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Effects of the endothelin receptor antagonist Bosentan on ischaemia/reperfusion injury in rat skeletal muscle

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Abstract

We examined the role of endothelin in ischaemia/reperfusion injury in skeletal muscle, using the endothelin receptor antagonist Bosentan. In the rat hindlimb tourniquet ischaemia model, one hindlimb was rendered ischaemic for 2 h at 36 °C, then blood flow was re-established for either 24 h to assess muscle survival or 1.5 h for a study of capillary perfusion. In the first set of rats, the gastrocnemius muscle was removed from the postischaemic limb and assessed for viability histochemically using the nitro blue tetrazolium stain. Tissue water content (a measure of oedema) and myeloperoxidase activity (a measure of neutrophil accumulation) were also assessed in the ischaemic muscle, the contralateral non-ischaemic muscle and the lungs. In the second set of rats, the hind limb was infused with India ink after 2-h ischaemia and 1.5-h reperfusion and the muscle was harvested, fixed and cleared. In control rats, muscle viability was $17 \pm 2\%$ (S.E.M.). In rats treated with Bosentan (10 mg/kg, i.p.) 30 min before release of the tourniquet, muscle viability ($48 \pm 7\%$) was significantly increased compared to the control group ($P < 0.01$). Bosentan treatment had no significant effect on tissue water content or myeloperoxidase activity in the ischaemic muscle, the contralateral non-ischaemic muscle or the lung. Immunoreactive endothelin levels in serum increased to a peak at 90 min of reperfusion and returned to control levels by 24-h reperfusion. India ink studies demonstrated a significantly increased functional capillary density in postischaemic Bosentan-treated muscles compared with postischaemic control muscles ($P < 0.05$). These results suggest that endothelin plays an important role in the necrosis which results from a period of ischaemia and reperfusion in skeletal muscle, by mediating a decrease in postischaemic microvascular perfusion.

Keywords: Endothelin; Ischaemia/reperfusion; Skeletal muscle; (Rat); Bosentan; No-reflow; Neutrophil; Oedema

1. Introduction

Tissue damage sustained following reperfusion of ischaemic tissue results from events in both the ischaemic and reperfusion phases (McCord, 1985). This ischaemia/reperfusion injury can occur in a range of clinical settings such as during transplantation surgery, tourniquet injury, free tissue transfer, acute compartment syndrome and replantation following accidental amputation, all of which entail unavoidable periods of ischaemia. The severity of ischaemia/reperfusion injury is directly related to the duration of ischaemia, the tissue temperature and the specific tissue sensitivity to ischaemic injury (Petrasek et al., 1994).

In addition to the direct injurious effects of ischaemia, on the resumption of an arterial blood supply, microvascular blood flow can be compromised to varying degrees (Strock and Majno, 1969). In the first hour of reperfusion, there is increased production and release of oxygen free radicals (Smith et al., 1989), Ca^{2+} influx and production of lipid mediators such as platelet-activating factor. The next few hours are characterised by the activation of endothelium, upregulation of inducible nitric oxide synthase (iNOS) (Zhang et al., 1997) and endothelin, expression of adhesion molecules (Zizzi et al., 1997), chemotaxis, adhesion of neutrophils (Smith et al., 1989) and microcirculation deficits such as the "no-reflow" phe-

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nomenon (Hickey et al., 1996). This latter condition effectively increases the duration of actual ischaemia in affected areas and therefore increases the severity of the injury. The inflammatory response is amplified after several hours of reperfusion due to increased production of cytokines such as tumor necrosis factor-α (TNF-α), interleukins 1α, 1β, 2 and 6, and interferon-γ (Seekamp et al., 1993) and further recruitment of leukocytes, resulting in increased tissue oedema (Zhang et al., 1997) and damaging effects to peripheral organs such as the lung (Seekamp et al., 1993). Each of these processes may contribute to the final tissue damage and necrosis (Grace, 1994).

Endothelin-1, a 21 amino acid peptide produced by endothelial cells, produces intense and long-lasting vasoconstriction (Yanagisawa et al., 1988; Roux et al., 1999). Although isolated vascular preparations contract in response to endothelin (Randall, 1991), intravenous administration produces a transient fall followed by a prolonged increase in systemic arterial blood pressure (MacLean et al., 1989). The fall in blood pressure results from activation of endothelin ET_B receptors located on endothelial cells, resulting in the release of prostacyclin and nitric oxide (NO) to cause vasodilatation (Newby and Webb, 1999).

