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RESEARCH ARTICLE

# Further evidence for a role of endothelin-1 (ET-1) in critical limb ischaemia

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**Abstract** Critical limb ischaemia (CLI), due to atherosclerotic arterial occlusion, affects over 20,000 people per year in the United Kingdom with many facing lower limb amputation and early death. A role for endothelin-1 (ET-1) in atherosclerosis is well-established and increased circulating and tissue levels of this peptide have been detected in patients with CLI. ET-1 and its receptors were identified in atherosclerotic popliteal arteries obtained from CLI patients undergoing lower limb amputation. In addition, plasma ET-1 levels were compared with those of non-ischaemic controls. ET-1 was associated with regions of atherosclerotic plaque, particularly in regions with high macrophage content. This peptide was also associated with endothelial cells lining the main vessel lumen as well as adventitial microvessels. $ET_A$ and $ET_B$ receptors were located within regions of plaque, adventitial microvessels and perivascular nerves. There was a statistically significant increase ($P< 0.001$) in plasma ET-1 in CLI patients when compared with controls. These results reveal sources of ET-1 in atherosclerotic popliteal arteries that potentially contribute to increased circulating levels of this peptide. Identification of variable receptor distributions in ischaemic tissue suggests a therapeutic potential of selective receptor targeting in patients with CLI.

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## Introduction

Peripheral arterial disease (PAD) affects the lower limb arteries and is a significant health care problem in the Western World. Atherosclerosis of the major arteries restricts blood flow to skeletal muscles causing intermittent claudication, chraracterised by muscle pain on walking. Further reduction in blood flow results in critical limb ischaemia (CLI), with ischaemic rest pain, ulceration and gangrene. CLI affects approximately 20,000 people in the UK, with an annual incidence of 400 per million per year. These patients suffer from high morbidity and mortality due to local disease and complications in the lower limb as well as overall cardiovascular disease (Schroeder 2007). Current treatments for modifying local progression and overall mortality have limited success. Although there is evidence that prostanoids delivered either intra-arterially or intravenously reduces rest pain, ulcer size and limb loss in CLI (Second European Consensus Document on chronic critical limb ischaemia 1991) evidence for the benefits of other pharmacological agents in effectively treating CLI is weak and their use is generally limited to inoperable disease in an attempt to control pain and avoid amputation (Schroeder 2007).

Most patients with CLI require revascularization to avoid major amputation, using endovascular and surgical techniques. In patients with unreconstructable disease, necrosis of significant areas of weight-bearing portions of the foot, fixed flexion contractures of the leg and limited life expectancy, primary amputation is indicated. Major amputations are usually performed below-knee or above-knee with eventual outcome being poor: over 20% below-knee and over 50% above-knee amputees do not return to independent ambula-

tion. Thirty percent of below-knee amputees require a major contralateral amputation within 5 years and 50% die within this time period (Schroeder 2007).

A pathophysiological role for ET-1 in ischaemia is well established with raised plasma ET-1 levels reported in both acute and chronic ischaemic conditions such as coronary syndromes (Yasuda et al. 1990), acute renal failure (Remuzzi and Benigni 1993), Stroke (Ziv et al. 1992) and PAD (Mangiafico et al. 1999; Tsui and Dashwood 2005). In patients with symptomatic atherosclerosis, plasma ET-1 concentrations correlated positively with the number of sites of atherosclerotic lesions (Lerman et al. 1991) and further studies confirmed ET-1 immunoreactivity in atherectomy specimens with significantly higher immunostaining in specimens from patients with unstable angina versus those with stable angina (Zeiher et al. 1995). In addition to its effect on tissue perfusion, ET-1 may contribute to tissue injury via its proliferative and proinflammatory actions (Rubanyi and Polokoff 1994).

Altered ET-1 levels have been described in patients with PAD (Tsui and Dashwood 2005) and marked increases in ET-1 and its receptors have been identified in skeletal muscle from CLI patients undergoing amputation (Tsui et al. 2002). Interestingly, altered ET-1 levels have been suggested to predict survival rate in these patients (Newton et al. 2005). ET antagonists have been shown to aid healing of skin ulcers in patients with systemic sclerosis (Korn et al. 2004) and to increase walking distance in patients with pulmonary hypertension (Denton et al. 2006; Barst 2007). Whilst the underlying pathology in connective tissue diseases and PAD are different, evidence that ET-1 plays a role in the pathophysiology of both these diseases, suggest ET antagonists may also have therapeutic potential in CLI.

