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Monophosphoryl lipid A provides biphasic cardioprotection against ischaemia-reperfusion injury in rat hearts

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1 We utilized a rat model of myocardial infarction to investigate whether cardioprotection by monophosphoryl lipid A (MLA) is provided in the early and late phases, as well as to determine whether this cardioprotection may be related to the activation of manganese superoxide dismutase (Mn-SOD), an intrinsic radical scavenger.

2 Pretreatment with MLA (0.5 or 1.0 mg kg^{-1}, i.v.) 24 h prior to 20-min left coronary artery (LCA) occlusion and 48-h reperfusion significantly decreased the incidence of ventricular fibrillation (VF) during ischaemia, as well as infarct size. Pretreatment with lower concentrations of MLA, however, was ineffective.

3 When we examined the time course of MLA (0.5 mg kg^{-1})-induced cardioprotection, both infarct size and the incidence of VF were significantly reduced in rats pretreated with MLA 0.5 h and 24 h before occlusion. We observed no differences, however, 2 and 72 h after MLA treatment.

4 The activity of Mn-SOD paralleled the cardioprotective effects of MLA. Mn-SOD activity in the myocardium was significantly enhanced in rats pretreated with MLA (0.5 mg kg^{-1}) 0.5 and 24 h before. Mn-SOD activity was not altered, however, in rats pretreated 2 or 72 h before. Lower MLA concentrations were not effective even 24 h after the treatment.

5 We conclude that MLA treatment induced a biphasic pattern of cardioprotection. The pattern of Mn-SOD activity suggests that this enzyme may play a major role in the acquisition of cardioprotection against ischaemia-reperfusion injury.

Keywords: Monophosphoryl lipid A (MLA); rat heart; myocardial infarction; ventricular fibrillation; manganese superoxide dismutase (Mn-SOD)

Abbreviations: iNOS, inducible nitric oxide synthase; LCA, left coronary artery; MLA, monophosphoryl lipid A; Mn-SOD, manganese superoxide dismutase; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; VF, ventricular fibrillation

Introduction

Classical ischaemic preconditioning is a phenomenon in which a brief episode of ischaemia protects the heart against the effects of an ensuing prolonged lethal ischaemia (Murry *et al*., 1986). While these cardioprotective effects of classical ischaemic preconditioning have been observed to last 1–2 h in dogs, we (Kuzuya *et al*., 1993) and others (Marber *et al*., 1993) have shown that the beneficial effects of preconditioning reappeared 24 h after the initial ischaemia in animal occlusion-reperfusion models of myocardial infarction. This phenomenon has been called delayed myocardial protection.

Monophosphoryl lipid A (MLA) is a derivative of lipopolysaccharide that lacks many of the endotoxic properties of the parent molecule. It possesses less than 1% of the pyrogenic activity of endotoxin (Ribi, 1984). Sublethal doses of endotoxin have been shown to increase myocardial tolerance to a subsequent challenge with ischaemia and reperfusion (Brown *et al*., 1989; Song *et al*., 1994). MLA has also been shown to retain some of the beneficial properties of endotoxin: MLA induces delayed myocardial protection against ischaemia-reperfusion induced infarction, arrhythmia and myocardial stunning in rats (Nelson *et al*., 1991), rabbits (Baxter *et al*., 1996; Zhao *et al*., 1997) and dogs (Yao *et al*., 1993a,b; Przyklenk *et al*., 1996; Vegh *et al*., 1996).

