Eur J Vasc Endovasc Surg 10, 31–35 (1995)

# Thromboxane and Neutrophil Changes Following Intermittent Claudication Suggest Ischaemia–Reperfusion Injury*

**H. S. Khaira[1], G. B. Nash[2], P. S. Bahra[2], K. Sanghera[3], P. Gosling[3], A. J. Crow[1] and C. P. Shearman[1]**

*[1]Department of Vascular Surgery, Queen Elizabeth Hospital, Edgbaston, Birmingham, U.K., [2]Department of Haematology, University of Birmingham, Edgbaston, Birmingham, U.K. and [3]Department of Biochemistry, Selly Oak Hospital, Selly Oak, Birmingham, U.K.*

**Objectives:** *It has been postulated that ischaemia–reperfusion occurs in intermittent claudication resulting in neutrophil activation and release of soluble mediators, increasing systemic vascular permeability and enhancing atherogenesis.*
**Methods:** *We measured neutrophil deformability, plasma thromboxane levels, and urinary microalbumin excretion in 30 male claudicants, and 10 age- and sex-matched controls, before and after exercise to maximum walking distance. Blood was taken from an antecubital vein.*
**Results** *There was an increase in urinary microalbumin excretion after exercise in claudicants. Statistically significant increases in the median and 90th percentile transit times (markers of neutrophil deformability) for isolated neutrophils from blood drawn 5 min after exercise in the claudicants were observed with no change in control subjects. Plasma thromboxane concentrations in claudicants increased within 10 min post-exercise. Plasma concentrations in controls were significantly lower throughout the study period. In the claudicant group, a positive correlation between the percentage change in the median transit time for neutrophils, and the percentage change in plasma thromboxane at 60 min post-exercise was found.*
**Conclusions:** *The results lend further support to the concept of ischaemia–reperfusion events in patients with intermittent claudication, leading to a systemic increase in vascular permeability as a result of endothelial injury or dysfunction (a crucial step in atherogenesis), associated with thromboxane production and neutrophil activation. We suggest that the above changes may contribute to the increased mortality seen in such patients.*

*Key Words: Neutrophils; Thromboxane; Intermittent claudication.*

## Introduction

It is accepted that severe tissue ischaemia followed by reperfusion has major adverse systemic effects,[1] especially on the lungs.[2,3] Evidence suggests a central role for neutrophils[2,4–7] and thromboxane $A_2$[8–10] in a sequence of events leading to vascular endothelial cell damage and resulting in a generalised increase in vascular permeability (quantified by small increases in renal permeability reflected by changes in urinary protein excretion - microalbuminuria[11–13]). A causal relationship between neutrophil activation and thromboxane $A_2$ has been demonstrated[14–17] and activated neutrophils may cause damage by release of oxygen-derived free radicals and proteolytic enzymes, and blockage of microvessels.[18]

Intermittent claudication, a common manifestation of peripheral occlusive arterial disease, affects at least 5% of men over 50 years[19,20] and is associated with a high cardiovascular mortality rate.[20–23] It has been postulated that patients with intermittent claudication undergo a series of ischaemia–reperfusion injuries and that the above neutrophil-related mechanisms may contribute to the increased mortality experienced by such patients.[18,24–28] Activated neutrophils undergo structural changes that result in decreased deformability.[29] Previous studies have used filtration rates of neutrophil suspensions to assess activation and changes in flow properties after intermittent claudication.[25,27] We used a cell transit analyser (CTA),[29,30] a sensitive indicator of white cell

*Presented at the 8th annual meeting of the European Society for Vascular Surgery, Berlin, Germany (September 1994).
Please address all correspondence to: Mr H. S. Khaira, Research Registrar, Department of Vascular Surgery, Queen Elizabeth Hospital, Edgbaston, Birmingham, U.K.

1078–5884/95/010031 + 05 $08·00/0 

activation, to measure transit times through a micropore filter for a large number of individual neutrophils. Thromboxane $A_2$ has been stated to rise in patients with peripheral occlusive arterial disease following exercise,[32] but no supporting data were presented in this paper, or correlated with other markers of endothelial or neutrophil response. Thromboxane is released during ischaemia in animal models,[10] and has a number of adverse actions including increasing vascular permeability by directly altering endothelial cell architecture, or indirectly through interaction with neutrophils.[8,10] Here, we have carried out measurements of thromboxane levels in parallel with studies on neutrophil rheology using systemic blood, and a marker of generalised endothelial permeability (microalbuminuria) before and after exercise in patients with intermittent claudication.