## Materials and methods

Studies were performed with local ethical committee approval and with patients' informed consent. Popliteal arteries ($n$=13) were obtained from patients undergoing lower limb amputation for CLI (age=73 [53–80; mean/range]). For in vitro autoradiography and immunohistochemistry, tissue was collected at surgery and frozen in liquid $N_2$ within 5 min. Cryostat-cut 10 μm serial transverse sections of popliteal artery were thaw-mounted onto polylysine-coated microscope slides and stored at−70°C until use. For in vitro autoradiography, following preincubation in 50 mM Tris HCl buffer, pH 7.4, sections were incubated in buffer containing [$^{125}$I]-PD151242 or [$^{125}$I]-BQ3020 (Amersham Biosciences, Buckinghamshire, UK) to identify $ET_A$ and $ET_B$ binding sites respectively as described previously (Dashwood et al. 1998). Non-specific binding was established by incubating paired sections in the presence of 1 μM unlabelled ET-1. $ET_A$/$ET_B$ receptor distributions were revealed by exposing incubated sections for up to 8 days against Hyperfilm $^3$H (Amersham). Receptor localization at the microscopic level was achieved by coating incubated sections in molten K2 nuclear emulsion (Ilford Photographic, Cheshire, UK), exposing for up to 14 days and processing according to the manufacturer's instructions (Dashwood et al. 1998). Sections were finally counterstained with haematoxylin and eosin for histological examination and images captured on a KS300 imaging system (Imaging Associates, Bicester, UK) via an Olympus BX50 microscope.

Standard immunohistochemistry was employed using Vector ABC or ABC/AP kits (Vector Labs, Lincs. UK) whereby, after initial blocking in 0.01 M PBS containing $H_2O_2$ (for ABC) or 10% normal horse serum (ABC/AP), vessel sections were incubated in primary antibodies to human ET-1 (Cambridge Research Biochemicals, Cheshire, UK, diluted 1:500) and $ET_A$ and $ET_B$ receptors (Alomone Labs, Israel, diluted 1:200) followed by incubation with biotinylated secondary antibody and Vectastain ABC-AP or ABC reagent. Finally, sections were incubated in alkaline phosphate substrate or 3,3′-diaminobenzidine (DAB) for up to 20 min (all kits from Vector Labs, Lincs, UK). Negative controls were incubated in PBS in place of the primary antibody. Endothelial cells, nerves and macrophages were

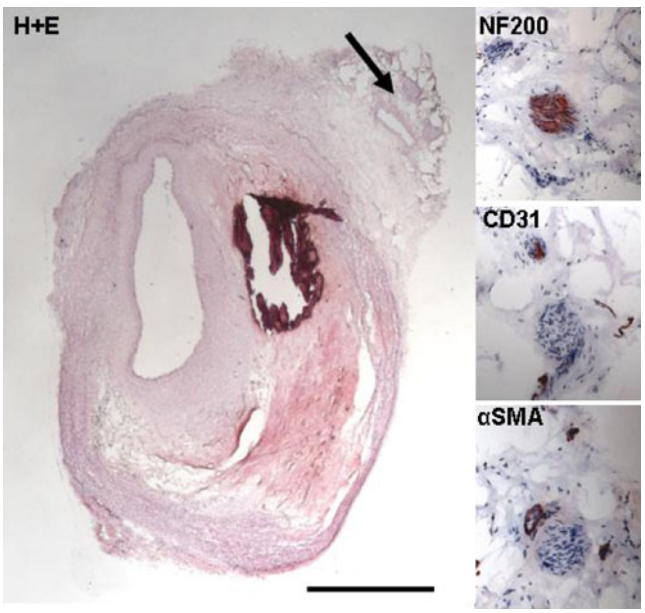


**Fig. 1** Example of atherosclerotic popliteal artery with perivascular immunostained structures. *Left*. Haematoxylin and eosin stained transverse section of an atherosclerotic popliteal artery from a CLI patients undergoing amputation. The lumen is reduced in size and a region of calcification is clearly visible. The arrow indicates the perivascular nerve and associated structures in consecutive sections shown in the panels on the right. *Right top*. Perivascular nerve identified using NF200. *Middle*. Microvessel endothelial cells identified using CD31. *Lower*. Vascular smooth muscle cells identified using anti-α smooth muscle actin. All immunostaining = brown DAB reaction. Scale bar=2 mm for left panel and 250 μm for right panels.

identified by incubating in primary antibodies CD31 (Dako Labs, Glostrup, Denmark, UK, diluted 1:200), NF200 (Dako, diluted 1:200), anti-α smooth muscle actin (Dako, diluted 1:200) and CD68 (Dako, diluted 1:200) respectively following the above protocol. Sections were lightly counterstained with haematoxylin, viewed under an Olympus BX50 microscope and photographed where appropriate. Macrophages and ET-1 +ve-staining cells (5 fields per section at×120 magnification) were assessed by an experienced histopathologist.

