The Effect of Acute Ischemia on ET-1 and Its Receptors in Patients with Underlying Chronic Ischemia of the Lower Limb

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Elevated plasma and tissue endothelin (ET)-1 levels in patients with critical limb ischemia (CLI) has been described. Here the effect of a period of acute ischemia and subsequent reperfusion on plasma ET-1 and tissue ET-1/ET receptors in skeletal muscle biopsies from CLI patients undergoing femoro-distal bypass surgery was studied. Peripheral and "local" blood and muscle biopsies were obtained from patients undergoing femoro-distal bypass surgery, at the start of the procedure (control), after a period of vascular clamping (ischemia), and after clamp release (reperfusion). Plasma ET-1 was determined by enzyme-linked immunosorbent assay. Tissue ET-1 was assessed by counting ET-1 immunostaining cells per unit area, and ET_A/ET_B receptors were identified on sections by *in vitro* autoradiography in which binding was quantitatively assessed by densitometry. There was no significant effect of ischemia or reperfusion on plasma ET-1 levels or on ET_A/ET_B receptor binding. However, tissue ET-1 increased during both acute ischemia and reperfusion ($P < 0.05$). A high proportion of positive ET-1 immunostaining was associated with microvessels and also exhibited a similar distribution to macrophages. Previously, it has been shown that both plasma ET-1 and tissue ET-1/ET receptors are increased in CLI patients compared with atherosclerotic controls. Also, increased muscle ET-1 levels have been described in acute ischemia caused by tourniquet application in nonischemic patients undergoing total knee replacement. In CLI patients, in whom ET-1 is already upregulated, this further increase may exacerbate existing pathologic processes and contribute to ischemia-reperfusion injury. ET-1 antagonists may therefore be useful adjuncts in CLI and other surgical procedures in which ischemia-reperfusion damage occurs. Exp Biol Med 231:802–805, 2006

Key words: ischemia; reperfusion; skeletal microvessels

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Introduction

Peripheral vascular disease (PVD) affecting the blood supply to the lower limb is a significant health care problem. Restriction of blood flow to the lower limb resulting from atherosclerotic disease leads to intermittent claudication, characterized by muscle pain on walking. Further reduction in blood supply results in critical limb ischemia (CLI), with ischemic rest pain, ulceration, and gangrene. In the UK, CLI affects about 20,000 people annually, and these patients suffer high morbidity and mortality from their local disease. Elevated plasma levels (1) and raised tissue endothelin (ET)-1 and ET_A/ET_B receptors in patients with CLI have recently been described (2). Despite improvements in surgical techniques, perioperative care, and adjuvant pharmacologic intervention, results of revascularization procedures for CLI remain poor. Nevertheless, bypass surgery remains the best option for limb salvage and improved quality of life.

Failure to reverse limb-threatening ischemia and consequential limb loss can occur despite a patent graft (3). This may be attributed to altered microvascular morphology and reactivity seen in patients with CLI (4), which prevents adequate perfusion of the ischemic tissue despite successful bypass grafting (5). Here we have studied the effect of a period of acute ischemia (vascular clamping) on plasma ET-1, "tissue" ET-1 and ET receptors in skeletal muscle biopsies obtained from patients undergoing femoro-distal bypass surgery for CLI.

Materials and Methods

Studies were performed with the local ethics committee approval and patients' informed consent. Peripheral venous blood samples were taken from the arm and "local" blood samples were taken from the femoral vein at the time of vascular clamping (control) in 10 patients (6 male, 4 female, median age 72, range 54–86) undergoing femoro-distal bypass surgery for CLI. Further samples were taken after vascular clamp application (acute ischemia; duration, 91 ± 12 minutes, mean ± SEM) and after clamp release

Table 1. Effect of Acute Ischemia on Plasma ET-1 and ET Receptor Levels in CLI Patients[a]