## Materials and Methods

Ethical committee approval and informed consent were obtained for this study. Thirty male patients with intermittent claudication (median age 63 years, range 43–73) were recruited from vascular outpatient clinics. Ten control subjects were selected from a local male choir (median age 64 years, range 49–71). Those with uncontrolled hypertension, diabetes mellitus, inflammatory rheumatological conditions, or on drugs likely to interfere with neutrophil function (e.g. calcium antagonists such as nifedipine) were excluded. The patients had a mean resting ankle brachial pressure index (ABPI) of 0.58 (range 0.42–0.78), compared to 1.18 (range 1.04–1.39) for controls.

Having emptied their bladders, patients rested for 1.5 h then emptied their bladders again, producing a mid-stream urine specimen (MSU). Fifteen millilitres of blood was taken from an antecubital vein, via an indwelling cannula, for neutrophil testing and 5ml for thromboxane $B_2$ ($TXB_2$). Blood was collected in sterile tubes containing EDTA[31] as anticoagulant. The patients were then exercised to their maximum walking distance on a treadmill at 3.2km/h and an incline of 12.5%. Controls exercised for a total of 4 min (200m) at the same settings. The mean maximum walking distance was 165m (range 83–310m) and all patients had a decrease in their ankle systolic pressures (mean of 61mmHg, range 28–114mmHg) after exercise relative to pre-exercise values. All the controls showed an increase in ankle systolic pressure after exercise. Blood was taken via the cannula at 5 min post-exercise for neutrophil testing and at 10, 20, and 60 min post-exercise for $TXB_2$ levels. Another MSU was taken 1h post-exercise. Blood for $TXB_2$ was immediately centrifuged at 2000*g* for 10 min; the plasma was aspirated and snap-frozen in liquid nitrogen. The urine specimens were also snap-frozen. Microalbuminuria was determined using a double-antibody $^{125}$I-radioimmunoassay (Diagnostic Products Corporation, Wallingford, U.K.). The result was expressed as an albumin/creatinine ratio (ACR) in mg/mmol to compensate for changes in urinary flow during the investigation.

$TXB_2$ levels were determined using a ELISA technique (Biotrack, Amersham Life Science, Amersham, U.K.).

Neutrophils were prepared within 30 min of sampling. Preparation followed standard procedures previously described[31,33] using a double density gradient of histopaque-1077 over histopaque-1119 (Sigma Diagnostics, Dorset, U.K.). This method yields >97% viable neutrophils (trypan blue exclusion). The neutrophils were suspended in 20% autologous plasma (in phosphate-buffered saline, Sigma Diagnostics, Dorset, U.K.) at a concentration of $2 \times 10^5$ cells/ml and allowed to equilibrate at 37°C for 20 min before analysis with the CTA. The CTA provides transit times in ms for a large number of individual neutrophils passing through a filter with thirty 8$\mu$m diameter pores.[29,30] A pressure of 400Pa was used to drive the flow of neutrophils through the pores, and each transit was electronically sensed and registered as a voltage pulse. The width of these pulses was analysed by computer and taken as a measure of transit time. Approximately 1000 cells were analysed in each sample and transit time percentiles calculated.

All results were expressed as mean ± S.E.M. (standard error of the mean) unless otherwise stated. Pre- and post-exercise values were compared using Student's paired *t*-test with $p < 0.05$ being taken as statistically significant. Thromboxane levels in claudicants and controls were compared using the Student's unpaired *t*-test. Spearman's rank correlation test was used to determine the correlation between percentage change in neutrophil transit times and percentage changes in thromboxane.

## Results

The CTA data for patients and controls is summarised in Table 1. The data showed statistically significant increases in the transit time for neutrophils in systemic blood taken from patients after exercise. There was a relatively small change in the median transit times representing the whole population of neutrophils, but there was a much greater shift within the sub-

