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Cardioprotective and endothelial protective effects of $[Ala\text{-}IL8]_{77}$ in a rabbit model of myocardial ischaemia and reperfusion

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1 We studied the effects of a form of interleukin-8 (i.e., $[Ala\text{-}IL8]_{77}$) on endothelial dysfunction and myocardial injury in rabbits. Pentobarbitone-anaesthetized rabbits were subjected to 1.5 h occlusion of the marginal coronary artery and 3.5 h reperfusion. $[Ala\text{-}IL8]_{77}$ (50 μg or its vehicle) was given i.v. as a bolus 10 min prior to reperfusion. $[Ala\text{-}IL8]_{77}$ was also studied in isolated perfused hearts of rabbits.

2 Myocardial ischaemia plus reperfusion in untreated rabbits produced severe endothelial dysfunction and myocardial injury, including marked myocardial necrosis, elevated cardiac myeloperoxidase (MPO) activity in ischaemic cardiac tissue, and loss of response of marginal coronary rings to the endothelium-dependent vasodilators, acetylchloline (ACh) and A23187.

3 Administration of $[Ala\text{-}IL8]_{77}$ 10 min prior to reperfusion resulted in significant protective effects in post-ischaemic reperfusion. Compared with untreated rabbits, $[Ala\text{-}IL8]_{77}$ caused a reduced necrotic zone ($P < 0.01$), lower MPO activity in the necrotic zone ($P < 0.05$), and significantly preserved vasorelaxant responses of marginal coronary artery rings to endothelium-dependent vasodilators, ACh ($P < 0.001$) and A23187 ($P < 0.001$).

4 These results indicate that myocardial ischaemia and reperfusion result in a severe endothelial dysfunction and myocardial injury which involved the interaction of neutrophils and endothelial cells. However, $[Ala\text{-}IL8]_{77}$ did not appear to exert a direct endothelial protective effect in the absence of neutrophils in rabbit isolated perfused hearts.

5 Inhibition of neutrophil accumulation in the myocardium, perhaps by prevention of endothelial dysfunction resulting from $[Ala\text{-}IL8]_{77}$, leads to significant protective effects in ischaemia and reperfusion in rabbits.

Keywords: Endothelial cell interleukin-8; granulocytes; endothelium-derived relaxing factor; reperfusion injury; myeloperoxidase activity; neutrophil adherence

Introduction

Prolonged myocardial ischaemia without reperfusion inevitably results in myocardial cell death. Although early reperfusion is a desired therapeutic goal, reperfusion itself may contribute to additional myocardial cell injury (Braunwald & Kloner, 1985). Recent evidence strongly implicates neutrophils in the genesis of reperfusion injury (Engler *et al.*, 1986; Lucchesi *et al.*, 1989; Forman *et al.*, 1990). Neutrophils promote cellular damage by releasing superoxide radicals, proteolytic enzymes, and cytotoxic cytokines. Experimental strategies which have involved prevention of activation (Bednar *et al.*, 1985; Simpson *et al.*, 1987; Simpson *et al.*, 1988a) or depletion (Romson *et al.*, 1983) of neutrophils have been shown to reduce myocardial reperfusion injury. A monoclonal antibody directed against one of the adhesive glycoproteins (i.e., CD-11b/CD-18) on the neutrophil cell surface has also been shown to reduce infarct size significantly in dogs (Simpson *et al.*, 1988b). Therefore, substances which inhibit neutrophil adherence would appear to have particular utility in preventing reperfusion injury.

Recently , a variety of interleukin-8 (i.e., $[Ala\text{-}IL8]_{77}$, a 77 amino acid peptide identical to the 72 amino acid sequence of IL-8, with a pentapeptide extension at the NH_2-terminus; Gimbrone *et al.*, 1989) which is produced by endothelial cells, has been shown, at nanomolar concentrations, to be a potent inhibitor of neutrophil adhesion to cytokine-activated endothelial monolayers and to protect these monolayers from neutrophil-mediated damage (Gimbrone *et al.*, 1989). It has been proposed that $[Ala\text{-}IL8]_{77}$ may attenuate inflammatory events at the interface between the blood and the vessel wall (Gimbrone *et al.*, 1989). However, the effects of $[Ala\text{-}IL8]_{77}$ on endothelial dysfunction and myocardial injury produced by myocardial ischaemia and reperfusion have not been studied. Since endothelial dysfunction has recently been found to be an important early event leading to myocardial cell injury (Tsao *et al.*, 1990; Lefer *et al.*, 1990), this interrelationship may be of fundamental pathophysiological significance.

The purposes of the present investigation were to examine the effects of $[Ala\text{-}IL8]_{77}$ in myocardial ischaemia plus reperfusion to determine its activity in (a) prevention of neutrophil accumulation in the myocardium, (b) preservation of endothelial function as assessed by release of endothelium-derived relaxing factor (EDRF), and (c) subsequent limitation of myocyte necrosis following myocardial ischaemia and reperfusion in the rabbit.

