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RESEARCH PAPER

Anti-inflammatory actions of aprotinin provide dose-dependent cardioprotection from reperfusion injury

JM Carter[1,3], U Buerke[1,3], E Rössner[1], M Russ[1], S Schubert[1], H Schmidt[1], H Ebelt[1], D Pruefer[2], A Schlitt[1], K Werdan[1] and M Buerke[1]

[1]*Department of Medicine III, Martin-Luther-University, Halle, Germany and* [2]*Department of Cardiothoracic and Vascular Surgery, Johannes-Gutenberg University Mainz, Mainz, Germany*

Background and purpose: Myocardial injury following ischaemia and reperfusion has been attributed to activation and transmigration of polymorphonuclear leukocytes (PMNs) with release of mediators including oxygen-derived radicals and proteases causing damage.
Experimental approach: We studied the serine protease inhibitor aprotinin in an *in vivo* rabbit model of 1 h of myocardial ischaemia followed by 3 h of reperfusion (MI + R). Aprotinin (10 000 Ukg^{-1}) or its vehicle were injected 5 min prior to the start of reperfusion.
Key results: Myocardial injury was significantly reduced with aprotinin treatment as indicated by a reduced necrotic area ($11 \pm 2.7\%$ necrosis as percentage of area at risk after aprotinin; $24 \pm 3.1\%$ after vehicle; $P<0.05$) and plasma creatine kinase activity (12.2 ± 1.5 and 17.3 ± 2.3 IU g^{-1} protein in aprotinin and vehicle groups, respectively, $P<0.05$). PMN infiltration (assessed by myeloperoxidase activity) was significantly decreased in aprotinin-treated animals compared to vehicle ($P<0.01$). Histological analysis also revealed a substantial increase in PMN infiltration following MI + R and this was significantly reduced by aprotinin therapy (44 ± 15 vs 102 ± 2 PMN mm^2 in aprotinin vs vehicle-treated animals, $P<0.05$). In parallel *in vitro* experiments, aprotinin inhibited neutrophil-endothelium interaction by reducing PMN adhesion on isolated, activated aortic endothelium. Finally, immunohistochemical analysis illustrated aprotinin significantly reduced myocardial apoptosis following MI + R.
Conclusions and implications: Inhibition of serine proteases by aprotinin inhibits an inflammatory cascade initiated by MI + R. The cardioprotective effect appears to be at least partly due to reduced PMN adhesion and infiltration with subsequently reduced myocardial necrosis and apoptosis.
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Keywords: myocardial reperfusion injury; ischaemia reperfusion; serine protease inhibitor; aprotinin; neutrophil adherence; apoptosis; leukocytes

Abbreviations: AAR, area at risk; ANAR, area not at risk; CK, creatine kinase; LAD, left anterior descending coronary artery; LV, left ventricle; MI + R, myocardial ischaemia followed by reperfusion; MPO, myeloperoxidase; PAR, protease activated receptor; PMNs, polymorphonuclear leukocytes; PRI, pressure rate index

Introduction

Reperfusion following myocardial ischaemia (MI) has been described as a double-edged sword (Hausenloy and Yellon, 2007). It is a vital step in managing myocardial infarction and following cardioplegic arrest during cardiac surgery, but it initiates a deleterious cascade of events leading to reperfusion injury. Reperfusion injury is characterized by endothelial dysfunction, neutrophil adhesion to the coronary endothelium (within 20 min of reperfusion) and accumulation within the myocardium (within 3 h of reperfusion Mullane *et al.*, 1988). The end result is myocardial injury by necrosis and apoptosis (Kunapuli *et al.*, 2006).

Polymorphonuclear leukocytes (PMNs) play a key role in ischaemia-reperfusion injury. They begin to roll along the endothelium following the rapid translocation of P-selectin by the endothelium and L-selectin expression on unactivated neutrophils (Hordijk, 2006). Rolling, followed by tight adhesion, results in neutrophil extravasation into the

Correspondence: Dr M Buerke, Department of Medicine III, Martin-Luther-University Halle-Wittenberg, Ernst-Grube-Str. 40, 06120 Halle, Germany.
E-mail: michael.buerke@medizin.uni-halle.de
[3]These authors contributed equally to this work.
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myocardium. As early as the endothelial adherence stage, the activated neutrophils release a variety of cytotoxic mediators, such as hydrogen peroxide, the superoxide anion, hydroxyl radicals and serine proteases, which all lead to tissue injury (Pham, 2006).

Serine proteases are among the key mediators of PMN-induced injury and their effects appear widespread. Elastase and cathepsin G are the two major neutral serine proteases in neutrophils (Weiss, 1989). They have direct effects such as elastase's degradation of molecules such as elastin, collagen, immunoglobulins, complement, clotting factors, proteoglycans, fibronectin and even intact cells. There are also indirect effects; both are chemoattractant and exhibit mutual positive feedback by further activation of leukocytes. Finally, these proteases are intimately involved in apoptosis (Thornberry *et al*., 1997; Sabri *et al*., 2003).

Thrombin is another serine protease with widespread downstream effects thought also to mediate reperfusion injury. The enzyme is versatile and has multiple functions including the conversion of fibrinogen to fibrin, platelet activation and aggregation and in eliciting responses from many cell types, including vascular smooth muscle cells, endothelium, monocytes, neurons, nephrons and osteoclasts (Day *et al*., 2006a). Thrombin is widely activated during coronary artery bypass graft surgery, myocardial infarction and other inflammatory states. Thrombin effects are mediated by the protease-activated receptors (PAR), of which types 1–4 have been identified thus far (Barnes *et al*., 2004). To date, inhibition of PARs (directly or with the use of serine protease inhibition) has been shown to reduce inflammation (Day *et al*., 2006b), to improve stroke volume and cardiac output following ischaemia and reperfusion (Jormalainen *et al*., 2007) and to reduce apoptosis in nephrons (Kher *et al*., 2005) and neutrophils (Park *et al*., 2007).

