Essential Role of Inducible Nitric Oxide Synthase in Monophosphoryl Lipid A–Induced Late Cardioprotection

Evidence From Pharmacological Inhibition and Gene Knockout Mice

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Background—Monophosphoryl lipid A (MLA), a nontoxic analogue of endotoxin, is a pharmacological agent that is known to have anti-ischemic effects. Mechanisms involved with the cardioprotection are still unclear. A role for inducible nitric oxide synthase (iNOS) was recently proposed. We tested this hypothesis using S-methylisothiourea (SMT), one of the specific pharmacological inhibitors of iNOS, as well as iNOS gene knockout mice.

Methods and Results—Adult male ICR or B6,129 mice were pretreated with either MLA 35 or 350 μg/kg IP (MLA35 or MLA350) or vehicle 24 hours before global ischemia/reperfusion, which was carried out in a Langendorff isolated perfused heart model (n=8 to 9 per group). Another group of MLA350 mice received SMT 3 mg/kg IP 30 minutes before heart perfusion. Ventricular contractile function and heart rate were not different between the groups during the preischemia and reperfusion periods (P>0.05). Preischemic basal coronary flow was significantly increased in all MLA350 but not MLA35 mice. Myocardial infarct size was reduced significantly, from 26.9±2.9% of risk area in vehicle-treated mice to 13.5±2.4% in the MLA350 group (mean±SEM, P<0.05). This reduction in infarct size was accompanied by augmented nitrite/nitrate accumulation, from 0.23±0.05 nmol/mg protein in the vehicle group to 0.97±0.27 nmol/mg protein in MLA350 mice (P<0.01). Infarct size increased significantly, to 22.2±2.8% after treatment with SMT in the MLA350 group. Furthermore, MLA350 failed to reduce infarct size in iNOS knockout mice (25.5±3.6%).

Conclusions—These results demonstrate a direct association of infarct size reduction with increased NO production with MLA350. An obligatory role for iNOS in mediating the cardioprotective effect induced by MLA was confirmed with the pharmacological inhibition and gene knockout mice. (Circulation. 1999;99:2157-2163.)

Key Words: ischemia • reperfusion • myocardial infarction • pharmacology • nitric oxide

Protection of the ischemic heart has been the subject of experimental and clinical research for more than 2 decades. A number of protection strategies have been developed, including the pharmacological approaches.1–2 The phenomenon called “preconditioning,” with its potent ability to enhance cellular endogenous mechanisms against ischemia/ reperfusion injury, has been extensively investigated by many investigators. Identification of novel pharmacological agents that can potentially induce long-lasting protection against ischemia and reperfusion injury is currently a major area of investigation. Among the several agents that can potentially precondition myocardium against ischemia, monophosphoryl lipid A (MLA) has been well investigated and is thought to be promising for future clinical applications in humans.3

MLA is an analogue of endotoxin, which was derived and purified from bacterial lipopolysaccharide in the 1980s. It retains several of the immunomodulatory properties of the parent endotoxin molecule without the associated toxicity. MLA appears to maintain many of the beneficial immunological activities of the parent molecule, including induction of tolerance to endotoxemia in both laboratory animals and human subjects.6 These beneficial effects of MLA may be achieved via its ability to induce cytokines, macrophage activation, and colony-stimulating factor induction with much less toxic effects than are associated with the parent endotoxin.7–9 To date, this less toxic and less pyrogenic agent has been investigated primarily for use as an immunotherapeutic,10 immunoprophylactic,11 or adjuvant for vaccines.12 Studies have shown that MLA also has cardioprotective effects when administered 24 hours before ischemia/reperfusion in rats, rabbits, dogs, and cultured adult rat cardiac myocytes.19

