Altered small artery morphology and reactivity in critical limb ischaemia

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ABSTRACT

Although the pathophysiology of critical limb ischaemia is poorly understood, there is evidence that the condition of the small arteries may determine the outcome of revascularization procedures. This study was designed to investigate the effects of critical limb ischaemia on the structure and function of the small arteries in the leg. Small arteries (< 500 μm) from proximal (non-ischaemic) and distal (ischaemic) sites were obtained from patients undergoing bypass surgery for critical limb ischaemia and mounted in a myograph. Reactivity and morphological measurements were carried out and compared with controls. Control vessels from the thigh and calf showed no difference in media to lumen ratio. However, a comparison of ischaemic and non-ischaemic vessels from the patients with critical limb ischaemia showed significant thinning of the ischaemic vessel wall. Contraction studies using noradrenaline and angiotensin II revealed a significant decrease in the response of ischaemic vessels compared with the non-ischaemic vessels from the same patient. Moreover, these differences in reactivity were still apparent after the responses were corrected for wall thickness. Endothelial function assessed using the endothelium-dependent agonists acetylcholine and bradykinin showed a significantly impaired relaxation response to acetylcholine but not to bradykinin in the ischaemic vessels, and acetylcholine-induced relaxation was not improved after incubation with indomethacin. There was no change in the response to the endothelium-independent cAMP-mediated vasodilator iloprost but a significant impairment to sodium nitroprusside which acts via cGMP. These results suggest that small arteries in critical limb ischaemia are altered in both structure and function, with vessel wall thinning and impaired responses to acetylcholine and sodium nitroprusside.

INTRODUCTION

The pathophysiology of critical limb ischaemia (CLI) is still poorly understood. In most patients, atherosclerotic disease produces occlusion or severe narrowing of the conducting arteries in the leg leading to a reduction in blood flow and perfusion pressure in the distal circulation. Preoperative investigations into the severity of ischaemia have concentrated mainly on measurement of pressure in the vasculature of the foot beyond the narrowing since these vessels have a profound effect on the ability of any bypass graft to deliver blood to the distal circulation.

Little is known about the state of the small arteries and arterioles in CLI; however, non-invasive studies have demonstrated an abnormal response to posture. The normal vasoconstrictor response on rising from the supine to the sitting position is diminished in patients with mild arterial disease [1] and is either absent or there is a paradoxical rise in skin perfusion in CLI [2]. More

Key words: nitric oxide, smooth muscle, subcutaneous small arteries, vascular structure.
Abbreviations: ACh, acetylcholine; ANGII, angiotensin II; BK, bradykinin; CLI, critical limb ischaemia; NA, noradrenaline.
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recently, studies by Jacobs et al. [3] using capillary microscopy and transcutaneous oximetry have shown that the microcirculation is severely compromised in CLI with a reduced capillary density and capillary dilation.

The failure of resistance vessel function may depend on a loss of structural integrity with vessel wall thinning or an abnormal contractile response of the vascular smooth muscle. Alternatively, there may be an increase in endothelium-mediated relaxation. Vasoactive substances produced by the vascular endothelium help maintain vascular tone and play a protective role by inhibiting platelet aggregation [4–6]. Vasodilators such as acetylcholine (ACh) and bradykinin (BK) cause vascular relaxation by stimulating the release of endothelium-derived relaxing factors, which include nitric oxide or closely related substances, prostacyclin [7] and endothelium-derived hyperpolarizing factor [8]. Additionally, physical stimuli such as changes in flow rate or shear stress produce endothelium-dependent relaxation [9]. Nitric oxide causes smooth muscle relaxation by producing a rise in the second messenger cGMP [10] whereas prostacyclin acts via cAMP [11]. Little is known about endothelium-derived hyperpolarizing factor, which causes smooth muscle cell membrane hyperpolarization, possibly by an action on calcium-dependent potassium channels [12].

The mechanisms underlying the abnormal vasoconstrictor responses in critical ischaemia are not known and therefore this study was designed to investigate the structure and reactivity of small arteries from patients with CLI.