A number of observations suggest that endogenously released endothelin contributes to ischaemia/reperfusion injury. Ischaemia is known to stimulate endothelin release (Rubanyi and Vanhoutte, 1985). In myocardial ischaemia in the rat, inhibition of endothelin synthesis with phosphoramidon decreases infarct size (Grover et al., 1992). Furthermore, raised plasma endothelin levels have been found in patients following myocardial infarction (Lam et al., 1991). In cat and dog brain, exogenously administered endothelin produces a vasoconstriction-related injury similar to that produced by ischaemia (Mima et al., 1989). In skeletal muscle, endothelin infusion into postischaemic muscle increases the severity of the reperfusion injury (Chang et al., 1992).

The aim of this study was to examine whether endothelin receptor antagonism during the postischaemic reperfusion of skeletal muscle increases survival following ischaemia/reperfusion injury. Bosentan, a low molecular weight, non-peptide, specific endothelin receptor antagonist, known to selectively block both endothelin ET_A and ET_B receptors (Clozel et al., 1994), was used in these experiments.

2. Materials and methods

2.1. Animal model

The model chosen for study of skeletal muscle ischaemia/reperfusion injury was the rat hindlimb tourniquet ischaemia model (Strock and Majno, 1969), used as described in our recent studies (Zhang et al., 1997). Male Sprague–Dawley rats, weighing 250–350 g, were used. All animal experiments were conducted with approval of the Animal Experimentation Ethics Committee, St Vincent's Hospital Melbourne. The animals were housed according to the National Health and Medical Research Council (Australia) guidelines for animal care.

2.2. Assessment of effect of Bosentan on systemic blood pressure

In order to determine the most appropriate dose of Bosentan, in initial experiments, the effects of Bosentan on the cardiovascular responses to increasing i.v. doses of endothelin were examined. Rats were anesthetized with pentobarbitone sodium (50 mg/kg intraperitoneal (i.p.)) supplemented with 20 mg/kg as required to maintain anesthesia. The internal carotid artery was cannulated with a 14-gauge cannula connected to a pressure transducer (Druck). The output of the pressure transducer was displayed and recorded on a MacLab recorder (ADI Instruments, Sydney, Australia) connected to a MacIntosh II ci computer. The external jugular vein was also cannulated to enable i.v. administration of endothelin. Bolus doses of endothelin of 30, 100, 300 and 1000 pmol/kg were administered and blood pressure responses were monitored for up to 30 min following each dose. Two groups of rats were examined: the first group received Bosentan (10 mg/kg, i.p.) ($n = 6$), and the second (control) group received vehicle (water for injection) at 1 ml/kg, i.p. ($n = 7$).

2.3. Ischaemia / reperfusion protocol

Rats were anesthetized, the left hind limb was shaved and a size 31 rubber band was applied on the thigh as high as possible. Pilot experiments using laser Doppler flowmetry and i.v. injection of India ink or fluorescein confirmed the effectiveness of the tourniquet applied in this manner in completely preventing blood flow during ischaemia ($n = 6$; data not shown). Ischaemia was maintained for 2 h, with the muscle temperature kept at 36 ± 1 °C using heat lamps. The temperature of the ischaemic limb was monitored using a probe (Sensortek Model BAT-12, Harvard Apparatus, South Natick, MA, USA) inserted subcutaneously in the lateral aspect of the gastrocnemius. The trunks and heads of the rats were shielded from the heat lamps to prevent hyperthermia and core temperature was maintained at 37.0 ± 1 °C. At the end of the ischaemic period, the rubber band was removed. Reperfusion was confirmed in all cases by the return of cutaneous circulation to the distal limb.

In the first series, the animals were divided into three experimental groups: one group ($n = 9$) received Bosentan (10 mg/kg, i.p.) 30 min prior to reperfusion, and the control group ($n = 17$) received the vehicle (water for injection) (1.0 ml/kg, i.p.). A sham group of five rats were anesthetised and placed under the heat lamps for the same period without application of the tourniquet. After

24-h reperfusion, the rats were sacrificed and the gastrocnemius muscle from the ischaemic limb was harvested and assessed for viability. The lungs and the anterior tibial muscles on the ischaemic and contralateral uninjured side were harvested and assayed for myeloperoxidase activity (indicative of neutrophil accumulation) and oedema (a measure of tissue water content) as in our previous studies (Zhang et al., 1997; Leong et al., 2000). All analytical procedures were carried out by persons blinded to drug pretreatments of the rats.

In the second series, the animals were divided into three experimental groups: a group ($n = 5$) receiving Bosentan (10 mg/kg, i.p.) 30 min prior to reperfusion, a control group ($n = 5$) receiving the vehicle and a sham group ($n = 5$) was prepared as described above. After 1.5-h reperfusion, the rats were sacrificed and the gastrocnemius muscle from the ischaemic limb was infused with India ink, as described in Sections 2.8 and 2.9 below. The decision to examine capillary perfusion at this stage of reperfusion was based on previous observations that capillary perfusion early in reperfusion correlates spatially with muscle survival 24 h after ischaemia (Hickey et al., 1996).