The mechanism of MLA-induced protection is not well understood, although several possibilities have been suggested. It was shown that lipopolysaccharide pretreatment induces heat shock protein 70i expression in rat myocardium that is associated with the delayed cardioprotection.20 However, MLA failed to induce a similar induction of this protein...
in rabbit heart. More recently, Zhao et al demonstrated that delayed cardioprotection with MLA can be abolished by aminoguanidine, an inhibitor of inducible nitric oxide synthase (iNOS). Unfortunately, pharmacological inhibitors do not always give satisfactory answers because many of these agents are not highly specific and do not entirely inhibit the target enzyme. Because NO is produced from l-arginine through a chemical reaction that is catalyzed by at least 3 major isoforms of NOS, ie, iNOS (inducible), eNOS (endothelial), and nNOS (neuronal), there is an unavoidable redundancy in functional actions among the different NOS isoforms. Therefore, the exact role of iNOS in MLA-induced cardioprotection requires further direct confirmation with more specific methods, such as state-of-the-art gene knockout technology. The iNOS gene knockout mice that were recently developed provide us an excellent opportunity to study the role of iNOS in the mechanisms of MLA-induced cardioprotection. The present study in a murine model focused on the following 3 specific aims: (1) to demonstrate that MLA induces delayed cardioprotection in the mouse heart, (2) to determine that MLA-induced cardioprotection is mediated by NO, and (3) to confirm that an intact iNOS system is obligatory for the MLA-induced cardioprotection.

Methods

Animals

Adult male outbred ICR mice were supplied by Harlan Sprague Dawley Co (Indianapolis, Ind), and the adult male iNOS gene knockout B6,129 mice were purchased from Jackson Laboratory (Bar Harbor, Me). The iNOS knockout mice were generated according to Laubach et al. In brief, to generate chimeric mice, C57BL/6J (B6) blastocysts were injected with the recombinant 129-derived ES cells and implanted into pseudopregnant females for development. Chimeric males were then mated with B6 females, and the resulting B6,129 F1 heterozygote mutant (+/−) mice were interbred to generate F2 homozygous mutant (−/−) mice for the iNOS disruption. Their progeny were genotyped by Southern analysis. The iNOS knockout mice were indistinguishable from wild-type mice in appearance, growth rate, reproduction, and histology.

Drugs

MLA and its vehicle solvent were provided by Ribi ImmunoChem Research Inc. The vehicle was composed of 40% propylene glycol and 10% ethyl alcohol in water. Mice were treated intraperitoneally with either MLA 35 or 350 μg/kg or volume-matched vehicle. S-Methylthiourea sulfate (SMT), a selective inhibitor of iNOS, was purchased from Sigma Chemical Co.

Langendorff-Perfused Isolated Heart Preparation

The methodology of isolated perfused mouse heart was described in detail previously.

Ischemia/Reperfusion Protocol

After a 30-minute stabilization period, hearts were subjected to 20 minutes of no-flow normothermic global ischemia and 30 minutes of reperfusion. The 7 experimental groups involved in this study are shown in Figure 1.

Exclusion Criteria

Hearts were excluded from further data analysis as a result of one of the following undesirable situations: (1) time delay in the aortic cannulation (>3 minutes), (2) damage of aorta during the cannulation, (3) sustained arrhythmia during the stabilization, and (4) depressed ventricular developed force (<0.1 g) at the end of stabilization.

Measurement of Infarct Size

At the end of the experiment, hearts were immediately removed from the Langendorff apparatus, weighed, and frozen at −20°C. The frozen heart was then cut manually into 7 or 8 transverse slices of approximately equal thickness (~0.8 mm) and stained by incubation in 10% triphenyl tetrazolium chloride (TTC) for 30 minutes. TTC buffer was then replaced by 10% formaldehyde, and the slices were fixed for 4 to 6 hours before measurement of the infarct area and the risk zone by computer morphometry (Biosquant System IV). The risk area was the sum of total ventricular area minus cavities. The infarct size was calculated as percent of risk area.