Methods

Experimental procedure

Twenty adult male New Zealand white rabbits (2.2 to 3.1 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} body weight), intravenously. An intratracheal tube was inserted through a midline incision and all rabbits were given intermittent positive-pressure ventilation via a Harvard small animal respirator (Harvard Apparatus, Inc., S. Natick, MA, U.S.A.). A polyethylene catheter was inserted into the external jugular vein, and the right femoral artery was cannulated and connected to a Statham P23C pressure transducer (Spectromed, Inc., Critical Care Division, Oxnard, CA, U.S.A.) for the measurement of arterial blood pressure. A midline

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thoratocomy was performed, the pericardium opened and the heart was exposed. A 3-0 silk ligature was carefully placed around the major marginal branch of the left circumflex coronary artery located on the dorsal surface of the heart, 10–12 mm from its origin. Heart rates and ST segment changes were obtained from standard lead II of the scalar electrocardiogram every 20 min. Arterial blood pressure and the electrocardiogram were continuously recorded on a Grass Model 7 oscillographic recorder (Grass Instrument Co., Quincy, MA, U.S.A.). The pressure-rate index, calculated as the product of the mean arterial blood pressure and heart rate divided by 1000, was employed as an approximation of myocardial oxygen demand.

Experimental protocol

After a 30 min period of stabilization following thoracotomy, myocardial ischaemia (MI) was initiated by complete ligation of the marginal coronary artery. This was designated as time 0. After 1.5 h of ischaemia, the ligature was untied and the ischaemic myocardium was reperfused for 3.5 h. Recombinant *E. coli* $[Ala\text{-}IL8]_{77}$ (J. Baker, Genentech, Inc., South San Francisco, CA, U.S.A., greater than 98% pure) was given as an intravenous bolus (50 μg) starting 10 min prior to reperfusion (6 rabbits). An additional series of 7 rabbits were given an equal volume of 0.9% NaCl as a vehicle. Five sham-operated control rabbits were subjected to all of the procedures except that the coronary artery ligature was not tightened. Two rabbits died due to surgical complications, one sham-operated control due to haemorrhage, and one myocardial ischaemic rabbit due to pulmonary failure prior to assignment to a group.

At the end of the 5 h experimental period, the ligature around the marginal coronary artery was retightened; 30 ml of 5% Evans blue dye was injected into the left atrium to stain the area of the myocardium perfused by the patent coronary arteries. The area-at-risk was therefore determined by negative staining. The atria, right ventricle, and major blood vessels were subsequently removed from the heart. The left ventricle was then sliced into sections 3 mm thick parallel to the atrioventricular groove. The unstained portion of myocardium (i.e., the area-at-risk) was separated from the stained portion (i.e., the area-not-at-risk). The unstained portion was again sliced into 1 mm thick sections and incubated in a 0.1% solution of nitroblue tetrazolium stain in phosphate buffer at pH 7.4 and 37°C for 15 min to detect the presence of coenzyme and dehydrogenase. The necrotic portion of the myocardium which did not stain was separated from the stained portion (i.e., the non-necrotic area-at-risk). Samples from all three portions of left ventricular cardiac tissue (i.e., non-ischaemic, ischaemic non-necrotic and ischaemic necrotic) were weighed and stored at −70°C for subsequent assay of myeloperoxidase activity.

Measurement of myeloperoxidase activity in cardiac tissue

Myeloperoxidase, an enzyme which is specific for neutrophils, was determined in cardiac tissue by the method of Bradley *et al.* (1982), as modified by Mullane *et al.* (1985). MPO activity was used as an index of neutrophil accumulation in the heart since it correlates closely with numbers of neutrophils present in the heart (Mehta *et al.*, 1989). Cardiac tissue samples were homogenized in 0.5% hexadecyltrimethyl ammonium bromide (HTAB) (Sigma Chemical Co., St. Louis, MO, U.S.A.), dissolved in 50 mM potassium phosphate buffer at pH 6 in a Polytron (PCU-2) homogenizer (Kinemutica GmbH, Luzern, Switzerland) for 15 s at 7000 r.p.m. Homogenates were centrifuged for 20 min at 12,000 *g* and 2°C. The supernatants were decanted and added to 0.167 mg ml^{-1} *o*-dianisodine dihydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 0.0005% H_2O_2 in 50 mM phosphate buffer at pH 6. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO activity is defined as that quantity of enzyme degrading 1 mmol of peroxide per min at 25°C.

