Up-regulation of MMP-2 and MMP-9 Leads to Degradation of Type IV Collagen During Skeletal Muscle Reperfusion Injury; Protection by the MMP Inhibitor, Doxycycline


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Objectives: to determine the role of matrix metalloproteinases, MMP-2 and MMP-9, in reperfusion injury following skeletal muscle ischaemia and whether inhibition of MMPs by doxycycline protects against tissue damage. Methods: rats were anaesthetised and a tourniquet applied to the proximal thigh to occlude blood flow. Four hours of ischaemia was followed by reperfusion for 0, 4, 24 or 72 h. Two further groups received doxycycline for 7 days prior to bilateral ischaemia and 24 h reperfusion. Skeletal muscle from both limbs, kidneys and lungs were harvested for zymography and immunohistochemical staining for type IV collagen. Results: upregulation of MMP-2 and MMP-9 was detected by zymography in the ischaemic leg and lung but not in the kidney. Quantitative immunohistochemical analysis showed marked degradation of type IV collagen in reperfused muscle, lung and kidney. Doxycycline-treated rats showed significant preservation of type IV collagen in skeletal muscle and a trend towards preservation in kidney and lung. Conclusions: MMP-2 and MMP-9 are strongly upregulated in skeletal muscle ischaemia/reperfusion injury and are also upregulated in remote organs, leading to degradation of basement membranes. Inhibition of MMP activity may therefore be potentially therapeutically useful in reducing the severity of reperfusion injury.

Key Words: Matrix metalloproteinases; Ischaemia; Reperfusion; Collagen degradation; Doxycycline.

Introduction

Lower extremity revascularisation, particularly following lengthy periods of profound ischaemia, frequently results in exacerbation of the injury to the limb as well as remote organ dysfunction. Reperfusion injury and remote systemic effects vary and the biochemical and molecular mechanisms mediating this damage remain unclear. A number of mechanisms of damage have already been implicated in skeletal muscle reperfusion injury, including complement activation and deposition, generation and activity of reactive oxygen species, increased microvascular permeability and an increase in intracellular calcium, which can activate Ca2+-dependent proteases, leading to cellular injury. Activation of leukocytes, which adhere to the endothelium and infiltrate the surrounding tissues, also plays a vital role in the induction of tissue damage.

The matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that have the ability to degrade all components of the extracellular matrix. Together with their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), they are the major physiological regulators of the turnover of the extracellular matrix (reviewed in 1). Previous work from one of the current authors has demonstrated that these extracellular antigens disappear during ischaemia and reperfusion, with a consequent loss of microvascular integrity. Studies by Rosenberg and co-workers demonstrated induction of both MMP-2 and MMP-9, also correlating with opening of the blood-brain barrier. The therapeutic utility of this knowledge was demonstrated in a rat model of focal stroke where inhibition of MMPs by an MMP-9-neutralising antibody resulted in a reduction in infarct volume. Similarly, inhibition of MMPs by doxycycline...
also significantly reduced infarct size in rats subjected to reversible middle cerebral artery occlusion.\textsuperscript{15}

The current study was therefore designed to determine whether MMPs -2 and -9 were activated during skeletal muscle reperfusion injury, both in the reperfused muscle and in distant organs involved in multi-system failure. Having demonstrated this, experiments were then performed to test the therapeutic efficacy of inhibiting MMP activity using doxycycline in reducing the pathological effects of reperfusion injury.

**Materials and Methods**

**Rat model of ischaemia and reperfusion in skeletal muscle of the hind limb**

A rat model of skeletal muscle ischaemia and reperfusion has previously been established in our laboratory.\textsuperscript{8} In brief, Sprague–Dawley rats (250–280 g; 5 rats/treatment) were anaesthetised by inhalation of halothane and oxygen. The area over the thigh was shaved, a photoplethysmography probe attached to the skin over the femoral artery and blood flow monitored on a Doppler ultrasound machine (Parks Medical Electronics; Aloha, Oregon, U.S.A.). To induce ischaemia, a tourniquet of cotton tape was tied tightly around the proximal thigh of the rat. Ischaemia was considered to be adequate if the Doppler trace remained flat and was maintained for 4 h before the tourniquet was released and the return of blood flow to the limb monitored by the return of the Doppler trace. To control for the effects of anaesthesia, sham-operated rats were treated identically to the ischaemic rats, except the tourniquet was not applied to the leg. Rats tolerated both the ischaemia and subsequent reperfusion well with no mortalities. The Animal Ethics Committees of the Queen Elizabeth Hospital and the University of Adelaide approved all experimental procedures used in this study.

