Preconditioning of Rat Heart with Monophosphoryl Lipid A: A Role for Nitric Oxide

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ABSTRACT

Preconditioning with monophosphoryl lipid A (MLA) protects rabbit hearts from prolonged ischemic reperfusion injury by a mechanism involving inducible nitric oxide synthase (iNOS) activation. This study was undertaken to determine whether MLA also could precondition rat hearts in a similar manner. Rats were injected with two different doses of MLA (300 μg/kg or 450 μg/kg s.c.) or vehicle (control), and after 24 hr the animals were sacrificed for preparation of isolated perfused rat hearts. Hearts were then perfused by working mode, and then made ischemic for 30 min followed by 30 min of reperfusion. Another group of hearts were treated simultaneously with a nitric oxide (NO) blocker, L-nitro-arginine-methyl-ester (L-NAME) (10 mg/kg) and MLA (450 μg/kg). For arrhythmia studies, 12 hearts were used in each group (total, 48 hearts). Cardiac functions were examined in a separate group of 24 hearts (n = 6/group). MLA-treated hearts (either dose) were tolerant to ischemic reperfusion injury as evidenced by improved postischemic ventricular recovery [coronary flow (ml/min) 19.1 ± 0.8 (300 μg/kg MLA), 22.6 ± 1.0 (450 μg/kg MLA) vs. 15.9 ± 0.7 (control); aortic flow (ml/min) 20.7 ± 1.8 (300 μg/kg MLA), 25.8 ± 1.4 (450 μg/kg MLA) vs. 11.0 ± 0.8 (control); left ventricular developed pressure (kPa) 13.3 ± 0.6 (300 μg/kg MLA), 14.6 ± 0.2 (450 μg/kg MLA) vs. 10.3 ± 0.7 (control)]. Incidences of ventricular fibrillation and ventricular tachycardia were decreased compared with the control group only in the 450 μg/kg dose of MLA-treated hearts (92% to 33%). Pretreatment of the hearts with L-NAME inhibited the preconditioning effect of MLA. To examine the induction of the iNOS expression, RNAs were extracted from the control and MLA-treated hearts (after 2, 4, 6, 8, 12 and 24 hr of treatment) and Northern blot analyses were performed with a specific cDNA probe for iNOS. A single band of approximately 4.6 kb corresponding to iNOS mRNA was detected after 4 hr of MLA treatment, whereas the maximal iNOS expression was found between 6 and 8 hr of MLA treatment. The results of this study demonstrated that MLA induced the expression of iNOS and protected the myocardium from ischemic reperfusion injury which is blocked by an inhibitor of NO synthesis, which suggests a role of NO in MLA-mediated cardioprotection.

Since the term ischemic preconditioning was introduced in 1986 (Murry et al.), a considerable amount of progress has been made in understanding this phenomenon. It now is accepted universally that a small amount of stress by repeated ischemia and reperfusion can delay the onset of further irreversible injury (Reimer et al., 1994) and reduce the subsequent postischemic ventricular dysfunction (Li et al., 1990; Kimura et al., 1992; Flack et al., 1991) and incidence of arrhythmias (Lawson et al., 1993; Tosaki et al., 1994) in hearts obtained from intact animals. Such preconditioning effects can be simulated by pretreating the hearts with adenine (Tsucida et al., 1994) or its receptor agonists (Liu et al., 1991), potassium channel openers (Gross et al., 1994), heat shock (Liu et al., 1992), oxidative stress (Maulik et al., 1993, 1995a) as well as by pharmacological manipulations (Maulik et al., 1995b). However, preconditioning is believed to be a species-specific phenomenon, and its mechanism of action varies between rats, rabbits, pigs and dogs (Li and Kloner, 1993, 1995). Recently, 24 hr pretreatment of rabbit hearts with MLA, a chemically modified nontoxic derivative of endotoxin, was