Thus, serine proteases are frequently encountered and are potent enzymes, with a central role in the mediation of reperfusion injury. They are key to coagulation control, platelet aggregation, leukocyte activation, infiltration, degranulation and hence inflammation and tissue injury.

Aprotinin is a powerful, naturally occurring (bovine) serine protease inhibitor which has a role in cardiothoracic surgery to limit perioperative blood loss (Dietrich *et al*., 2006) MI (Asimakopoulos *et al*., 2001), and cerebral protection (Harmon *et al*., 2004). Animal studies have revealed a variety of effects including cardioprotection, reduced endothelial dysfunction and leukocyte migration (Bull and Maurer, 2003; Khan *et al*., 2004). Despite recent debate over its use sparked by two recent papers reporting an association with renal failure and other complications (Karkouti *et al*., 2006; Mangano *et al*., 2006), the serine proteases (and their inhibition with aprotinin and other compounds) are attracting substantial interest for their potential in the therapy of ischaemia reperfusion injury and a wider spectrum of inflammatory disease states. Nevertheless, although the emerging data implicate anti-neutrophil and anti-inflammatory mechanisms in the cardioprotective effects of serine protease inhibition, the details remain unclear. Therefore, we conducted the first comprehensive investigation using parallel *in vivo* and *in vitro* studies to examine the effect of aprotinin (administered just before reperfusion) at multiple points of neutrophil inflammatory activation and the eventual extent of tissue injury including the degree of necrosis and apoptosis in a well-established rabbit model of MI and reperfusion.

Materials and methods

Test systems and experimental design

All animal procedures and experimental protocols were approved by the State and University Animal Care Committee. Full details are published elsewhere (Buerke *et al*., 2006). Briefly, adult male rabbits were anaesthetized with sodium pentobarbital (30–60 mg kg^{-1} i.v. with supplemental bolus injection for maintenance) and placed on artificial ventilation while receiving complete haemodynamic and electrocardiographic monitoring including the pressure rate index (PRI), an approximation of myocardial oxygen demand. A thoracotomy was performed to allow access to the left anterior descending (LAD) coronary artery.

Myocardial ischaemia was induced by tightening a reversible LAD ligature so that the vessel was occluded (time point 0). Aprotinin (Trasylol 10 000 U kg^{-1}, Bayer, Leverkusen, Germany) or its vehicle (0.9% saline) was given intravenously as a bolus 55 min after the coronary occlusion (that is, 5 min before reperfusion (R)). Five minutes later (that is, after a total of 60 min ischaemia) the LAD ligature was released and the ischaemic myocardium was reperfused for 3 h.

The rabbits were randomly divided into four major groups. Six 'sham' MI + R rabbits received 'vehicle' (that is, saline, 1 mL kg^{-1}), Six 'sham' MI + R rabbits received aprotinin (10 000 U kg in 1 mL kg^{-1} saline), seven MI + R rabbits received 'vehicle' (that is, saline, 1 mL kg^{-1}), seven MI + R rabbits received 'high-dose aprotinin' (10 000 U kg^{-1} in 1 mL kg^{-1} saline) and four MI + R rabbits received 'low-dose aprotinin' (1000 U kg^{-1} in 1 mL kg^{-1} saline). The sham MI + R group rabbits were subjected to the same surgical procedure as all other groups except that the LAD coronary artery was not occluded.

Measurements made

Determination of myocardial necrosis. At the end of the 3 h reperfusion period, the ligature around the LAD was again tightened. As previously published (Buerke *et al*., 2001), the Evans blue/nitroblue tetrazolium technique was used to identify the zones of MI and infarction. The myocardium was divided into the 'area not at risk' (ANAR) and the 'AAR'. The 'AAR' was further subdivided into viable and necrotic portions. Results for the three portions of myocardium are expressed once indexed to the total LV or 'AAR' mass.

Plasma creatine kinase analysis. Arterial blood samples (2 mL) were drawn immediately before LAD occlusion and every 60 min thereafter. Samples were assayed for total protein and creatine kinase (CK) according to the previously published methods (Buerke *et al*., 2006). CK results are expressed as IU g^{-1} protein.

Determination of myocardial myeloperoxidase activity. The myocardial activity of myeloperoxidase (MPO), an enzyme occurring virtually exclusively in neutrophils, was determined using the methods described previously (Pruefer *et al*., 2002). Results give an approximation of neutrophil accumulation in the myocardium and one unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide min^{-1} at 25 °C.

Histological analysis of myocardial neutrophil accumulation, necrosis and apoptosis. Five additional rabbits, each receiving vehicle or high-dose aprotinin (10 000U kg^{-1}), were similarly subjected to 60 min of ischaemia and 180 min of reperfusion to determine the presence of infiltrating neutrophils or apoptotic myocytes by histopathological and immunohistological techniques. Following histological preparation and staining as detailed elsewhere (Pruefer *et al*., 2002), the total number of infiltrating neutrophils was analysed in 10 separate fields for each tissue section. The number of PMNs was counted for each field and expressed as PMN mm^{-2}.

In situ *determination of myocardial apoptosis.* Immunohistochemical procedures for apoptosis were performed using the direct immunoperoxidase detection of digoxigenin-labelled genomic DNA (TdT-mediated dNTP nick end labeling assay) (Pruefer *et al*., 2002). The number of apoptotic myocytes (that is with a peroxidase reaction in the cell nucleus) was counted for each field. The number of stained (apoptotic) myocytes was divided by the total number of myocytes and then multiplied by 100 to yield the percentage of apoptotic myocytes, that is, (stained myocytes/total myocytes) × 100.

Rabbit PMN isolation and labelling. In six additional rabbits, peripheral blood was collected from the femoral artery and centrifuged to produce a >95% pure and >95% viable PMN sample, which was labelled with fluorescent dye (Sigma Chemical Co., Deisenhofen, Germany) as described previously (Buerke *et al*., 1994).