	Control	Ischemia	Reperfusion	*P*
Plasma ET-1—"local"	1.53 (0.4–8.6)	1.86 (0.8–9.6)	1.40 (0.3–8.0)	0.97
Plasma ET-1—peripheral	1.15 (0.1–8.9)	1.32 (0.5–8.3)	1.30 (0.7–8.9)	0.37
ET_AR (dpm $\times$ 10^3/mm^2)	9.6 (1.1–13.8)	9.5 (1.0–11.7)	9.4 (2.1–14.4)	0.57
ET_BR (dpm $\times$ 10^3/mm^2)	10.3 (6.9–14.6)	12.2 (5.4–13.6)	11.7 (3.1–14.1)	1.00

[a] "Local" and peripheral plasma ET-1 (fmol/ml) and tissue ET receptor binding in CLI patients (median and range, $n = 6$–10). Effect of acute ischemia and reperfusion. There was no significant difference in plasma ET-1 or ET receptors during ischemia or reperfusion. Although "local" plasma ET-1 levels were higher than peripheral levels this did not reach significance ($P = 0.77$, $P = 0.1$, and $P = 1.0$ for control, ischemia, and reperfusion, respectively).

(reperfusion; time 21 $\pm$ 2 minutes). Plasma ET-1 was determined by enzyme-linked immunosorbent assay (Biomedica, Vienna, Austria). Gastrocnemius muscle biopsies were also obtained at the same time points. Tissue ET-1 was identified by standard immunohistochemistry, using a monoclonal anti–ET-1 antibody (Peninsula Laboratories, St. Helens, UK, diluted 1:500) and the avidin-biotin-complex method (Vector Laboratories, Peterborough, UK). Immunostaining was assessed by counting ET-1–positive cells within a 100 μm $\times$ 150 μm grid at $\times$40 magnification (five random areas per section) on two sections per biopsy. Macrophages and endothelial cells were identified using CD68 and CD31, respectively (Dako Labs, Glostrup, Denmark, both diluted 1:200). ET_A and ET_B receptors were also identified on sections by *in vitro* autoradiography using 150 p*M* [^{125}I]-PD151242 and [^{125}I]-BQ3020 (Amersham