2.4. Muscle viability assessment

Muscle viability was assessed by nitro blue tetrazolium staining (Labbe et al., 1988; Novikoff et al., 1961). Previous studies from this laboratory using the rabbit rectus femoris muscle have shown that 24-h reperfusion is sufficient to avoid false positive staining in skeletal muscle (Hickey et al., 1992). The gastrocnemius muscle was sliced into 3-mm transverse sections. The muscle slices were incubated in a phosphate buffer (pH 7.4) containing 0.033% nitro blue tetrazolium (Sigma, St. Louis, MO) and 0.13% NADH (Sigma) for 25 min at room temperature, and then fixed in 10% buffered formalin. After fixation, each slice was weighed and the proportion of viable (labelled) tissue on both sides of each slice was quantified using computerized planimetry (Video Pro 32, Faulding Imaging, Clayton, Victoria, Australia). The percentage of viable tissue for the whole muscle was calculated from the proportion of viable tissue and the weight of each slice. Results are expressed as percentage of viable muscle tissue (mean ± standard error of the mean (S.E.M.)).

2.5. Radioimmunoassay of plasma endothelin levels

The endothelin radioimmunoassay (RIA) was carried out using a commercial assay kit (Peninsula Laboratories, Belmont, CA, USA) according to the manufacturer's instructions.

A standard curve was prepared such that a duplicate set of tubes contained 1, 2, 4, 8, 16, 32, 64, 128 pg/tube of the standard endothelin peptide. Incubation was carried out at room temperature for 90 min, after which endothelin was precipitated by the addition of goat anti-rabbit immunoglobulin G and centrifugation at $1700 \times g$ for 20 min at 4 °C. The supernatant, containing unbound ^{125}I-endothelin, was then aspirated and the amount of ^{125}I measured in a Packard Gamma Counter. The endothelin-like immunoreactivity of the unknown samples was determined by reference to the standard curve which was fitted by linear regression. Results were expressed as the mean ± the standard error of the mean (S.E.M.). All comparisons between multiple groups were evaluated using the one-way analysis of variance (ANOVA). P values < 0.05 were considered to be significant. Where significant differences were noted, Fisher's LSD comparison was performed to assess which groups were the source of the difference.

2.6. Oedema assessment

The ischaemic and contralateral anterior tibial muscle and the lungs were weighed before (wet weight) and after (dry weight) freeze-drying. Tissue water content (ml water/g dry weight muscle) was calculated as (wet weight − dry weight)/dry weight.

2.7. Myeloperoxidase assay

Myeloperoxidase content was assessed using a modification of the assay described by Bozeman et al. (1990). Between 20 and 30 mg of freeze-dried muscle was pulverized using a mortar and pestle and then homogenised in 2 ml of 20 mM of potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA (Ajax Chemicals, Australia). The homogenate was centrifuged at $14\,000 \times g$ for 3 min at 4 °C and the supernatant was resuspended in 1 ml of 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% cetyltrimethylammonium bromide (Serva, Heidelberg, Germany). The samples were freeze/thawed three times by immersion in cold acetone followed by sonication at 37 °C and then centrifuged at $1900 \times g$ for 7 min at 15 °C. A 250-μl aliquot of each sample was mixed with 8 μl of 0.1 M tetramethylbenzidine (Sigma) in dimethylformamide (Merck Chemicals, Australia) in a 37 °C water bath. The reaction was started with 20 μl of 7.88 mM hydrogen peroxide (Merck Chemicals), and after 3 min was stopped with 1.75 ml of 1 M acetic acid (Ajax Chemicals). The samples were centrifuged at $14\,000 \times g$ for 10 min at 4 °C. The absorbance was read on a spectrophotometer (Hitachi U-2000, Tokyo, Japan). One unit (U) of enzyme activity was defined as the change in absorbance of 1.0 per min at a wavelength of 655 nm.

2.8. India ink infusion technique

Heparinised India ink solution was prepared as previously described by Hickey et al (1996). Immediately prior to the end of the reperfusion period, the abdominal aorta was catheterized in an anterograde direction. The catheter was connected to a Miniplus 2 peristalsis pump (Gilson, Villiers Le Bel, France) with a reservoir containing the

biological ink prepared for infusion, with a side branch connected to a pressure transducer linked to a MacLab system to monitor infusion pressure. Infusion pressure was maintained between 90 and 110 mm Hg throughout by modulation of the infusion rate. This method allows rapid perfusion of both the postischaemic limb and the contralateral (sham-operated) limb. After infusion for approximately 5 min, the gastrocnemius muscles were rapidly isolated, removed and fixed in 10% buffered formalin.