Peripheral blood samples from CLI patients ($n$=15, age 76 [48–87], median/range) and controls ($n$=12 non-ischaemic patients, age 75 [64–89], median/range) were obtained from the brachial vein and 'local' levels were measured in blood obtained from the femoral vein of CLI patients ($n$=12) during surgery. Plasma ET-1 levels were determined by enzyme-linked immunosorbent assay following the manufacturer's instructions (Biomedica, Vienna, Austria).

## Statistics

Quantitative values were expressed as median (range) or mean (SEM) and the Wilcoxon signed rank test used with statistical significance inferred at $p<0.05$. GraphPad Prism version 3.02 software was used for statistical analyses (GraphPad Software, San Diego, CA, USA).

## Results

### Tissue

All popliteal artery sections from CLI patients undergoing lower limb amputation ($n$=13) exhibited pronounced atherosclerotic involvement (Figs. 1 and 2). Perivascular nerves, identified using NF200, were closely associated with microvessels in the adventitia. ET-1 immunostaining cells (15.6±1.9 [mean/SEM]) per unit area (Fig. 2) were similar in number and distribution to CD68-positive macrophages (15.4±1.8 [mean/SEM]) (Fig. 2). In addition, positive ET-1 immunostaining was associated with the luminal endothelium (not shown) as well as endothelial cells of the adventitial vasa vasorum and neural microvessels, the vasa nervorum (Figs. 2 and 3). $ET_A$ and $ET_B$ receptors were also identified in the same microvessels by immunohistochemistry. Within regions of plaque, $ET_A$ receptor immunostaining was strong and similar in distribution to ET-1, co-localising with macrophages. $ET_B$ receptor staining within regions of plaque was relatively weak, but dense staining was associated with the luminal endothelium. Regions of the adventitia showed positive immunostaining for both endothelin receptor subtypes, associated in particular with the vasa vasorum and the vasa nervorum. In general, $ET_A$ staining

