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Myocardial protection after monophosphoryl lipid A: studies of delayed anti-ischaemic properties in rabbit heart

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1 Monophosphoryl lipid A (MLA) is a non-pyrogenic derivative of *Salmonella* lipopolysaccharide. Administration of this agent at high doses to rats and at low doses to dogs was previously shown to confer marked protection against ischaemia-reperfusion 24 h later, although the cellular mechanisms of this delayed protection are obscure. We hypothesized that MLA pretreatment causes the induction of the 70 kDa cytoprotective stress protein HSP70i in the myocardium. If this were the case, protection against ischaemia-reperfusion injury would be observed both *in vitro* and *in vivo*.

2 Rabbits were pretreated with MLA 0.035 mg kg^{-1}, i.v. or vehicle solution. For the *in vitro* study, hearts were isolated 24 h later and Langendorff-perfused with Krebs-Henseleit buffer at 37°C. Global ischaemia was induced for 20 min followed by 120 min reperfusion. Recovery of post-ischaemic left ventricular function and lactate dehydrogenase efflux was similar in MLA and vehicle pretreated hearts and there was no significant difference in the percentage of infarction of the left ventricle determined by triphenyltetrazolium staining (MLA $22.4 \pm 5.2\%$, vehicle $24.8 \pm 5.1\%$).

3 When 30 min regional ischaemia and 120 min reperfusion was instituted in pentobarbitone-anaesthetized rabbits 24 h after pretreatment with MLA or vehicle, the percentage infarction within the risk zone was reduced from $42.6 \pm 5.7\%$ in vehicle pretreated animals to $19.6 \pm 4.4\%$ in MLA pretreated animals ($P < 0.01$).

4 Determination of myocardial HSP70i content by Western blot analysis showed that MLA treatment did not increase HSP70i immunoreactivity.

5 We conclude that MLA at this dose confers protection only against ischaemia-reperfusion injury *in vivo* and that this protection is not related to induction of HSP70i. Because protection was observed only *in vivo* it seems possible that the delayed protection conferred by MLA is mediated by effects on humoral or blood-borne factors.

Keywords: Endotoxin; HSP70; ischaemia-reperfusion; lipopolysaccharide; monophosphoryl lipid A; myocardial infarction; myocardial ischaemia; myocardial protection; stress protein

Introduction

Sublethal doses of Gram-negative bacterial endotoxins (lipopolysaccharides) can increase myocardial tolerance to a subsequent challenge with ischaemia and reperfusion (Brown *et al.*, 1989; Song *et al.*, 1994). This protection is not observed immediately but is seen many hours later. The development of enhanced tolerance to ischaemia-reperfusion may be related to the augmentation of intracellular anti-oxidant defences since endotoxin has been shown to increase catalase activity in rat myocardium (Brown *et al.*, 1989), superoxide dismutase (SOD) activity in rat lung (Frank *et al.*, 1980) and mitochondrial manganese-dependent SOD (Mn-SOD) activity in rat liver (Dougall & Nick, 1991). The enhancement of anti-oxidant activity following endotoxin treatment is a feature of the cellular stress response shared by other oxidative stress stimuli (see Donati *et al.*, 1990), especially sublethal heat shock (Currie *et al.*, 1988; Karmazyn *et al.*, 1990; Steare & Yellon, 1994; Yamashita *et al.*, 1994a) and sublethal hypoxia or ischaemia-reperfusion (Hoshida *et al.*, 1993; Yamashita *et al.*, 1994b). These stresses have been shown to confer delayed myocardial protection against ischaemia-reperfusion and in addition to changes in intracellular anti-oxidant defences are also associated with elevation of heat shock proteins (HSP's) including the inducible 72 kDa HSP (HSP70i) (Currie *et al.*, 1988; 1993; Hutter *et al.*, 1994; Marber *et al.*, 1993).

Monophosphoryl lipid A (MLA) is obtained by modification of the lipid A domain of *Salmonella minnesota* lipopolysaccharide and is reported to be 1000 times less pyrogenic than endotoxin (see Yao *et al.*, 1994 for review). MLA has been shown to retain some of the beneficial properties of endotoxin. Nelson *et al.* (1991) showed in the rat isolated heart that pretreatment with MLA 5 mg kg^{-1} 24 h prior to global ischaemia resulted in better preservation of post-ischaemic ventricular function, and that the protection was associated with an increase in myocardial catalase activity. Two subsequent studies (Yao *et al.*, 1993a,b) examined delayed cardioprotection in the dog with much lower doses of MLA (0.035 to 0.1 mg kg^{-1}). MLA at these low doses was shown to reduce infarct size if administered 24 h, but not 1 h, prior to coronary occlusion (Yao *et al.*, 1993b).