METHODS

Patients

Sixteen normotensive (mean blood pressure 134 ± 3.1/75 ± 2.8 mmHg), non-diabetic patients [12 male and 4 female (10 smokers and 6 non-smokers), mean age 64 ± 3 years] undergoing femorodistal or below-knee femoro-popliteal bypass surgery took part in the study. All patients fulfilled the criteria for critical leg ischaemia as defined by the 1991 European Consensus Document [12], i.e. ‘persistently recurring ischaemic rest pain requiring regular analgesia for more than 2 weeks, with an ankle systolic pressure < 50 mmHg and/or a toe systolic pressure of < 30 mmHg, or ulceration or gangrene of the foot or toes, with an ankle systolic pressure of < 50 mmHg or a toe systolic pressure of < 30 mmHg.’

Of these patients, two had suffered previous cerebral vascular accidents, one had rheumatoid arthritis, five also suffered from ischaemic heart disease (one of whom also suffered from asthma) and eight had no other illnesses. The mean haemoglobin of the group was 12.7 ± 2.5 (range 9.2–17.4) g/dl and all but one had normal renal function with a mean serum creatinine of 102 ± 8.0 μmol/l. The one patient with a raised serum creatinine of 208 μmol/l recovered back to normal levels after surgery. All but two of the patients with CLI were receiving medication. Six patients were on heparin therapy (three on heparin alone, one with anti-anginal treatment, one also on aspirin and one on both anti-anginal therapy and aspirin), five patients received aspirin treatment (two on aspirin alone, one with anti-anginal treatment, one also on diuretics and one on both diuretics and anti-anginal therapy), and the three remaining patients received diuretic therapy (one on diuretics alone and two also on anti-anginal therapy). None of the patients with CLI was taking an angiotensin-converting enzyme inhibitor.

Control data were obtained from 24 age-matched (mean age 67 ± 3 years) ‘non-ischaemic’ normotensive, non-diabetic patients balanced for sex [12 male and 12 female (11 smokers and 3 non-smokers)] who were undergoing surgery on the thigh or calf for either orthopaedic or cardiac reconstruction. None of the controls had clinical evidence of peripheral vascular disease, 14 were completely free from disease, eight (patients undergoing coronary artery bypass grafting) had ischaemic heart disease, one suffered from Parkinson’s Disease and one suffered from anaemia, asthma, chronic bronchitis and osteoporosis. Twelve of the control patients were on no medication, seven were on anti-anginal therapy (two of whom also received diuretics and one angiotensin-converting enzyme therapy for congestive heart failure), two patients received diuretic therapy alone and three received diuretics with aspirin (one of whom also received angiotensin-converting enzyme therapy for congestive heart failure). All patients received inhalational anaesthesia for operation. One sample was obtained from each control patient. The proximal and distal control groups each contained 12 patients (6 male, 6 female).

All patients gave their informed consent and the study was approved by Leicestershire Health Authority Ethics Committee.

Preparation of vessels

In the operating theatre two samples (approx. 1 cm³) of subcutaneous fat were obtained from each patient with critical ischaemia. The first was taken from an incision above the knee, representing a non-ischaemic sample, while the second was obtained from a distal calf incision, representing an ischaemic sample. These samples were designated the CLI group (proximal and distal). One sample (either proximal or distal) was obtained from each patient in the control group. Small arteries (< 500 μm) were dissected from the fat under the microscope and mounted as ring segments on two 40-μm stainless steel wires in a Mulvany myograph [13]. One wire was attached to a force transducer and the other was con-
Angiotensin II (ANGII; 10−7 M) was incubated with indomethacin (10−5 M), before and after incubation with indomethacin (10−5 M), and BK (10−9–10−6 M). Endothelium-independent vasodilatation was studied using sodium nitroprusside (10−8–10−4 M) and iloprost (10−8–10−6 M). The order of the experiments was fixed for each vessel in all groups to control for time and order effects. At the end of the study, vessels were fixed in formalin overnight. Ten-percent formalin was used for routine histology and 3% formalin for a preliminary confocal microscopy study.