Measurement of NO Products

Thirty-six ICR mice were pretreated with vehicle or MLA 35 or 350 μg/kg IP. Twenty-four hours later, hearts were isolated and subjected to either 5 minutes of aerobic perfusion (37°C) for washing out the Langendorff apparatus, weighed, and frozen at −20°C. The frozen tissue was immediately collected, frozen, and stored at −80°C. For preparation of tissue extracts, 2 mL of ice-cold homogenization buffer (0.1 mmol/L phosphate buffer, pH 7.4) was added to the powdered tissue sample, and the mixture was homogenized in a Polytron equipped with a PT10 probe. The homogenate was then spun in a microcentrifuge for 10 minutes, and the supernatant (representing the cytosol) was transferred to a fresh tube and kept frozen at −80°C until analyzed. Protein concentration...
was determined with a BIO-RAD protein assay kit. Total NO oxidation products were measured with a SIEVERS nitric oxide analyzer (model 280NOA). NO undergoes a series of reactions with several molecules present in biological fluids, leading to the accumulation of the final products, nitrite and nitrate. Thus, the index of total NO production is the sum of both nitrite and nitrate accumulated in the tissue samples. The reducing agent used for the analysis was a saturated solution of vanadium (III) chloride (VCl₃) in 1 mol/L HCl. To minimize foaming from the heart tissue samples containing protein, 100 µL of a 1:30 dilution of Dow Corning Antifoam C was added to the VCl₃ reagent. Five milliliters of the reagent was used in the purge vessel for analysis of 30 to 50 samples. At a temperature of 90°C, VCl₃ reagent quantitatively converts nitrite, nitrate, and S-nitroso compounds to NO, which is then measured by the NO analyzer.

Data Analysis and Statistics
Each experimental group consisted of 8 or 9 animals. The group means and their SEMs for each parameter are presented. One-way ANOVA was used to compare the values of groups. If a significant value of F was obtained, the Student-Newman-Keuls post hoc test was subsequently used to make pairwise comparisons among the groups. Paired t test was also used to compare any pretreatment and posttreatment values for any given parameter. A value of P < 0.05 was considered statistically significant.

Results
Exclusions
A total of 78 hearts were subjected to the ischemia/reperfusion protocol (Figure 1) in the 7 experimental groups (n = 8 to 9 each) for the assessment of ventricular function as well as infarct size. Among them, 16 hearts (ie, 21% of the 78 perfused hearts) were excluded according to the exclusion criteria described under Methods. An additional 36 hearts were used for measurement of nitrite levels in the myocardial tissue extracts.

Baseline Cardiac Hemodynamics and Contractile Function
Morphometric characteristics of the mice as well as the preischemic baseline values are summarized in the Table. There was no significant difference in the preischemic basal value of heart rate and ventricular contractile parameters (ie, developed force, rate-force product, and resting tension) between the groups, although pretreatment with high-dose MLA exhibited a positive inotropic effect during the first 20 minutes of stabilization (Figure 2). This effect on myocardial contractility persisted in the SMT-treated group but was absent in the iNOS knockout group. The coronary flow rate was significantly increased in all MLA350-treated groups compared with the vehicle group (P < 0.05, Figure 4).

Postischemic Cardiac Hemodynamic and Contractile Function
After 20 minutes of global ischemia, ventricular developed force and rate-force product were significantly depressed in all experimental groups, regardless of the pretreatment conditions during reperfusion (Figure 2). The resting tension and...
heart rate were not significantly different between the preischemic and reperfusion periods (Figure 3). Postischemic coronary flow was not significantly different from its preischemic values for all the groups \((P > 0.05;\) Figure 4). The average coronary flow rate was generally higher in MLA-treated groups than in the vehicle group, although these differences were not significant \((P > 0.05)\).

**Infarct Size**

Myocardial infarction was evident in the mouse hearts after ischemia/reperfusion. Pretreatment with MLA reduced the infarct size in a dose-dependent manner (Figure 5A). The high-dose MLA (ie, 350 μg/kg) caused a significant reduction in infarct size, to 13.5±2.4% of risk zone compared with 26.9±2.9% in the vehicle-treated group \((P < 0.05)\). The MLA-induced reduction in infarct size was abolished by pretreatment with SMT (22.2±2.8%) and was completely absent in the iNOS knockout mice (25.5±3.6%). The area at risk for the globally ischemic hearts was not different between the groups (Figure 5B). Representative samples of the TTC-stained heart slices are shown in Figure 6. The viable (area in red color) and necrotic (area in pale color) tissues were clearly distinguishable in these pictures.