The mechanism of protection following low-dose MLA treatment is not clear. Small, but statistically insignificant, rises in tissue anti-oxidant levels were reported in the dog (Yao *et al.*, 1993a) but other possible mechanisms have also been identified. These workers showed that neutrophil infiltration into the ischaemic border zone was significantly decreased following MLA pretreatment in dogs (Yao *et al.*, 1993a). Additionally, they found that vascular endothelial and/or smooth muscle responses were decreased with 1 h pretreatment with MLA, but returned to normal after 24 h and concluded that these effects on endothelium-dependent responses could have been mediated by the L-arginine/nitric oxide (NO) pathway. Thus, it seems that low-dose MLA may exert several biological effects and that the protection observed against ischaemia-reperfusion injury *in vivo* may not be ascribed completely, if at all, to increases in endogenous antioxidant activity. A preliminary report suggests that expression of

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HSP70i occurs in rat myocardium following endotoxin treatment *in vivo* (Brown *et al.*, 1993). It has been conjectured that MLA could stimulate the synthesis of HSP's (Yao *et al.*, 1994), and these cytoprotective proteins might be responsible, at least in part, for delayed cardioprotection. To date, however, no study in the literature has assessed the myocardial HSP response following MLA treatment. The aims of the current study were two fold. We sought: (1) to compare responses to ischaemia-reperfusion *in vitro* and *in vivo* following MLA treatment in this species and thus to determine the relative importance of direct enhancement of myocardial defences in protection following low-dose MLA; and (2) to examine if myocardial content of the principal inducible HSP associated with tissue protection, HSP70i, is augmented by MLA treatment in the rabbit.

Data contained in this paper were presented to the British Pharmacological Society at its summer 1995 meeting in Oxford (Baxter *et al.*, 1995).

Methods

Materials and Animals

MLA (supplied as a 0.3 mg ml^{-1} solution in a vehicle consisting of 40% polyethylene glycol and 10% ethanol in water) was the gift of Ribi ImmunoChem Research (Hamilton, Montana, U.S.A.). Zinc-cadmium sulphide fluorescent microspheres (1 to 10 μm) were from Duke Scientific (Palo Alto, CA, U.S.A.). *Salmonella minnesota* lipopolysaccharide and triphenyltetrazolium chloride were from Sigma Chemical. A monoclonal antibody to HSP70i (code C92F3A-5) was supplied by Stressgen (Victoria, British Columbia, Canada). All other chemicals were of analytical reagent quality.

Barrier-raised male New Zealand White rabbits (2.2 to 3.0 kg) were used for these studies. The care and use of animals in this work were in accordance with UK Home Office guidelines on the Animals (Scientific Procedures) Act 1986.

Preparation of animals for ischaemia-reperfusion studies

Twenty four hours before each experiment animals were pretreated with i.v. boluses of either MLA 0.035 mg kg^{-1} or vehicle 0.12 ml kg^{-1}. The dose was diluted to approximately 0.8 ml with 0.9% sodium chloride injection BP immediately before injection. Animals received no other manipulation and were returned to their pens immediately.

In vitro *study: Langendorff perfusion and ischaemia-reperfusion protocol*

Twenty four hours after pretreatment, animals were heparinized (500 i.u.) and deeply anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.v.). Hearts were excised and immediately perfused in the non-recirculating Langendorff mode and subjected to the global ischaemia-reperfusion protocol described below. Krebs-Henseleit buffer of the following composition (mmol l^{-1}) was used for the isolated heart study: NaCl 118, $NaHCO_3$ 25, KCl 4.2, KH_2PO_4 1.8, $CaCl_2$ 1.8, $MgSO_4$ 1.2, D-glucose 10 (pH 7.35–7.45 when equilibrated with 95% O_2/5% CO_2). Hearts were perfused at a constant flow rate of 45 ml min^{-1} (determined by timed collection of coronary effluent) with endocardial temperature maintained at 37°C.

A compliant fluid-filled latex balloon was connected to a pressure transducer (Gould Instruments) and introduced into the left ventricle through an incision in the left atrium. The volume of the balloon was adjusted to give a left ventricular end diastolic pressure of ~10 mmHg and this preload was maintained throughout the experiment. Pacing electrodes were positioned on the aortic cannula and the right atrial appendage. Hearts were paced during ischaemia at 200 beats min^{-1}.

After stabilization, global ischaemia was induced by cessation of aortic flow. During global ischaemia temperature was maintained at 37 ± 0.5°C by surrounding the heart in a water-jacketed chamber. Global ischaemia was maintained for 20 min. Following the period of global ischaemia, flow was re-instated at the pre-ischaemic value of 45 ml min^{-1} for 120 min. During the first 60 min of reperfusion end diastolic pressure and left ventricular developed pressure was recorded. Samples of coronary eluent were collected during stabilization and during the first 60 min of reperfusion for estimation of lactate dehydrogenase by an optimised spectrophotometric assay (catalogue number DG 1340-K, Sigma Chemicals). After 120 min reperfusion, hearts were frozen at −18°C and infarct size was assessed with triphenyltetrazolium staining as described below. Previous work in this laboratory has shown that maximum post-ischaemic functional recovery is seen between 30 and 60 min of reperfusion but that 120 min reperfusion is required for optimal delineation of the infarcted area by triphenyltetrazolium (Jenkins *et al.*, 1995).