PMN adherence to vascular endothelium. Aortas from the same group of six animals (which had PMNs isolated) were harvested and prepared for stimulation with thrombin (by incubation for 10 min with 0.5–2.0 U mL^{-1} thrombin (Sigma Chemical Co.) as described previously (Buerke *et al*., 1994). The fluorescent-labelled PMNs (400 000 PMN mL^{-1}) were added to the thrombin-stimulated vascular endothelium alone or in combination with increasing concentrations of aprotinin (that is, 10–500 U mL^{-1}) and incubated for 20 min in a metabolic shaker bath at 37 °C. After the incubation period, the aortic rings were removed and placed onto glass microscope slides. PMNs were counted using epifluorescence microscopy (Zeiss, Göttingen, Germany). Adherent neutrophils were counted on five fields from each vessel segment and expressed as PMN mm^{-2}.

Data analysis and statistical procedures

All values in the text and figures are presented as means ± s.e.mean of *n* independent experiments. All data were subjected to ANOVA followed by Fisher's PLSD test. Probabilities of 0.05 or less were considered to be statistically significant.

Results

Effect of aprotinin on PMN adherence to vascular endothelium in vitro

To upregulate P-selectin on the endothelial surface, we stimulated the aortic vascular endothelium with thrombin (2 U mL^{-1}) for 10 min. Few PMNs adhered to unstimulated endothelium. The addition of PMNs to thrombin-stimulated aortic endothelium resulted in a significant ($P<0.001$) six- to ninefold increase in adherence. Addition of aprotinin (1–500 U mL^{-1}) inhibited PMN adherence in a concentration-dependent manner (Figure 1). At a concentration of 100 U mL^{-1}, a maximal inhibitory effect was obtained (that is, a 60% inhibition of thrombin-induced adherence). Increasing the concentration of aprotinin above 100 U mL^{-1} did not produce additional inhibition. These results indicate that aprotinin effectively inhibits PMN adherence to the thrombin-activated vascular endothelium in a dose-dependent manner.

Cardiac and systemic haemodynamic changes

There were no significant differences in any haemodynamic, electrocardiographic or biochemical parameters between the two sham groups. Thus, sham groups (which were identical other than the use of aprotinin or saline vehicle) illustrate the absence of any impact of aprotinin on these baseline variables in the rabbit model state. The results for the pressure rate index, an approximation of myocardial oxygen demand were similar between all groups. Data from one sham and both MI + R groups are shown in Figure 2. Thus, aprotinin at the dose regimen employed, appeared devoid of any significant side effects, which may interfere with the study. In the two groups of MI + R rabbits, there were no significant differences in any of the variables observed before coronary occlusion. However, several minutes after LAD occlusion, the ST segment of the ECG became significantly elevated, peaking at 30 min after coronary occlusion. There was no significant difference in peak ST-segment elevation between the two MI + R groups (that is, 0.16 ± 0.02 mV in vehicle group vs 0.15 ± 0.03 mV in aprotinin group). After reperfusion, the ST segment fell almost to control values, suggesting that coronary reperfusion was effective. During reperfusion, there was a noticeable increase in premature ventricular contractions in all groups. Three rabbits in the vehicle group and one rabbit in the high-dose aprotinin group developed ventricular fibrillation following reperfusion, which was not successfully converted to a normal sinus rhythm by using cardiac defibrillation, and these animals were excluded. In the MI + R groups, the PRI decreased 60 min after coronary occlusion and gradually returned to baseline during reperfusion. There were no significant differences between the two MI + R groups for any of the PRI values, suggesting that aprotinin did not alter myocardial oxygen demand (Figure 2). The observation that aprotinin had no effect on measured variables between the

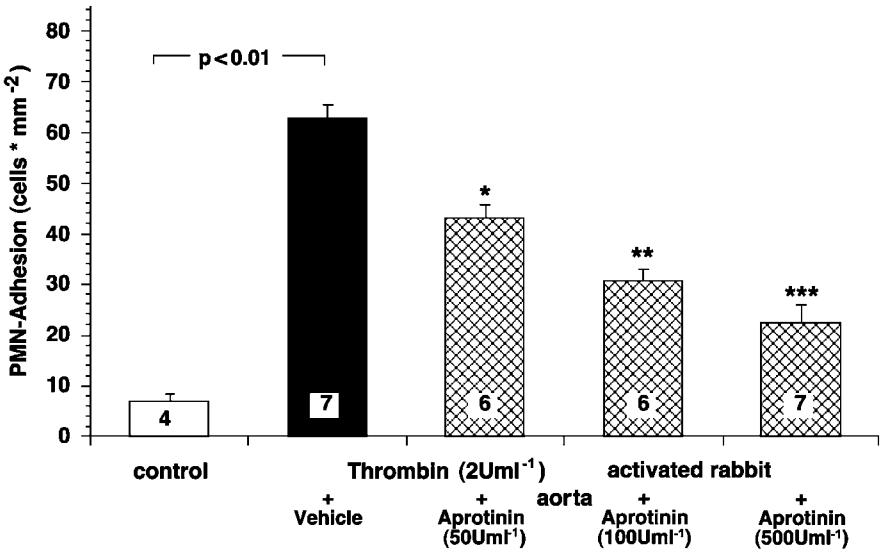

Figure 1 Effect of aprotinin (50, 100 and 500 U kg^{-1}) on polymorphonuclear leukocyte (PMN) adhesion to rabbit aortic endothelium stimulated with thrombin (2 U mL^{-1}). Aprotinin and PMNs were simultaneously added to the aortic preparation and incubated for 20 min in a shaker bath at 37 °C. Adhesion is expressed as number of PMN mm^{-2}. Bar heights are means; brackets indicate ± s.e.mean. Numbers at the base of bars are the number of aortic rings studied. (*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to vehicle).