**Drugs**

Acetylcholine hydrochloride, ANGII, BK, cocaine hydrochloride, indomethacin, NA and sodium nitroprusside were obtained from Sigma Chemical Company, Poole, Dorset, U.K. Iloprost was a gift from Schering U.K. All drugs except indomethacin (dissolved in absolute ethanol) were dissolved in distilled water and diluted to the final bath concentration with physiological salt solution.

### Statistical analysis

Cross-sectional area (CSA) of the vessel media was calculated using the equation

\[
CSA = \pi(d m + d^2)
\]

where \(d\) = internal diameter and \(m\) = media thickness.

Tension is expressed as milliNewtons per millimetre vessel length (mN/mm), media stress is expressed as tension divided by media thickness (mN/mm²) and relaxation data as percentage change from maximum NA-induced contraction. Results are expressed as means ± S.E.M. Statistical comparison of morphology data, maximum response and \(ED_{50}\) was performed using Student’s \(t\)-test between proximal and distal in the CLI group; unpaired \(t\)-test between proximal and distal in the control group. Dose–response curves were analysed by one-way ANOVA for repeated measures with a \(P\) value < 0.05 being considered statistically significant.

### RESULTS

#### Morphology

The mean vessel diameter, media thickness and media cross-sectional area were similar in the proximal and distal vessels in both the control and CLI groups (Table 1). The media to lumen ratio of the vessels (media thickness over internal diameter), which takes into account differences due to vessel selection, was compared. The proximal and distal (above and below the knee) samples from the controls were of similar thickness but in the CLI patients the media to lumen ratio of the distal ischaemic vessels was significantly reduced. Routine histological examination of vessels fixed in the myograph demonstrated normal endothelium and no pathological changes in the smooth muscle. In addition, a preliminary confocal microscopy study of vessels fixed

#### Table 1 Morphological characteristics of vessels from controls and patients with CLI

<table>
<thead>
<tr>
<th></th>
<th>Proximal</th>
<th>Distal</th>
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<tbody>
<tr>
<td>Internal diameter (μm)</td>
<td>291 ± 23</td>
<td>253 ± 28</td>
</tr>
<tr>
<td>Media thickness (μm)</td>
<td>23.07 ± 2.95</td>
<td>24.14 ± 2.68</td>
</tr>
<tr>
<td>Media CSA (μm²)</td>
<td>19.643 ± 2972</td>
<td>19.300 ± 2730</td>
</tr>
<tr>
<td>Media/lumen (%)</td>
<td>10.54 ± 2.1</td>
<td>11.87 ± 1.6</td>
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Table 2  Results of contraction studies using NA and ANGII on control vessels and vessels from patients with CLI

*P < 0.05, proximal versus distal (Student’s paired/unpaired t-test). †P < 0.05, proximal versus distal analysis of curve; one-way ANOVA for repeated measures. ††P < 0.01, proximal versus distal analysis of curve; one-way ANOVA for repeated measures.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 12)</th>
<th>Ischaemic (n = 16)</th>
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<tbody>
<tr>
<td></td>
<td>Proximal Distal</td>
<td>Proximal Distal</td>
</tr>
<tr>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum response (mN/mm)</td>
<td>2.26 ± 0.33 2.43 ± 0.44</td>
<td>2.59 ± 0.45 1.74 ± 0.21††</td>
</tr>
<tr>
<td>ED50 (nM)</td>
<td>0.22 ± 0.05 0.13 ± 0.04</td>
<td>0.24 ± 0.10 0.39 ± 0.20</td>
</tr>
<tr>
<td>Media stress (mN/mm²)</td>
<td>72.1 ± 15 66.8 ± 10</td>
<td>90.90 ± 12.30 77.15 ± 8.25††</td>
</tr>
<tr>
<td>ANGII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum response (mN/mm)</td>
<td>1.47 ± 0.22 1.04 ± 0.17†</td>
<td>1.89 ± 0.33* 0.96 ± 0.10††</td>
</tr>
<tr>
<td>ED50 (nM)</td>
<td>2.90 ± 1.4 3.56 ± 0.95</td>
<td>1.97 ± 0.52 6.52 ± 2.62</td>
</tr>
<tr>
<td>Media stress (mN/mm²)</td>
<td>37.8 ± 10 20.4 ± 4.9</td>
<td>73.90 ± 10.10 50.69 ± 7.82††</td>
</tr>
</tbody>
</table>