**Table 1. Pore transit times (milliseconds) for neutrophils pre- and post-exercise for claudicants and controls**

| | Transit timepre-exercise(ms) | Transit timepost-exercise(ms) | Percentage change | *p*Student's paired*t*-test |
|---|---|---|---|---|
| Patients ($n = 30$) | | | | |
| Median | 4.6 ± 0.2 | 5.0 ± 0.2 | 9.0 ± 4.2 | 0.040 |
| 90th percentile | 12.3 ± 0.9 | 14.6 ± 1.3 | 20.2 ± 8.0 | 0.018 |
| Controls ($n = 10$) | | | | |
| Median | 3.2 ± 0.2 | 3.4 ± 0.2 | 7.6 ± 4.2 | 0.11 |
| 90th percentile | 9.0 ± 1.1 | 9.1 ± 1.0 | 4.2 ± 6.1 | 0.50 |

population of slower flowing cells (90th percentile). There was no statistically significant percentage change in transit times for controls after exercise. The data shows a difference between controls and patients for the median and 90th percentile transit times pre- and post-exercise. However, valid comparison is not possible because a filter malfunction meant that the filter used for the patients could not be used for the whole of the control group. Comparing post-exercise to pre-exercise transit times is valid.

The percentage change in ACR (microalbuminuria) showed a statistically significant increase 1 h after exercise of 32.0 ± 14.4% ($p = 0.034$, Student's paired *t*-test). Controls showed no change in ACR post-exercise. The data showing median ACR values are summarised in Table 2.

**Table 2. Microalbuminuria (ACR) for claudicants and controls**

| | Patients ($n = 30$) | Controls ($n = 10$) |
|---|---|---|
| ACR pre-exercise* | 0.57 (0.36–1.41) | 0.50 (0.45–0.72) |
| ACR post-exercise* | 0.68 (0.33–2.49) | 0.52 (0.32–0.79) |

*data is not normally distributed and therefore median and interquartile range given.

Data for thromboxane $B_2$ levels are shown graphically in Figure 1. Plasma thromboxane concentrations in claudicants rose for all time points after exercise ($p < 0.001$ compared to pre-exercise; Student's paired *t*-test). Plasma concentrations in controls were lower than those in claudicants ($p < 0.0005$; Student's unpaired *t*-test) throughout the study period but showed a rise at 10, 20, and 60 min post-exercise ($p < 0.05$ compared to pre-exercise; Student's paired *t*-test). There was a statistically significant positive correlation between the percentage change in the median transit time for the whole population of neutrophils, and the percentage change in peak plasma thromboxane at 60 min post-exercise (Spearman's rank correlation coefficient = 0.423; $0.05 > p > 0.02$).

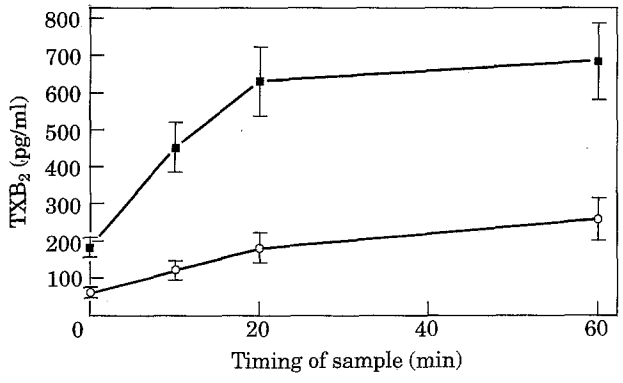


**Fig. 1.** Systemic plasma thromboxane levels (mean ± S.E.M.) at pre-exercise (time point 0) and at 10, 20, and 60 min post-exercise in patients with intermittent claudication [(■)$n = 30$] and controls[(○)$n = 10$].

## Discussion

Intermittent claudication is a common presentation of peripheral vascular disease and although the disease in the lower extremities seems to follow a benign course,[20,23] it is associated with a cardiovascular mortality 2–3 times that of the general population[20–23] reflecting the systemic nature of atherosclerosis. The calf muscles of patients with intermittent claudication have been shown to become ischaemic on exercise as gauged by changes in transcutaneous oxygen pressure[34] and blood pH[35] measurements. It has been postulated therefore that these patients undergo a series of ischaemia–reperfusion events that activate neutrophils[25,27] and release soluble mediators (such as thromboxane[10]), both causing systemic increases in vascular permeability[24,28] and enhanced atherogenesis.[36] A link between neutrophils and thromboxane has been shown in animal models of severe ischaemia.[14–17]

Microalbuminuria has been used as a measure of increased vascular permeability[11,12] and therefore an indirect measure of the systemic effects of ischaemia–reperfusion injury.[13] Our results confirm the previous findings of increased microalbuminuria in patients with intermittent claudication after exercise.[26,28]

Neutrophil activation in intermittent claudication has previously been examined using filtration methods[25,27] and intravital microscopy.[28] Filtration methods rely on structural changes in the neutrophils, reducing their deformability after activation.[32] We

have used a CTA because it combines the advantages of filtration methods and single pore or pipette methods by providing data on individual cells at a reasonable rate of acquisition.[30] Our results have confirmed previous findings of decreased deformability of neutrophils, manifest as increased transit times, after exercise. They show, moreover, that a subpopulation of cells are more responsive than the whole population (i.e. the 90th percentile transit time increases more than the median), suggesting that not all neutrophils react equally to claudication.