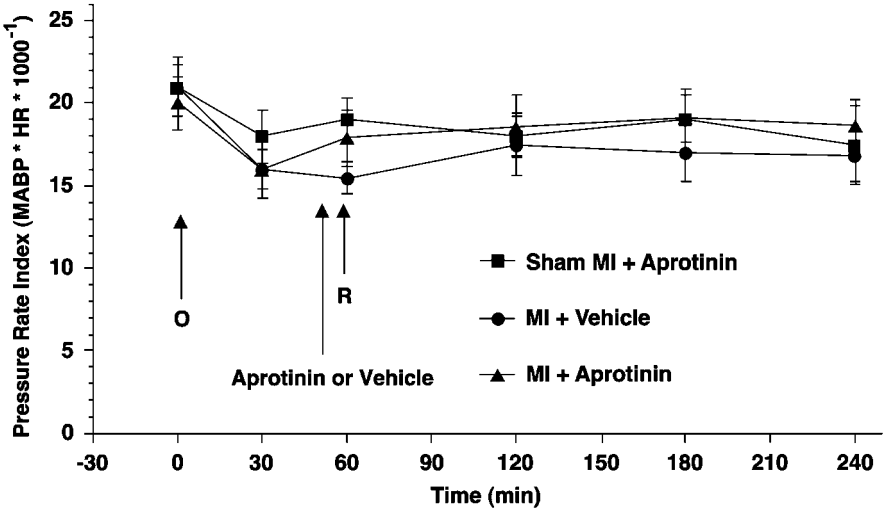

Figure 2 Pressure rate index (expressed as MABP (mm Hg) × HR (beats per min) 1000^{-1}) as an indicator of myocardial oxygen demand during the myocardial ischaemia followed by reperfusion (MI + R) procedure. The index fell in MI groups 30 min after coronary occlusion and appeared to recover more quickly in the aprotinin-treated group, but none of the observed differences reached statistical significance. The aprotinin dose was 10 000 U kg^{-1}. All values are means ± s.e.mean for six sham MI and aprotinin rabbits and seven rabbits in each MI group (O = LAD occlusion and R = reperfusion).

sham groups but had a significant impact between MI + R groups suggest that the effects of aprotinin are dependent on the presence of MI and reperfusion.

Effect of aprotinin on myocardial injury following reperfusion

To ascertain the effects of aprotinin on the degree of actual myocardial salvage, we measured the amount of necrotic cardiac tissue expressed as a percentage of either the area at risk (ischaemic area) or of total LV mass. There was no significant difference in the wet weights of the areas at risk expressed as a percentage of total LV between the two ischaemic groups (Figure 3), indicating that a comparable area of myocardium had been rendered ischaemic in both groups.

About 30% of the ischaemic myocardium became necrotic in the vehicle group when indexed to the AAR or 10% when indexed to the total left ventricle (LV). However, in the aprotinin-treated group, necrotic myocardium was less than 15 or 5% of the AAR or LV, respectively ($P<0.01$). Therefore, aprotinin (10 000 U kg^{-1}) significantly protected against necrotic injury in MI + R rabbits (Figure 3). Among the low-dose aprotinin (1000 U kg^{-1}) animals, the area of necrosis (as % AAR) was similar to that seen in vehicle-treated animals (28 and 30%, respectively). Results were similar when the necrotic area was related to total LV mass.

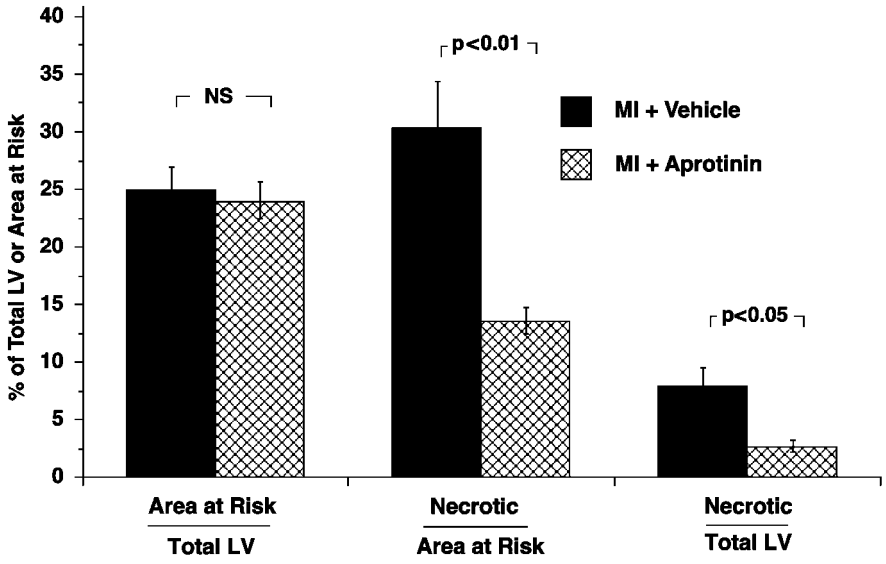

Figure 3 The effect of aprotinin (10 000 U kg^{-1}) on infarct size (as tissue wet weight). The 'area at risk' relative to the total left ventricle (LV) was similar in all groups indicating a similarly sized ischaemic area in all myocardial ischaemic (MI) groups. The necrotic area relative to the area at risk and the total LV was significantly reduced by aprotinin administration just before reperfusion. Height of bars are means, brackets represent ± s.e.mean. Each group had seven rabbits.

Therefore, aprotinin exerted its cardioprotective effect in a dose-dependent manner.