ABBREVIATIONS: MLA, monophosphoryl lipid A; iNOS, inducible nitric oxide synthase; L-NAME, L-ω-nitro-ω-arginine methyl ester; NO, nitric oxide; cDNA, complementary deoxynucleic acid; VF, ventricular fibrillation; VT, ventricular tachycardia; HR, heart rate; CF, coronary flow; AF, aortic flow; LVDP, left ventricular developed pressure; LVmax/dp/dt, maximum first derivative of left ventricular developed pressure; cGMP, cyclic guanosine monophosphate; IFN, interferon; LPS, lipopolysaccharides; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ECG, electrocardiogram.
found to render the hearts more tolerant to ischemic reperfusion injury (Zhao et al., 1997). Such cardioprotection was attributed to MLA-induced synthesis of iNOS. Because pathophysiology of preconditioning varies from species to species, it was of considerable interest to examine whether MLA also could induce iNOS in the rat heart and simultaneously provide cardioprotection. The results of our study demonstrate in rats treated with MLA, at the described dose, the induction of the expression of iNOS mRNA in myocardium simultaneously adapting the hearts to ischemia reperfusion injury, which suggests a role of NO in MLA-mediated cardioprotection.

**Materials and Methods**

**Working Rat Heart Preparation**

Male Sprague Dawley rats (320–350 g b.w.t.) were used for all studies. All animals received human care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication no. 85–23, revised 1985). Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg b.wt.) and then given intravenous heparin (500 IU/kg). After thoracotomy, the heart was excised and placed in ice-cold perfusion buffer. Immediately after preparation, the aorta was cannulated, and the heart was perfused by the Langendorff method for a 5-min washout period at a constant perfusion pressure equivalent to 100 cm of water (10 kPa). The perfusion medium consisted of a modified Krebs-Henseleit bicarbonate buffer ([millimolar concentration] sodium chloride, 118; potassium chloride, 4.7; calcium chloride, 1.7; sodium bicarbonate, 25; potassium biphosphate, 0.36; magnesium sulfate, 1.2; and glucose, 10). The Langendorff preparation was switched to the working mode after the washout period as described previously by Tosaki and Hellegouarch (1994). Essentially, it is a left-heart preparation, and oxygenated Krebs-Henseleit buffer at 37°C enters the cannulated left atrium at a pressure equivalent to 17 cm of H2O. The perfusion fluid then passes to the left ventricle, from which it is ejected spontaneously through the aortic cannula against a pressure equivalent to 100 cm of H2O.

Aortic flow was measured by an in-line calibrated rotameter. Coronary flow rate was measured by a timed collection of the coronary effluent that dripped from the heart. Continuous cardiac pressure measurements were recorded. All measurements were analyzed in real time with a data acquisition, analysis and presentation system. Direct measurements of heart rate, developed pressure (defined as the aortic systolic minus end-diastolic pressure) and the maximum first derivative of LVDP (LVmax dp/dt) were made at each point. After a 10-min aerobic perfusion of the heart, the left atrial inflow and aortic outflow lines were clamped at a point close to their origin. For isolated working rat heart, a 10-min stabilization period was enough to obtain stable cardiac function. Reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines. To prevent the myocardium from drying out during normothermic global ischemia, the thermostated glassware (in which hearts were suspended) was covered and the vapor content was kept at a constant level (90–100%).