2.9. Muscle clearing

Following fixation, the muscles were dehydrated in graded ethanol and cleared in cedar wood oil (Auroma, Australian Botanical Products, Hallam, Victoria, Australia). Using a dissecting microscope, hand-cut 5-mm-thick slices of the cleared muscles were examined to determine the pattern of perfusion and to identify the type and morphology of individual labelled vessels.

2.10. Quantitation of muscle cross-sectional area containing capillary label

Five slices from each muscle were embedded in paraffin, and 10-μm sections prepared and stained with tartrazine. With the operator blinded to the treatment, one tartrazine-stained section from each muscle slice was examined. The section was projected onto tracing paper using a Micro Promar projecting microscope (Leitz Wetzlar, Germany). Initially, using a 2.5× objective lens, a tracing was made of the complete cross-section of the muscle. Then, under higher magnification, the distribution of the ink-perfused capillaries adjacent throughout the muscle cross-section was determined. Perfused and non-perfused areas were identified and mapped onto the muscle tracing. Video images of the muscle tracing were taken using a WV-CL700/A CCD video camera (Panasonic, Osaka, Japan) connected to an IBM-compatible computer. The images were processed using the Video Pro 32 image analysis software package (Leading Edge, Hove, South Australia). This software was used to quantify (i) the cross-sectional area of the muscle slice, and (ii) the area in which capillary perfusion was present. The absolute areas of the muscle sections and the perfused areas were measured, and a ratio between the two was derived for each section. The percentage of muscle area perfused was derived from each muscle by calculating the mean of the ratios from the five sections examined.

2.11. Histology

Representative sections of nitro blue tetrazolium-stained muscle, fixed in buffered 10% formalin, were processed, embedded in paraffin, and 5-μm sections were cut and stained with haematoxylin and eosin (H & E).

3. Results

3.1. Time course of increases in plasma endothelin levels following reperfusion of ischaemic skeletal muscle

Following 2-h hindlimb ischaemia, plasma endothelin levels were assessed after 0-, 0.25-, 1.5-, 4- and 24-h reperfusion. Endothelin levels were significantly increased after 1.5 h of reperfusion ($P < 0.05$, ANOVA) (Fig. 1), but not at the other time points.

3.2. Effect of Bosentan on systemic blood pressure changes induced by endothelin

In order to establish a suitable dose of Bosentan, the potency of Bosentan as an antagonist of endothelin's vascular effects was examined. Endothelin (30–1000 pmol/kg, i.v.) elicited dose-related and biphasic changes in systemic arterial blood pressure, comprising a transient depressor response lasting 1 min, followed by a prolonged pressor response which reached a plateau level after 10 min.

Bosentan (10 mg/kg), which had no effect on resting blood pressure (data not shown), significantly attenuated both the depressor (Fig. 2A) and pressor (Fig. 2B) responses induced by 300 and 1000 pmol of intravenously administered endothelin ($P < 0.05$, one-way ANOVA, compared to control group). Therefore, this dose of Bosentan was chosen to be in the mid range of those used to assess the contribution of endothelin to ischaemia/reperfusion injury.

3.3. Effect of ischaemia on muscle viability

In the vehicle-treated (control) rats, 2-h normothermic ischaemia and 24-h reperfusion reduced muscle viability to

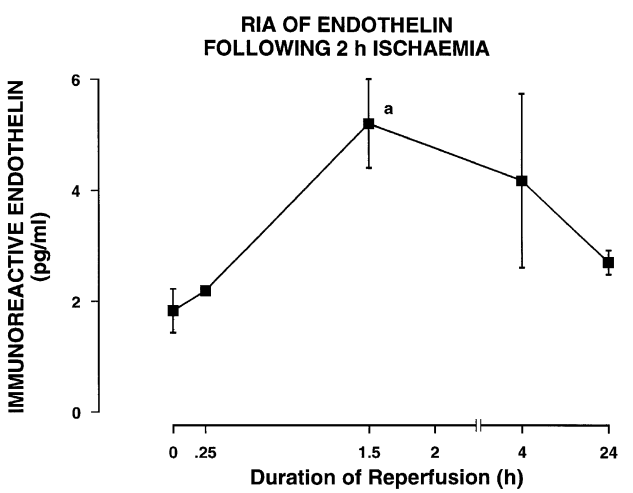

Fig. 1. Immunoreactive endothelin levels in plasma following 2-h hindlimb ischaemia and 0-, 0.25-, 1.5-, 4- and 24-h reperfusion. ([a]denotes statistical significance. $P < 0.05$, one-way ANOVA with repeated measures).