Figure 1  Dose–response curves to noradrenaline (A) and angiotensin II (B) in control vessels (n = 12)

*P < 0.05, proximal compared with distal curves.

Contraction studies

The results of cumulative dose–contraction curves to NA and ANGII are shown in Table 2. No difference was
found between the proximal and distal vessel responses to NA in the controls, with the dose–response curves, maximum response and dose required to produce 50% of the maximum response (ED$_{50}$) being similar (Figure 1A). There was also no difference in maximum response or ED$_{50}$ to ANGII in the controls; however, a small reduction in response was observed in the dose–response curve from the distal controls compared with the proximal controls ($P < 0.05$) (Figure 1B). In the CLI group, both NA and ANGII produced substantially reduced contractile responses in those vessels originating from the distal ischaemic region compared with their paired proximal vessels ($P < 0.01$) (Figure 2). Similarly, the maximum contraction produced by the distal ischaemic vessels in response to ANGII was significantly reduced when compared with the proximal vessels ($P < 0.05$). The ED$_{50}$ values for proximal and distal vessels were similar in CLI and control groups for both vasoconstrictors (Table 2). When the contraction data were corrected for differences in vessel thickness and expressed as media stress the difference between the curves produced by NA and ANGII in the CLI group remained significant (Table 2, $P < 0.05$).

**Endothelium-dependent relaxation**

The controls showed similar maximum relaxation responses to ACh in the proximal (41±13%) and distal (44±10%) vessels (Figure 3A). In the CLI vessels the maximum ACh-induced relaxation responses in both proximal (30±9%) and distal (17±8%) vessels were poor but not significantly different from each other. However, comparison of the curves showed that the

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**Figure 3** Relaxation dose–response curves to acetylcholine in controls (A; $n = 12$) and critical ischaemic vessels (B; $n = 14$)

$^*P < 0.05$, proximal compared with distal curves.

**Figure 4** Relaxation dose–response curves to bradykinin in controls (A; $n = 12$) and critical ischaemic vessels (B; $n = 16$)
Figure 5  Relaxation dose–response curves to sodium nitroprusside in controls (A; n = 12) and critical ischaemic vessels (B, n = 14)

$^* P < 0.05$, proximal compared with distal curves.

Distal ischaemic vessels produced a significantly poorer relaxation than the proximal vessels ($P < 0.05$) in response to ACh (Figure 3B). The maximum relaxation response of either the proximal or distal vessels was not significantly improved by incubation with indomethacin (results not shown).

No difference in the response to BK was found between the proximal and distal controls or CLI vessels (Figure 4).

Endothelium-independent relaxation

Experiments using the endothelium-independent vasodilator sodium nitroprusside revealed similar responses in the control vessels with both achieving similar maximum relaxations: proximal, $79 \pm 5\%$; distal, $74 \pm 5\%$ (Figure 5A). However, although the CLI vessels also produced identical maximum relaxations (proximal $79 \pm 5\%$ and distal $74 \pm 5\%$), the distal ischaemic vessels were less sensitive than the proximal vessels ($P < 0.05$) (Figure 5B). No difference in the response to iloprost was found between the proximal and distal controls or CLI vessels (Figure 6).