Reperfusion was allowed to proceed for 0, 24 or 72 h before rats were sacrificed. A transverse block of skeletal muscle from both legs, containing all the muscles of the calf, was harvested distal to the tourniquet.\textsuperscript{8} Tissue crushed by the tourniquet was excluded from analysis. Lungs and kidneys were also collected. Tissues were either processed fresh for zymography, snap-frozen in liquid nitrogen or fixed in 10% buffered formalin. In a second series of experiments, ischaemia was induced for 4 h in both legs of the rats followed by 0, 4 or 24 h reperfusion (5 rats/group) before tissues were collected for analysis.

**Zymography**

Gelatin zymography was carried out using a modification of the method of Porter et al.\textsuperscript{16} Fresh tissue was homogenised in lysis buffer (0.1 mM PMSF, 50 mM Tris HCl (pH 7.6), 2 M Urea, 0.1% NaCl, 0.1% EDTA, 0.1% Brij 35 (Sigma Aldrich, St Louis, MO, U.S.A.) and the supernatant dialysed overnight in a Membra-Cel MD25-14 dialysis membrane (14 000 kDa cut-off) against 25 mM Tris HCl (pH 8.5), 10 mM CaCl\textsubscript{2}, 0.1% Brij 35 and 0.1 mM PMSF. Protein concentration was determined using a Bradford assay according to the manufacturer’s instructions (BioRad, Hercules, CA, U.S.A.). Cellular extracts (40 \mu g protein/lane) were electrophoresed through 10% non-reducing polyacrylamide gels containing 0.1% gelatin (Sigma Aldrich). Gels were then incubated for 15–18 h at 37 °C in the zymogram buffer (Tris HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl\textsubscript{2}, 0.02% Brij 35) to allow development of gelatinolytic activity before being stained with Coomassie Brilliant Blue R (Sigma Aldrich). Zymograms were assessed descriptively without any quantitation of bands.

**Western blotting**

Protein samples (40 \mu g protein/lane) were electrophoresed under reducing conditions through a 10% polyacrylamide gel and then transferred to Hybond ECL membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.) by electrotransfer in 25 mM Trizma base, 192 mM glycine 10% methanol, pH 8.3. Membranes were blocked in 5% skim milk powder in phosphate-buffered saline (PBS) before overnight incubation in a 1/2000 dilution of a polyclonal rabbit anti-rat MMP-9 antibody (Chemicon, cat no AB19016, Temecula, Ca, U.S.A.). Secondary detection was carried out with a 1/1000 dilution of an ECL reagents (Amersham Biosciences) according to the manufacturer’s instructions. An MMP control-1 standard (Sigma Aldrich) was also analysed on the blots to verify the identity of the immunoreactive bands.

**Immunohistochemical analysis of degradation of type IV collagen**

Frozen sections (10 \mu M, skeletal muscle; 3 \mu M, lung; 5 \mu M, kidney) were fixed in 20% aceton at −20 °C for

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20 min, then incubated 3 times for 5 min in 0.1% bovine serum albumin (BSA) in PBS to block non-specific binding. Sections were incubated for 15 h at 4°C with a 1/100 dilution of rabbit polyclonal anti-human type IV collagen antibody (Rockland, Gilbertsville, PA, U.S.A.) in 0.1% BSA/PBS. A slide from which the primary antibody was omitted was included in all experiments as a negative control. After washing 3 times in 0.1% BSA/PBS, sections were incubated for 1 h at room temperature with a 1/400 dilution in 0.1% BSA/PBS of biotinylated anti-rabbit IgG antibody (Vector Laboratories; Burlingame, CA, U.S.A.). Sections were then incubated for 1 h at room temperature with a 1/200 dilution in 0.1% BSA/PBS, of fluorescein-conjugated streptavidin (Amersham Biosciences), washed and mounted under fluorescent mounting medium (Dako, Australia). Tissues were examined using an Olympus BH2 fluorescence microscope with maximum excitation wavelength of 494 nm and maximum emission wavelength of 518 nm.