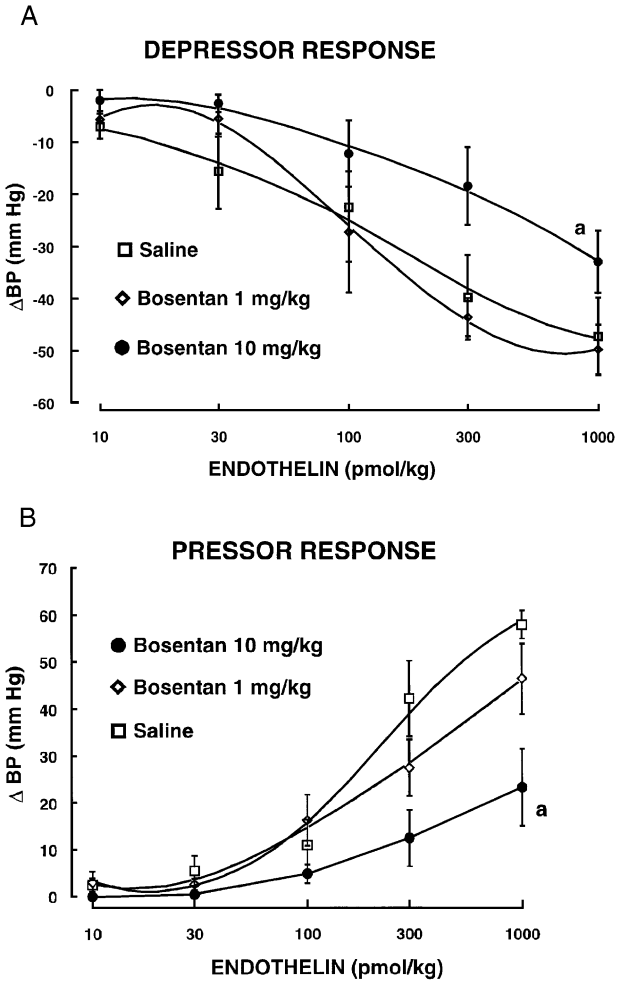

Fig. 2. Effect of Bosentan (10 mg/kg) on (A) the depressor and (B) the pressor responses induced by a range of doses of endothelin. (Control rats—open circles/Bosentan-treated rats—closed squares). Results are shown as the mean ± S.E.M. of at least five observations at each dose. [a]Bosentan significantly attenuated both the pressor and depressor effects of endothelin at 300 and 1000 pmol/kg ($P < 0.05$, one way ANOVA with repeated measures).

17 ± 2%. Treatment with Bosentan (1–100 mg/kg, i.p.) significantly increased muscle viability at the two highest doses, 10 and 100 mg/kg ($P < 0.01$, unpaired Student's *t*-test) (Fig. 3).

3.4. Effect of ischaemia on water content and myeloperoxidase activity

Ischaemia and reperfusion of the rat hind limb induced significant ($P < 0.05$, unpaired Student's *t*-test) increases in tissue water content (Table 1) and myeloperoxidase activity (Table 2) compared with muscle from sham-operated rats. Local hindlimb ischaemia did not significantly affect tissue water content and myeloperoxidase activity in the contralateral non-ischaemic muscle, compared with muscle from the sham group. In the lungs of rats subjected to local hindlimb ischaemia, there was no change in tissue water content compared with unoperated rats, but lung

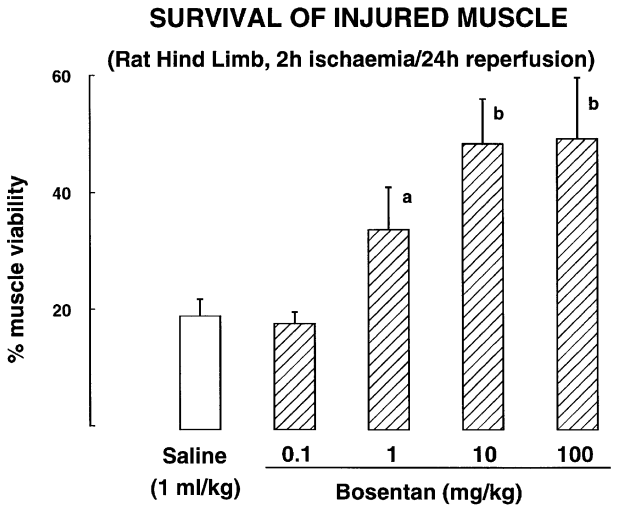

Fig. 3. Muscle viability following 2-h ischaemia (at 36 °C) and 24-h reperfusion in control (open bar) and Bosentan-treated (cross hatched bar) rats. Results are shown as the mean ± S.E.M. of at least seven (control) and six (Bosentan) observations. Bosentan treatment increased muscle viability significantly compared to control animals ([a]$P < 0.05$, [b]$P < 0.07$ unpaired Student's *t*-test).

myeloperoxidase activity was significantly increased relative to that of the sham group ($P < 0.05$, unpaired Student's *t*-test).