In vivo *study: infarction procedure and infarct size assessment*

The infarction protocol described by Baxter *et al.* (1994) was followed. Approximately 24 h after MLA or vehicle-treatment rabbits were anaesthetized with a combination of pentobarbitone sodium 40 mg kg^{-1}, i.v. and Hypnorm 0.15 ml kg^{-1}, i.m. (Hypnorm contains fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1}). Surgical anaesthesia was maintained by administration of Hypnorm 0.1 ml ml^{-1}, i.m. at hourly intervals and pentobarbitone sodium 5–10 mg kg^{-1}, i.v. when required. Animals were ventilated with 100% O_2 and the right common carotid artery was cannulated for periodic haemodynamic and arterial blood gas and pH measurements. Tidal volume was adjusted as necessary throughout the procedure to maintain arterial pH between 7.35 and 7.50. The thorax was opened by mid-line sternotomy, the pericardium incised and an anterolateral branch of the circumflex coronary artery was identified. A 3/0 silk ligature (Mersilk type 504, Ethicon) was passed underneath the vessel at a point approximately half-way between the apex and the insertion of the left atrial appendage. The ends of the suture were threaded through a 1.5 cm polypropylene tube to form a snare. Following stabilization, heparin sodium 500 iu was given i.v. before coronary occlusion. The artery was occluded for 30 min by pulling the ends of the suture taut and clamping the snare onto the epicardial surface. Coronary occlusion caused epicardial cyanosis and regional dyskinesis within 20 to 30 s and was accompanied by ST segment deviation in the surface ECG within 2 min. After 30 min occlusion, reperfusion was instituted by releasing the snare. Successful reperfusion was confirmed by conspicuous blushing of the previously ischaemic myocardium and gradual resolution of the ST segment.

At the end of 120 min reperfusion, heparin sodium 500 iu was given i.v. before the heart was excised and Langendorff-perfused with saline solution to remove blood. The ligature was tightened once more and zinc-cadmium sulphide microspheres were infused through the aorta to delineate the myocardium at risk under ultraviolet light. After freezing at −18°C, the heart was sliced transversely from apex to base in 2 mm sections. The slices were defrosted, blotted and incubated at 37°C with 1% w/v triphenyltetrazolium chloride in phosphate buffer (pH 7.4) for 10–20 min and fixed in 4% v/v formaldehyde solution to distinguish clearly stained viable tissue and unstained necrotic tissue (Fishbein *et al.*, 1981). The volumes of the infarcted tissue (I) and the tissue at risk (R) were determined by a computerised planimetric technique ('Summa Sketch II', Summa Graphics, Seymour, Conneticut, U.S.A.).

Preparation of animals for HSP70i analysis

Six animals were pretreated with MLA or vehicle as described above. Two animals were pretreated with *S. minnesota* lipopolysaccharide 0.05 mg kg^{-1}, i.v. dissolved in saline and another two with saline (lipopolysaccharide controls). The

animals treated with lipopolysaccharide became sluggish within an hour of its administration but did not become tachypnoeic, ataxic or develop diarrhoea. Twenty four hours later they appeared well. Two animals were heat-stressed under pentobarbitone anaesthesia as reported previously (Marber *et al.*, 1993). Briefly, anaesthetized animals were closely wrapped in a warming blanket and rectal temperature was raised to 42°C. This temperature was maintained for 15 min after which the animals were allowed to cool and recover from anaesthesia.

Twenty four hours following these pretreatments, animals were deeply anaesthetized with pentobarbitone and the hearts removed and washed with cold saline to remove blood. The left ventricular tissue (free wall and interventricular septum) was dissected quickly and frozen in liquid nitrogen until prepared for Western blot analysis.

Determination of myocardial HSP70i content by Western blot analysis

Frozen left ventricular tissue (100 mg) was powdered in liquid nitrogen, suspended in 1.0 ml SDS–PAGE sample buffer (20% w/v glycerol; 6.0% w/v SDS; 120 mmol l^{-1} Tris HCl, pH 6.8) and hand homogenized in 2×500 μl aliquots. 2-Mercaptoethanol was added to a final concentration of 10% v/v and samples heated over a boiling water bath for 5 min. Samples were cooled, centrifuged at 11,000 *g* for 5 min to pellet cell debris and supernatants transferred to clean microcentrifuge tubes; 8% w/v bromophenol blue in ethanol (10 μl ml^{-1} of sample) was added and samples stored at −20°C prior to electrophoresis.

Samples were electrophoresed on 10% polyacrylamide SDS–PAGE gels according to Laemmli (1970). For visualization of proteins, gels were stained in Coomassie Brilliant Blue R250 (0.3% w/v in 40% v/v methanol: 7% v/v glacial acetic acid) and destained in the same solvent without Coomassie Blue. For Western blotting following SDS–PAGE, gels were placed directly into a Biorad Transblot cell and proteins transferred electrophoretically onto Hybond-C membranes (Amersham, UK) overnight at 180 mA and 4°C using 250 mmol l^{-1} Tris HCl, pH 8.3, 192 mmol l^{-1} glycine and 20% v/v methanol. Following transfer, filters were blocked using dried milk powder (Marvel) (0.1% w/v in phosphate-buffered saline, pH 7.2; 0.05% (v/v) Tween 20) for 1 h at room temperature. Filters were then incubated with a monoclonal antibody for HSP70i (C92F3A-5; Stressgen) at a dilution of 1:1000 (in 0.1% w/v Marvel; PBS; 0.05% v/v Tween 20) for 1 h at room temperature. Filters were washed for 3×5 min in the same buffer (no antibody) and then exposed to horseradish peroxidase-conjugated rabbit-anti-mouse IgG second antibody (DAKO) at a dilution of 1:2500 in the same buffer for 1 h at room temperature. Filters were washed as above and developed using an enhanced chemiluminescence detection system (Amersham) and exposed to Kodak X-Omat AR film for between 30 s and 3 min after allowing >3 min for maximum light emission to develop.

Statistical analysis

The data are presented as mean ± s.e.mean. The significance of differences in mean values with respect to time was evaluated by 2-way analysis of variance followed by Fisher's protected least significant difference test. Differences in infarct size were assessed by Student's unpaired *t* test. The null hypothesis was rejected when $P \leqslant 0.05$.