The development of endotoxin induced tolerance to ischaemia-reperfusion may be related to its ability to augment the intracellular antioxidant defences. 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 SOD (Mn-SOD) in rat liver (Dougall & Nick, 1991). In addition, delayed myocardial protection following various stimuli was found to correlate with the induction of Mn-SOD (Hoshida *et al*., 1993; Yamashita *et al*., 1994; 1996; 1997a) and with increased myocardial catalase activity (Nelson *et al*., 1991). In contrast, statistically significant increases in either catalase or total SOD activity were not detected following low-dose MLA treatment in dogs (Yao *et al*., 1993b). Treatment of humans and mice with MLA has been observed to produce transient increases in tumor necrosis factor-α, which is thought to be a potent inducer of endogenous mitochondrial Mn-SOD (Fujii & Taniguchi, 1991; Nelson *et al*., 1995). However, the effect of MLA on myocardial Mn-SOD activity is not known. We therefore utilized a rat model of myocardial infarction to investigate the mechanism by which MLA protects against injury. Specifically, we sought to determine whether MLA induces early phase, as well as later phase, cardioprotection,

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and whether this protection may be related to the activation of endogenous antioxidant enzymes, especially Mn-SOD.

Methods

Experimental protocol

Male wistar rats (300–350 g) were maintained in a 12-h dark/light cycle, housed at 23±1.5°C with 45±15% relative humidity, and fed and watered *ad libitum*. MLA was dissolved in saline containing 0.2% triethylamine according to its solubilization instruction. The animals were lightly anaesthetized with sodium pentobarbitone (5–10 mg kg^{-1}, i.p.), and were pretreated with i.v. boluses of either MLA (0.035, 0.10, 0.5, and 1.0 mg kg^{-1}) or vehicle *via* the right femoral vein. Rats were allowed to recover from the administration of MLA or vehicle for various time intervals (0.5, 2, 24 or 72 h) before being subjected to myocardial infarction. The myocardial activity of Mn-SOD was determined 0.5, 2, 24, or 72 h after the administration of MLA or vehicle. All the experiments were conducted sequentially and the treatment was undertaken in a randomized fashion.

The infarct procedure was performed as previously described (Hoshida *et al*., 1996; Yamashita *et al*., 1997b). Aseptic surgical procedures were used throughout this protocol. At various time intervals (0.5, 2, 24 or 72 h) after the administration of MLA or vehicle, the rats were anaesthetized with sodium pentobarbitone (25 mg kg^{-1}, i.p.); additional sodium pentobarbitone (5–10 mg kg^{-1}, i.p.) was given as required. They were subsequently intubated and ventilated with a small-animal respirator (model SN-480-7-10; Shinano Seisakusyo, Tokyo, Japan), at a rate of 60–70 cycles min^{-1} and a tidal volume of 1 ml 0.1 kg^{-1} body weight, which volume can maintain arterial pH between 7.35 and 7.50. Using polyethylene tubes, the left femoral artery was cannulated for the continuous measurement of arterial blood pressure with a pressure transducer (TP-300T; Nihon Kohden, Tokyo, Japan). The heart rate, the incidence of arrhythmias, and ST-segment changes were monitored and the haemodynamic variables were continuously recorded (model WT-645G recorder; Nihon Kohden, Tokyo, Japan).

Silk thread (7-0) was passed around the left coronary artery (LCA) of each rat, about 3–4 mm distal to the LCA origin. After a 10-min stabilization period, the arterial pressure was measured with a transducer *via* the femoral artery cannula, and the LCA was ligated. After 20 min of coronary occlusion, the snare was released; reperfusion was indicated by a change in the colour of the ventricular surface. The surgical wounds were repaired 60 min after reperfusion, and the rats were returned to their cages to recover. Benzylpenicillin (30,000 u kg^{-1}) was injected intramuscularly as prophylaxis against infection.

Arrhythmias were monitored by ECG. Ventricular fibrillation (VF) was defined according to the criteria of the Lambeth Conventions (Walker *et al*., 1988). If VF did not spontaneously resolve within 3 s, manual cardioversion was attempted by gentle flicking of the nonischaemic region of the heart. Rats in which VF continued for more than 6 s or for which cardioversion had to be performed more than three times, were excluded from analysis of infarct size.