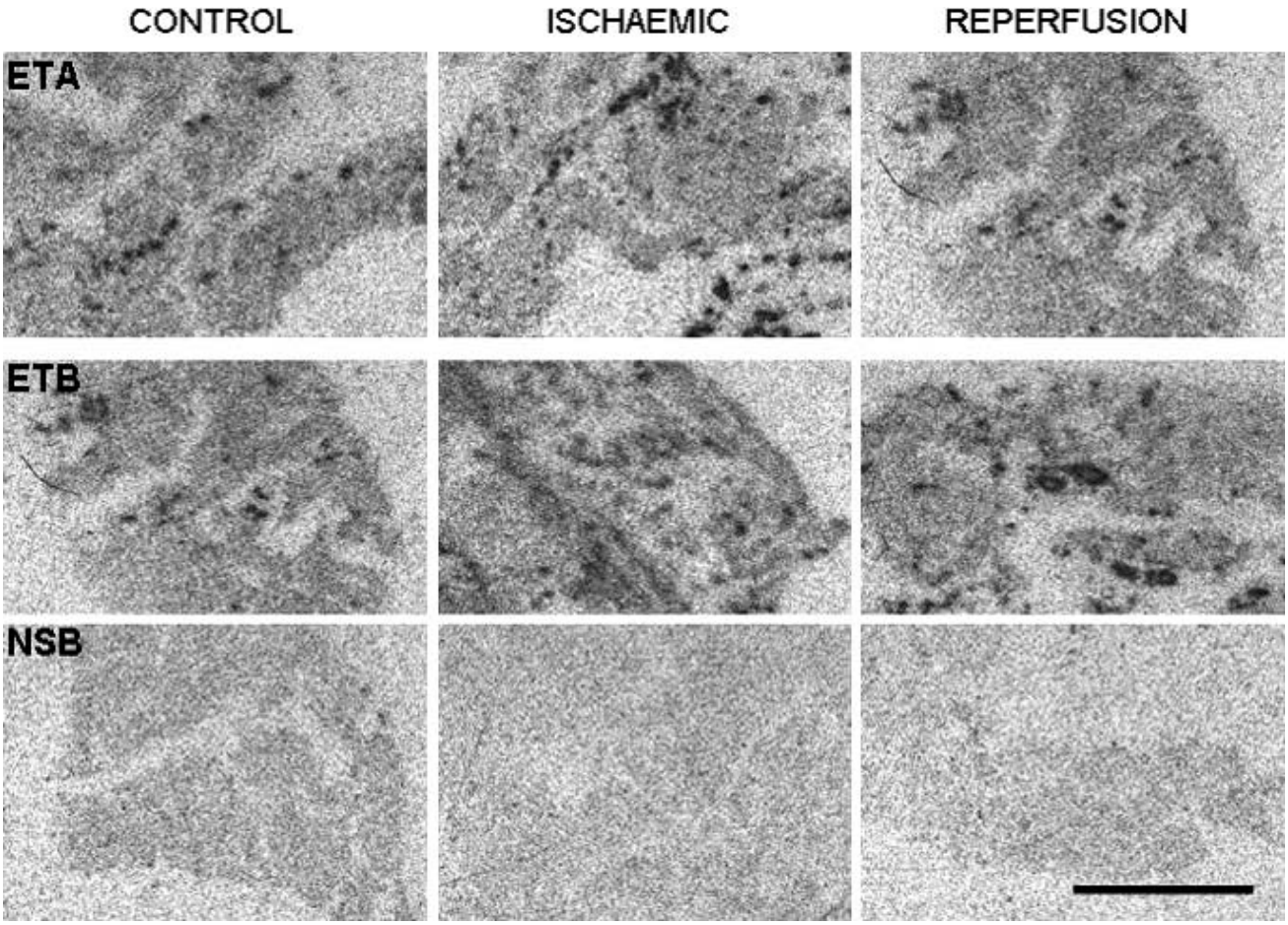

Figure 1. Autoradiographic distribution of ET_A and ET_B receptor binding to muscle biopsies. Representative film autoradiographs of ET_A (top panels) and ET_B (middle panels) receptor binding to control, ischemic, and reperfused muscle biopsies taken from a CLI patient undergoing femoro-politeal bypass surgery. Apart from binding to skeletal muscle strong, binding was associated with microvessels (dense patches) that were identified on stained underlying tissue. Lower panels are representative of nonspecific binding for both radioligands. Densitometric analysis shown in Table 1 was derived from film autoradiographs in which specific binding was established by subtracting nonspecific from total binding. Scale bar = 2.5 mm.

