-1 decreases progressively in the period following MI in the rat. Loss of responsiveness is not associated with increased local or systemic ET-1 synthesis but is temporally linked to upregulation of ET_B receptor expression in the smooth muscle medial layer. Early after MI ET_B receptors play a role in mediating contraction in response to low concentrations of ET-1, but with time post-MI the upregulated ET_B receptor appears to become linked to pathways that inhibit rather than potentiate contraction.

In small mesenteric arteries from sham-operated animals the dominance of ET_A receptor mRNA in the smooth muscle medial layer is reflected in the effectiveness of the ET_A receptor antagonist, BQ-123 as an inhibitor of ET-1 mediated contraction. Thus, the ET_A receptor is the major player in regulation of ET-1 induced contraction in these arteries, as in most arteries studied (Davenport *et al*., 1995). However, responses to very low concentrations of ET-1 persist in the presence of BQ-123 in these vessels consistent with previous studies showing that, in addition to ET_A receptors, high affinity ET_B receptors can mediate arterial smooth muscle contraction (Sumner *et al*., 1992; Teerlink *et al*., 1994; Mickley *et al*., 1997a; McCulloch *et al*., 1998). Binding studies have shown that ET_B receptors are relatively few in number in smooth muscle, even when ET_B mediated responses are much more significant than those found here (Gray *et al*., 1994; Teerlink *et al*., 1994; Davenport *et al*., 1995). The lack of detectable ET_B receptor mRNA or immunoreactivity in the medial layer of arterial sections from sham-operated rats is consistent with the inability of the selective ET_B receptor agonist SRTX S6c to evoke reproducible contraction in small mesenteric arteries. In these arteries the ET_B receptor is localized almost exclusively to the endothelial layer consistent with the presence of ET_B receptors on these cells. However, these endothelial receptors have no relevance to the functional studies presented here as the endothelium was removed prior to commencement of the studies.

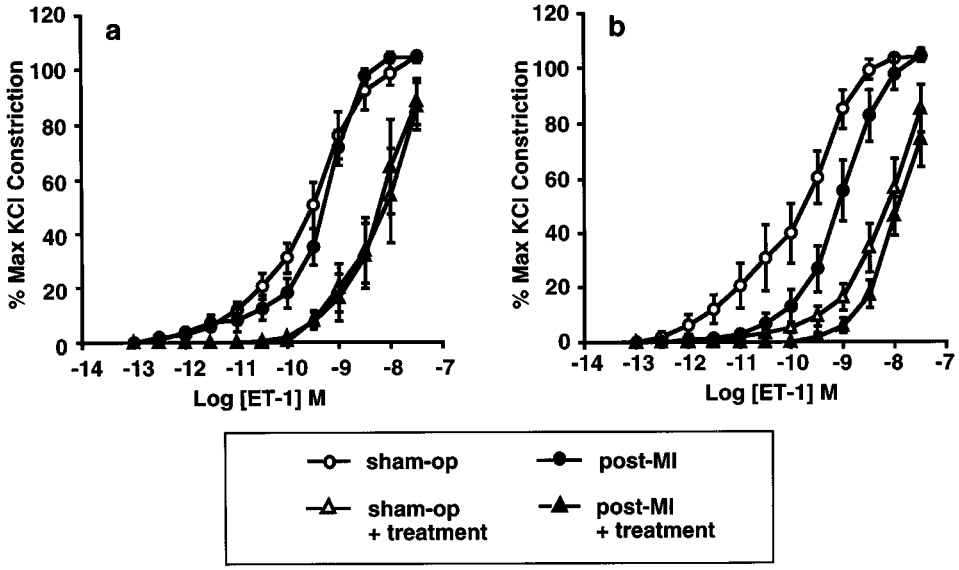

Figure 5 The effect of combined ET_A receptor antagonism, by BQ-123 (10^{-6} M), and ET_B receptor desensitization, by exposure to SRTX S6c (3×10^{-8} M), on cumulative concentration-response curves to ET-1 in small mesenteric arteries from rats 5 weeks (a) or 12 weeks (b) after coronary artery ligation (post-MI) or sham-operation (sham-op). Agonist responses are expressed as a percentage of the maximal contraction to KCl (60 mM) and are means ± s.e.mean ($n = 6-8$).

In vessels from rats at 5 weeks post MI, responses to ET-1 were similar to those seen in the age-matched arteries from sham-operated animals. However the ET_A antagonist resistant portion of the response to low concentrations of ET-1 was more marked in post-MI than in sham-op arteries. In these vessels, the ET-1 CRC was shifted in parallel only when ET_A receptor blockade was combined with ET_B receptor desensitization, suggesting a role for contractile ET_B receptors. However, the SRTX S6c response was not increased in the 5 week post-MI vessels, nor was there any evidence for increased immunoreactive ET_B receptor or ET_B receptor mRNA in the smooth muscle media at this time point. This result and the fact that ET_B desensitization alone had no effect on responses to ET-1 suggests that existing ET_B receptors may regulate the activation of the ET_A receptor through intracellular crosstalk (Fukuroda *et al*., 1994b; Clozel & Gray, 1995; Mickley *et al*., 1997a; Ozaki *et al*., 1997), rather than by direct activation of receptor signalling mechanisms that lead to contraction.