Determination of infarct size

The size of the infarct was measured by previously reported method (Yamashita *et al*., 1997a; 1998). Forty eight hours after reperfusion, the rats were reanaesthetized with sodium pentobarbitone (25 mg kg^{-1}, i.p.), intubated and ventilated with a respirator. After the heart was exposed and the LCA was reoccluded, Evans blue dye (2%) was injected *via* the right femoral vein to estimate the area perfused by the occluded artery (ischaemic region). The left ventricle was then cut into six pieces perpendicular to the apex-base axis, and the specimens were incubated with 1% triphenyltetrazolium chloride at 37°C to stain the noninfarcted region. The ischaemic, infarcted, and nonischaemic areas of tissue were separated with scissors and weighed. The area at risk and the infarct size were defined as the ratios of the mass of the ischaemic region to the left ventricular mass and the mass of the infarct region to the mass of the ischaemic region, respectively, and are expressed as percentages.

Sampling of the myocardial tissue

To obtain tissue samples for measurement of SOD activity, rats that were treated with MLA or vehicle but did not receive coronary occlusion/reperfusion were sacrificed at the appropriate times with an overdose of sodium pentobarbitone. The myocardial tissue was rinsed in phosphate-buffered saline (PBS), and the atria and right ventricle were removed. The left ventricular myocardial samples were rapidly frozen in liquid nitrogen and stored at −80°C. To measure myocardial SOD activity, blood was washed out of the left and right coronary arteries from the ascending aorta with an adequate volume of PBS before taking myocardial tissue samples.

Measurement of myocardial SOD activities

Total SOD activity of the myocardial samples was determined by the nitroblue tetrazolium (NBT) method (Hoshida *et al*., 1993; Yamashita *et al*., 1994). Myocardium was homogenized in 20 mM PBS, 1 mM EDTA and centrifuged at 900×*g* for 15 min. The supernatant was sonicated and incubated with NBT and xanthine-xanthine oxidase, and the SOD activity in the supernatant was measured colorimetrically. To determine the Mn-SOD activity, the assay was repeated in the presence of potassium cyanide (1 mM), an inhibitor of copper, zinc-superoxide dismutase (Cu, Zn-SOD). Mn-SOD activity was calculated by subtracting Cu, Zn-SOD activity from total SOD activity. Mn-SOD activity was corrected for the concentration of protein in the supernatant.

Materials

MLA was purchased from Ribi ImmunoChem Research (Hamilton, MT, U.S.A.). Other chemicals were purchased from Sigma Immunochemicals (St. Louis, MO, U.S.A.), and Wako (Osaka, Japan).

Statistics

All values are expressed as means±s.e.mean. Differences in haemodynamic parameters during the ischaemia-reperfusion protocol were assessed with respect to time and treatment by repeated measures analysis of variance (ANOVA) followed by Fisher's protected least significant difference method. Comparisons in infarct size, area at risk, and Mn-SOD activity between groups were assessed by one-way ANOVA with Bonferroni's *post hoc* test for multiple comparisons. Incidence of ventricular fibrillation was compared using a chi-squared test with Yates' correction. A level of $P<0.05$ was defined as statistically significant.

Results

Exclusion because of VF or death

Six animals (three in the vehicle group, three in the MLA group) developed intractable VF and were excluded from evaluation of myocardial infarct size, and nine animals (four in the vehicle group, five in the MLA group) died before completion of the experimental protocol.

Haemodynamic data, area at risk, and rectal temperature

We observed no significant differences in rate-pressure product (Tables 1 and 2) or in the rectal temperature during the infarct protocol among the group of rats before ischaemia, at the end of the ischaemic period, and 0.5 h after reperfusion (data not shown). In addition, the size of the anatomical area at risk, expressed as a percentage of the left ventricular area did not differ significantly among these groups of animals (Figures 1 and 2).