Thromboxane has been shown to be associated with ischaemia in animal models.[10] Our results show a rise in thromboxane after exercise. Moreover, a positive correlation between neutrophil activation and thromboxane changes following claudication has been shown, analogous to that seen in animal models of more severe ischaemia. Whether the elevation of thromboxane directly induces the change in neutrophil transit times is uncertain because in the present study thromboxane levels continue to rise for 60 min, whereas neutrophil changes have been shown to reverse over this time.[27] However, we[25] have previously found that neutrophil filterability is still significantly decreased at 60 min post-exercise. Inhibition of cyclooxygenase and thromboxane synthetase have been shown, however, to reduce neutrophil accumulation in skin abrasion preparations in rabbits,[15] and thromboxane receptor antagonists inhibit the ischaemia-induced oxidative burst of neutrophils.[14] The rise in thromboxane in controls may be due to an element of small vessel atherosclerosis in these patients, some of whom have been smokers and as a process of ageing, or may indicate that a degree of muscle ischaemia during exercise is normal. The site of production of thromboxane in ischaemia–reperfusion is not clear. Ketoconazole, a specific inhibitor of platelet thromboxane synthesis, does not affect thromboxane levels in plasma after ischaemia–reperfusion.[9] Other potential sources include white blood cells, endothelial cells, and fibroblasts.

What is the significance of the above findings? Microalbuminuria is an indicator of systemic vascular permeability and has been noted to be proportional to the severity of injury in burns and trauma.[11–13] It is a marker of morbidity and death from cardiovascular disease.[37,38] Epidemiological studies have shown a correlation between the white cell count (especially neutrophils) and the risk of myocardial infarction and stroke.[39] Activation of neutrophils magnifies the effects of limb ischaemia by microvascular obstruction and endothelial damage by the release of oxygen-derived free radicals and proteases.[7,18] This effect is not limited to the local microcirculation, and the neutrophil plays a central role in the pathogenesis of ischaemia–reperfusion injury and adult respiratory distress syndrome.[18,40,41] Thromboxane has a number of adverse actions including vasoconstriction, enhanced capillary permeability, platelet aggregation, membrane destabilisation, and cytolysis of endothelial cells.[42] It may modulate permeability directly by altering endothelial cell architecture and cytoskeleton or indirectly through interaction with neutrophils.[8,10] Thromboxane is believed to be an "aggressive" mediator of lethality in sepsis and septic shock.[43]

This study, therefore, adds further support to the suggestion that patients with intermittent claudication undergo repeated bouts of ischaemia and reperfusion which may cause systemic injury on a small scale, and may lead to enhanced atherogenesis and increased mortality. It has been previously proposed that intermittent claudication can be treated with five words: "stop smoking and keep walking".[44] There would seem, therefore, to be a conflict between the methods to improve the local circulatory compromise and the possible adverse systemic effects resulting from such treatment. Thus, we would suggest that in addition to optimising local treatment, either in the form of a structured exercise programme or a procedure to reopen the occluded artery (e.g. angioplasty), some form of systemic protective drug is also required.

In this study we have looked only at systemic changes following intermittent claudication, but the pulmonary microcirculation may modify the systemic neutrophil response by trapping less deformable cells coming from the ischaemic limb. If such an effect does exist one might expect neutrophils isolated from femoral vein blood to have longer transit times than those from systemic blood, at least immediately after exercise.[27] If a soluble mediator is responsible for systemic activation of neutrophils one would not expect to find such a difference. Further studies to answer these questions are currently underway.

## Acknowledgements

This work was supported by The Wellcome Trust (Grant No. 036800) and Bayer Plc. The authors wish to thank the patients who volunteered to take part and the Warley male choir for providing the control group.