**Myocardial Nitrite/Nitrate Content**

Pretreatment with MLA caused a moderate but statistically insignificant decrease of myocardial nitrite/nitrate in the nonischemic hearts 24 hours later. The nitrite/nitrate levels increased significantly only in the high-dose MLA-treated mice after ischemia/reperfusion \((0.97±0.27 \text{ nmol/mg protein})\) compared with the vehicle group \((0.23±0.05; P < 0.01;\) Figure 7). The postischemic myocardial accumulation of
nitrite/nitrate was associated with the reduction in infarct size with MLA (Figure 5).

Discussion

Novel Findings

The salient findings of this study are summarized as follows. (1) Higher doses of MLA (ie, 350 μg/kg), when administered 24 hours before ischemia/reperfusion, resulted in a significant reduction of myocardial infarct size and improvement in coronary flow. The antinecrotic effect was not associated with improvement in the postischemic ventricular contractile function. (2) The cardioprotective effect was abolished by SMT, a specific inhibitor of iNOS, and was completely absent in iNOS knockout mice. (3) The cardioprotective dose of MLA resulted in a significant increase in nitrite/nitrate accumulation in the ischemic/reperfused heart. Taken together, our results suggest that high-dose MLA induces a significant anti-ischemic protection in the mouse heart, which was associated with increased accumulation of NO products. These data, coupled with a lack of protection in iNOS knockout mice and blockade of cardioprotection with iNOS inhibitor in normal mice, strongly suggest a cause-and-effect relationship of NO in the pharmacological protection induced by MLA. To the best of our knowledge, this is the first study to establish a direct role of NO in delayed pharmacological preconditioning in the ischemic mouse heart.

Anti-Ischemic Effects of MLA in Heart

MLA has been shown to induce such immunostimulatory effects as cytokine production, macrophage stimulation, and a variety of other effects on both humoral and cell-mediated immune response.5,7-9 In vivo studies demonstrated the ability of MLA to protect the heart during ischemia/reperfusion.2,15-17 The present study clearly demonstrates a dose-dependent antinecrotic effect of MLA (Figure 5). The lower dose of MLA (35 μg/kg) did not reduce infarct size. This finding is in accordance with a previous report15 in which a similar dose of MLA reduced postischemic infarct size in the in situ rabbit heart after regional ischemia but not in the isolated heart subjected to global ischemia. In addition, it seems that rodents require a much higher drug concentration to exert the cardioprotective effects. Tosaki et al14 reported significant antiarrhythmic effects of MLA with 300 and 450 μg/kg MLA in the isolated working rat heart. Similar species-related differences in induction of late cardioprotection were observed in the mouse heart,25 in which whole-body heat shock failed to induce anti-ischemic effects. However, MLA-induced infarct size reduction was comparable to “acute” ischemic preconditioning in the isolated mouse heart.24

NO and Delayed Cardioprotection

NO is an essential modulator of biological systems, including the cardiovascular system.21 It is critical in the signal transduction of ischemic myocardium.26 Numerous studies have shown the beneficial and harmful effects of NO in the physiological regulation and control of the cardiovascular system. On one hand, NO is a free radical itself and can also form peroxynitrite, a more potent oxidant that can potentially cause cellular membrane lipid peroxidation, which may lead to myocardial dysfunction.27-30 In contrast, NO is the modulator of vascular smooth muscle tone, and its biological action can be cardioprotective against ischemia/reperfusion injury through coronary vasodilation and reduction in myocardial oxygen consumption via upregulation of cGMP.31 Pretreatment with NO donors has been reported to be beneficial in the ischemic myocardium. Both antiarrhythmic and anti-infarction32 effects of the NO donors have been well documented. More recently, NO has been appreciated as the possible key trigger and mediator for ischemic preconditioning.33

NO may enhance myocardial protection by cGMP-dependent as well as cGMP-independent mechanisms (Figure 8). NO is a unique messenger because 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.26 NO may also modulate K