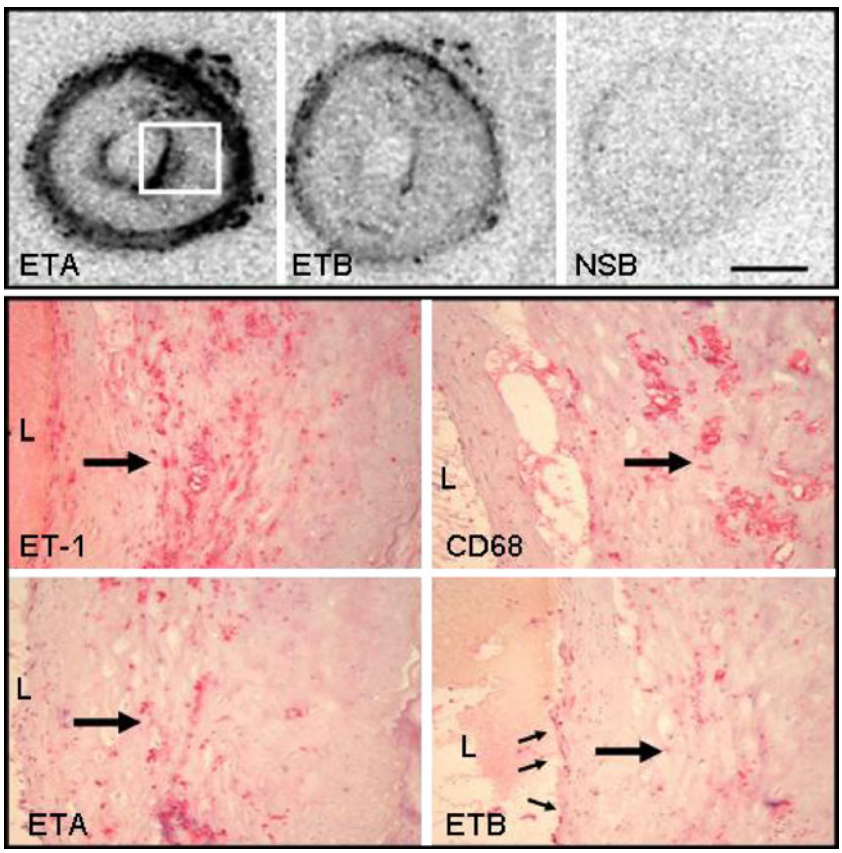


**Fig. 2** ET-1, $ET_A$ and $ET_B$ receptor localization on atherosclerotic human popliteal artery. *Top panels*. Film autoradiographs of [$^{125}$I]-PD151242 ($ET_A$, left) and [$^{125}$I]-BQ3020 ($ET_B$, middle) binding to human atherosclerotic popliteal artery from a CLI patient undergoing lower limb amputation. *Right panel*. Non-specific binding. *Lower panels*. Higher magnification illustrations of regions shown within the rectangle in the top panels. *Top left*. ET-1 immunostaining. *Top right*. Macrophages on an adjacent section identified using CD68. *Bottom left*. $ET_A$ receptor immunostaining. *Bottom right*. $ET_B$ receptor immunostaining. There is strong ET-1 and $ET_A$ receptor immunostaining exhibiting a similar distribution pattern to macrophages (CD68). $ET_B$ receptor immunostaining is also associated with macrophages and to the luminal endothelium (arrowed). L = lumen (containing thrombus). Scale bar=2 mm for top panels and 0.5 mm for lower panels

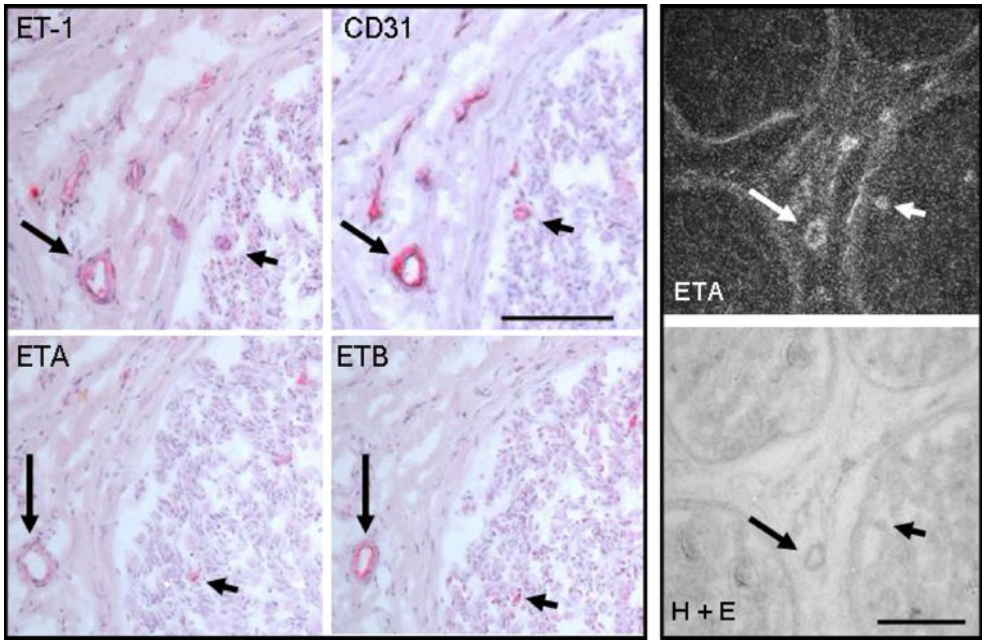


**Fig. 3** ET-1, $ET_A$ and $ET_B$ receptors located on perivascular nerve of atherosclerotic human politeal artery. *Left panels*. ET-1, $ET_A$ and $ET_B$ receptor immunostaining at regions of perivascular nerve of a popliteal artery from a CLI patient undergoing lower limb amputation. ET-1, $ET_A$ and $ET_B$ receptors are all associated with nerve microvessels (vasa nervorum) with $ET_A$ receptors localised to vascular smooth muscle cells and ET-1/$ET_B$ receptors to the lining endothelium of these microvessels (identified using CD31). Scale bar=0.2 mm. Right panels. Top autoradiograph showing $ET_A$ receptor binding ($[^{125}I]$-PD151242) localised to the perivascular nerve of an atherosclerotic popliteal artery from a CLI patient undergoing amputation (dark-field illumination where white grains indicate binding sites). *Lower panel*. H and E-stained underlying tissue showing neural microvessels (arrowed)

was associated with the vascular smooth muscle cells and $ET_B$ staining with the endothelium of these microvessels (Fig. 3).