To further demonstrate preservation of myocardial tissue, we assayed CK activity, a biochemical marker of myocardial tissue injury. In the two sham groups, CK activity increased marginally throughout the 4 h observation period, due to surgical preparation. There were no significant differences between the CK levels in the two sham groups (as the CK rise in sham groups is caused by the surgical preparation of the chest wall, a process we expect to be unaffected by aprotinin). For ease of comparison with the MI + R groups, only the sham and aprotinin group CK values are represented in Figure 4. In the MI + R vehicle-treated animals, plasma CK activity increased slightly during the ischaemic period but there was a marked washout of CK into the circulation within the first hour following reperfusion. This progressed substantially during the remaining 2 h of reperfusion. This additional increase in CK in MI + R over sham groups arises due to myocardial injury additional to the 'background' CK release, following surgical preparation of the chest wall in this model. In contrast, high-dose aprotinin-treated animals had significantly lower plasma CK activities throughout the reperfusion period ($P<0.05$). These results further suggest that aprotinin significantly attenuates myocardial reperfusion injury (Figure 4). The CK values among the low-dose aprotinin animals were not significantly different to those of the vehicle-treated group (19 IU g^{-1} protein vs 21 IU g^{-1} protein CK activity at 3 h, respectively), again suggesting a dose-related effect of aprotinin.

Myocardial neutrophil accumulation

Figure 5 summarizes data relating to myocardial PMN infiltration. MPO activity in the ANAR was similarly low in both MI + R groups, confirming the absence of PMN infiltration into the non-ischaemic myocardium. The viable portion of the AAR showed modest PMN infiltration in the vehicle group. This effect was less pronounced in the aprotinin group but these differences were not significant.

The necrotic myocardium exhibited a marked increase of MPO activity (to 1.451 ± 0.26 IU per 100 mg of tissue in vehicle-teated animals). In contrast, high-dose aprotinin significantly reduced the MPO activity in the necrotic myocardium (0.82 ± 0.12 IU per 100 mg of tissue, $P<0.01$). These results indicated that aprotinin treatment retarded neutrophil accumulation in the myocardium after MI + R.

In keeping with the myocardial salvage data, the use of low dose aprotinin did not significantly reduce MPO activity in the necrotic area when compared to the vehicle treated group (0.88 IU per 100 mg of tissue vs 0.92 IU 100 mg^{-1} P = NS).

In parallel to the biochemical data, we analysed histologically the myocardium (AAR and ANAR) from MI + R animals treated with either 10 000 U kg^{-1} aprotinin or vehicle. In the non-ischaemic myocardium (that is, the ANAR) there was almost no neutrophil-endothelial adherence, no neutrophil infiltration and no myocardial cell damage visible in either group. However, vehicle-treated animals exhibited a marked increase in PMN accumulation in the ischaemic region. A representative photomicrograph is shown in Figure 6. Adherence of PMNs to the coronary endothelium and infiltration into the extravascular tissue is associated with marked myocardial cell injury shown by loss of cross-striation and cell integrity. In contrast, aprotinin-treated rabbits exhibited significantly lower neutrophil accumulation in the AAR and markedly reduced histological evidence of myocardial necrosis. Data for neutrophil infiltration for vehicle- and aprotinin-treated groups are summarized in Figure 7. The results indicate that accumulation of neutrophils occurred in rabbit myocardium after MI + R and was markedly inhibited by aprotinin (Figure 7).

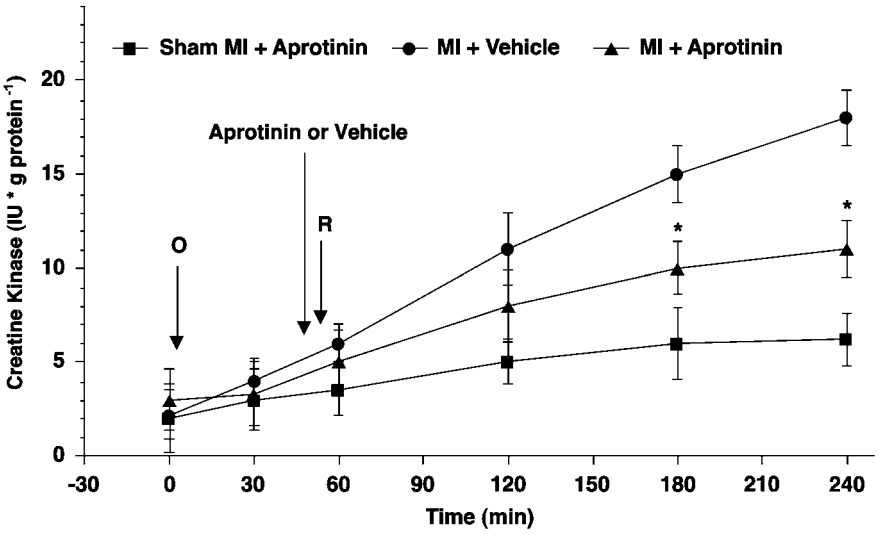

Figure 4 Effect of aprotinin (10 000 U kg^{-1}) on creatine kinase (CK) release into the circulation during myocardial ischaemia followed by reperfusion (MI + R). Plasma CK activity (expressed as International Units (IU) g^{-1} protein) was measured throughout the experiment for all groups. CK release was significantly higher in the MI + R vehicle-treated group compared to the MI + R aprotinin-treated group at the later time points (*$P<0.05$). All values are means ± s.e.mean for seven rabbits in each ischaemic group and six rabbits in the sham MI and aprotinin group (O = LAD occlusion and R = reperfusion).