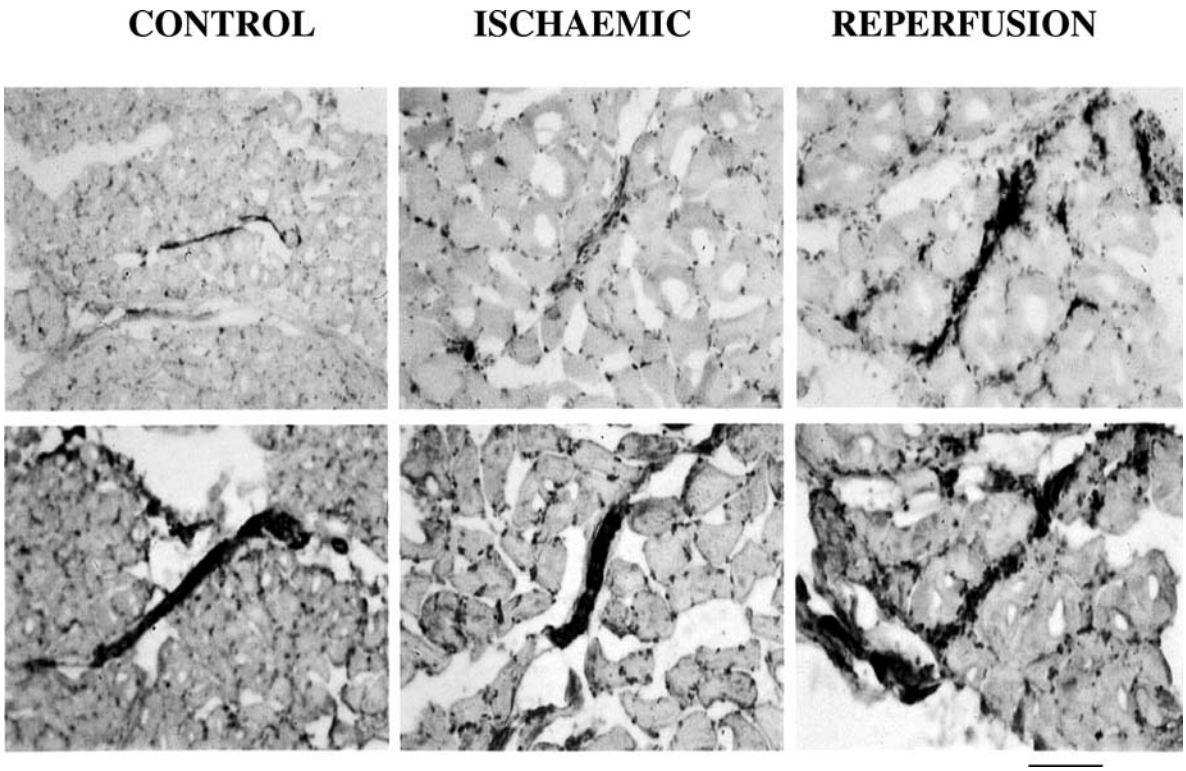

Figure 2. ET-1 immunoreactivity in control, ischemic, and reperfused muscle from CLI patient. ET-1 immunoreactivity was more pronounced in reperfused (top right) compared with control (top left) and ischemic (top center) sections. The bottom panel shows CD68 immunoreactivity on adjacent sections. The similar pattern of ET-1 and CD68 immunostaining suggests that macrophages may be a source of increased ET-1. Note also the histologic deterioration in the ischemic and reperfused sections compared with control, showing tissue damage under these conditions. Scale bar = 50 μm.

Biosciences, Buckinghamshire, UK) with nonspecific binding being established in the presence of 1 μ*M* unlabeled ET-1. Autoradiographs were generated after 4–8 days of exposure to Hyperfilm ^{3}H (Amersham), and binding was determined densitometrically (6). Selected sections were then stained with hematoxylin and eosin for histologic examination.

Statistics. Quantitative data were analyzed using the Friedman test and the Wilcoxon signed rank test, followed by Dunn's post hoc test. Statistical analyses were performed using GraphPad Prism version 3.02 (GraphPad, San Diego, CA). Statistical significance was inferred at $P < 0.05$.

Results

No significant change in "local" or peripheral plasma ET-1 levels were found over the period of acute ischemia and reperfusion studied (Table 1). In addition, although median "local" levels were higher than peripheral levels, this difference did not reach significance (Table 1). Autoradiographic studies revealed ET_A and ET_B receptor binding to skeletal muscle and microvessels (Fig. 1), which, again, did not significantly alter over the period of acute ischemia or reperfusion studied (densitometric data shown in Table 1).

However, tissue ET-1 immunostaining increased slightly during ischemia and was more pronounced after reperfusion (Fig. 2). Apart from ET-1 immunostaining being associated with microvessels, a high proportion of staining exhibited a similar distribution to macrophages (Fig. 2) increased during acute ischemia and was elevated further during reperfusion (Figs. 2 and 3).