Experimental Groups

Figure 7. Effect of MLA on nitrite/nitrate accumulation in tissue extracts of nonischemic and ischemic/reperfused mouse hearts (mean ± SEM; n = 6 each). *P < 0.05 vs vehicle.
dependent protein kinases may be capable of phosphorylating K<sub>ATP</sub> channels and priming the channel to offer cardioprotection. Cameron et al. provided direct evidence that NO enhances K<sub>ATP</sub> channel activity in hypertrophied ventricular myocytes. Opening of the K<sub>ATP</sub> channel appears to be protective because of the increase in outward potassium current, resulting in shortening of the action potential, which in turn may spare ATP, thereby allowing less entry of calcium into the myocyte through the voltage-sensitive calcium channel. Decreased intracellular calcium overload may reduce ischemic injury and lead to better myocyte preservation. There is mounting evidence supporting the involvement of K<sub>ATP</sub> channels in the mechanism of ischemic preconditioning and pharmacological protection with MLA.

iNOS and Delayed Cardioprotection

Three major isoforms of NOS, ie, iNOS, eNOS, and nNOS, are able to catalyze the chemical reaction that produces NO from L-arginine. eNOS and nNOS are constitutively expressed in normal biological systems and are important mediators of cellular signal transduction. iNOS is expressed under such pathophysiological conditions as endotoxin challenge and stress and is capable of producing large amounts of NO, which tends to be cytotoxic. However, it has been increasingly recognized that iNOS can be very important in the immune reaction against microbacterial and environmental insults. In the present investigation, we observed complete lack of MLA-induced protection in the iNOS knockout mice, suggesting an obligatory role of this isoform in the protective process. Furthermore, the cardioprotection was significantly abolished by SMT, which was reported to be 10- to 30-fold more potent as an inhibitor of iNOS than the L-arginine analogues, as well as aminoguanidine. Therefore, the abrogation of MLA-induced anti-ischemic effect could be at least in part due to the inhibition of iNOS in the ischemic heart. The cardioprotective dose of MLA significantly increased NO production after ischemia/reperfusion but not in the nonischemic hearts, suggesting that iNOS was functional only in the ischemic hearts. Similarly, Zhao et al. observed that no increase of iNOS enzyme activity occurred in the nonischemic heart tissue in MLA-treated rabbits. This suggests that posttranslational modifications of the iNOS enzyme are required before it is capable of generating NO. It is possible that ischemia may activate certain kinases (such as protein kinase C and tyrosine kinase) or inhibit phosphatas that may promote phosphorylation-dependent activation of the inactive iNOS induced by MLA.

The role of constitutive forms of NOS in MLA-induced protection is still not clear. We observed a consistent improvement in preischemic coronary flow in all MLA-treated groups (Figure 4). Blocking iNOS with SMT did not reverse the improvement of coronary flow in MLA-treated mice. Also, SMT only partially (although significantly) blocked the antinecrotic effect of MLA (Figure 5). These data suggest that MLA may have stimulated eNOS in the heart and that the drug may be improving vascular endothelial function independently of the iNOS enzyme. Future studies in eNOS knockout mice will provide insights on the role of eNOS in MLA-induced cardioprotection.

Conclusions and Future Directions

We have shown that MLA induces a dose-dependent cardioprotection against myocardial infarction in the ischemic mouse heart. This antinecrotic effect was associated with enhanced accumulation of nitrite/nitrate in the ischemic tissue, was blocked by selective inhibition of iNOS, and was completely absent in iNOS knockout mice. To the best of our knowledge, this is the first direct evidence supporting an obligatory role of iNOS in mediating the delayed cardioprotection by MLA. Further investigations in the murine model are necessary to elucidate (1) the potential role of eNOS or nNOS in MLA-induced cardioprotection, (2) the key type(s) of cytokines and their receptors that may be responsible in activation of iNOS, and (3) other pharmacological agents that could induce late preconditioning via signal transduction pathways similar to those of MLA.

Acknowledgments

This work was funded in part by grants from the NHLBI (HL-51045, HL-59469) to Dr Kukreja. Dr Xi was supported by a NRSA fellowship from the NIH (HL-07537) and a Research Fellowship from the American Heart Association, Mid-Atlantic Affiliate (F98273V). We thank Dr R. Hutte of Sievers Instruments, Inc. for his help in measuring tissue nitrite/nitrate concentrations and Dr G.T. Elliott of Ribi ImmunoChem Research Inc for providing MLA.

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Circulation. 1999;99:2157-2163
doi: 10.1161/01.CIR.99.16.2157

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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