Results

Exclusions

A total of 58 rabbits were used for these studies. All animals were well 24 h after the pretreatment protocols. Thirty rabbits were used for the *in vitro* study, 16 rabbits were used for the *in vivo* study and 12 rabbits were used for HSP70i analysis. In the *in vitro* study, 8 hearts were excluded for technical reasons including failure to develop stable rhythm, loss of temperature control during ischaemia, myocardial trauma on excision and an unusually high baseline coronary perfusion pressure. In the *in vivo* study, 2 animals were excluded (one due to haemmorhage and another due to slippage of the coronary artery snare during ischaemia). The final numbers in the *in vitro* study were: 11 MLA; 11 vehicle. In the *in vivo* study, final numbers were: 7 MLA; 7 vehicle. For HSP70i analysis the numbers in each group were: 3 MLA; 3 vehicle; 2 lipopolysaccharide; 2 saline; 2 heat shock.

In vitro *study*

Mechanical function Left ventricular end diastolic pressure was identical in MLA and vehicle-treated groups at the end of the stabilization period (MLA 9.8 ± 0.4 mmHg, vehicle 9.8 ± 0.6 mmHg) and developed pressure was not different in the two groups (MLA 102.7 ± 3.4 mmHg, vehicle 94.3 ± 5.4 mmHg). A rise in end diastolic pressure during ischaemia, continued during reperfusion, and was similar in the two groups (Figure 1a). At the end of 60 min reperfusion, end diastolic pressure was 61.8 ± 8.2 mmHg in MLA-pretreated hearts and 64.6 ± 7.4 mmHg in vehicle-pretreated hearts. Recovery of myocardial contractility was expressed as

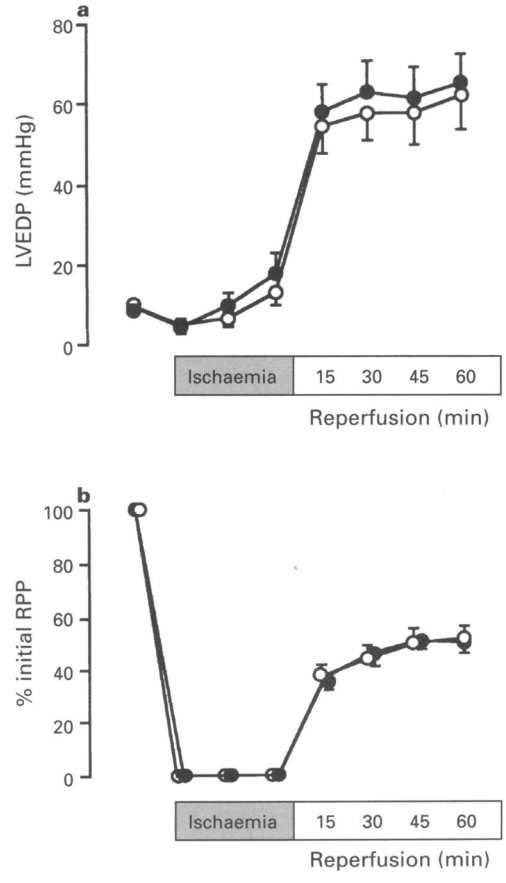

Figure 1 Changes in (a) left ventricular end diastolic pressure (LVEDP) and (b) rate pressure product (RPP) during 20 min ischaemic and 60 min reperfusion in buffer-perfused hearts from vehicle (●) and MLA (○) pretreated rabbits. Twenty four hours after pretreatment, hearts were Langendorff-perfused at 45 ml min^{-1}. After stabilization, zero-flow global ischaemia was induced for 20 min with endocardial temperature maintained at 37°C. At all points during the ischaemia-reperfusion protocol these mechanical parameters were similar in the two groups. Data points are mean ± s.e.mean ($n = 11$ in each group).

rate pressure product (i.e. heart rate × developed pressure) to normalize for slight variations in heart rate between the experimental groups. At all timepoints this index was similar in both groups (Figure 1b). At 60 min reperfusion there was 50.7±5.2% recovery of rate-pressure product in MLA-pretreated hearts compared with 49.8±3.3% recovery of rate pressure product in vehicle-pretreated hearts.

Coronary perfusion pressure Despite identical coronary flow rate (45 ml min^{-1}), coronary perfusion pressure was significantly lower in MLA pretreated hearts compared with vehicle-treated hearts. This difference was observed at baseline (MLA 53.8±1.9 mmHg, vehicle 68.7±2.7 mmHg, $P<0.01$) and remained significant throughout the period of reperfusion when coronary perfusion pressure rose steadily in both groups (Figure 2).

Myocardial lactate dehydrogenase efflux and infarction Lactate dehydrogenase leakage was similar at the end of the stabilization period and during the first 60 min of reperfusion in both groups (Figure 3a). The volume of infarction expressed as a percentage of left ventricle volume was similar in each group (MLA 22.4±5.2%, vehicle 24.8±5.1%, Figure 3b). The volume of myocardium at risk i.e. left ventricular volume, was not different in the two groups (MLA 2.7±0.1 cm^3, vehicle 2.9±0.1 cm^3).