Thirty-five images were captured from each slide (1 slide/tissue sample) in a stepwise fashion across the section, excluding the outside of the section to avoid artefactual edge effects. Quantitation of type IV collagen was carried out using VideoPro® 4 software by measuring “brightness”, which is a function of both pixel number and area. All comparisons between treatment protocols were carried out within the one experiment to avoid inter-experimental variation.

**Statistical analysis**

In pilot experiments (data not shown), it was determined the cumulative mean brightness measurement became stable when approximately 25 images had been collected. It was therefore decided to collect 35 measurements per slide, providing a total of 175 measurements of brightness of collagen immunostaining for each group of 5 rats. Further preliminary analysis demonstrated that these measurements of brightness for each group of rats were normally distributed, therefore data were displayed using mean ± 1 standard deviation. Data were then analysed using an unbalanced 2-way ANOVA 3 × 4 factorial design with interaction with p values of <0.05 considered significant.

Median scores for the degree of histological damage in reperfused tissues were ranked and analysed using analysis of variance. A p value of <0.05 was considered significant.

**Results**

**Zymographic detection of gelatinolytic activity**

Figure 1 illustrates the induction of zymographic activity of MMP-2 and MMP-9 in tissues harvested from rats. Baseline low levels of the pro-form of MMP-2 (72 kDa) were observed in the skeletal muscle of sham-operated rats but there was no MMP-9 activity in these tissues (Fig. 1A). A similar result was observed when the limbs were subjected to 4 h ischaemia only without reperfusion. However, when the rats were subjected to 4, 24 or 72 h reperfusion, there was a marked induction of activity of pro-MMP-9 (92–97 kDa) as well as elevated levels of pro-MMP-2 (72 kDa). Skeletal muscle from rats subjected to 72 h reperfusion also showed a qualitative increase in the levels of the 62 kDa active form of MMP-2 and a reduction in the amount of MMP-9 activity when compared to 24 h reperfusion.

Zymographic analysis of lung tissue (Fig. 1B) also demonstrated increased gelatinolytic activity, although less marked. Sham-operated animals showed zymographic activity corresponding to both pro-MMP-2 and MMP-9 with highest activity in the animals sacrificed immediately after 4 h anaesthesia. Gelatinolytic activity decreased as the interval between anaesthesia and euthanasia increased, presumably reflecting recovery from anaesthetic damage. In animals subjected to 4 h ischaemia and 0–72 h reperfusion, levels of MMP-2 and MMP-9 were elevated with shorter times
of reperfusion and had returned to lower levels following 72 h reperfusion. However, samples of renal tissue only expressed baseline levels of pro-MMP-2, which did not alter following ischaemia and reperfusion (Fig. 1C).

Western blotting (Fig. 1D) confirmed that levels of MMP-9 protein were elevated in skeletal muscle and lung tissues following 4 h ischaemia and 24 h reperfusion. The identity of the gelatinolytic 92–97 kDa band as MMP-9 was also confirmed by Western blotting when compared to a commercial MMP standard (Fig. 1D). Incubation of zymograms with the selective zinc chelator, (1,10)-Phenanthroline (100 μM), abolished all gelatinolytic activity (data not shown), confirming the identity of these bands as metalloproteinases.

Type IV collagen is one of the major substrates of the proteolytic activity of MMP-2 and MMP-9 and also forms a key component of the basement membrane. The amount of degradation of type IV collagen was therefore chosen as an indicator of the degree of tissue damage induced by MMP activity in vivo during reperfusion injury. Figure 2 shows representative images of immunohistochemical staining patterns for type IV collagen in tissues subjected to 4 h ischaemia (unilateral or bilateral) and 24 h reperfusion when compared to sham-operated rats subjected to a 4 h anaesthetic and sacrificed 24 h later. There was a marked reduction in the intensity of staining of the basement membrane in skeletal muscle, lungs and kidney tissue following 4 h unilateral ischaemia and 24 h reperfusion. The degree of collagen breakdown in all tissues was further increased when the rat was subjected to a greater degree of ischaemia by having both limbs occluded. Specificity of antibody binding was confirmed by omitting the primary antibody (data not shown).
Fig. 3. Rats were subjected to 4 h bilateral ischaemia followed by 0, 4, 24 or 72 h reperfusion before tissues were harvested. Quantitative immunohistochemical analysis was carried out using VideoPro® software and the mean brightness ± 1 standard deviation calculated. Data were analysed using an unbalanced 2-way ANOVA 3 x 4 factorial design with interaction. * p<0.05; ** p<0.01. □ Skeletal muscle; ■ Lung; □ Kidney.