Bosentan treatment had no effect on the increased tissue water content or myeloperoxidase activity in the ischaemically injured and reperfused muscle. Neither tissue water content nor myeloperoxidase activity was significantly altered by Bosentan treatment in the contralateral non-ischaemic muscle of rats, in which the left hindlimb had been made ischaemic and reperfused for 24 h (Tables 1 and 2).

3.5. Intramuscular distribution and morphology of carbon-perfused vessels

These results were based on analysis of muscles which had undergone 2-h ischaemia, 90-min reperfusion, India

Table 1
Effects of Bosentan (10 mg/kg) on water content (ml/g) of sham-operated muscle, non-ischaemic contralateral muscle and postischaemic muscle, and of lung from rats subjected to 2-h hindlimb tourniquet ischaemia and 24-h reperfusion, or a sham procedure

Tissue	Control	Bosentan
Sham-operated muscle	3.33 ± 0.02	ND
Contralateral, non-ischaemic muscle	3.15 ± 0.05	3.04 ± 0.13
Postischaemic muscle	5.27 ± 0.09 *	5.08 ± 0.15 *
Sham lung	3.59 ± 0.27	ND
Lung (ischaemic limb)	3.91 ± 0.14	4.09 ± 0.14

Results are shown as mean ± S.E.M. of at least six observations per group.
ND—not done.
* Significantly greater ($P < 0.05$) than contralateral, non-ischaemic muscle.

Table 2
Effects of Bosentan (10 mg/kg) on myeloperoxidase activity (U/g) of sham-operated muscle, non-ischaemic contralateral muscle and postischaemic muscle, and of lung from rats subjected to 2 h of hindlimb tourniquet ischaemia and 24-h reperfusion, or a sham procedure

Tissue	Control	Bosentan
Sham-operated muscle	1.6 ± 0.8	ND
Contralateral, non-ischaemic muscle	0.9 ± 0.2	0.8 ± 0.3
Postischaemic muscle	11.0 ± 1.3[a]	7.9 ± 1.2[a]
Sham lung	6.3 ± 0.6	ND
Lung (ischaemic limb)	12.5 ± 3.7[b]	11.2 ± 1.1[b]

Results are shown as mean ± S.E.M. of at least six observations per group.
[a]Significantly greater ($P < 0.05$) than contralateral non-ischaemic muscle.
[b]Myeloperoxidase levels in lung from rats undergoing hindlimb ischaemia and reperfusion, significantly higher than from sham-operated rats.

ink perfusion, formalin fixation and clearing in cedar wood oil. Five-millimeter cross-sections were used for qualitative analysis, and quantitative analysis was performed on 10-μm histological sections (Fig. 4). In the sham-operated group ($n = 5$), muscles were completely perfused in most cases, with a functional capillary density (the number of perfused capillaries in a defined area) so high that minimal light was transmitted through the section. In the postischaemic control group ($n = 5$), only larger vessels were generally perfused by India ink (Fig. 5). In some cases, artery and vein pairs were both labelled, indicating that some India ink was reaching the venous side of the circulation. Despite this, in most muscles, minimal capillary label was present, apart from some small peripheral zones of the muscle (Fig. 5). One of five muscles differed from the rest of the group in that approximately 50% of the muscle contained capillary perfusion, but at a significantly reduced functional capillary density, relative to the sham muscles. In the postischaemic Bosentan-treated muscles, three of five in the group showed capillary label in the core of the muscle, normally at a reduced functional capillary density relative to the sham-operated muscles, but higher than the untreated postischaemic muscles (Fig. 6). The volume of the muscle perfused in these three muscles ranged from 30% to 60%. The remaining muscles were only perfused in the larger arteries and arterioles.

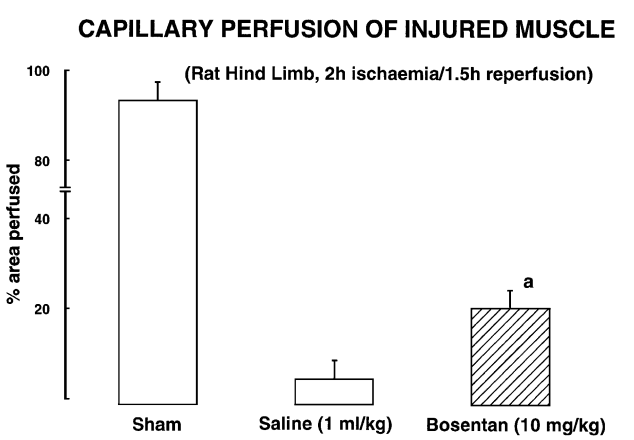

Fig. 4. Graph of degree of carbon perfusion of muscle following 2-h ischaemia (at 36 °C) and 1.5-h reperfusion in control (open bar) and Bosentan-treated (cross-hatched bar) rats, as well as sham-operated muscles. Results are shown as the mean ± S.E.M. of at least five (sham), five (control) and five (Bosentan) observations. [a]Bosentan treatment (10 mg/kg) increased muscle perfusion significantly compared with controls ($P < 0.05$).