Discussion

Increased plasma ET-1 has previously been reported in patients with peripheral arterial occlusive disease (1). The data from the present study support these findings because control peripheral plasma ET-1 levels are considerably higher in CLI patients than those in nonischemic subjects undergoing orthopedic surgery measured in our laboratory (7).

Reduced blood flow in CLI leads to muscle ischemia, tissue hypoxia, and increased shear stress of the microvasculature, all stimuli for ET-1 production. Clinically, elevated tissue and circulating levels of ET-1 have been reported in acute and chronic ischemic conditions such as acute renal failure (8), acute coronary syndromes (9), and stroke (10). During surgical revascularization, a period of acute ischemia is induced by application of vascular clamps during anastomoses. On clamp release and on completion of the revascularization procedure, reactive hyperemia occurs and contributes to postoperative leg swelling (11). Reperfusion injury may also occur, resulting in further cellular injury, compromising tissue perfusion.

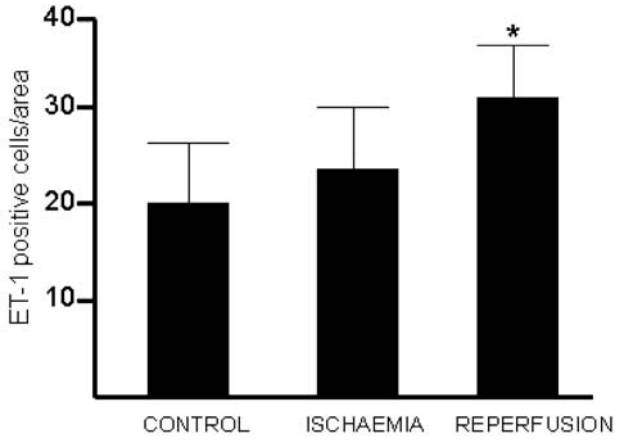

Figure 3. Histogram showing tissue ET-1 levels in muscle biopsies of CLI patients. Tissue ET-1 assessed as positive ET-1 immunostaining cells per unit area in control, ischemic, and reperfused samples. Mean ± SD, $n = 10$ patients. Increased tissue ET-1 in the reperfused biopsies was statistically higher than control and ischemic muscle, $P < 0.05$.

Previously we had shown that both plasma ET-1 and tissue ET-1 and ET receptors are increased in patients with CLI compared with controls (2, 12). Although plasma ET-1 levels and ET_A/ET_B receptors located on skeletal muscle are not affected by acute tourniquet-induced ischemia in nonischemic control subjects, there is an increase in tissue ET-1 during acute ischemia and reperfusion (7). In the present study, a slight increase in tissue ET-1 during clamp-induced acute ischemia with a further increase after reperfusion was again found, despite the elevated baseline ET-1 levels from underlying ischemia in CLI patients. As a proinflammatory peptide, increased ET-1 tissue levels during reperfusion may result in edema, microvascular vasoconstriction, and cellular necrosis, effectively exacerbating the hypoxic damage sustained during ischemia.

Potential sources of ET-1 within ischemic skeletal muscle include existing microvessels, migrating macrophages and microvascular endothelium at areas of angiogenesis that has been reported in CLI muscle (2). "Overspill" of ET-1 from chronically ischemic muscle, as well as from atherosclerotic femoral arteries, may contribute to the increased plasma ET-1 levels described in CLI patients.

Our data show that, in CLI patients in whom ET-1 is already upregulated, acute ischemia from vascular clamping and subsequent reperfusion both result in further elevation of tissue ET-1. This further increase may exacerbate existing pathologic processes and contribute to ischemia-reperfusion injury. ET-1 antagonists may therefore be useful adjuncts to such surgical procedures to reduce ischemia-reperfusion damage.

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