DISCUSSION

This study demonstrates that CLI results in a reduced media to lumen ratio in subcutaneous small arteries and preliminary studies using confocal microscopy indicate that this occurred with no change in smooth muscle cell
density. Moreover, it was not associated with a change in compliance as assessed by examining length–tension characteristics before and after deactivating the smooth small muscle by incubation with potassium cyanide, iodoacetate and dinitrophenol (C. Hillier and A. Thurston, unpublished work). Previous detailed studies of human essential and renovascular hypertension have suggested that the maintenance of vascular smooth muscle structure is dependent upon a complex control system involving haemodynamic factors [14,15], endogenous mitogens or growth factors [16] and sympathetic innervation [17]. Critical ischaemia results in a low-flow, low-pressure environment in which the normal shear stress, stretch and perfusion pressures experienced by the vascular endothelial and smooth muscle cells is altered [18,19].

It is likely that mechanisms which, in the high-flow, high-pressure environment of hypertension, cause vascular smooth muscle hypertrophy are reversed in the critical ischaemic condition. The reduction in media to lumen ratio found in this study may be the result of three different structural alterations: hypoplasia, i.e. a reduction in the number of smooth muscle cells; atrophy, in which there is a reduction in cellular size or extracellular components such as collagen; or a remodelling which involves a re-orientation of the tissue components so that the lumen is increased and the media thickness reduced without any change in cross-sectional area [20]. Comparison of proximal and distal vessels within both CLI and control groups showed that the media cross-sectional area was not significantly reduced in CLI with no change in diameter, suggesting that no loss of smooth muscle had occurred, and therefore observed differences in media to lumen ratio could represent remodelling. No comparison was made between CLI and control vessels.

The complex interactions between flow, pressure and the physical characteristics of the vessels (differences in compliance and active tension) have made the primary determinant of vascular structure difficult to identify. Previous studies have concentrated on the effects of pressure. Bund et al. [21] showed that when the hind limb vascular bed of hypertensive rats was protected from high pressures by ligation of the external iliac artery, the resistance arteries adjusted their structure according to the pressure to which they were exposed. However, in that study the effects of the concomitant reduction in flow rate and the consequent alterations in the endothelial release of vasoactive factors were not investigated. Furthermore, analyses were based on measurements of mean arterial pressure despite evidence to suggest that pulse pressure rather than mean arterial pressure is more closely related to vascular structural changes [22] although this is still the subject of some debate [23]. Experimental investigations into the effects of alterations in haemodynamics on structure are hampered by the difficulty in altering one factor without changing many others. The complexity of the system is compounded by the role of the vascular endothelium. Changes in the architecture of the vessel wall can be mediated by endothelium-dependent release of potent growth factors including platelet-derived growth factor and heparin sulphate, as well as a variety of cytokines associated with pathological conditions, e.g. interleukin and insulin-like growth factor [24].

In this study there was a reduction in the pharmacological responses of the vessels taken from the distal ischaemic area to the vasoconstrictor agents NA and ANGII and both the endothelium-dependent relaxing vasodilator ACh and the endothelium-independent vasodilator sodium nitroprusside. A reduction in media thickness provides an obvious explanation for an alteration in the ability of a vessel to contract in response to pressor stimuli. However, expression of the contraction data for NA and ANGII in terms of media stress, which corrects for the differences in media thickness, did not explain completely the observed differences between the proximal and distal vessels from the CLI group. This suggests that there is an alteration in the agonist/receptor/contraction process per se, although it is not possible from this study to determine at what level the changes have occurred.