Incidence of VF

The incidence of VF at reperfusion was low (20%) in the control group in this *in vivo* rat infarct model. On the other hand, the incidence of VF during ischaemia was very high (100%, Figure 2). Therefore, we examined whether tolerance to VF during ischaemia was acquired 24 h after MLA treatment. We found that the incidence of VF during ischaemia was markedly reduced after administration of 0.5 or 1.0 mg kg^{-1} MLA, whereas lower concentrations were not effective (Figure 1).

When we assayed the time course of tolerance to VF during ischaemia, we observed that treatment with 0.5 mg kg^{-1} MLA resulted in a biphasic tolerance curve (Figure 2). After 0.5 h, the incidence of VF was reduced by 70%, but returned to control levels after 2 h. Tolerance to VF during ischaemia reappeared 24 h after MLA treatment and again disappeared at 72 h (Figure 2). In the rats administered vehicle, however, the incidence of VF did not change significantly over time (Figure 2).

Size of myocardial infarction

When we measured the size of the myocardial infarction 24 h after MLA treatment, we found that administration of 0.5 or 1.0 mg kg^{-1} MLA markedly reduced infarct size (Figure 1). In contrast, lower concentrations of MLA had no effect on the size of the infarct (Figure 1).

We also observed that, when assessed as a function of time following administration of 0.5 mg kg^{-1} MLA, the size of the infarct was reduced in a biphasic pattern (Figure 2). As with the incidence of VF, the first phase of protection occurred 0.5 h after MLA treatment, disappeared at 2 h, reappeared after 24 h, and again disappeared 72 h after MLA administration (Figure 2). In the rats administered vehicle, infarct size was not significantly altered as a function of time (Figure 2).

Activation of Mn-SOD after MLA treatment

When we assayed Mn-SOD activity in rat myocardium 24 h after MLA treatment, we found that the activity of this enzyme was significantly higher in rats administered 0.5 or 1.0 mg kg^{-1} MLA (Figure 3). In contrast, Mn-SOD activity was not altered in rats treated with 0.035 or 0.10 mg kg^{-1} MLA (Figure 3).

As a function of time, we observed a biphasic alteration in myocardial Mn-SOD activity. The activity of this enzyme increased 0.5 h after administration of MLA, declined after 2 h, increased again after 24 h, and then decreased after 72 h (Figure 4). In contrast, Mn-SOD activity remained constant over time in rats administered vehicle (Figure 4). The activity of the cytosolic isoform of SOD (Cu, Zn-SOD) did not change

Table 1 Rate-pressure product with time in the groups pretreated with MLA or vehicle 24 h prior to coronary occlusion

Group	*Rate-pressure pre-occlusion*	*Product 20-min occlusion*	(mmHg $min^{-1} \times 10^3$) *30-min reperfusion*
Vehicle control	45.6±3.7	40.3±3.7	44.1±4.4
MLA (0.035 mg kg^{-1})	46.2±3.4	43.0±4.6	45.8±4.3
MLA (0.10 mg kg^{-1})	43.2±3.0	38.9±3.5	44.1±3.1
MLA (0.5 mg kg^{-1})	47.7±2.9	39.3±3.9	40.1±3.2
MLA (1.0 mg kg^{-1})	42.1±3.7	40.3±4.1	42.6±3.4

Data are means±s.e.mean. *n*: 7–10 in each group. There was no significant difference between groups at each time point.

Table 2 Rate-pressure product with time in the groups pretreated with MLA (0.5 mg kg^{-1}) or vehicle 0.5–72 h prior to coronary occlusion

Group	*Rate-pressure pre-occlusion*	*Product 20-min occlusion*	(mmHg $min^{-1} \times 10^3$) *30-min reperfusion*
Control	48.1±4.4	43.6±4.6	45.8±3.9
Vehicle 0.5 h	44.4±3.6	40.9±2.9	43.5±3.4
MLA 0.5 h	41.5±3.4	39.6±3.7	39.3±3.7
Vehicle 2 h	43.6±3.2	39.4±3.6	42.8±4.6
MLA 2 h	42.6±3.0	37.3±4.0	40.0±4.3
Vehicle 24 h	45.6±3.7	40.3±3.7	44.1±4.4
MLA 24 h	47.7±2.9	39.3±3.9	40.1±3.2
Vehicle 72 h	47.2±3.9	44.9±3.7	45.2±3.9
MLA 72 h	45.8±3.9	38.3±4.4	43.2±4.3

Data are means±s.e.mean. *n*: 5–10 in each group. There was no significant difference between groups at each time point.