**Experimental Protocol**

Ninety rats were assigned randomly to four groups: Control, injected with vehicle; MLA (300 µg/kg); MLA (450 µg/kg); and simultaneous treatment with MLA (450 µg/kg) and L-NAME (10 mg/kg) (fig. 1). All injections were given intravenously 24 hr before the initiation of the experiment. Hearts (n = 12 in each group for arrhythmia studies, total 60 hearts; n = 6 in each group for cardiac function studies, total 30 hearts) were subjected to 30 min of normothermic global ischemia at 37°C followed by 30 min of reperfusion. Myocardial function (HR, CF, AF, LVDP and LVmax dp/dt) was measured before ischemia and after 30 min of reperfusion. In MLA-treated groups, the drug (300 or 450 µg/kg) was injected intravenously 24 hr before the induction of ischemia, and pre- and postischemic cardiac function was recorded. An epicardial ECG was recorded by a polygraph throughout the experimental period by two silver electrodes attached directly to the heart to record ECG. ECGs were recorded by a high-speed Gould ECG recorder and analyzed to determine the incidence of VF and VT. After 1 min of sustained VF hearts were defibrillated and myocardial function was recorded. The heart was considered to be in VF if an irregular undulating baseline was apparent on the ECG. VT was defined as five or more consecutive premature ventricular complexes, and this classification included repetitive monomorphic VT, which is difficult to dissociate from rapid VT. In each instance, VT switched spontaneously to sinus rhythm or VF; therefore, VT was considered nonsustained. The heart was considered to be in sinus rhythm if normal sinus complexes occurring in a regular rhythm were apparent on the ECG. Before ischemia and during reperfusion HR, CF and AF rates were registered. LVDP and LVmax dp/dt were also recorded by the insertion of a Millar catheter into the left ventricle via the left atrium and mitral valve. The hemodynamic parameters were registered by a Cordat II acquisition system as described previously (Engelman et al., 1995).

**Exclusion criteria.** Preselected exclusion criteria for the present studies demanded that hearts were excluded if: 1) ventricular arrhythmias occurred during the period before the induction of global ischemia, and 2) CF and AF were less than 19 ml/min and 35 ml/min, respectively, before the initiation of ischemia. Thus, one heart was excluded from the control group; two hearts were excluded from the...
300 μg/kg MLA-treated group; one heart was excluded from the 450 μg/kg MLA-treated group; and one heart was excluded from the L-NAME group.

**RNA Preparation and Northern Blot Analysis of iNOS**

For RNA extraction, rats were sacrificed after 0, 2, 4, 6, 8, 12 and 24 hr after MLA treatment. Hearts were excised, instantly frozen in liquid N2 and stored at −70°C for RNA preparation. At a later date, total RNA was extracted from the heart by the acid-guanidinium-thiocyanate-phenol-chloroform method as described previously (Maulik et al., 1993). For Northern blot analysis, total RNA was electrophoresed in 1% agarose-formaldehyde-formamide gel and transferred to Gene Screen Plus. After prehybridization, membranes were hybridized with a 1.8-kb fragment of mouse macrophage iNOS cDNA obtained from Cayman Chemical Co. (Ann Arbor, MI). Each hybridization was repeated at least three times with different membranes. After each hybridization, the iNOS cDNA was removed and rehybridized with GAPDH cDNA probe, the results of which served as loading controls.

The autoradiograms were evaluated quantitatively by a computerized β-scanner. The results of densitometric scanning were normalized relative to the signal obtained for the GAPDH cDNA probe.

**Statistics**

The data for myocardial function were expressed as the mean ± S.E.M. One-way analysis of variance first was carried out to test for any differences between the mean values of all groups. If differences were established, the values of the MLA-treated groups were compared with those of the drug-free control group by a modified t test. An analog procedure was followed for distribution of discrete variables such as the incidence ofVF and VT. An overall χ² test for a 2 × n table was constructed followed by a sequence of 2 × 2 χ² tests to compare individual groups. A change of P < .05 was considered significant.

**Results**

**Arrhythmias in ischemic/reperfused hearts.** Hearse and Tosaki (1987) previously reported that the vulnerability to reperfusion-induced arrhythmias in the rat heart is determined by the duration of the preceding ischemic period and that a complex bell-shaped time-response relationship exists. In the present studies, we required that the control group exhibits a high vulnerability to reperfusion-induced arrhythmias to demonstrate any antiarrhythmic effects. To ensure this within the experimental time course and condition defined for this study, 30 min of normothermic global ischemia followed by 30 min reperfusion was selected. The results demonstrate (fig. 2A) that in rats subjected to ischemia/reperfusion protocol, the incidence of reperfusion-induced VF was reduced from its control value of 92% to 75% and 33% (P < .05), respectively, with the concentrations of 300 and 450 μg/kg MLA. The incidence of reperfusion-induced VT showed the same pattern (fig. 2B). Incidence of arrhythmias was not affected for the L-NAME group (10 mg/kg) (fig. 2, A and B).