In vivo *study*

Systemic haemodynamics during infarct protocol Table 1 describes alterations in heart rate, mean arterial pressure, RPP (=systolic arterial pressure × heart rate), arterial pH and blood gas tensions during the infarction protocol. Twenty four hours after pretreatment with MLA or vehicle, there were no differences in any of these variables between the experimental groups. There was a decrease in blood pressure in both groups within a few minutes of coronary occlusion. RPP remained similar in each group throughout the infarct protocol. At one time point (5 min ischaemia) a significant difference was observed in heart rate between the groups but the reason for this difference and its implications are unknown. The overall similarity of haemodynamic and blood gas parameters in the two groups suggest that these did not contribute to the marked differences in infarct size described below.

Infarct limitation Absolute infarct volume was reduced from 0.48±0.07 cm^3 in vehicle-pretreated animals to 0.26±0.08 cm^3 in MLA-pretreated animals ($P=0.06$). Figure 4 is a graphic representation of the percentage of infarction within the risk zone (I/R) which was reduced from 42.6±5.7% to 19.6±4.4% ($P<0.01$). This 54% reduction in infarct size in the MLA pretreated animals clearly indicates delayed protection 24 h after MLA. The reduction in infarct size observed in the MLA pretreated hearts was not related to the volume of myocardium at risk during ischaemia since this was very similar in the two groups (vehicle 1.21±0.16 cm^3, MLA 1.12±0.07 cm^3).

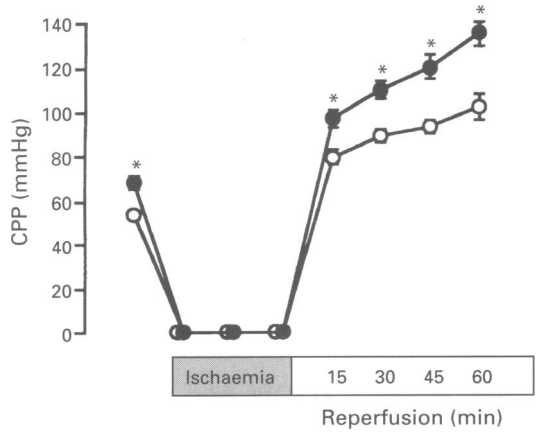

Figure 2 Changes in coronary perfusion pressure (CPP) during perfusion protocol. During stabilization, CPP was significantly lower in hearts from MLA-pretreated rabbits (○) compared with vehicle-treated controls (●). In both groups, reperfusion was associated with a steady increase in CPP although this was consistently lower in MLA-pretreated hearts. Data points are mean±s.e.mean ($n=11$ in each group). *$P<0.01$ (2-way ANOVA and Fisher's test).

Myocardial HSP70i analysis

Figure 5a is an immunoblot of left ventricular myocardial samples harvested 24 h after pretreatments and probed against HSP70i. It is clear that there was no induction of HSP70i 24 h after MLA or vehicle treatments. Positive controls for the Western blot analysis consisted of left ventricular tissue from

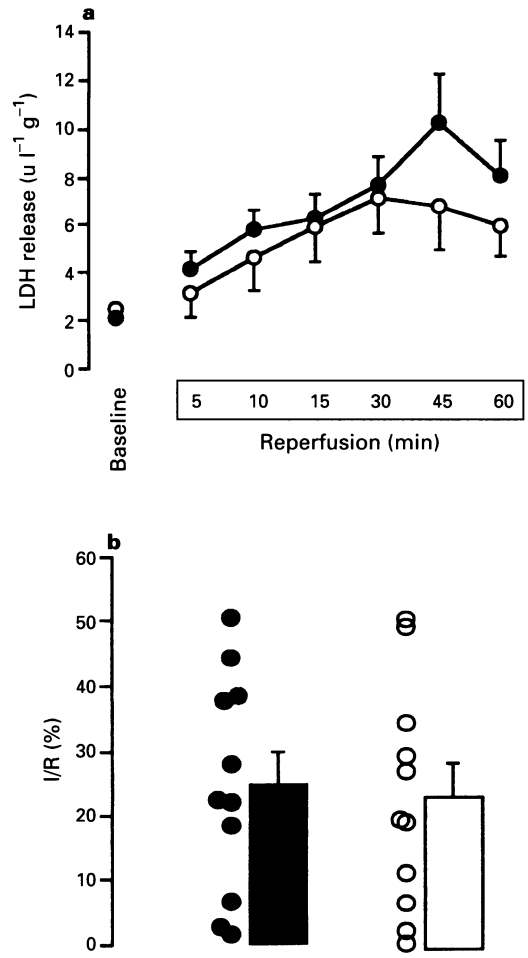

Figure 3 (a) Lactate dehydrogenase (LDH) efflux into the coronary eluent during post-ischaemic reperfusion of isolated buffer-perfused hearts was determined by spectrophotometric assay. Concentration of the enzyme in coronary eluent increased during the first 60 min reperfusion and this rise was similar in both groups (● vehicle, ○ MLA pretreated). Data points are mean±s.e.mean ($n=11$ in each group). (b) Percentage infarction of the left ventricle (I/R) determined by triphenyltetrazolium staining. During global ischaemia the whole of the left ventricle was at risk of infarction. After 20 min global ischaemia and 120 min reperfusion, hearts were stained with triphenyltetrazolium chloride and the infarcted area determined by computerised planimetry. There was no limitation of infarct size in MLA pretreated hearts (○ and open columns) compared with vehicle pretreated hearts (● and filled columns). Histograms represent mean±s.e.mean. Circles show individual data values within each group.