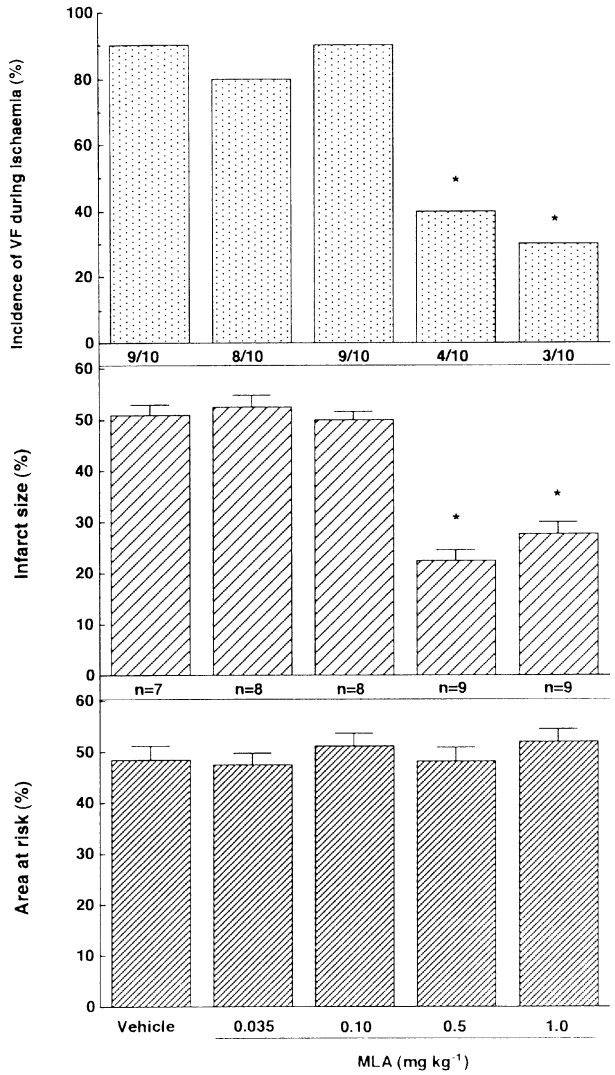

Figure 1 Cardioprotective effects of MLA administered 24 h prior to coronary occlusion. Twenty-four hours after administration of MLA or vehicle, the coronary artery was ligated for 20 min and reperfused. (Upper panel) Incidence of VF during ischaemia. (Middle panel) Myocardial infarct size. (Lower panel) Area at risk in each group. Seven to ten rats were examined in each group. $*P<0.05$ vs vehicle group.