Protection against reperfusion injury by doxycycline

Effectiveness of prior treatment of the rats with doxycycline before the induction of ischaemia and reperfusion was assessed by determining if the type IV collagen in the basement membranes in skeletal muscle, lungs and kidneys was protected from degradation induced by reperfusion injury. The effect of doxycycline treatment on the severity of reperfusion injury was also assessed by gelatin zymography, and by qualitative histological alterations in the degree of injury to the muscle, lungs and kidneys.

Figure 5A illustrates qualitatively, the marked reduction in gelatinolytic activity of MMP-2 and MMP-9 in skeletal muscle following treatment of the rats with doxycycline before 4 h bilateral ischaemia and 24 h reperfusion. The gelatinolytic activity of MMP-9 was virtually abolished and the levels of both pro-MMP-2 and active MMP-2 were also reduced. The lower dose of doxycycline (50 mg/kg) was sufficient to inhibit gelatinolytic activity and increasing the dose to 200 mg/kg did not cause a further decrease in activity. In contrast, there was no detectable change in MMP-2 and MMP-9 activity in lung tissue following administration of doxycycline (Fig. 5B). The levels of gelatinolytic activity of pro-MMP-2 in the kidneys also did not change after doxycycline treatment (Fig. 5C).

Immunohistochemical staining for type IV collagen showed that administration of doxycycline partially protected against collagen degradation. Figure 2 (fourth column) illustrates staining of representative tissue sections from animals pre-treated with 50 mg/kg doxycycline compared to animals subjected to 4 h ischaemia and 24 h reperfusion only. The intensity of collagen staining in tissues from doxycycline-treated
animals was brighter than in the animals subjected to bilateral ischaemia and reperfusion only but collagen staining did not return to the baseline intensity seen in the sham-operated animals. Quantitation of brightness of staining is illustrated in Figure 6, which shows statistically significant protection against collagen degradation in the skeletal muscle of rats administered 50 mg/kg doxycycline ($p<0.05$). The higher dose of doxycycline did not confer any additional protection against collagen degradation, correlating with no change in the gelatinolytic activity of MMP-2 and MMP-9 in skeletal muscle. Changes in the degree of brightness in lung and kidney tissues were less evident following doxycycline treatment.

**Histological analysis**

To determine the degree of gross histological damage following ischaemia/reperfusion (I/R) and to examine whether doxycycline could reduce this damage, haematoxylin and eosin-stained sections were graded by an independent investigator according the criteria of Carter *et al.* Table 1 illustrates the median scores in tissues following 4 h bilateral ischaemia and 24 h reperfusion and following pretreatment with either 50 mg/kg or 200 mg/kg doxycycline as described above. Figures in brackets represent the range of scores for the five rats in each treatment group. When compared to the sham-operated animals, a highly significant increase in the degree of damage was observed in both skeletal muscle ($p=0.0044$) and kidney ($p<0.001$) following 4 h bilateral ischaemia and 24 h reperfusion. Doxycycline (50 mg/kg) significantly reduced the degree of I/R-induced damage in kidney ($p<0.05$) but not in skeletal muscle. Lung tissue showed a mild degree of damage in the sham-operated rats, presumably reflecting injury caused by 4 h of inhalational halothane anaesthesia and the degree of gross pulmonary damage was not significantly increased following ischaemia/reperfusion.
Fig. 6. Rats in groups of five were given 50 mg/kg or 200 mg/kg doxycycline twice daily by gavage for 7 days, before being subjected to 4 h bilateral ischaemia and 24 h reperfusion. Tissues were then harvested and frozen sections immunostained for type IV collagen. Quantitative immunohistochemical analysis was carried out using VideoPro® software and the mean brightness \( \pm 1 \) standard deviation calculated. Data were analysed using an unbalanced 2-way ANOVA 3 × 4 factorial design with interaction. * \( p < 0.05 \).