Table 1 Haemodynamic parameters and arterial blood gas and pH measurements during *in vivo* ischaemia-reperfusion protocol (30 min left coronary occlusion followed by 120 min reperfusion)

		Ischaemia		*Reperfusion*		
	Pre-occlusion	*5 min*	*29 min*	*30 min*	*60 min*	*120 min*
Heart rate (beats min^{-1})						
Vehicle	223±6	249±6	259±10	257±12	257±9	259±11
MLA	212±8	227±5*	240±8	231±10	240±12	223±16
Mean arterial pressure (mmHg)						
Vehicle	78.4±4.4	54.7±7.2	62.1±4.1	54.6±5.4	54.9±5.2	56.1±5.5
MLA	78.0±3.0	64.3±6.6	68.7±4.1	63.4±2.0	62.1±3.0	57.6±2.6
Rate pressure product ($mmHg\,min^{-1} \times 10^3$)						
Vehicle	19.9±1.2	16.2±2.3	19.5±1.6	16.7±2.0	17.6±1.4	18.6±1.7
MLA	18.5±1.0	16.7±1.5	19.3±1.6	17.4±1.0	17.7±1.2	17.1±1.5
Arterial pH						
Vehicle	7.40±0.01	7.43±0.01	7.37±0.04	7.42±0.02	7.44±0.01	7.44±0.02
MLA	7.44±0.02	7.43±0.02	7.44±0.01	7.45±0.01	7.46±0.01	7.44±0.01
Arterial P_{O_2} (kPa)						
Vehicle	49.3±5.8	51.6±3.9	49.8±3.8	55.9±3.6	56.8±4.1	63.9±4.0
MLA	49.6±5.8	48.7±5.7	54.1±4.5	57.8±2.2	52.4±1.9	59.6±2.2
Arterial P_{CO_2} (kPa)						
Vehicle	4.6±0.3	4.3±0.2	4.4±0.3	4.1±0.3	4.0±0.2	4.1±0.2
MLA	4.4±0.5	3.4±0.2*	3.4±0.2*	3.7±0.2	3.4±0.3	3.8±0.2

Data are mean±s.e.mean. $n = 7$ in each group. *$P<0.05$ compared with vehicle pretreated control (2-way ANOVA and Fisher's test)

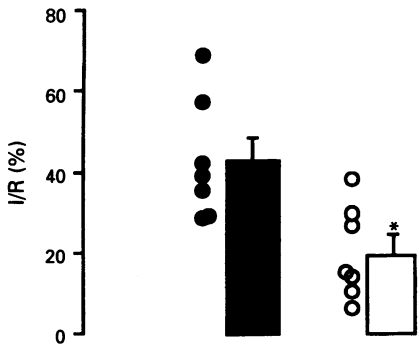

Figure 4 Percentage infarction of the risk zone (I/R) determined by triphenyltetrazolium staining after 30 min ischaemia and 120 min reperfusion *in vivo*. Risk volume was determined by fluorescent microsphere exclusion and was similar in both groups. Significant reduction in I/R was seen in animals pretreated with MLA (○ and open columns) compared with vehicle-treated controls (● and filled columns). Histograms represent mean±s.e.mean. Circles show individual data values within each group. $n=7$ in each group. *$P<0.01$ (Student's unpaired t test).

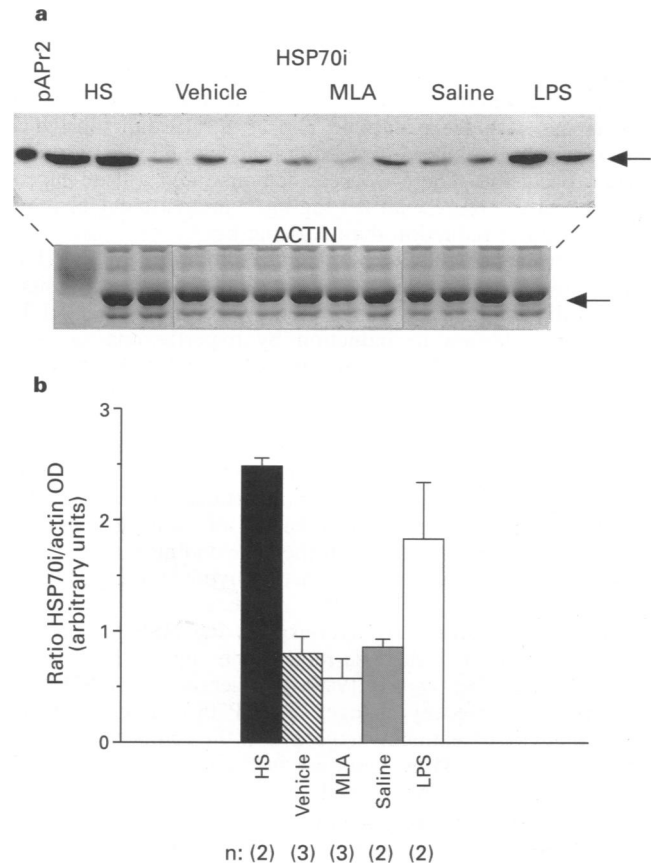

Figure 5 (a) Western blot probed against HSP70i. Left ventricular samples were obtained 24 h after treatment with vehicle, MLA, lipopolysaccharide (LPS), saline or heat shock (HS) (for details see text). Total protein loading was similar in all lanes (Coomassie blot not shown). HSP70i band and actin band are marked with arrows. (b) The histogram shows mean±s.e.mean optical density readings for each group (ratio of HSP70i band to actin band). MLA did not increase HSP70i immunoreactivity whereas there was clear induction of HSP70i following LPS and HS treatments.

rabbits pretreated with either heat shock or *S. minnesota* lipopolysaccharide, 0.05 mg kg^{-1}. In contrast to MLA, both heat stress and lipopolysaccharide pretreatments caused marked induction of HSP70i compared with respective saline-treated controls.