Studies on coronary ring responses

After injection of Evans blue, hearts were excised and placed in warmed, oxygenated Krebs-Henseleit (K-H) buffer. The marginal coronary artery was carefully isolated and 10–12 mm long segments were removed both above and below the ligature. The segment above the ligature (i.e., the proximal segment) was used as a non-ischaemic, non-reperfused control, and the portion of the vessel which had undergone ischaemia and reperfusion (i.e., the distal segment) was used as an ischaemic vessel. These segments were placed in warmed K-H consisting of (in mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 12.5 and glucose 10.0. Isolated coronary vessels were cut into rings of 2 to 3 mm in length. The rings were suspended in 20 ml tissue baths filled with K-H buffer warmed to 37°C and connected to Grass FT-03 force-displacement transducers (Grass Instrument Co., Quincy, MA, U.S.A.). Responses were recorded on a Grass Model 7 oscillographic recorder. Coronary rings were initially stretched to give a preload of 0.5 g of force and allowed to equilibrate for 1 h. Preloads of 1.0 g or higher caused injury to the endothelium and thus could not be used. Coronary rings were then exposed to 100 nM U-46619 (9,11-methanoepoxy PGH_2) (The Upjohn Co., Kalamazoo, MI, U.S.A.), a thromboxane-mimetic, to generate about 0.5 g of developed force. Once a stable contraction was observed, ACh was added to the bath to achieve the following final concentrations: 0.1, 1, 10 and 100 nM. After the response stabilized, the rings were washed and allowed to equilibrate to baseline once again. The procedure was repeated with A-23187 (1, 10, 100 and 1000 nM) and then to $NaNO_2$ (0.1, 1, 10 and 100 μM), final concentrations. $NaNO_2$ was dissolved in 0.1 N HCl and the solution titrated to pH 2.0. Distilled water at pH 2.0 added to the bath did not produce any vasorelaxation.

Rabbit isolated perfused hearts

Adult male rabbits were anaesthetized with pentobaritone sodium (30 mg kg^{-1}) injected intravenously, and then given heparin (1000 u kg^{-1}) intravenously. Hearts were rapidly isolated following a midsternal thoracotomy and placed in oxygenated Krebs-Henseleit (K-H) solution at pH 7.3 to 7.4. Following rinsing of all blood, hearts were transferred to a Langendorff perfusion apparatus, and perfused retrogradely through the aorta. Hearts were perfused with K-H solution at 37°C oxygenated with 95% O_2 plus 5% CO_2. Hearts were equilibrated for 5 min at a coronary perfusion pressure of 50 mmHg, and then constant flow perfusion was instituted at 50 ml min^{-1}. Coronary perfusion pressure (CPP) was then continuously recorded from a side-arm in the aortic inflow tract via a Statham P23AC pressure transducer (Spectramed, Inc., Oxnard, CA) and a Grass Model 7 oscillographic recorder (Grass Instrument Co., Quincy, MA).

After 5 min of constant flow perfusion, a bolus injection of the coronary vasoconstrictor U-46619 was given at 1 μg ml^{-1} of flow into the aortic inflow line. When CPP reached a stable plateau, the endothelium-dependent dilator acetylcholine (ACh), was injected at doses of 2, 20 and 200 ng ml^{-1} of flow. Following washout of all vasoactive agents, the process was repeated for U-46619 followed by the endothelium-independent dilator nitroglycerin at 0.5, 5 and 50 μg ml^{-1} of flow. Hearts were then shifted to a recirculation mode of perfusion with 200 ml of K-H solution. Hearts were then tested for vasodilator responses 50 min later, either after a period of control flow, or ischaemia (i.e., coronary flow reduced to 15% of control for 30 min), followed by 20 min of reperfusion. Ischaemic hearts received 2 ml of either 0.9% NaCl or 50 μg of $[Ala\text{-}IL8]_{77}$ 10 min prior to reperfusion. Myeloperoxidase analysis of rabbit hearts at the end of the experiments failed to show any detectable MPO activity.

Generation of superoxide radicals

Superoxide radicals were generated synthetically from a xanthine-xanthine oxidase system from a solution consisting of 50 mmol l^{-1} potassium phosphate (pH 7.8), 10 mmol l^{-1} EDTA, 10 μmol l^{-1} ferricytochrome C type III, 50 μmol l^{-1} xanthine, 200 μmol ml^{-1} potassium cyanide and 1 μg ml^{-1} dithionite. Xanthine oxidase (12.3 u ml^{-1}) was added to reaction mixture at 30°C and absorption measured every 15 s at 550 nm according to the method of McCord & Fridovich (1969).

Statistical analysis

All values in the text and figures are presented as means ± s.e. mean of n independent experiments. Data were analyzed by Kruskal-Wallis non-parametric analysis of variance due to inequalities of variances among the groups. Differences between specific means were tested for post-hoc analysis by the Mann-Whitney U test. A value of $P < 0.05$ was accepted as being statistically significant.