Table 1. Paraffin sections were stained with haematoxylin and eosin and scored by an independent investigator blinded to the nature of the treatment. Median scores plus, in brackets, the range of scores, for each tissue (5 rats/group) are given in the table.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sham-Operated</th>
<th>Bilateral I/R</th>
<th>Doxycycline 50 mg/kg</th>
<th>Doxycycline 200 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left leg</td>
<td>6.5 (4–7)</td>
<td>6 (4–6)</td>
<td>7 (6–7)</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>2 (2–3)</td>
<td>3 (3–4)</td>
<td>3 (3–4)</td>
<td>3 (2–4)</td>
</tr>
<tr>
<td>Kidney</td>
<td>1 (0–1)</td>
<td>3</td>
<td>2</td>
<td>3 (2–3)</td>
</tr>
</tbody>
</table>

Discussion

In the current study, we have shown that gelatinolytic activity corresponding to MMP-2 and MMP-9 is markedly increased during ischaemia-reperfusion in a rat model of skeletal muscle lower limb occlusion. Elevated activity of MMPs was observed both in the skeletal muscle that had undergone ischaemia and reperfusion and, to a lesser extent, in the lungs of these animals. However, zymographic activity in the kidneys appeared unchanged. The increase in activity of MMP-2 and MMP-9 correlated with a reduction in basement membranes of the levels of type IV collagen, widely recognised as a major substrate for the proteolytic activity of a number of the MMPs including MMP-2 and MMP-9.25–27 The increase in gelatinolytic activity also correlated with a marked increase in the severity of tissue damage as determined by histological scoring, confirming the systemic nature of the ischaemic insult and demonstrating a similar pattern to the organ damage observed in the clinical situation.

The identity of the gelatinolytic activities observed on the zymograms was defined by the molecular weights of the species observed, in accordance with published sizes. The pro-form of MMP-2 (gelatinase A) is ubiquitously expressed and has a molecular weight of 72 kDa, with an active form of 64 kDa.23,24 Pro-MMP-9 has been widely reported as being 92–96 kDa along with an 84 kDa activated form.26,27 Incubation of zymograms with the zinc chelator, (1,10)-phenanthroline, abolished all gelatinolytic activity, confirming the identity of these bands as metalloproteinases. Western blotting, using a commercially-available standard for MMPs, also confirmed that the 92–96 kDa band observed on the zymograms was MMP-9. Interpretation of MMP levels in the zymograms was descriptive only and no quantitative analysis was carried out.

Our data is in agreement with that reported by Frisdal and co-workers who used a rat model of permanent ligation of the femoral artery and demonstrated an increase in gelatinolytic activity of MMP-2 and MMP-9, accompanied by degradation of basement membrane components, including type IV collagen and laminin in the soleus muscle.28 Although collateral circulation in the limb would provide some level of perfusion, ischaemia was permanent and there was no formal reperfusion phase in these studies. This model is less relevant to the clinical situation and they were not able to examine mechanisms of damage to distant organs, which are also commonly affected in reperfusion injury.

The patterns of expression of MMP-2 and MMP-9 reported above are also in agreement with the results of studies in both cerebral and cardiac ischaemia/reperfusion (I/R). In an in vitro model of I/R in isolated...
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rat hearts, reperfusion resulted in increased levels of MMP-2 in the perfusate as well as in the ventricular tissue. This rise in MMP-2 levels was partially inhibited by doxycycline, resulting in improved cardiac function. However, in an in vivo pig model of cardiac ischaemia (90 min) followed by brief reperfusion (90 min), marked elevation of MMP activity was observed but without degradation of collagen, presumably a reflection of the brief duration of reperfusion. Increased levels of both collagenase (MMP-1) and gelatinase (MMP-9) activity were also observed following cardiac I/R in a pig model, with MMP-9 immunolocalised to infiltrating leukocytes in the ischaemic myocardium.