Discussion

The current studies were undertaken to explore the mechanisms of low-dose MLA-induced cardioprotection further. Our hypothesis was that MLA acted directly on the myocardium to confer protection against ischaemia-reperfusion injury, through direct enhancement of the expression of HSP70i. If this were so it would be expected that protection would be evident both in an isolated heart preparation and *in vivo*. These

studies show that pretreatment of the rabbit with this dose of MLA, previously shown to be protective in the dog, conferred protection against ischaemia-reperfusion injury *in vivo* but not *in vitro*. Reduction in infarct size *in vivo* was not related to myocardial risk volume nor to systemic haemodynamic status. Furthermore, this delayed protection was not associated with increased HSP70i immunoreactivity in the myocardium.

The failure to observe cardioprotection in the rabbit isolated heart in this study is in contrast to the work of Nelson *et al.* (1991) who showed that there was significant protection against ischaemia-reperfusion injury in the isolated rat heart 24 h after MLA pretreatment. There are two possible reasons for this discrepancy. First, the susceptibility of rat and rabbit myocardium to ischaemia is known to differ (Hearse *et al.*, 1976; Ytrehus *et al.*, 1994). Secondly, and we feel more importantly, the MLA dose used by Nelson and co-workers (5 mg kg^{-1}) was 150 fold greater than that used in the present study. The dose selected in our study (0.035 mg kg^{-1}) was within the range shown to confer protection in the dog *in vivo* (Yao *et al.*, 1993a,b) and in the rabbit *in vivo* (Zhao *et al.*, Ribi ImmunoChem, unpublished observations). Ribi (1984) reported that MLA was 100 times less pyrogenic than the parent endotoxin molecule. It is possible therefore that at the dose used by Nelson *et al.* (1991) the toxicity, or at least the pyrogenicity of MLA, would be of the same order of magnitude as that associated with endotoxin. We would suggest that the protection observed in that study was associated with the oxidative stress response normally induced by sublethal doses of conventional endotoxin. This conclusion is further supported by the observation that myocardial catalase activity was increased in the study of Nelson *et al.* (1991) whereas Yao *et al.* (1993a,b) did not observe statistically significant increases in either catalase or SOD activity following low-dose MLA treatment in dogs. The results of our ischaemia-reperfusion study conducted *in vivo*, showing marked infarct size reduction following 30 min coronary artery occlusion, are fully in agreement with the results of Yao *et al.* (1993a,b) in which infarct size was reduced in the dog following 60 min coronary artery occlusion. These workers demonstrated a dose-dependent effect of MLA with 0.1 mg kg^{-1} pretreatment giving a greater infarct reduction than 0.03 mg kg^{-1}.

The protection that we observed *in vivo* following low-dose MLA treatment was clearly not associated with HSP70i since myocardial content of this protein was not augmented by MLA. In addition to induction by hyperthermia as mentioned earlier, HSP70i is known to be induced in myocardium by several other stimuli including ischaemia (Dillmann *et al.*, 1986; Knowlton *et al.*, 1991; Marber *et al.*, 1993) and is a marker of the stress response in tissues. Moreover, there is accumulating evidence that the protein is directly cytoprotective and is able to confer resistance to myocardial ischaemia. For example, transfection of isolated myocytes and myocyte-derived cells with the gene coding for HSP70i is associated with enhanced tolerance to hypoxic injury (Mestril *et al.*, 1994; Heads *et al.*, 1995), and hearts from transgenic mice which constitutively over-express the HSP70i gene are more resistant to ischaemia-reperfusion injury (Radford *et al.*, 1994; Marber *et al.*, 1995; Plumier *et al.*, 1995). The failure to observe any changes in HSP70i content following low-dose MLA treatment suggests that the compound did not induce the classic stress response in the myocardium of pretreated animals. It was noted that treatment with conventional endotoxin (*S. minnesota* lipopolysaccharide) and thermal stress, used here as positive controls for the HSP70i assay, *did* markedly elevate the protein content. Our observation that endotoxin treatment elevates myocardial HSP70i content confirms the report of Brown *et al.* (1993). These workers demonstrated that treatment of rats with endotoxin 0.5 mg kg^{-1} (a dose 10 fold greater than that used here) was associated with the elevation of myocardial HSP70i mRNA and protein. Three days following endotoxin pretreatment, isolated hearts were found to be more resilient to ischaemia-reperfusion and this protective effect was lost when endotoxin pretreatment was preceded by cycloheximide treatment, suggesting that the protection was dependent on *de novo* protein synthesis (Brown *et al.*, 1993).