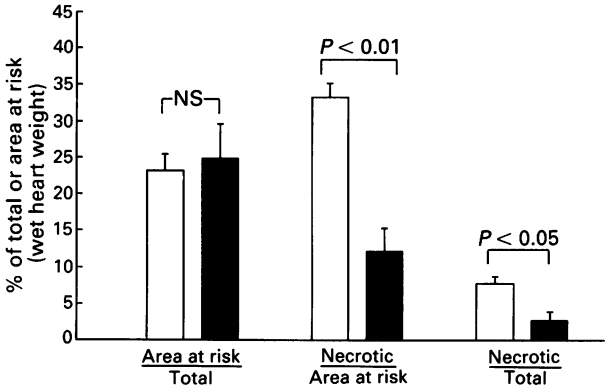

Figure 1 Area-at-risk indexed to total left ventricle (area-at-risk/total left ventricle × 100) and necrotic area indexed to area-at-risk (necrotic area/area-at-risk × 100) and to total left ventricle (necrotic area/area-at-risk × 100) in wet weight percent. Heights of columns are means and vertical bars indicate s.e.mean Treatment with [Ala-IL8]$_{77}$ (solid columns) significantly reduced the size of the necrotic area when compared to the untreated MI group (open columns).

Results

All sham MI rabbits maintained stable mean arterial blood pressures and heart rates over the course of the experimental period, so that the pressure-rate index did not change. Neither was there any change in ST segment of the electrocardiograms (Table 1). However, ST segments were significantly elevated in both of the MI groups (0.3 to 0.4 mV at 20–40 min post-occlusion) relative to the sham MI group. Moreover, there were no significant differences between the MI groups in ST segment elevation indicating that the severity of ischaemia was similar between the groups of rabbits subjected to myocardial ischaemia.

There were no significant differences between the MI groups for heart rates, mean arterial blood pressures (MABP) or the pressure-rate product, which was used as an index of myocardial oxygen demand. The initial MABP for all ischaemic rabbits combined was 100 ± 2 mmHg and the final MABP for all ischaemic rabbits combined was 89 ± 2 mmHg. Heart rate did not change over time in any of the experimental groups. Moreover, there were no statistically significant changes in the pressure-rate index (PRI) among any of the three experimental groups (Table 1). Therefore, changes in myocardial oxygen demand cannot account for any subsequent differences observed in any of the indices of severity of myocardial ischaemia.

Myocardial necrosis was assessed by staining techniques following 90 min of ischaemia and 3.5 h reperfusion in order to determine an anatomical index of the myocardial area jeopardized and that which became necrotic. The area-at-risk determined by negative staining following perfusion with Evan's blue stain, showed no significant differences between either of the MI groups (Figure 1), indicating that a similar amount of tissue was jeopardized by occlusion of the marginal coronary artery in both MI groups. In contrast, the necrotic area, which was measured by negative staining with nitroblue tetrazolium, indicated that a relatively large amount of the myocardial tissue at risk became necrotic (33%) in the untreated MI group. However, treatment with [Ala-IL8]$_{77}$ 10 min prior to reperfusion significantly reduced myocardial necrosis. This significant reduction in necrosis was observed whether the necrotic area was expressed as a percentage of the area-at-risk or as a percentage of the total left ventricle. Thus, [Ala-IL8]$_{77}$ afforded significant cardioprotection.

Figure 2 summarizes the cardiac myeloperoxidase activities for the groups of rabbits studied. Very low MPO activities were observed in the sham MI group, while significantly elevated MPO activities were observed in the area-at-risk in both MI groups. However, treatment with [Ala-IL8]$_{77}$ resulted in a significantly lower MPO activity in the necrotic area. MPO activities in the area-not-at-risk were very low for all three of the groups and there were no significant differences in MPO activities among any of them. Thus, these data suggest that [Ala-IL8]$_{77}$ can significantly limit the amount of neutrophil infiltration into the necrotic zone of the ischaemic myocardium.

In an effort to assess the extent of coronary vascular endothelial dysfunction, we isolated marginal coronary artery rings and assessed the ability of endothelium-dependent vasodilators to promote release of endothelium-derived relaxing factor (EDRF) in these preparations. U-46619 was used to contract marginal coronary artery rings, prior to testing with endothelium-dependent (e.g., ACh, A-23187) and endothelium-independent (i.e., $NaNO_2$) vasodilators. Figure 3 illustrates typical responses to ACh and $NaNO_2$ of isolated coronary artery rings taken from the ischaemic marginal artery segment below the site of occlusion. ACh added to rings from sham MI rabbits produced a full relaxation response. In marked contrast, rings isolated from the untreated MI rabbits were essentially unresponsive to addition of ACh. However, coronary artery rings isolated from rabbits treated with [Ala-IL8]$_{77}$ relaxed normally to ACh compared with the untreated MI group. Responses of these same coronary artery rings to the endothelium-independent vasodilator acidified $NaNO_2$ were