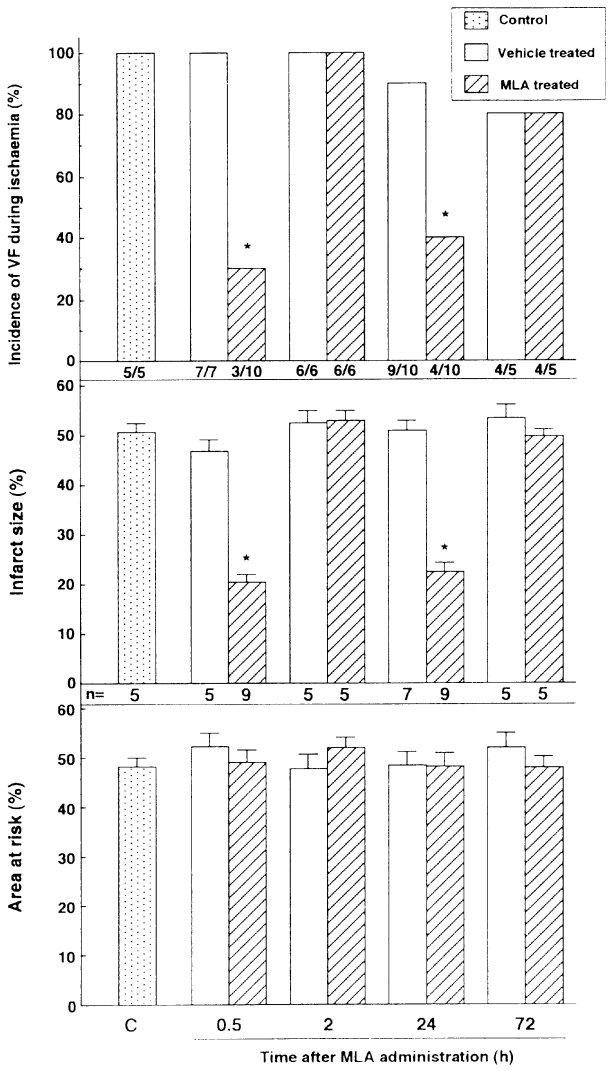

Figure 2 Time course of MLA-induced cardioprotective effects. At various times after treatment with MLA (0.5 mg kg^{-1}) or vehicle, the coronary artery was ligated for 20 min and reperfused. Open columns: vehicle groups; hatched columns: MLA-treated groups. C, control group. Five to ten rats were examined in each group. $*P<0.05$ vs corresponding vehicle group.

over time in the rats treated with either MLA or vehicle (data not shown).

Discussion

We have shown here that the pretreatment of rats with MLA induces biphasic protection against myocardial ischaemia-reperfusion injury, evidenced by reduced incidence of ischaemia-induced VF and a decrease in myocardial infarct size. This pattern of MLA-induced cardioprotection in which protection occurred 0.5 and 24 h after MLA administration but not at intermediate times was similar to that observed in ischaemic preconditioning (Kuzuya *et al.*, 1993; Marber *et al.*, 1993) and whole-body hyperthermia (Yamashita *et al.*, 1998).

In this study, a significant reduction in infarct size and a marked protection against ischaemia-induced VF were observed 0.5 h but not 2 h (early phase), and 24 h but not 72 h (delayed phase) after MLA administration in a biphasic manner. We also examined whether delayed protection was still present if MLA had been given 48 h before ischaemia in some animals. The cardioprotection already disappeared 48 h after MLA administration (vehicle treated: $n=3$, MLA treated: $n=3$, data not shown). However, Baxter *et al.* (1997) have reported that delayed cardioprotection induced by ischaemic preconditioning in *in vivo* rabbit model was prolonged, extending between 24 and 72 h after ischaemic preconditioning. The apparent discrepancy may be explained by differences in the nature and magnitude of the prior stimuli. For example, we have recently reported that the late-phase cardioprotection induced by whole-body hyperthermia shifted to a later period as the magnitude of the prior heat stress increased (Yamashita *et al.*, 1998).

Using isolated rat hearts, Nelson *et al.* (1991) have shown that treatment with MLA 24 h prior to global ischaemia resulted in better preservation of post-ischaemic ventricular function, however, that cardioprotective effect was not observed 2 h after MLA treatment. Moreover, in a canine