## References

1 Fiddian-Green RG. Splanchnic ischaemia and multiple organ failure in the critically ill. *Ann R Coll Surg Engl* 1988; **70**: 128–134.

2 Schmeling DJ, Caty MG, Oldham KT, Guice KS, Hinshaw DB. Evidence for neutrophil-related acute lung injury after intestinal ischaemia-reperfusion. *Surgery* 1989; **106**: 195–202.
3 Paterson IS, Klausner JM, Pugatch R *et al.* Noncardiogenic pulmonary edema after abdominal aortic aneurysm surgery. *Ann Surg* 1989; **209**: 231–236.
4 Heflin AC, Brigham KL. Prevention by granulocyte depletion of increased vascular permeability of sheep lung following endotoxaemia. *J Clin Invest* 1981; **68**: 1253–1260.
5 Anner H, Kaufman RP, Kobzik L, Valeri CR, Shepro D, Hechtman HB. Pulmonary hypertension and leukosequestration after lower torso ischaemia. *Ann Surg* 1987; **206**: 642–648.
6 Anner H, Kaufman RP, Kobzik L, Valeri CR, Shepro D, Hechtman HB. Pulmonary leckosequestration induced by hind limb ischaemia. *Ann Surg* 1987; **206**: 162–167.
7 Welbourn CRB, Goldman G, Paterson IS, Valeri CR, Shepro D, Hechtman HB. Pathophysiology of ischaemia reperfusion injury: central role of the neutrophil. *Br J Surg* 1991; **78**: 651–655.
8 Huval WV, Dunham BM, Lelcuk S, Valeri CR, Shepro D, Hechtman HB. Thromboxane mediation of cardiovascular dysfunction following aspiration. *Surgery* 1983; **94**: 259–266.
9 Lelcuk S, Alexander F, Valeri CR, Shepro D, Hechtman HB. Thromboxane A2 moderates permeability after limb ischaemia. *Ann Surg* 1985; **202**: 642–646.
10 Klausner JM, Paterson IS, Goldman G *et al.* Thromboxane A2 mediates increased pulmonary microvascular permeability following limb ischaemia. *Circ Res* 1989; **64**: 1178–1189.
11 Shearman CP, Gosling P, Simms MH. Glomerular permeability to large molecules: a marker of vascular permeability? *Br J Surg* 1988; **75**: 1273.
12 Gosling P, Shearman CP. Increased levels of urinary proteins: markers of vascular permeability? *Ann Clin Biochem* 1988; **25**(Suppl): 150s–151s.
13 Smith FCT, Gosling P, Sanghera K, Green MA, Paterson IS, Shearman CP. Microproteinuria predicts the severity of systemic effects of reperfusion injury following infrarenal aortic aneurysm surgery. *Ann Vasc Surg* 1994; **8**: 1–5.
14 Paterson IS, Klausner JM, Goldman G *et al.* Thromboxane mediates the ischaemia-induced neutrophil oxidative burst. *Surgery* 1989; **106**: 224–229.
15 Palder SB, Huval W, Lelcuk S *et al.* Reduction of polymorphonuclear leukocyte accumulations by inhibition of cyclooxygenase and thromboxane syntase in the rabbit. *Surgery* 1986; **99**: 72–81.
16 Paterson IS, Klausner HB, Hechtman HB, McCollum CN. Mediation by thromboxane of the white blood cell activation induced by ischaemia. *Br J Surg* 1990; **77**: A708.
17 Goldman G, Welbourn R, Klausner JM, Valeri CR, Shepro D, Hechtman HB. Thromboxane mediates diapedesis after ischaemia by activation of neutrophil adhesion receptors interacting with basally expressed intercellular adhesion molecule-1. *Circ Res* 1991; **68**: 1013–1019.
18 Nash GB, Shearman CP. Neutrophils and Peripheral Arterial Disease. *Critical Ischaemia* 1992; **2**: 5–20.
19 Dormandy J, Mahir M, Ascady G *et al.* Fate of the patient with chronic leg ischaemia. A review article. *J Cardiovasc Surg* 1989; **30**: 50–57.
20 Dormandy JA. Natural history of intermittent claudication. *Hosp Update* 1991; **April**: 314–320.
21 Bloor K. Natural history of arteriosclerosis of the lower extremities. *Ann R Coll Surg Engl* 1961; **28**: 36–52.
22 Reunanen A, Takkunen H, Aromaa A. Prevalence of intermittent claudication and its effect on mortality. *Acta Med Scand* 1982; **211**: 249–256.