Table 1 Peak ST-segment elevation, initial and final pressure-rate index (PRI)

Group	*ST-segment* (mV)	*Initial PRI* (HR × MABP/1000)	*Final PRI* (HR × MABP/1000)
Sham MI + Vehicle (5)	0 ± 0	29.3 ± 1.6	27.1 ± 2.3
MI + vehicle (7)	0.3 ± 0.06	26.2 ± 2.0	20.9 ± 1.9
MI + [Ala-IL8]$_{77}$ (6)	0.4 ± 0.07	28.2 ± 0.9	22.0 ± 0.8

All values are means ± s.e.mean.
Numbers in parentheses are numbers of rabbits studied in each group.
HR = heart rate; MABP = mean arterial blood pressure. MI = mycardial ischaemia.

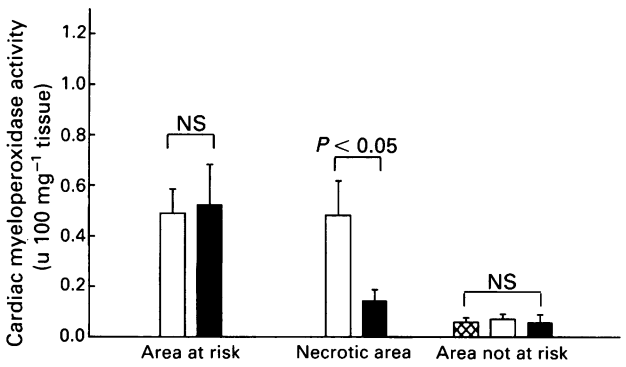

Figure 2 Myeloperoxidase (MPO) activity in the area-at-risk, necrotic area and area-not-at-risk in units $100\,mg^{-1}$ tissue. Heights of columns are means and vertical bars indicate s.e.mean. Treatment with $[Ala\text{-}IL8]_{77}$ (solid columns) significantly reduced the MPO activity in the necrotic zone when compared to the untreated MI group (open columns). The cross-hatched columns represent the Sham MI + vehicle group.

not different among the three groups studied. All groups relaxed fully in response to acidified $NaNO_2$ indicating that the coronary vascular smooth muscle was fully capable of relaxing to a vasodilator.

Figure 4 illustrates the mean responses to ACh and to $NaNO_2$ which were shown by the representative tracings in Figure 3. In addition, responses to the endothelium-dependent, non-receptor mediated vasodilator, A-23187 (calcium ionophore), are also shown. The pattern of responses to A-23187 was similar to that of ACh in all groups. Coronary rings isolated from sham MI rabbits relaxed fully, while significant impairment was observed in the untreated ischaemic MI rings. Treatment with $[Ala\text{-}IL8]_{77}$ produced virtually a complete relaxation which was significantly greater than the

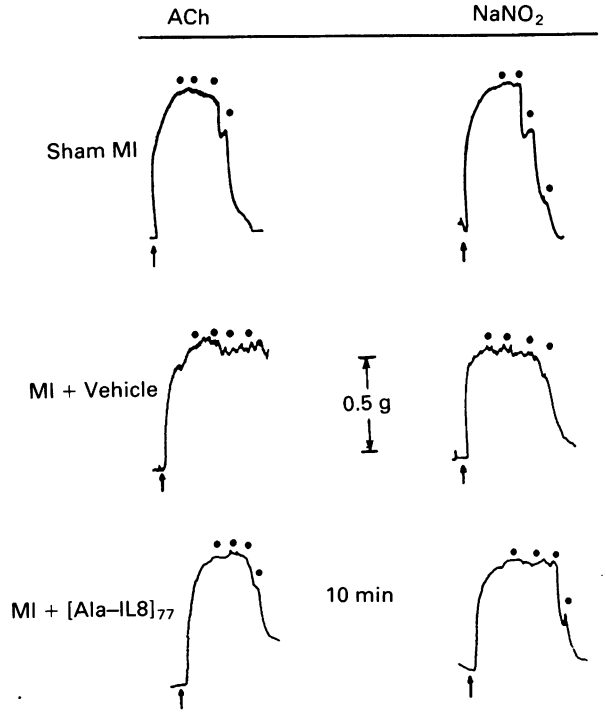

Figure 3 Representative recordings of marginal coronary artery rings of rabbit from the three groups studied in the presence of the endothelium-dependent vasodilator acetylcholine (ACh) and the non-endothelium-dependent vasodilator acidified $NaNO_2$. Rings were precontracted with U-46619, a thromboxane mimetic, as shown by the arrows. Dots above the tracings indicate addition of vasodilator substances. The endothelium-dependent relaxation was abolished by ischaemia/reperfusion in the untreated MI group, whereas treatment with $[Ala\text{-}IL8]_{77}$ significantly preserved this response.