**Effects of MLA and L-NAME on cardiac functions.** Table 1 shows the absolute values for HR, CF, AF, LVDP and LV max dp/dt before the induction of ischemia; no statistically significant difference was found between the drug-free, MLA- and L-NAME-treated groups. Table 2 shows the postischemic recovery of cardiac function after 30 min ischemia followed by reperfusion in the drug-free, MLA- and L-NAME-treated groups. During reperfusion LVDP dropped significantly to 10.3 ± .7 kPa in the control group. For rats treated with 300 and 450 μg/kg of MLA, LVDP was improved significantly from its control value of 10.3 ± 0.7 kPa to 13.3 ± 0.6 kPa (P < .05) and 14.6 ± 0.2 kPa (P < .05), respectively. Similar results were obtained for LV max dp/dt. AF in the MLA-treated (300 μg/kg) hearts after reperfusion was significantly higher (20.7 ± 1.8 ml/min vs. 11.0 ± 2.2 ml/min for the control group) than the AF of the controls. CF followed the same pattern. Thus, rats treated with 300 or 450 μg/kg of MLA, a significant recovery in CF, AF, LVDP and LV max dp/dt was observed in comparison with the drug-free control group. L-NAME failed to modify postischemic cardiac functions (table 2).

**Effects of L-NAME on MLA-preconditioned hearts.** MLA at both doses improved the postischemic cardiac functions (table 2) and reduced the incidence of arrhythmias only at 450 μg/ml dose (fig. 2); hence, we used the higher dose, i.e., 450 μg/kg, for this study. Rats were injected simultaneously with MLA and L-NAME, and after 24 hr they were sacrificed for the isolated working heart preparation. Results are de-
picted in figure 3. L-NAME completely blocked the beneficial effects of MLA. Improved CF, AF and ventricular functions were reversed by L-NAME. Incidence of VF and VT were 83% and 100%, respectively, after L-NAME treatment (fig. 4).

**Effects of MLA on the induction of iNOS expression.**
In MLA-pretreated animals, iNOS mRNA was detected by Northern analysis as a single band of about 4.6-kb size. iNOS mRNA was detected first after 4 hr, the maximum expression was noticed at 6 hr and it remained the same up to 8 hr (fig. 5). The induction of the expression of iNOS began to decline after 12 hr and reached the base-line value (negligible) after 24 hr (not shown).

**Discussion**
Numerous studies from different laboratories have demonstrated that MLA can provide a cardioprotective effect by its ability to precondition hearts against lethal ischemic injury. MLA when injected 24 hr before the experiment can reduce myocardial ischemic reperfusion injury in a variety of species including dogs (Yao et al., 1993, 1995), rabbits (Yoshida et al., 1996; Zhao et al., 1996, 1997) and rats (Maulik et al., 1995b).

In rabbits, enhancement of iNOS enzyme activity and reduction of polymorphonuclear leukocytes infiltration in the infarcted tissue was found to be associated with cardioprotection (Zhao et al., 1997). In dogs and rabbits, the ATP-

**TABLE 1**
Cardiac function before the induction of ischemia (preischemic values)°

<table>
<thead>
<tr>
<th>Function</th>
<th>Control</th>
<th>MLA (300 µg/kg)</th>
<th>MLA (450 µg/kg)</th>
<th>l-NAME (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>320 ± 6</td>
<td>315 ± 3</td>
<td>320 ± 5</td>
<td>313 ± 4</td>
</tr>
<tr>
<td>CF</td>
<td>27.3 ± 0.7</td>
<td>27.2 ± 0.7</td>
<td>27.0 ± 0.7</td>
<td>26.5 ± 0.8</td>
</tr>
<tr>
<td>AF</td>
<td>51.3 ± 1.3</td>
<td>50.0 ± 0.7</td>
<td>50.6 ± 1.1</td>
<td>49.7 ± 1.5</td>
</tr>
<tr>
<td>LVDP</td>
<td>17.9 ± 0.2</td>
<td>18.0 ± 0.2</td>
<td>18.0 ± 0.2</td>
<td>17.6 ± 0.3</td>
</tr>
<tr>
<td>LVdp/dtmax</td>
<td>806 ± 24</td>
<td>798 ± 24</td>
<td>802 ± 21</td>
<td>788 ± 18</td>
</tr>
</tbody>
</table>