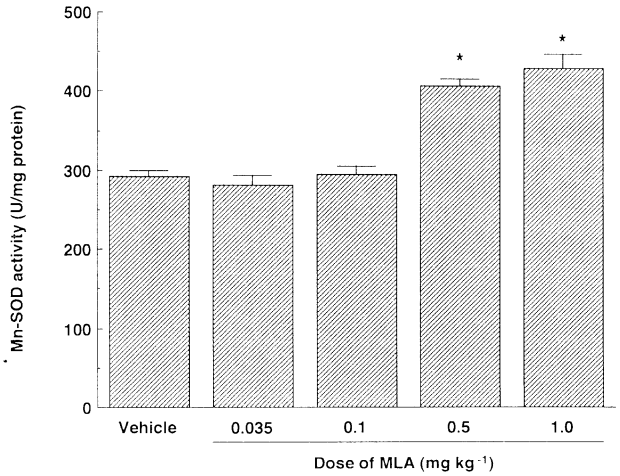

Figure 3 Manganese superoxide dismutase (Mn-SOD) activity in myocardial tissue 24 h after MLA treatment. Myocardial Mn-SOD was measured in rats 24 h after administration of vehicle or various concentrations of MLA. Four to five rats were examined in each group. $*P<0.05$ vs vehicle group.

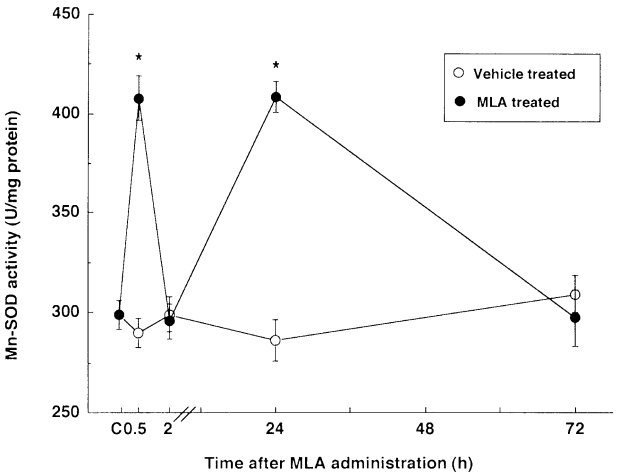

Figure 4 Time course of MLA-induced alterations in myocardial Mn-SOD activity. Myocardial Mn-SOD activity was measured in rats at various times after administration of 0.5 mg kg^{-1} MLA. Open circles, vehicle group; closed circles, MLA-treated group. C, control group. Each point represents the means ± s.e.mean of four to five rats. $*P<0.05$ vs corresponding vehicle group.

model pretreatment with MLA 1 h before ischaemia did not produce a significant reduction in myocardial infarct size (Yao *et al*., 1993b). These results, together with our finding that cardioprotection occurred 0.5 h after MLA treatment but disappeared after 2 h, suggest that as in ischaemic preconditioning, the early phase of MLA-induced cardioprotection may be transient. Elliott's group has also reported transient early protection following MLA in the rabbit heart (Weber *et al*., 1997).

The mechanism underlying carioprotection occurring after MLA treatment may be related to an increase in Mn-SOD activity. We found that the activity of this enzyme paralleled cardioprotection induced by MLA. We and others (Hoshida *et al*., 1993; Yamashita *et al*., 1994; Zhou *et al*., 1996) have shown that a brief ischaemic or anoxic insult increases Mn-SOD activity and induces cardioprotection or myocyte protection in a biphasic manner, both immediately and 24 h later. Presumably the late increase in Mn-SOD activity is related to increased content of Mn-SOD protein. Total SOD activity, however, did not increase 24 h after low-dose MLA treatment in dogs (Yao *et al*., 1993b). Treatment with MLA has been observed to produce transient increases in tumour necrosis factor-α in humans and mice (Henricson *et al*., 1990; Astiz *et al*., 1995). Cytokines have been shown to induce Mn-SOD in myocardium (Eddy *et al*., 1992; Maulik *et al*., 1993; Nelson *et al*., 1995). However, the causal relation between the MLA-induced cardioprotection and the elevation of Mn-SOD activity remains to be elucidated in the present study.