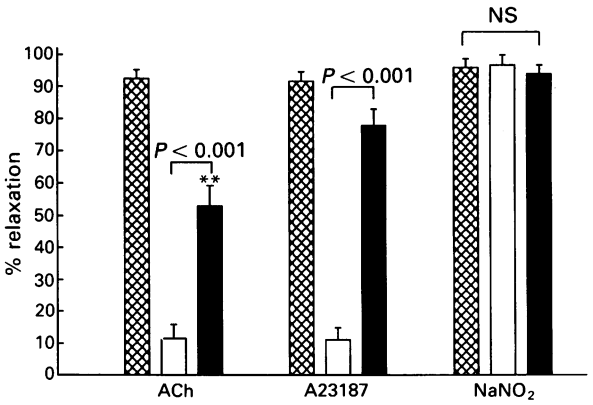

Figure 4 Group data from ischaemic coronary artery rings are summarized for the four groups studied following addition of the endothelium-dependent receptor mediated vasodilator acetylchloline (ACh), the endothelium-dependent nonreceptor-mediated vasodilator A-23187, and the endothelium-independent vasodilator acidified $NaNO_2$. Responses are expressed as percentage relaxation of a U-46619-induced contraction. Significant response decrements were observed in the untreated MI group (open columns) in response to ACh and A-23187 which were significantly preserved in the MI group treated wh $[Ala\text{-}IL8]_{77}$ (solid columns). $^{**}P < 0.001$ compared with Sham MI group (cross-hatched column).

untreated ischaemic MI rings and was not significantly different from the sham MI rings. This indicates that both receptor-mediated and non-receptor-mediated endothelium-dependent responses were fully protected by $[Ala\text{-}IL8]_{77}$ treatment. All ischaemic rings fully relaxed in the presence of $NaNO_2$, indicating that the vascular smooth muscle had not been damaged during the course of the experiment.

Figure 5 illustrates the responses of the control coronary artery rings which were taken from the proximal portion of the marginal coronary artery above the site of occlusion. There was no significant difference among the groups in the relaxation to ACh. There was a small but significant difference in response to A-23187 between the untreated MI control rings and the sham MI control rings. This difference is probably of no physiological consequence, and no other group comparisons were significant with regard to responses to A-23187. Finally, there were no significant differences in

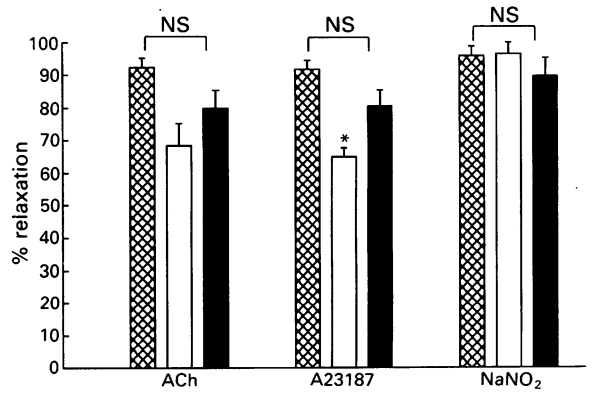

Figure 5 Group data from control coronary artery rings are summarized for the four groups studied following addition of the endothelium-dependent receptor mediated vasodilator acetylcholine (ACh), the endothelium-dependent non-receptor-mediated vasodilator A-23187, and the endothelium-independent vasodilator acidified $NaNO_2$. Responses are expressed as percentage relaxation of a U-46619-induced contraction. The only significant response decrement observed was between the sham MI group (hatched columns) and the untreated MI group (open columns) in response to A-23187. $^{*}P < 0.05$ compared with Sham MI group. The solid columns represent the MI + $[Ala\text{-}IL8]_{77}$ group.

Table 2 Effect of [Ala-IL8]$_{77}$ on vasodilator responses in rabbit isolated perfused hearts

Group	n	*% response to acetylcholine*[a]	*% response to nitroglycerin*[b]
Control hearts	5	104 ± 7	103 ± 5
MI + Vehicle	6	44 ± 8*	100 ± 7
MI + [Ala-IL8]$_{77}$ (50 μg)	6	55 ± 7*	99 ± 5

All values are means ± s.e. mean. The numbers represent the second response (i.e., 20 min post-reperfusion or sham reperfusion) calculated as a percentage of the initial control response (i.e., prior to ischaemia or sham ischaemia). Coronary vascular resistances decreased to ACh by about 65% of their U-46619 added tone.

[a] Concentration = 200 ng ml^{-1}.

[b] Concentration = 50 μg ml^{-1}.

n = number of hearts studied; MI = myocardial ischaemia.

