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


1Department of Vascular Surgery, Queen Elizabeth Hospital, Edgbaston, Birmingham, U.K., 2Department of Haematology, University of Birmingham, Edgbaston, Birmingham, U.K. and 3Department 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, especially on the lungs.2,3 Evidence suggests a central role for neutrophils4-7 and thromboxane A28-10 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 - microalbuminuria11-13). A causal relationship between neutrophil activation and thromboxane A2 has been demonstrated14-17 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 years19,20 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.30 We used a cell transit analyser (CTA),29,30 a sensitive indicator of white cell
activation, to measure transit times through a micropore filter for a large number of individual neutrophils. Thromboxane A₂ 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 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 5 ml for thromboxane B₂ (TXB₂). Blood was collected in sterile tubes containing EDTA³¹ 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 TXB₂ levels. Another MSU was taken 1 h post-exercise. Blood for TXB₂ was immediately centrifuged at 2000g 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 ¹²⁵I-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.

TXB₂ 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 described³¹,³³ 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 × 10⁵ 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 8μm diameter pores.³⁵,³⁰ 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 claudinants 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-
Thromboxane and neutrophil changes in claudicants

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

<table>
<thead>
<tr>
<th></th>
<th>Transit time pre-exercise (ms)</th>
<th>Transit time post-exercise (ms)</th>
<th>Percentage change</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients (n = 30)</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Median</td>
<td>4.6 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>9.0 ± 4.2</td>
<td>0.040</td>
</tr>
<tr>
<td>90th percentile</td>
<td>12.3 ± 0.9</td>
<td>14.6 ± 1.3</td>
<td>20.2 ± 8.0</td>
<td>0.018</td>
</tr>
<tr>
<td><strong>Controls (n = 10)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>3.2 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>7.6 ± 4.2</td>
<td>0.11</td>
</tr>
<tr>
<td>90th percentile</td>
<td>9.0 ± 1.1</td>
<td>9.1 ± 1.0</td>
<td>4.2 ± 6.1</td>
<td>0.50</td>
</tr>
</tbody>
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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

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 30)</th>
<th>Controls (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR pre-exercise*</td>
<td>0.57 (0.36-1.41)</td>
<td>0.50 (0.45-0.72)</td>
</tr>
<tr>
<td>ACR post-exercise*</td>
<td>0.68 (0.33-2.49)</td>
<td>0.52 (0.32-0.79)</td>
</tr>
</tbody>
</table>

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

Data for thromboxane B2 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).

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, it is associated with a cardiovascular mortality 2–3 times that of the general population 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 and blood pH measurements. It has been postulated therefore that these patients undergo a series of ischaemia–reperfusion events that activate neutrophils and release soluble mediators (such as thromboxane), both causing systemic increases in vascular permeability and enhanced atherogenesis. A link between neutrophils and thromboxane has been shown in animal models of severe ischaemia.

Microalbuminuria has been used as a measure of increased vascular permeability and therefore an indirect measure of the systemic effects of ischaemia–reperfusion injury. Our results confirm the previous findings of increased microalbuminuria in patients with intermittent claudication after exercise. Neutrophil activation in intermittent claudication has previously been examined using filtration methods and intravital microscopy. Filtration methods rely on structural changes in the neutrophils, reducing their deformability after activation. 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 is 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, we25 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 re-open 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.

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Thromboxane and neutrophil changes in claudicants


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