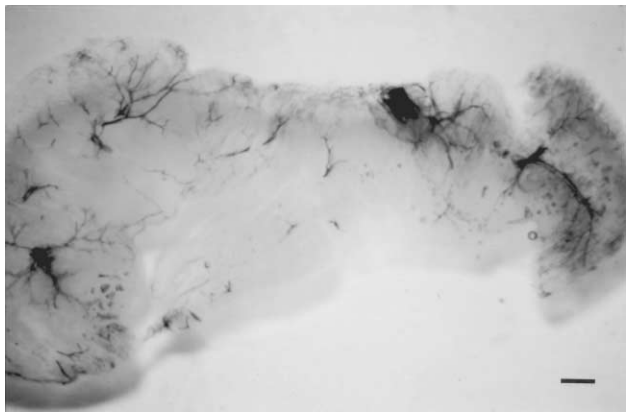

Fig. 5. Photograph of a slice of India ink-perfused control ischaemic muscle after 2-h ischaemia/1.5-h reperfusion. Scale bar = 50 mm.

3.6. Histology summary

These muscles were examined after 2-h ischaemia, with or without Bosentan treatment (10 mg/kg), and 24-h reperfusion.

The postischaemic control group tissues displayed a heterogeneous appearance. Some areas displayed histology typical of infarcted tissue: loss of muscle nuclei, capillaries congested with erythrocytes (suggestive of no-reflow) and minimal leukocyte infiltrate. In other areas, often adjacent to infarcted areas, there was significant haemorrhage, oedema and neutrophil infiltration. In addition, muscle

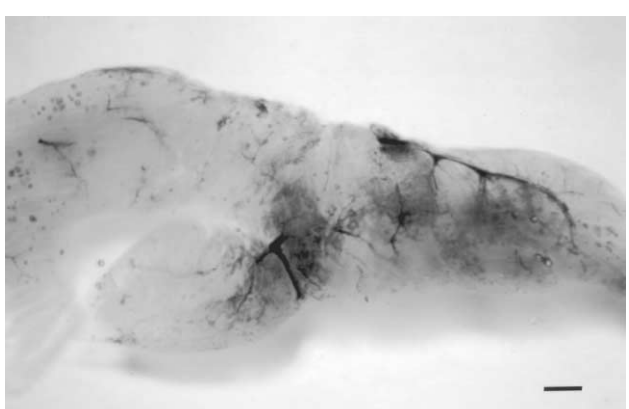

Fig. 6. Photograph of a slice of India ink-perfused Bosentan-treated (10 mg/kg) muscle after 2-h ischaemia/1.5-h reperfusion. Scale bar = 50 mm.

fibres in these areas were often necrotic. Occasional small areas of tissue displayed healthy muscle fibre histology.

The postischaemic Bosentan-treated muscles, compared with controls, appeared to contain relatively less neutrophil infiltrate and fewer congested capillaries. In support of the nitro blue tetrazolium findings, a greater proportion of the muscle fibres displayed normal histological appearance. In addition, individual necrotic muscle fibres were often observed within areas of viable fibres, whereas in control tissues, large areas of necrotic fibres were consistently observed. However, there was still evidence of an inflammatory insult as neutrophil infiltrate and oedema were present, often in areas of viable muscle fibres.

4. Discussion

Three separate lines of evidence support a role for endothelin in the pathogenesis of skeletal muscle ischaemia/reperfusion injury. The injury results in an increase in circulating endothelin levels following reperfusion of ischaemic muscle; prior treatment with Bosentan, an endothelin receptor antagonist, significantly increases skeletal muscle survival following ischaemia/reperfusion injury; and postischaemic infusion of endothelin exacerbates ischaemia/reperfusion injury (Chang et al., 1992). Studies of cerebral (Giuffrida et al., 1992; Willette et al., 1993), hepatic (Goto et al., 1994) and renal ischaemia (Kusumoto et al., 1994; Sandok et al., 1992) also support a role for endothelin in ischaemia/reperfusion injury. However, the extent to which endothelin contributes to ischaemia/reperfusion injury may be organ-specific, as some studies of the ischaemic myocardium show no effect of endothelin receptor antagonism (Richard et al., 1994).

While hypoxia itself is an obvious stimulus to endothelin formation, a number of other stimuli for endothelin synthesis, such as thrombin, are known to be active during reperfusion (MacLean et al., 1989; Nakagomi et al., 1989). Endothelin synthesis has been detected following myocardial infarction in humans (Lam et al., 1991; Ray et al., 1992) and endothelin antagonism has already been shown to decrease reperfusion injury in a range of tissues (Kusumoto et al., 1994; Grover et al., 1993). In the present study, elevated levels of immunoreactive endothelin were detected at all times during the first 24-h reperfusion, with maximal levels at 1.5-h reperfusion.