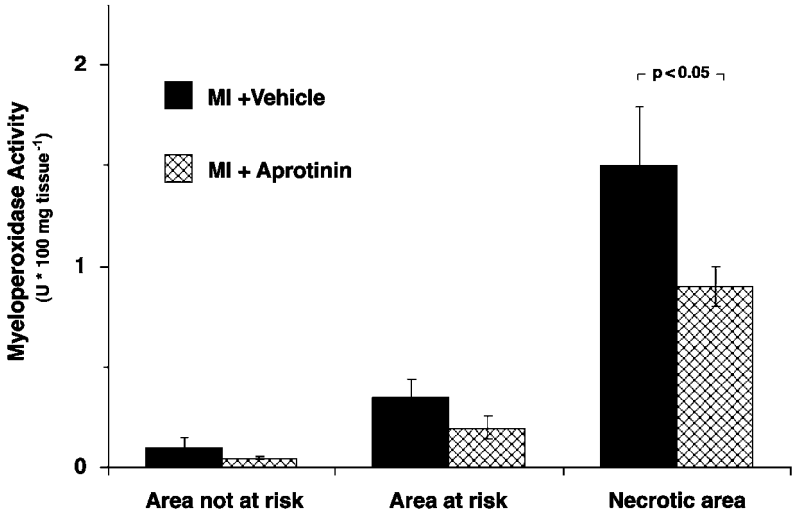

Figure 5 Cardiac myeloperoxidase activity in the area at risk, the necrotic area and the non-jeopardized myocardium in both myocardial ischaemia followed by reperfusion (MI + R) groups expressed as Units per 100 mg tissue wet weight. Aprotinin therapy (10 000 U kg^{-1}) before reperfusion significantly reduced leukocyte accumulation into the necrotic myocardium compared to vehicle. Heights of bars are means, brackets represent ± s.e.mean, $n=7$ in each group.

Effects of aprotinin on circulating white cells

To determine whether aprotinin exerted any neutropenic effects, which could contribute to any cardioprotection, we assessed circulating white blood cell (WBC) counts. WBC counts did not change significantly over the course of the experiment in either the vehicle or the aprotinin-treated MI + R groups (Table 1). Differential WBC counts showed neutrophils accounted for a consistent 45–55% of the total WBC count. There were no significant differences between these groups at any time point (Table 1). These results indicate that aprotinin does not induce cardioprotection by systemic leukopenia in rabbits.

Apoptosis following MI and reperfusion

The induction of apoptosis following MI and reperfusion may contribute to impaired LV function. The TdT-mediated dNTP nick end labeling assay was used to assess the degree of *in situ* apoptosis. Non-ischaemic sections of heart tissue (or any tissue section from sham rabbits) did not undergo any immunostaining (that is, less than 3% of the myocytes stained, Figure 9). Similarly, immunohistological preparations in which either the digoxigenin-dUTP or the antibody peroxidase conjugate was replaced with non-immune serum did not exhibit any labelling of myocardial or other cells. In contrast to these control portions of myocardium, apoptosis

was clearly evident in ischaemic-reperfused myocardial sections obtained from vehicle-treated hearts (that is, 23 ± 3% myocytes, $P<0.05$ compared to sham MI rats) (Figures 8a and 9). As DNA degradation also occurs non-specifically in necrotic myocardium, we evaluated apoptosis only in areas without typical histological signs of necrosis (that is, death of cell groups, loss of membrane integrity, and cell swelling and lysis). No reaction product was detectable in non-apoptotic cells. Intense immunostaining (that is, apoptosis) was evident on myocytes, endothelial cells as well as on infiltrating leukocytes in 'AAR' tissue from vehicle-treated animals. 'AAR' tissue from high-dose aprotinin-treated animals had markedly diminished immunostaining (13.1 ± 2.5%) compared to vehicle-treated rabbits ($P<0.05$), indicating a reduced occurrence of apoptosis (Figures 8b and 9). This analysis indicates that reperfusion of the ischaemic myocardium results in induction of apoptosis in cardiac tissue and that aprotinin treatment appears to be an effective inhibitor of such apoptosis.

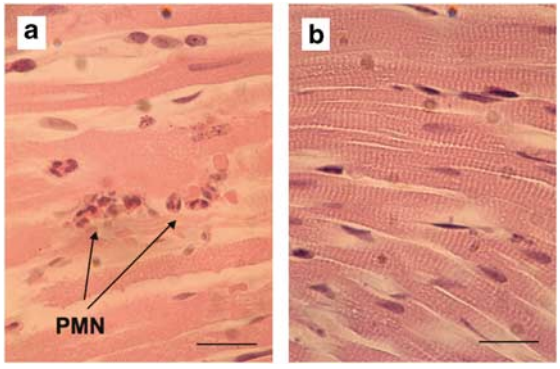

Figure 6 Typical photomicrographs of rabbit myocardium after myocardial ischaemia followed by reperfusion (MI + R), treated with either vehicle (left) or 10 000 U kg^{-1} aprotinin (right). (**a**) MI + R and vehicle-treated tissue exhibiting numerous neutrophils sequestered into coronary venule and infiltrating the myocardium. Cardiomyocytes are injured, lacking striations and showing myofibrillary contraction bands. (**b**) MI + R- and aprotinin- (10 000 U kg^{-1}, 5 min before reperfusion) treated tissue exhibits reduced coronary venule neutrophil adhesion, less myocardial polymorphonuclear leukocyte (PMN) infiltration, and no visible signs of cardiac myocyte injury. Magnification × 250.

Discussion

This study presents novel data using parallel *in vitro* and *in vivo* studies to show the effect of aprotinin in reducing neutrophil adhesion and transmigration following MI and reperfusion. The data support an antithrombin/P-selectin/PAR effect of aprotinin by showing suppression of the adhesion of neutrophils to thrombin-stimulated aortic endothelium. We add to the substantial evidence base demonstrating the cardioprotective effects of aprotinin (illustrated by a reduction in necrotic area and CK release) and we demonstrate an aprotinin-related reduction in cardiomyocyte apoptosis following reperfusion.

The effect of aprotinin on neutrophil activation and accumulation
One important component of the myocardial salvage afforded by aprotinin is very likely to be mediated by its

Table 1 Circulating WBC counts in rabbits during MI + R

Groups	*Time points*			
	Initial	*10 min pre–R*	*60 min post-R*	*180 min post-R*
MI + R + vehicle	4520 ± 221	4045 ± 217	4221 ± 274	5558 ± 353
MI + R + aprotinin	4210 ± 246	5012 ± 212	4493 ± 174	5138 ± 179

Abbreviations: MI, myocardial ischaemia; R, reperfusion, WBC, white blood cell.
All values are mean (± s.e.mean) circulating WBCs μL^{-1} for 4–5 rabbits in each group.