* $P < 0.01$ from control hearts.

response to $NaNO_2$ among any groups. These data indicate that there was no significant endothelium impairment in the non-ischaemic segment of the marginal coronary artery. The endothelium dysfunction occurred only in the ischaemic segment of the marginal coronary artery of rabbits subjected to myocardial ischaemia.

Rabbit hearts perfused with Krebs-Henseleit solution without any blood plasma or blood cells responded normally to ACh and nitroglycerin initially, and also after 50 min of perfusion at control flow (Table 2). However, rabbit hearts subjected to 30 min of global ischaemia (i.e., to 15% of control flow) and 20 min of reperfusion at control flows experienced a significant impairment in vasodilatation to ACh but not to nitroglycerin, indicating an endothelial dysfunction. Moreover, addition of [Ala-IL8]$_{77}$ to the perfusate 10 min prior to reperfusion failed to restore vasodilator responsiveness to ACh, indicating that [Ala-IL8]$_{77}$ under the conditions of the buffer-perfused heart, failed to exert a direct endothelium protecting effect in the rabbit coronary vasculature. Additional studies are necessary to determine the precise interaction between neutrophils and the endothelium in the presence of all of the substances present in blood during myocardial ischaemia.

Finally, [Ala-IL8]$_{77}$ added to the synthetic xanthine-xanthine oxidase mixture at a concentration of 0.3 μg ml^{-1} did not significanly alter the absorbance at 550 nm from 0.124 ± 0.013 to 0.126 ± 0.015 whereas recombinant human superoxide dismutase (hSOD) reduced the absorbance to 0.019 ± 0.006 ($P < 0.001$ from control). Thus, [Ala-IL8]$_{77}$ clearly lacks intrinsic superoxide radical scavenging activity.

Discussion

Myocardial ischaemia produced by the occlusion of a large coronary artery results in functional alterations due to the prolonged interruption of blood flow. Although reperfusion restores flow, it initiates generation of reactive oxygen metabolites and stimulates release of pro-inflammatory mediators (McCord, 1985; Hess & Manson, 1984; Zweier, 1988). Prominent targets for post-ischaemic reperfusion injury include both endothelial cells and cardiac myocytes. Damage to the endothelium may impair control of vascular tone by inhibiting release of vasodilator substances such as endothelium-derived relaxing factor (EDRF) which can result in a loss of vasodilator reserve (Van Benthuysen *et al.*, 1987; Mehta *et al.*, 1989; Johnson *et al.*, 1990). This defect has been shown to occur within 10–20 min after reperfusion in cats and rats (Tsao *et al.*, 1990; Lefer *et al.*, 1990).

Neutrophils have been shown to accumulate in the post-ischaemic myocardium (Sommers & Jennings, 1964; Romson *et al.*, 1982; Johnson *et al.*, 1990) and can cause cellular dysfunction by a variety of mechanisms. Neutrophils release cytotoxic oxygen metabolites, enzymes (e.g., proteases and collagenases), and cytokines such as tumor necrosis factor (TNF) which has been shown to inhibit EDRF release from the vascular endothelium (Aoki *et al.*, 1989). Neutrophils also release superoxide radicals which have been shown to inactivate EDRF (Rubanyi & Vanhoutte, 1987) and may disrupt cellular membranes through lipid peroxidation reactions, thus promoting increased microvascular permeability. In addition, neutrophil aggregates may participate in microvascular plugging leading to the 'no-reflow' phenomenon (Kloner *et al.*, 1974; Engler *et al.*, 1986). Experimental strategies which have involved prevention of activation of neutrophils (Bednar *et al.*, 1985; Simpson *et al.*, 1987; Simpson *et al.*, 1988a) or neutrophil depletion (Romson *et al.*, 1983) have been shown to reduce reperfusion injury.

Adherence of neutrophils to the endothelium appears to represent a critical step in the pathogenesis of ischaemia-reperfusion injury and can lead to endothelial damage (Harlan, 1987). Cytokines such as IL-1 and TNF can act directly on endothelial cells to induce the expression of endothelial-leukocyte adhesion molecules (ELAMs) (Bevilacqua *et al.*, 1987). The expression of these adhesion molecules promotes adherence of neutrophils to the surface of the endothelium, which is a prerequisite for diapedesis of these cells into ischaemic reperfused tissues.