The role played by endothelin during reperfusion may be related to the intensity and duration of its vasconstrictor effects. Arteriolar vasoconstriction is considered to contribute to the no-reflow phenomenon (Strock and Majno, 1969; Zamboni et al., 1993), which extends the period of ischaemia during reperfusion of affected tissues. Previous measurements of carbon perfusion in the hamster dorsal skinfold showed that functional capillary density (a measure of no-reflow) was reduced to 30% of preischaemic levels in the first hour of reperfusion and only partially recovered by 24 h. Red blood cell velocity was significantly reduced throughout this period (Menger et al., 1992). In the present experiments, the impairment in functional capillary density in the first 1.5 h of reperfusion in untreated postischaemic muscle was significantly attenuated by Bosentan treatment. These observations raise the possibility of a causal link between endothelin and vasoconstriction-mediated no-reflow. Thus, the increased viability of muscle in Bosentan-treated rats may have resulted from inhibition of endothelin-mediated vasoconstriction. It has also been suggested that a failure of the vasodilator response to endothelin could contribute to ischaemia/reperfusion injury by further intensification of the vasoconstrictor response mediated by endothelin ET_A receptors (Newby and Webb, 1999). Thus, it is possible that selective block of endothelin ET_A receptors would improve the protection from ischaemia/reperfusion injury to a greater extent than non-selective blockers, such as Bosentan, if postischaemic endothelin retained the capacity to release vasodilators.

In many studies of ischaemia/reperfusion injury in skeletal muscle (Seekamp et al, 1993) and other tissues (Ma et al., 1992), oedema and neutrophil infiltration have been measured as correlates of the intensity of the injury. However, the results of the present study suggest that use of these parameters as indices of reperfusion injury may not accurately predict tissue protection when measured after 24-h reperfusion, since despite the substantial increase in muscle survival, neither parameter was influenced by Bosentan treatment. This dissociation of oedema, neutrophil influx and ultimate tissue survival is supported by other studies, which show that tissue oedema and neutrophil numbers, especially after extended reperfusion, do not always accurately reflect the degree of postischaemic tissue injury in skeletal muscle (Petrasek and Walker, 1994; Breidahl et al., 1996; Skjeldal et al., 1994; Leong et al., 2000; Lazarus et al., 2000) or other tissues (Thornton et al., 1989; Lemasters and Thurman, 1991; Kohout et al., 1995; Zizzi et al., 1997). Endothelin may have no effect on vascular permeability in postischaemic skeletal muscle, as suggested by Chang et al. (1992), even though it has been shown to induce an increase in vascular permeability in a variety of other tissues (Lehoux et al., 1992). However, the pronounced vasoconstrictor effect of endothelin may limit fluid loss from capillaries made leaky by endothelin itself, or by other mediators of ischaemia/reperfusion injury including tumor necrosis factor-α, interleukin-1, complement C5a, histamine, thrombin and thromboxane A_2 (Rubanyi and Polokoff, 1994). The failure of Bosentan to reduce the oedema in ischaemia/reperfusion injury muscle suggests either that there is a balance between the oedema-promoting and inhibiting activities of this peptide or that other chemical mediators play a more dominant role in this response. However, the results of the present study do not exclude the possibility that Bosentan influences muscle oedema at an earlier stage of reperfu-

sion. This is not reflected in tissue water content measured at 24-h reperfusion.

Several studies of ischaemia/reperfusion injury in skeletal muscle have provided evidence for a role of neutrophils in tissue necrosis. Conversely, several studies have found that the absence of neutrophils does not affect survival of skeletal muscle following ischaemia/reperfusion injury (Skjeldal et al., 1994; Breidahl et al., 1996). Although there is a potential link between endothelin and neutrophil activation (Gomez-Garre et al., 1992), the failure of Bosentan to markedly decrease neutrophil accumulation suggests that other mediators account for neutrophil influx in postischaemic skeletal muscle. The muscle protection achieved by Bosentan was incomplete and was not accompanied by inhibition of neutrophil infiltration. Thus, other mechanisms of reperfusion injury, such as neutrophil infiltration (Korthuis et al., 1988), may make a separate endothelin-independent contribution to the overall level of muscle necrosis. Experiments combining endothelin antagonism with anti-neutrophil treatments are required to establish whether further salvage of postischaemic muscle is possible.

In summary, these findings raise the possibility that antagonists of endothelin receptors or inhibitors of its synthesis will be of value in reducing muscle necrosis in ischaemia/reperfusion injury (see also Roux et al., 1999). It remains to be determined whether muscle survival can be increased to a greater extent by combining Bosentan pretreatment with inhibition of neutrophil infiltration and oedema formation.

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