In the kidney, despite considerable degradation of type IV collagen which was partially reversed by doxycycline (suggesting MMP involvement), no activation of gelatinolytic activity was evident following I/R, although constitutive low levels of pro-MMP-2 were observed. This observation is in agreement with studies by Jain and co-workers who showed that, in a rat model of renal ischaemia/reperfusion, MMP-2 levels were only elevated very late (8 weeks after 45 min of renal ischaemia) in the reperfusion phase. It therefore appears likely that early renal collagen loss observed in the current study is mediated by MMPs other than MMP-2 and MMP-9. Only very low levels of leukocyte infiltration were observed in kidneys of rats subjected to I/R, correlating with the absence of zymographically-active MMP-9 in these tissues.

The patterns of gelatinolytic activity observed in the lungs are also consistent with published data reporting patterns of expression in various lung cell types. Human bronchial epithelial cells secrete both 68 and 72 kDa forms of MMP-2 and 92 kDa MMP-9. Rat alveolar macrophages constitutively express MMP-2 as do lung fibroblasts, endothelial cells and vascular smooth muscle cells. All these MMP species were observed in the studies reported above, with an increase in MMP-9 activity following I/R, which correlated with leukocyte infiltration into the tissues. Increases in lung MMP-2 and MMP-9 activity, along with neutrophil migration into the tissues, were also observed in lung transplantation models of I/R in the rat.

The elevated activity of MMP-9 in damaged tissues, demonstrated above, is in agreement with previous studies by ourselves and others, showing leukocyte infiltration during skeletal muscle reperfusion injury. Schlag and co-workers demonstrated considerable extravasation of leukocytes into muscle tissue, which increased with increasing reperfusion times, and correlated closely with the degree of tissue injury. These infiltrating leukocytes are capable of secreting high levels of MMP-9, confirming the elevation in gelatinolytic activity observed on zymography.

The molecular and cellular mechanisms which activate MMP-2 and MMP-9 during skeletal muscle reperfusion injury remain to be elucidated. Elevated levels of tissue MMP-9 are presumed to derive from degranulation of infiltrating neutrophils, which are a major feature of reperfusion injury, both in the skeletal muscle and in remote organs including the lungs. The mechanisms inducing the elevated levels of MMP-2 and the nature of the cells which synthesise and secrete MMP-2 are not yet clear. It has been reported that human skeletal muscle satellite cells in culture secrete MMP-2 and can also be induced to express MMP-9 by treatment with phorbol ester. The MMP-2 promoter contains a p53 consensus binding site and expression of p53 will cause transcriptional activation of MMP-2. Elevated levels of expression of p53 can be detected during ischaemia/reperfusion, suggesting a possible mechanism which would result in the induction of elevated expression of MMP-2 in these tissues.

Other matrix metalloproteinases and their inhibitors, the TIMPs, are also likely to participate in the cascade of events which leads to the induction of tissue damage during reperfusion injury. For example, the proteolytic activity of MMP-3 has been implicated in the breakdown of basement membrane proteins including laminin and fibronectin as well as displaying some proteolytic activity against type IV collagen. MMP-3 is also one of the primary activators of MMP-9 from its inactive proenzyme form which would make it highly likely to play a role in tissue damage during reperfusion injury. Other likely candidates include MT1-MMP which is involved in a trimolecular cell surface complex with both MMP-2 and TIMP-1, leading to activation of MMP-2. Further investigations are required to delineate the pathways leading to proteolytic damage of tissues during reperfusion injury.

The above study has demonstrated that doxycycline was partially protective in reducing the degree of breakdown of type IV collagen, particularly in skeletal muscle, but did not alter the degree of gross histological damage. Doxycycline is a broad-spectrum MMP inhibitor but has only moderate inhibitory activity. We observed a reduction in gelatinolytic activity and protection against type IV collagen breakdown but no significant reduction in the degree of tissue damage observed by histological assessment. A clinically-effective therapy is likely to involve blocking multiple pathways leading to tissue damage, which will include MMP activation, complement deposition and activation and neutrophil activation and adhesion.
Acknowledgments

We would like to thank Mr Matthew Thompson for providing discussion and early impetus in the project and Mr Richard Knowling for advice and discussion. Ms Nicole Pratt, Dept of Public Health, Adelaide University carried out the statistical analysis. We also thank Dr Michael Teder for consultation and advice with the histopathological scoring system and Mr Ken Porter and the staff of the Animal House for their care of the rats. D. M. Roach was funded by a scholarship from the Royal Australasian College of Surgeons.

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Accepted 5 January 2002