The pyrexia produced by conventional endotoxins is well recognised and is probably related to the induction of cytokines (Kluger, 1990). Pretreatment with cytokines, including tumour necrosis factor and interleukin-1α have been demonstrated to enhance myocardial anti-oxidant defences and lead to reduced ischaemia-reperfusion injury (Brown *et al.*, 1992; Maulik *et al.*, 1993). Pyrexia *per se* might also be associated with the induction of HSP's. MLA 0.065 mg kg^{-1} was found by Yao *et al.* (1993b) to cause a very mild and transient pyrexia in dogs (approximately 1°C) and they have suggested that this elevation of temperature could lead to the induction of HSP's. While this is a possibility, experimental heat shock protocols that result in HSP70i induction usually involve elevations of core temperature of $\geqslant$3°C above normal body temperature (Currie *et al.*, 1988; 1993; Marber *et al.*, 1993; Hutter *et al.*, 1994). We were not able to measure core temperature following MLA in the conscious rabbits in our study but close examination of the animals after MLA treatment revealed no perceptible alterations in behaviour, heart rate, or respiratory pattern. The two rabbits treated with *S. minnesota* lipopolysaccharide became sluggish within 30–45 min of its administration but did not show any of the characteristics of severe pyrexia that we observed during heat shock, namely profound vasodilatation, tachycardia, tachypnoea and sweating. Thus, while endotoxin treatment increased myocardial HSP70i content it is not clear if this was a consequence of pyrexia. It may well be the case that higher doses of MLA, like endotoxin, induce HSP70i and anti-oxidant synthesis as part of a generalized cell stress response but we would expect these higher doses to be associated with manifestations of endotoxin-like toxicity.

The facts (1) that we did not observe any elevation of HSP70i content after MLA and (2) that we observed protection against ischaemia-reperfusion *in vivo* but not *in vitro*, lead us to believe that the delayed myocardial protection induced by MLA is not the consequence of some direct enhancement of myocardial resilience to ischaemia but, rather, the protection may be the result of actions affecting neuronal, humoral or blood-borne factors that play some role in the evolution of myocardial infarction *in vivo*. In this connection, two studies by Yao *et al.* (1993a,b) are of interest for they suggest a possible attenuation of neutrophil activity 24 h after MLA treatment. The role of the neutrophil in ischaemia-reperfusion injury, particularly with regard to the development of irreversible ischaemic injury, has been an area of keen debate during the last decade (Reimer *et al.*, 1989; Lucchesi *et al.*, 1989; Hanssen 1995). Yao and colleagues demonstrated that 24 h pretreatment of dogs with MLA (0.03–0.1 mg kg^{-1}) conferred enhanced tolerance to 60 min left circumflex occlusion and 5 h reperfusion. Marked reductions in infarct size (determined by triphenyltetrazolium staining) were observed. After 5 h reperfusion, tissue myeloperoxidase activity in the infarct border zone, an index of neutrophil infiltration in ischaemic myocardium (Mullane *et al.*, 1985), was determined. Significant reductions in border zone myeloperoxidase activity were seen 24 h after treatment with MLA 0.03 and 0.1 mg kg^{-1} (Yao *et al.*, 1993a) and MLA 0.065 mg kg^{-1} (Yao *et al.*, 1993b). These data suggested that MLA may confer protection by attenuating neutrophil activity at the border zone of the evolving infarct. The interesting observation that border zone myeloperoxidase activity was not attenuated in the hearts of dogs treated 1 h before ischaemia-reperfusion (Yao *et al.*, 1993b) corresponds with the fact that no anti-infarct effect was observed following this short pretreatment.

The mechanism by which MLA exerts an anti-neutrophil and anti-infarct effect 24 h, but not 1 h after treatment, is not clear but this time-course would be consistent with an effect involving protein synthesis. Treatment with conventional endotoxin is known to increase the *de novo* synthesis of the in-

ducible form (calcium-independent) of nitric oxide synthase (iNOS) (Knowles *et al.*, 1990a; Julou-Schaeffer *et al.*, 1991), an effect which can be inhibited by dexamethasone (Knowles *et al.*, 1990b; Song *et al.*, 1994). Recently, Song *et al.* (1994) have shown that *Escherichia coli* endotoxin administration to rats 8 h, but not 1 h, before coronary artery occlusion *in vitro* was markedly anti-arrhythmic. This protection was abolished by pretreatment with dexamethasone suggesting that the induction of iNOS may be a key event in the mediation of this protection. If it is the case that MLA shares this ability of endotoxin to increase iNOS synthesis, this action could contribute to the apparent anti-neutrophil effect of the compound (Yao *et al.*, 1993b). To date there is no direct evidence that MLA increases NOS activity in myocardium or coronary vessels. However, the observation in our *in vitro* study that MLA pretreated hearts exhibited a modest but significantly reduced coronary perfusion pressure throughout the Langendorff perfusion procedure suggests that there was a marked coronary vasodilatation in these hearts compared with hearts from vehicle-treated animals. Further studies are warranted to explore this observation, to determine the effects of MLA on iNOS activity, and to examine the effects of dexamethasone on MLA-induced cardioprotection *in vivo*.

In conclusion, we have shown that 24 h pretreatment of rabbits with low-dose MLA reduces infarct size in a rabbit *in vivo* model of ischaemia-reperfusion. This protective effect was not associated with changes in myocardial HSP70i content. No reduction in ischaemic injury was observed in a model of ischaemia-reperfusion *in vitro* and this observation provides strong support for the proposal that the delayed myocardial protection observed after low-dose MLA treatment could be mediated by actions on a blood-borne factor, possibly the neutrophil. Previous studies with higher doses of MLA observed protection in the rat heart *in vitro* and it may well be the case that the pattern of myocardial protection observed with this compound are related to species, dose and model. However, the protection observed *in vivo* after low-dose treatment in the rabbit suggests that MLA could be amenable to clinical use in man prior to procedures involving scheduled ischaemia, such as cardiac surgery. Further studies are required to explore the potential of this compound as an anti-ischaemic agent and its intriguing mechanisms of action.

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