The immunohistochemical observations were supported by the results of the in vitro autoradiographic studies. There was a patchy distribution of $[^{125}I]$-PD151242 binding within regions of neointima and plaque of atherosclerotic popliteal arteries that mirrored the distribution of macrophages as well as binding to vascular smooth muscle cells in media, adventitia and those of the vasa vasorum and vasa nervorum (Figs. 2 and 3). $ET_B$ receptor binding ($[^{125}I]$-BQ3020) was barely detectable within regions of plaque, but was associated with the luminal endothelium as well as to the endothelium of adventitial microvessels and those of the perivascular nerves (Fig. 3).

### Plasma

There was a marked increase in peripheral ET-1 levels in plasma obtained from CLI patients compared with non-ischaemic controls (>4-fold, $p<0.001$; Fig. 4). 'Local' CLI plasma ET-1 levels, obtained from the femoral vein, were also significantly higher than controls ($p<0.001$) and although median levels were approximately 35% higher than peripheral CLI levels, this did not reach significance (Fig. 4).

## Discussion

Although original interest focussed on the potent vasoconstrictor action of ET-1, it was not long before other actions of this peptide were described, including its potent proliferative and pro-inflammatory effects (Rubanyi and Polokoff 1994). These properties of ET-1 may contribute to the pathophysiology of

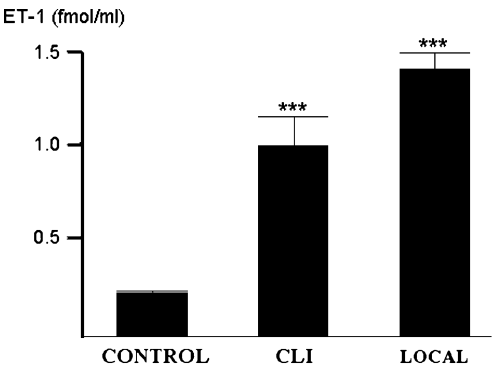


**Fig. 4** ET-1 plasma levels in CLI versus control patients. Histogram showing median (range) plasma ET-1 levels (fmol/ml) in non-atherosclerotic controls and CLI patients obtained from the brachial vein. 'Local' plasma levels were determined in blood obtained from the femoral vein of CLI patients during surgery. Both CLI local and peripheral ET-1 plasma levels were significantly higher than controls ($p<0.001$). Although 'local' CLI levels were higher than peripheral CLI levels this did not reach significance

coronary artery disease by causing vasoconstriction resulting in myocardial ischaemia, as well as neointima formation and progression of atherosclerosis. In coronary artery disease, ET-1 has been found in association with inflammatory cells such as macrophages within atherosclerotic coronary vessels (Zeiher et al. 1995). Our findings in CLI patients appear to be similar, in that inflammatory cells may be an important source of ET-1. In addition, $ET_A$ receptors exhibit a similar distribution to ET-1 within atherosclerotic regions of popliteal arteries from CLI patients, suggesting an autocrine/paracrine role of this peptide in this condition. Whereas these observations indicate that the distribution of ET-1 and $ET_A$ receptors may be associated with the pathophysiology of CLI, the $ET_B$ receptor distribution in 'diseased' popliteal arteries implies that this receptor subtype may be associated more with certain beneficial effects attributed to ET-1. For example, $ET_B$ receptors may play a role in ischaemia-induced angiogenesis of skeletal microvessels seen in CLI patients which may, along with $ET_B$-receptor-mediated dilatation, represent an attempt to improve perfusion to the ischaemic tissue (Tsui et al. 2002).

Here, we have also identified a potentially important localisation of ET-1 and its receptors in perivascular nerves of popliteal arteries from CLI patients. Previous studies have shown an association of ET-1 with the microvascular supply of nerves associated with coronary vessels (Dashwood et al. 1996) and with vasa nervorum of the sural nerve (Dashwood and Thomas 1997). ET-1 receptor localisation on nerve microvessels reveals a novel 'neural' site of action of this peptide. In an experimental model of diabetes $ET_A$ receptor antagonism increases blood flow of the rat sciatic nerve. The authors suggest that in diabetic patients endogenous ET-1-induced ischaemia may have deleterious effects on nerve conduction (Cameron et al. 1994). Is it possible therefore that neuronal control of blood vessels may be affected via ET-1-induced nerve ischaemia?

Our data suggests that $ET_A$ receptor antagonists may have therapeutic potential in lowering the incidence of CLI in patients with PVD.

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