[Ala-IL8]$_{77}$ is a recently discovered 10-kD protein which is produced by the endothelium and acts as a soluble leukocyte adhesion inhibitor (Gimbrone *et al.*, 1989). It has been sequenced and shares considerable sequence identity over 72 amino acids (with an additional pentapeptide added) with mononuclear leukocyte-derived IL-8 [(Ser)-IL8]$_{72}$ produced by cells of the immune system. When activated by IL-1 or TNF, endothelial cells produce this leukocyte adhesion inhibitor [Ala-IL8]$_{77}$ which attenuates the adhesive interaction between leukocytes and endothelial cells (Wheller *et al.*, 1988; Strieter *et al.*, 1989; Schroder & Christophers, 1989). [Ala-IL8]$_{77}$ has been shown to be a potent inhibitor of neutrophil adhesion to cytokine-activated endothelial monolayers (Gimbrone *et al.*, 1989) and to protect these monolayers from neutrophil-mediated damage (Gimbrone *et al.*, 1989). It has been proposed that [Ala-IL8]$_{77}$ may attenuate inflammatory events at the interface between the blood and the vessel wall (Gimbrone *et al.*, 1989). Therefore, an important new strategy in prevention of reperfusion injury may be the identification of substances which protect the endothelium from damage by inactivation of neutrophil adherence mechanisms, thus inhibiting neutrophil induced endothelial dysfunction which later leads to infiltration of neutrophils into the myocardium.

There is evidence for a reduced neutrophil accumulation in ischaemic rabbit cardiac tissue evidenced by the MPO data. This anti-inflammatory effect of intravenous [Ala-IL8]$_{77}$ confirms the earlier report of this phenomenon by Hechtman *et al.* (1990). Although [Ala-IL8]$_{77}$ significantly retarded MPO activity in the necrotic zone, it did not do so in the non-necrotic area-at-risk. This may be due to two different neutrophil pools, a circulating pool migrating to the necrotic area, and another pool (e.g., marginated or rolling neutrophils) which behave differently and which adhere to the endothelium or migrate into the extravascular compartment

in the area-at-risk. Further work is necessary to clarify these relationships.

We have shown a remarkable degree of endothelial protection by $[Ala\text{-}IL8]_{77}$ delivered 10 min prior to reperfusion compared to untreated MI rabbits. In this connection, $[Ala\text{-}IL8]_{77}$ maintained EDRF effectiveness during the reperfusion phase of myocardial ischaemia and reperfusion. This endothelial protective effect may retard neutrophil adherence to endothelial cells and the progressive infiltration of neutrophils into deeper areas of the myocardium. This is supported by the finding that EDRF (i.e., nitric oxide) prevents neutrophil adherence to endothelial cells (McCall *et al.*, 1988). Thus, protection and preservation of endothelial cell function by prevention of neutrophil adherence would appear to be a major mechanism of action which could explain the protective effects of $[Ala\text{-}IL8]_{77}$ observed in this model. However, it is unlikely that a direct endothelial preservation by $[Ala\text{-}IL8]_{77}$ could explain our results since $[Ala\text{-}IL8]_{77}$ failed to prevent endothelial dysfunction in rabbit isolated perfused hearts in the absence of blood or blood cells. Moreover, $[Ala\text{-}IL8]_{77}$ did not scavenge superoxide radicals in a totally synthetic xanthine-xanthine oxidase system.

Nevertheless, several possibilities still exist with regard to the mechanism of action of $[Ala\text{-}IL8]_{77}$ in myocardial ischaemia and reperfusion. $[Ala\text{-}IL8]_{77}$ may exert (a) direct effects on neutrophils to reduce the number of these leukocytes accumulating in myocardial tissue following reperfusion; (b) direct inhibitory effects on chemotactic function of neutrophils to prevent their migration along chemotactic gradients; (c) direct inhibitory effects on the ability of neutrophils to express or upregulate adhesion molecules on their surface; (d) direct or indirect inhibitory effects on neutrophils to prevent damage to endothelial cells by inhibition of cytotoxic mediator release; and (e) other, as yet unknown effects, such as cell membrane stabilizing activity. These possibilities remain for future investigation.

In summary, we have observed that coronary artery occlusion for 1.5 h followed by 3.5 h reperfusion resulted in significant endothelial dysfunction, neutrophil accumulation in the myocardium and myocardial injury in the anaesthetized rabbit. Intravenous administration of $[Ala\text{-}IL8]_{77}$, a novel endothelial cell-derived leukocyte adhesion inhibitor, preserved endothelial function, reduced neutrophil infiltration into the necrotic myocardium and reduced myocardial necrosis, thus providing a significant degree of endothelial and myocardial protection in this model. Since the rabbit is known to have very little collateral coronary flow (Schaper, 1981) it is unlikely that $[Ala\text{-}IL8]_{77}$ could protect by opening collateral vessels. This is even more remote since $[Ala\text{-}IL8]_{77}$ failed to exert any direct coronary vasoactivity in isolated coronary rings (data not shown), and did not exert any systemic vasodilation *in vivo* (i.e., pressure-rate index (PRI) was not decreased). Despite the beneficial effects observed in our short term model of myocardial ischaemia and reperfusion in rabbits, it should be emphasized that we have no information on long term effects of infarction (e.g., 24–48 h) nor do we know whether these results would be directly applicable in clinical cases of human myocardial ischaemia where the time of onset of ischaemia or of reperfusion may not be clearly defined.

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