In this study incubation with the non-specific cyclooxygenase inhibitor indomethacin had no effect on the reduced response to ACh suggesting no role for vasoconstrictor prostanooids. However, it has been shown previously that ACh-stimulated release of nitric oxide can be attenuated by oxygen-derived free radicals [25], possibly by the breakdown of the nitric oxide molecule [26]. More recently, Omar et al. [27] have presented evidence that suggests a protective role for superoxide dismutase, which acts as a scavenger of the superoxide anion, in vascular tissue. However, superoxide dismutase levels do not appear to be critical in endothelium-dependent relaxations that do not require guanylate cyclase activity resulting in increases in intracellular cGMP, such as prostacyclin-induced relaxation. Similarly, endothelium-independent relaxations to nitroglycerine and nitroprusside (both acting via increases in cGMP) are also modulated by intracellular superoxide dismutase activity, whereas relaxations to papaverine and isoprotefenol, which are associated with adenylate cyclase activity and increases in intracellular cAMP, are unaffected by superoxide dismutase inhibition [27]. This implies that superoxide radicals may produce a selective impairment of guanylate cyclase-mediated relaxation. The data from this study show an impairment in responses to both ACh and sodium nitroprusside, but not to BK or the stable prostacyclin analogue iloprost. There is evidence to suggest that BK produces the release of both nitric oxide and prostacyclin [28,29]. Inhibition of the nitric oxide component by superoxide radicals may result in an increase in prostacyclin activity resulting in
no apparent loss of relaxation. Our study does not test this directly but does implicate a failure in cGMP-mediated responses as an explanation for the observed differences.

The cellular signalling system from membrane receptor to second messenger is mediated via guanine-nucleotide-binding regulatory proteins (G-proteins). There is evidence to suggest that atherosclerotic disease leads to a selective impairment of G-protein signal transduction [30,31]. The muscarinic receptor response to ACh is mediated via the pertussis toxin-sensitive G\(i\) protein [32] whereas BK is mediated via the G\(q\) protein which activates phospholipase C [30]. It has been suggested that early atherosclerotic changes can result in a selective impairment of G\(i\)-protein-mediated responses. As the disease process progresses the responses to other G-protein-linked responses become impaired. Finally, with severe atherosclerotic lesions the ability of the vessel to relax is completely abolished [30]. The overall poor responses to ACh in the control and ischaemic vessels observed in this study may therefore represent the specific effects of underlying undetected atherosclerosis in the controls but more marked effects in the patients with critical ischaemia with more severe atherosclerotic disease.

This investigation into the effects of critical ischaemia on small arteries assumed that subcutaneous tissue below a major arterial occlusion would be, if not completely ischaemic, at least subject to an altered environment. Previous studies have attempted to determine the extent of ischaemia present in the skin at various levels of the leg in patients with CLI. A variety of techniques have been used including radioisotope clearance studies [33,34], laser Doppler flowmetry [35,36] and transcutaneous oximetry (TcPO\(_2\)) [37,38]. However, few studies have shown agreement between these methods of assessment of ischaemia and the clinical outcome of the patient. Similarly, when these methods are used to predict the optimal level of amputation there is little correlation between those areas categorized as ischaemic and those failing to heal after amputation. One study has reported healing in an area with a measured TcPO\(_2\) of zero [39]. No measure of ischaemia was made at the site of incision in this study, but the obvious inconsistencies in the available techniques suggest that they may have been of limited relevance for the size and site (depth) of vessels taken for this in vitro study.

In conclusion, we have shown that CLI is associated with an alteration in both morphology and reactivity in small arteries. There is a significant decrease in the vessel wall to lumen ratio which is probably due to remodelling rather than loss of vascular smooth muscle. However, the reduction in vascular wall thickness does not explain the poor contractile responses to NA and ANGII since the media stress (force per unit of wall thickness) is also abnormal in the ischaemic vessels. The impaired responses to both the endothelium-dependent vasodilator ACh and the endothelium-independent agonist sodium nitroprusside but not to BK or iloprost suggests an impairment of relaxation mechanisms specific for guanylate cyclase-mediated responses. The study of the distal circulation in CLI is in its infancy and the processes affecting vascular structure are still largely unknown. The vascular abnormalities demonstrated in this study show that these vessels may not be able to respond to normal physiological stimuli in an appropriate way. Therefore, the restoration of systemic perfusion pressure after bypass surgery may result in damage to the capillary beds and reduced gas exchange to the tissues. These results may explain the incidence of post-operative oedema and poor skin healing observed in many patients.

ACKNOWLEDGMENT

Supported by a grant from the British Heart Foundation.

REFERENCES