° n = 12 in each group, mean ± S.E.M. Comparisons were made with the control (vehicle-treated) group. HR (beats/min); CF (ml/min); AF (ml/min); LVDP (kPa); LVdp/dtmax (kPa/s).

**TABLE 2**
Cardiac function after 30 min ischemia followed by 30 min reperfusion (postischemic values)°

<table>
<thead>
<tr>
<th>Function</th>
<th>Control</th>
<th>MLA (300 µg/kg)</th>
<th>MLA (450 µg/kg)</th>
<th>l-NAME (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>296 ± 6</td>
<td>294 ± 4</td>
<td>293 ± 6</td>
<td>286 ± 7</td>
</tr>
<tr>
<td>CF</td>
<td>15.9 ± 0.7</td>
<td>19.1 ± 0.8°</td>
<td>22.6 ± 1.0°</td>
<td>16.3 ± 0.6</td>
</tr>
<tr>
<td>AF</td>
<td>11.0 ± 0.8</td>
<td>20.7 ± 1.8°</td>
<td>25.8 ± 1.4°</td>
<td>10.5 ± 0.8</td>
</tr>
<tr>
<td>LVDP</td>
<td>10.3 ± 0.7</td>
<td>13.3 ± 0.6°</td>
<td>14.6 ± 0.2°</td>
<td>10.2 ± 0.4</td>
</tr>
<tr>
<td>LVdp/dtmax</td>
<td>447 ± 11</td>
<td>537 ± 13°</td>
<td>600 ± 13°</td>
<td>430 ± 16</td>
</tr>
</tbody>
</table>

° n = 12 in each group, mean ± S.E.M. ° P < .05. Comparisons were made with the control (vehicle) group. HR (beats/min); CF (ml/min); AF (ml/min); LVDP (kPa); LVdp/dtmax (kPa/s).

Fig. 3. Effect of l-NAME on MLA-mediated postischemic functional recovery. Rats were injected with MLA (450 µg/kg i.p.), and 24 hr later hearts were isolated for the working heart preparation. Hearts (n = 6 in each group) were subjected to 30 min global ischemia followed by 30 min reperfusion, and myocardial functions were monitored as described under "Materials and Methods." Group 1, drug-free ischemic-reperfused hearts; group 2, hearts treated with 450 µg/kg MLA; group 3, hearts treated with 450 µg/kg MLA and 10 mg/kg of l-NAME. °P < .05 compared with control.
dependent potassium channel (K\textsubscript{ATP}) was shown to play a role in MLA-induced preconditioning (Elliott \textit{et al.}, 1996). Although the beneficial effect of preconditioning is recognized universally, its mechanism of action remains controversial. Furthermore, the pathophysiology of preconditioning apparently varies from one species to another. For example, adenosine A\textsubscript{1} receptor seems to be involved in the preconditioning of rabbit heart, but it does not play an important role in the preconditioning of rat heart (Li and Kloner, 1993). Protein kinase C has been instrumental for preconditioning in rat and rabbit hearts, but it is not involved in the preconditioning of dog heart (Przyklenk \textit{et al.}, 1995). As mentioned earlier, enhancement of iNOS activity seems to contribute to MLA-mediated preconditioning in rat hearts. This study was undertaken to examine whether iNOS could play a role in the preconditioning of rat hearts.