Pretreatment with 5 mg kg^{-1} MLA 24 h prior to global ischaemia was shown to result in a better preservation of post-ischaemic ventricular function in isolated rat hearts (Nelson *et al*., 1991). In the dog (Yao *et al*., 1993a,b; Przyklenk *et al*., 1996) and rabbit (Baxter *et al*., 1996; Zhao *et al*., 1997) models of myocardial infarction, however, delayed cardioprotection was acquired at much lower doses of MLA (0.035–0.1 mg kg^{-1}). In our rat infarct model, these low doses of MLA were unable to reduce infarct size or to increase Mn-SOD activity. Both the cardioprotection and the increase in Mn-SOD activity were acquired only when higher doses (0.5–1.0 mg kg^{-1}) of MLA were administered. There are two possible reasons for this discrepancy among species. First, the susceptibility of myocardium to ischaemia is known to differ among these species (Hearse *et al*., 1976; Ytrehus *et al*., 1994). Tosaki *et al*. (1998) also have shown that pretreatment with at least 0.45 mg kg^{-1} dose of MLA 24 h prior to global ischaemia was needed to be obtained cardioprotection against both left ventricular function and VF after ischaemia-reperfusion injury in rat. This dose of MLA was very similar to that used in this study. Second, the signal transduction occurring during cardioprotection in the rat may be different than that in rabbits and dogs. For example, ischaemic preconditioning in the rat does not depend on adenosine activation of A_1-receptors (Li & Kloner, 1993), whereas it does in rabbits (Liu *et al*., 1991) and dogs (Auchampach & Gross, 1993).

Recent evidence has suggested that KATP channels may play a role in the delayed cardioprotection induced by MLA. For example, this delayed effect can be inhibited by KATP channel blockers (Elliott *et al*., 1996; Mei *et al*., 1996). In addition, MLA-induced delayed cardioprotection was decreased by treatment with the inducible nitric oxide synthase (iNOS) inhibitor, aminoguanidine (Zhao *et al*., 1997). These findings suggest that MLA may induce iNOS, thereby increasing nitric oxide signalling, which in turn, modulates KATP channels by increasing the second messenger, cyclic GMP (Cameron *et al*. 1996; Maulik *et al*., 1996). Although opening of KATP channels has been suggested to be the end effector in ischaemic preconditioning (Gross & Auchampach, 1992), it has been reported recently, in isolated human atrial muscle, that ischaemic preconditioning abolished the protection against simulated ischaemia and reoxygenation induced by KATP channel openers (Carr & Yellon, 1997). These findings suggest that further elucidation of the relationship between KATP channel opening and Mn-SOD activation in MLA-induced biphasic cardioprotection is necessary.

Although other stimuli that induce delayed cardioprotection such as sublethal ischaemia and whole-body hyperthermia can be associated with increases in Mn-SOD and heat shock protein 72 (Hoshida *et al*., 1993; Marber *et al*., 1993; Yamashita *et al*., 1998), previous work has established that increased heat shock protein 72 content is probably not

involved in MLA protection (Baxter *et al*., 1996). This dissociation of the two proteins in MLA protection remains to be clear, but a signalling pathway for cardioprotection induced by MLA may be somewhat different from that by other sublethal stimuli.

In summary, pretreatment of MLA protected against VF during ischaemia and limited the extent of myocardial infarction after reperfusion both immediately and 24 h after administration. The time course of the protective effect is similar to that for increases in myocardial Mn-SOD activity. This pattern of Mn-SOD activity suggests that activation of this enzyme may play a major role in the acquisition of cardioprotection against ischaemia-reperfusion injury. However, it remains to be clarified if Mn-SOD is directly associated with the cardioprotection against ischaemia-reperfusion in *in vivo* myocardial infarct model. Further studies are needed to elucidate signal transduction of cytoprotection evoked by pharmacological preconditioning with MLA.

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