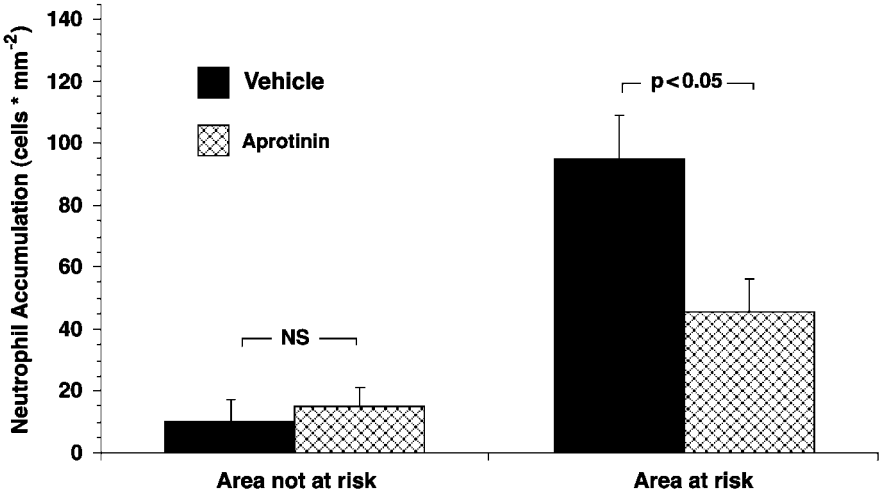

Figure 7 The impact of aprotinin on myocardial neutrophil accumulation following myocardial ischaemia followed by reperfusion (MI + R). The number of infiltrating neutrophils (measured after 3 h reperfusion and expressed as PMN mm^{-2}) is substantially higher in the 'at risk' myocardium compared to the 'not at risk tissue'. Aprotinin (10 000 U kg^{-1}) significantly reduces 'area at risk' infiltration compared to vehicle-treated animals. All values are means ± s.e.m with four rabbits in each group.

antineutrophil effects. Clearly, neutrophils are involved in rabbit MI + R, as we observed substantial PMN involvement, by *in vitro* adherence, histological assessment of infiltration or by MPO assay. Adherent and activated neutrophils release a variety of cytotoxic mediators such as hydrogen peroxide (H_2O_2), superoxide anion (O^-), hydroxyl radical (OH^-), cathepsin G, and elastase, all of which are potent contributors to myocardial injury (Williams, 1996). In addition to directly injurious effects, these enzymes produce an element of positive feedback, as they may further aggravate neutrophil activation and endothelial dysfunction.

The effect of aprotinin on neutrophil–endothelial function has been examined by several groups. The adhesion of neutrophils to the endothelium following reperfusion is surprisingly rapid and is known to take less than 20 min. Changes in gene expression are unlikely to underlie such rapid effects, and it has been proposed that the release of P-selectin stores from Weibel–Palade bodies may drive such PMN adhesion. Thrombin, acting through the PARs (Day *et al*., 2006a), is a recognized and potent activator of P-selectin release and hence of PMN rolling and subsequent adhesion. We postulate that the *in vitro* reduction in thrombin-stimulated PMN–endothelial adhesion by aprotinin may have arisen through an effect of aprotinin on this process of PAR-mediated, thrombin-induced P-selectin expression. This study found a parallel increase in PMN adhesion infiltration and eventually myocardial injury, all of which were reduced by aprotinin use. It seems this recognized stepwise process of rolling, adhesion, transmigration and hence infiltration of neutrophils may not always take place as aprotinin is able to limit leukocyte accumulation independently of leukocyte adhesion (Asimakopoulos *et al*., 2000).

The widespread proinflammatory sequelae of PAR activation by thrombin may explain why the antithrombin effects of serine proteases such as aprotinin act to reduce inflammation over and above its effects on P-selectin-mediated neutrophil activation. Recent data from a renal ischaemia-reperfusion study shows a significant aprotinin-related reduction in interleukin-1 and 6 mRNA and a trend towards lower tumour necrosis factor mRNA expression and P38 Map kinase activity (Kher *et al*., 2005). Even among CABG patients, PAR-1 inhibition by aprotinin reduced markers of systemic inflammation (Day *et al*., 2006a), whereas thrombin inhibition improved cardiac function with an increase in stroke volume and cardiac output following ischaemia-reperfusion (Jormalainen *et al*., 2007). Conversely, pretreatment with thrombin increased isolated cardiomyocyte death following ischaemia and reperfusion (Mirabet *et al*., 2005). Thus, the emerging trend is one of serine protease inhibitor suppression of leukocyte activation but also a more wide-

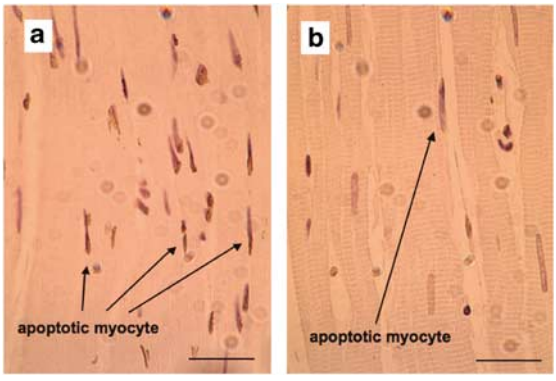

Figure 8 Photomicrographs of rabbit myocardial tissue (following myocardial ischaemia followed by reperfusion (MI + R)) analysed for apoptosis by the TdT-mediated dNTP nick end labeling assay. A brown reaction product is present at sites of myocyte apoptosis (arrows). (**a**) Myocardium from animals subjected to MI + R and vehicle treatment demonstrates significant numbers of apoptotic cardiomyocytes, whereas (**b**) myocardium from MI + R- and aprotinin- (10 000 U kg^{-1}) treated animals has substantially fewer apoptotic cardiomyocytes. Both panels are at a magnification of × 250.