The major novel finding of this study is that MLA pretreatment at cardioprotective doses induces iNOS mRNA expression in rat hearts. The stimulation of iNOS induction occurred as early as 4 hr after the MLA pretreatment, reaching a peak between 6 and 8 hr and then declined progressively to the base-line level. Both endotoxin and lipid A previously were shown to stimulate NO production (Traylor \textit{et al.}, 1996; Rees \textit{et al.}, 1990) and increased the level of cGMP (Fleming \textit{et al.}, 1990), a second messenger for NO signaling. Evidence for endotoxin-mediated NO production via inducible iNOS is increasing. The promoter region of the cloned murine iNOS gene was found to contain transcriptional regulatory elements responsive to IFN\textgamma and LPS (Martin \textit{et al.}, 1994). Recently, LPS was shown to induce expression of constitutive Ca\textsuperscript{2+}-dependent iNOS activity (Mayeux \textit{et al.}, 1995). More recently, lipid A was found to stimulate NO production by isolated rat proximal tubules in a time-dependent manner (Traylor \textit{et al.}, 1996). By use of the rabbit infarct model, Zhao \textit{et al.} (1997) noticed that stimulation of iNOS enzyme activity did not occur in the nonischemic heart tissue when rabbits were treated with MLA. These investigators concluded that iNOS protein is present in rabbit hearts in an inactive form which requires activation by ischemia. Thus, ischemia by activating kinases or inhibiting phosphatases may promote phosphorylation of the inactive form of iNOS induced by MLA. It is possible, as implicated from the results of our present study, that iNOS mRNA translated into protein promotes NO formation during ischemia. However, endothelial constitutive nitric oxide synthase also could be involved in the cardioprotection 24 hr after treatment.

NO recently has been implicated in cardioprotection (Lefer \textit{et al.}, 1993; Maulik \textit{et al.}, 1996a, b; Engelman \textit{et al.}, 1995). A recent study from our laboratory demonstrated that NO enhanced myocardial protection by cGMP-dependent as well as cGMP-independent mechanisms (Maulik \textit{et al.}, 1995a, b, c). Much of the NO action in biological systems is mediated by the second messenger, cGMP. NO is an unique messenger in that it is produced in one cell and diffuses into adjacent target cells to activate cytosolic guanylate-cyclase-bound...
heme to generate the NO-heme adduct of guanylate cyclase. In the ischemic myocardium, NO also functions by a GMP-independent mechanism by virtue of its antioxidant effects toward oxygen free radicals as well as o xofermyoglobin radicals, the important causative factors for ischemia reperfusion injury (Maulik et al., 1995a, b, 1996a). It has been believed generally that NO affords cardioprotection by its ability to quench free radicals generated during the reperfusion of ischemic myocardium. Thus, NO seems to serve both as an intracellular antioxidant and as a messenger molecule in the ischemic myocardium. Finally, NO also can promote cardioprotection by reducing endothelial inflammation during ischemia and reperfusion by virtue of its ability to decrease soluble intracellular adhesion molecule-1, endothelial leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1 (Engelman et al., 1995).

MLA is derivatized from the minimal pharmacophore of endotoxin (lipid A) by removing a phosphoester from reducing sugar of disaccharide followed by saponification of a long-chain β-hydroxy ester from the 3-position hydroxy group of reducing glucosamine (Qureshi et al., 1982). Pretreatment 12 to 24 hr before ischemia with a single i.v. bolus injection of MLA was found to cause a 50% to 75% reduction of infarct size in canine and rabbit hearts (Yao et al., 1995; Yao et al., 1993; Yoshida et al., 1996). In the present study, MLA pretreatment caused the improved postischemic ventricular recovery and at both doses, but a higher dose (450 μg/kg) was necessary to observe a reduction in the incidence of ventricular fibrillation and ventricular tachycardia.

In summary, treatment with MLA 24 hr before ischemia improved contractile function in the ischemic reperfused working rat heart model. Corroborated with these findings, MLA also induced the expression of iNOS mRNA in the hearts after 4 hr of treatment. The MLA-mediated cardioprotection was inhibited by reducing nitric oxide synthesis suggesting that MLA may exert its cardioprotection at least in part by inducing NO synthesis.

References


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