23 Jelnes R, Gaardsting O, Hougaard Jensen K, Baekgaard N, Tonnesen KH, Schroeder T. Fate in intermittent claudication: outcome and risk factors. *BMJ* 1986; **293**: 1137–1140.
24 Shearman CP, Gosling P, Gwynn BR, Simms MH. Systemic effects associated with intermittent claudication. A model to study biochemical aspects of vascular disease? *Eur J Vasc Surg* 1988; **2**: 401–404.
25 Hickey NC, Gosling P, Baar S, Shearman CP, Simms MH. Effect of surgery on the systemic inflammatory response to intermittent claudication. *Br J Surg* 1990; **77**: 1121–1124.
26 Hickey NC, Shearman CP, Gosling P, Simms MH. Assessment of intermittent claudication by quantitation of exercise-induced microalbuminuria. *Eur J Vasc Surg* 1990; **4**: 603–606.
27 Neumann F, Waas W, Diehm C *et al.* Activation and decreased deformability of neutrophils after intermittent claudication. *Circulation* 1990; **82**: 922–929.
28 Hickey NC, Hudlicka O, Gosling P, Shearman CP, Simms MH. Intermittent claudication incites systemic neutrophil activation and increased vascular permeability. *Br J Surg* 1993; **80**: 181–184.
29 Buttrum SM, Drost EM, MacNee W *et al.* Rheological response of neutrophils to different types of stimulation. *J Appl Physiol* (In Press).
30 Fisher TC, Wenby RB, Meiselman HJ. Pulse shape analysis of RBC micropore flow via new software for the cell transit analyser (CTA). *Biorheology* 1992; **29**: 185–201.
31 Nash GB, Jones JG, Mikita J, Christopher B, Dormandy JA. Effects of preparative procedures and of cell activation on flow of white cells through micropore filters. *Br J Haematol* 1988; **70**: 171–176.
32 DiPerri T, Pasini FL, Ralli L *et al.* Effect of controlled physical activity on haemorheological and metabolic changes in PAOD patients. *Clinical Hemorheology* 1988; **8**: 737–750.
33 English D, Andersen BR. Single-step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of ficoll-hypaque. *J Immunol Methods* 1974; **5**: 249–252.
34 Caillard P, Mouren X, Pujade B, Blanchemaison P, Elbeze Y, Cloarec MP. Objectifying exercise ischaemia in peripheral vascular disease: a study in 120 patients. *Angiology* 1990; June: 469–478.
35 Hands LJ, Sharif MH, Payne GS, Morris PJ, Radda GK. Muscle ischaemia in peripheral vascular disease studied by 31P-magnetic resonance spectroscopy. *Eur J Vasc Surg* 1990; **4**: 637–642.
36 Jellinek H, Detre Z. Role of the altered transmural permeability in the pathomechanism of arteriosclerosis. In 2 Parts *Path Res Pract* 1986; **181**: 693–712.
37 Winocour PH. Microalbuminuria. *BMJ* 1992; **304**: 1196–1197.
38 Kannel WB, Stampfer MJ, Castelli WP, Verter J. The prognostic significance of proteinuria: The Framingham Study. *Am Heart J* 1984; **108**: 1347–1352.
39 Ernst E, Hammerschmidt DE, Bagge U, Matrai A, Dormandy JA. Leukocytes and the risk of ischaemic disease. *JAMA* 1987; **257**: 2318–2324.
40 Windsor ACJ, Mullen PG, Fowler AA, Sugerman HJ. Role of the neutrophil in adult respiratory distress syndrome. *Br J Surg* 1993; **80**: 10–17.
41 Bradbury AW, Murie JA, Ruckley CV. Role of the leucocyte in the pathogenesis of vascular disease. *Br J Surg* 1993; **80**: 1503–1512.
42 Homer-Vanniasinkam S, Crinnion JN, Gough MJ. Role of the thromboxane $A_2$ in muscle injury following ischaemia. *Br J Surg* 1994; **81**: 974–976.
43 Klosterhalfen B, Horstmann-Jungemann K, Vogel P *et al.* Time course of various inflammatory mediators during recurrent endotoxaemia. *Biochem Pharmacol* 1992; **43**: 2103–2109.
44 Housley E. Treating claudication in five words. *BMJ* 1988; **296**: 1483–1484.


*Accepted 25 November 1994*