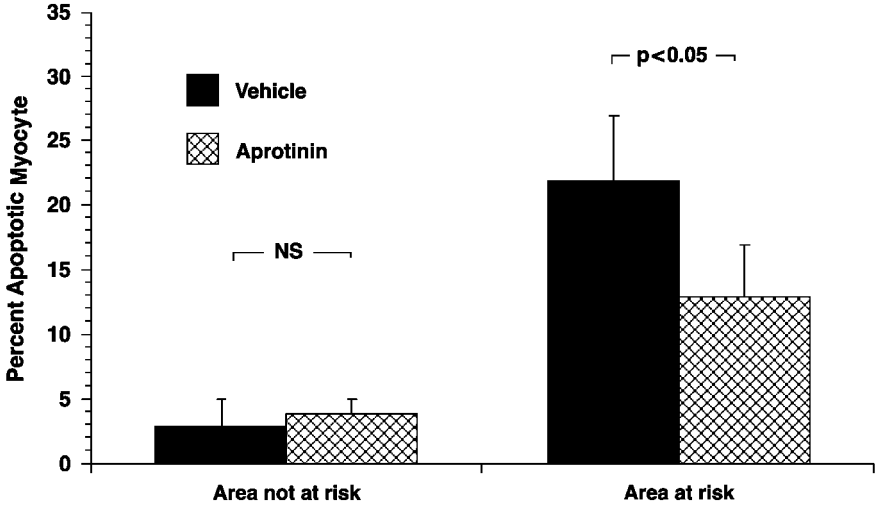

Figure 9 Summary of the protective effect of aprotinin treatment against apoptosis in rabbit hearts after myocardial ischaemia followed by reperfusion (MI + R). The number of apoptotic myocytes as a percentage of the total number of myocytes in the 'area not at risk' and 'area at risk' myocardium from vehicle- and aprotinin- (10 000 U kg^{-1}) treated animals is shown. Aprotinin significantly reduces the proportion of apoptotic myocytes compared to the effects of vehicle treatment. All values are means ± s.e.mean with five rabbits in each group.

spread inhibition of inflammatory activation by suppression of multiple thrombin—PAR receptor-mediated cascades.

The effect of aprotinin on myocardial injury and apoptosis

The cardioprotection exerted by aprotinin in this study was characterized by a 50% reduction in necrotic myocardium and by a marked attenuation of plasma CK activity compared to treatment with vehicle alone. These findings are in keeping with previous data from a number of animal models (Pruefer *et al*., 2002; Khan *et al*., 2004; Lazar *et al*., 2005). Our data are novel in that they demonstrate cardioprotection using parallel techniques and these show that firstly, the effects of aprotinin are dose-dependent (with no significant effect using 'low-dose' aprotinin) and, secondly, that aprotinin is effective when given at the end of the ischaemic period (thus, indicating an effect on reperfusion injury). Cardioprotective capability even when given just before reperfusion is important, as this is the time point closely analogous to the clinical situation (for example, in PCI centred therapy of myocardial infarction or with reperfusion following cardioplegic arrest for cardiopulmonary bypass). The smaller necrotic area in aprotinin-treated animals cannot be attributed to differences in the severity of ischemia, as all MI + R groups had a similar ischaemia area (indexed to LV size) and similar ST-segment elevation. Moreover, comparable PRI values in both ischaemic groups lessen the possibility that the cardioprotective effect of aprotinin was related to alterations in myocardial oxygen demand.

The importance of apoptosis as a cause of cell death and organ dysfunction in the cardiovascular system is becoming clearer (Clarke *et al*., 2007) and is induced following MI + R (Kunapuli *et al*., 2006). We have demonstrated a reduction of cardiomyocyte apoptosis by aprotinin when given just before reperfusion. This finding supports a relationship between apoptosis, tissue injury and the inhibition of both by serine protease inhibitors. Aggressive activation of platelets by thrombin causes their apoptosis, a phenomenon which is inhibited by thrombin inhibition (Leytin *et al*., 2007). Ischaemia-reperfusion of the renal parenchyma produced nephron apoptosis, which was reduced by pre-treatment with aprotinin (Kher *et al*., 2005). Serine protease inhibition has even been shown to reduce neutrophil apoptosis (Park *et al*., 2007). Apoptosis is triggered by a wide variety of compounds including cytokines such as tumour necrosis factor-α, interleukin-1 and oxygen-derived free radicals (Raff, 1992). Indeed, inhibition of intermediaries, such as insulin-like growth factor-1 (Buerke *et al*., 1995b), the classical complement pathway with exogenous C1 esterase inhibitor (Buerke *et al*., 1995a) and capase inhibition (Yaoita *et al*., 1998), have all been shown to inhibit apoptosis following MI + R. Previous work from our laboratory was the first to identify a reduction in myocyte apoptosis by aprotinin in a rat model of ischaemia and reperfusion (Pruefer *et al*., 2002). It seems hardly surprising then that nonspecific inhibition of the large serine protease family (with all their attendant proinflammatory effects) might also inhibit apoptosis in a variety of cell types.

We hypothesize that the antiapoptotic effect of aprotinin may have arisen through a complex interplay of events including (i) a reduction of leukocyte adhesion and subsequent accumulation and (ii) a reduction of injury by inhibition of harmful proteases once released by leukocytes and (iii) inhibition of thrombin and the wider serine protease family with subsequent reduction of their capacity for local and systemic inflammatory activation.

In conclusion, we have demonstrated an aprotinin-mediated reduction in successive steps of leukocyte activation and transmigration using parallel *in vitro* and *in vivo* techniques. These effects also extend to thrombin-stimulated endothelial–neutrophil interaction. We demonstrated a comprehensive and substantial reduction in cardiac injury and an antiapoptotic effect of aprotinin, when delivered just before reperfusion in an *in vivo* setting. Taken together, our data support an antineutrophil and anti-inflammatory effect of aprotinin, which may point to a clinical role beyond its current use as a haemostatic agent in cardiothoracic surgery.

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Conflict of interest

The authors state no conflict of interest.

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