Investigation of ET-1 induced contraction in arteries from 12 week post-MI rats demonstrated progressive loss of sensitivity, corresponding with previous studies *in vitro* and *in vivo* that have demonstrated reduced constrictor and pressor responses to ET-1 post-coronary artery ligation (Fu *et al*., 1993). Loss of contractile responsiveness to ET-1 is unlikely to be due to smooth muscle dysfunction as the maximum responses to potassium and phenylephrine were conserved. The ET_A receptor antagonist, BQ-123, caused a parallel shift in the ET-1 concentration-response curve demonstrating that responses to ET-1 in these vessels were mediated predominantly by ET_A receptors. Down-regulation of ET receptor binding sites has been observed in cultured vascular smooth muscle cells following incubation with high concentrations of ET-1 (Hirata *et al*., 1988) and in conditions where local ET-1 concentration is increased *in vivo* (Schiffrin *et al*., 1997). However, in the present study the circulating concentration of ET-1 was not significantly increased and there was no evidence for recruitment of vascular smooth muscle cells as a site for ET-1 synthesis, as has been shown in other pathologies (Schiffrin *et al*., 1996; 1997; Rossi *et al*., 1999). Quantification of receptor expression was not possible in the small mesenteric arteries, but no gross alteration in the amount of ET_A receptor mRNA present in the smooth muscle medial layer was evident. However, in contrast to sham-operated controls there was a marked increase in ET_B receptor mRNA and protein associated with the smooth muscle of these arteries. In previous studies increased ET_B receptor expression has been associated with increased contractile responsiveness to ET_B selective agonists like SRTX S6c (Barber *et al*., 1996; Adner *et al*., 1998). In the present study, SRTX S6c induced contractions were not increased in arteries from 12-week post-MI rats relative to sham-operated controls. In fact, fewer arteries in this group responded to SRTX S6c than in controls. The role of the up-regulated ET_B receptor became clear after desensitization of the ET_B receptor, a procedure which we have shown previously to induce similar effects to ET_B receptor blockade using a specific antagonist (Mickley *et al*., 1997a). In arteries from 12-week post MI rats, ET_B receptor desensitization caused a leftward shift in the ET-1 concentration-response curve, restoring responses to control levels. These results suggest that the up-regulated ET_B receptor is linked to mechanisms that inhibit rather than potentiate contraction. Involvement of the endothelial ET_B receptor can be ruled out as a mechanism as removal of the endothelium was confirmed by complete loss of relaxation to ACh. Inhibitory ET_B receptors have been suggested previously based on the actions of ET_B receptor desensitization or antagonists in endothelium-denuded human gastroepiploic (Seo, 1996) or rat mesenteric arteries (Mickley *et al*., 1997a). In a recent study, Iwasaki *et al*. (1999) demonstrated endothelium-independent ET_B receptor mediated dilatation in rabbit mesenteric arteries. Dilatation was inhibited by indomethacin suggesting a role for vasodilator prostaglandins. This mechanism is however unlikely to be involved in the present study as indomethacin was present throughout all experiments. Intracellular crosstalk has emerged as a mechanism for modulation of ET_A receptor mediated actions by ET_B receptors (Fukuroda *et al*., 1994b; Clozel & Gray, 1995; Mickley *et al*., 1997a; Ozaki *et al*., 1997). However, in the present study, intracellular modification of ET_A receptor-mediated mechanisms seems unlikely as BQ-123 had similar effects in the presence or absence of ET_B receptor desensitization. There is evidence for a role of ET_B receptors in the clearance of ET-1 (Fukuroda *et al*., 1994a). It is possible that ET_B receptors have this role in CHF, and it cannot be ruled out in the present experiments that desensitization might have increased the local ET-1 concentration resulting in augmented responses. Clearly, further investigation of the mechanisms that cause ET_B receptor up-regulation and their role in pathophysiology is required.

Our data show that vascular responsiveness to ET-1 in small arterioles is progressively reduced after MI. Loss of responsiveness is related to up-regulation of an ET_B receptor in the smooth muscle media that is linked to inhibition of contraction stimulated *via* the ET_A receptor. There is no evidence for additional production of ET-1 by smooth muscle cells in these arteries. Increased systemic vascular resistance is believed to contribute to the development of CHF post MI by increasing afterload (Love & McMurray, 1996). If the mechanisms described in this study were to persist during progression to CHF then one would predict that an ET_A receptor antagonist would be of more benefit than an $ET_{A/B}$ antagonist in reducing constrictor responses to the increased circulating concentrations of ET-1 that accompany CHF.

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