Myocardial ischemia/reperfusion protection using monophosphoryl lipid a is abrogated by the ATP-sensitive potassium channel blocker, glibenclamide

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Abstract

Objectives: Monophosphoryl lipid A (MLA), a detoxified derivative of the lipid A portion of the endotoxin molecule, given as a pretreatment 24 h prior to cardiac ischemia/reperfusion reduces myocardial stunning and infarction in dogs. This study was undertaken to evaluate the ability of MLA pretreatment to reduce infarct size in a rabbit model of in situ regional myocardial ischemia and reperfusion. Secondly, the potential role of modulation of ATP-sensitive potassium (K\(_{ATP}\)) channel in MLA's cardioprotection was evaluated using in vivo pharmacologic antagonism with a K\(_{ATP}\) channel blocker, as well as the role of tumor necrosis factor using an enzyme-linked immunosorbent assay method of serum cytokine analysis.

Methods: Rabbits were pretreated intravenously with MLA or vehicle injection 24 h prior to initiation of 30 min in situ left anterior descending coronary artery occlusion followed by 3 h reperfusion. In animals receiving glibenclamide, the potassium channel antagonist was administered 30 min prior to inducing ischemia. Animals receiving glibenclamide, which possesses hypoglycemic effects, underwent serial blood glucose evaluation prior to drug and throughout the ischemia and reperfusion periods. Hemodynamics were monitored; infarct size and area at risk were assessed by contrast dye staining (triphenyltetrazolium chloride). Serum tumor necrosis factor was measured by enzyme-linked immunosorbent method in animals administered cardioprotective doses of MLA as well as pyrogenic doses of MLA and endotoxin (positive control) to determine if elaboration of this cytokine could be associated with the cardioprotective effect of MLA.

Results: MLA administered as a single intravenous dose 24 h prior to ischemia reduced infarct size, expressed as a percent of the area at risk, 64 and 71% at doses of 35 and 10 \(\mu\)g/kg, respectively. Lower doses of MLA (2.5 and 5 \(\mu\)g/kg) did not significantly reduce infarct size. Administration of glibenclamide (300 \(\mu\)g/kg) 30 min prior to ischemia completely blocked the ability of MLA pretreatment to limit infarct size, while MLA vehicle-glibenclamide-treated control rabbits displayed infarcts not significantly different from MLA-vehicle-treated control rabbits. A cardioprotective dose of MLA (35 \(\mu\)g/kg) did not induce the elaboration of tumor necrosis factor into rabbit serum (within the limits of assay sensitivity).

Conclusions: Single-dose pretreatment with MLA administered intravenously to rabbits substantially reduces infarct size when administered 24 h prior to ischemia. Pharmacologic preconditioning with MLA appears to be mediated through K\(_{ATP}\) channels as the channel blocker, glibenclamide, reversed the cardioprotective activity of MLA when administered 1 day following MLA pretreatment, yet 30 min prior to ischemia. In this model the cardioprotective activity of MLA does not appear to be associated with increases in serum tumor necrosis factor.

Keywords: Preconditioning; Potassium channel; ATP-sensitive; Sulphonylureas; Monophosphoryl lipid A; Myocardial infarction; Myocardial ischemia; Rabbit, anesthetized

1. Introduction

Ischemia preconditioning, first described by Murray, Jennings and Reimer in 1986 as an endogenous cardioprotective mechanism rapidly induced following transient ischemic episodes [1], has come to be recognized as a potent means of limiting infarct size and additionally reducing contractile dysfunction and ventricular arrhythmias resulting from ischemic episodes followed by reperfusion [1–4].
Protection afforded by ischemic preconditioning was initially recognized to develop rapidly, within the first 5 to 10 min of reperfusion following transient ischemic episodes of 5 to 15 min in duration. This rapidly developing protection appears to be transient in nature with protection lasting for no longer than 2 h in anesthetized animal models. More recently a ‘second window’ of preconditioning (delayed preconditioning) has been described where protection reappears 12 to 24 h following ischemic preconditioning and which lasts for at least 48 h following the transient ischemic event [5,6].

It is fairly well accepted that the first window of ischemic preconditioning results following adenosine release from vascular endothelium and/or cardiomyocytes during the transient preconditioning ischemia or subsequent reperfusion [7,8]. It is proposed that this extracellular adenosine binds to A<sub>1</sub> and/or A<sub>3</sub> receptors in cardiac tissue and leads to G protein coupled activation of adenylylate cyclase, and protein kinase C with preconditioning resulting thereafter [8,9]. There appears to be redundancy in the endogenous mediators playing a role in eliciting ischemic preconditioning, with bradykinin and norepinephrine in addition to adenosine, for example, playing contributory roles in posts ischemic signaling in the rabbit [10].

Among the 9 classes of potassium channels found on myocytes, one channel found in very high density is the ATP-sensitive potassium (K<sub>ATP</sub>) channel [11]. This channel, which when opened results in an intracellular to extracellular flow of potassium, is normally inhibited from opening by ATP [11,12]. During ischemia, possibly as a result of ATP depletion or lactate buildup, the K<sub>ATP</sub> channel opens, resulting in a shortening of the monophasic action potential and also a reduction in myocyte metabolic rate [11–13]. As a result of K<sub>ATP</sub> channel activation, ATP levels remain higher, and lactate accumulation is reduced during ischemia and calcium overload during ischemia, and early reperfusion is reduced [11,14,15].

Pharmacologic activation of K<sub>ATP</sub> channel with drugs such as bimakalim, cromakalim and nicorandil results in cardiac protection in models of ischemia/reperfusion with reduction in infarct size and myocardial stunning reported in various animal species [15,16,17].

It appears ultimately that activation of K<sub>ATP</sub> channel during ischemic preconditioning may be a result of signaling through adenosine, acetylcholine, bradykinin–nitric oxide pathways. [10,18,19–21] To illustrate the idea that K<sub>ATP</sub> channel activation is a late event in ischemic preconditioning signal transduction, pharmacologic blockade of K<sub>ATP</sub> channel with glibenclamide is reported to abrogate the cardioprotective effects of ischemic preconditioning and, additionally, pharmacologic preconditioning with adenosine, acetylcholine or bradykinin [13,18,19].

Monophosphoryl lipid A (MLA), a non-toxic derivative of endotoxin [22,23], has been reported to possess cardioprotective activity in various animal models of ischemia/reperfusion injury and is currently undergoing clinical study in coronary artery bypass graft surgery patients. Previous reports indicate that MLA when administered 12 to 24 h but not 1 h prior to ischemia reduces infarct size in rabbits and dogs. Regional myocardial stunning or global contractile dysfunction in dogs subjected to either regional ischemia or global ischemia (associated with cardiopulmonary bypass), respectively, is also reduced with MLA pretreatment [24–31]. Dose–response studies of MLA in the rabbit infarct model have up to this point in time not been reported and, therefore, were undertaken.

The observation that MLA’s cardioprotection develops between 1 and 12 h following administration suggests that the mechanism of delayed preconditioning and that of pharmacologic preconditioning with MLA may share common features. Although it is presently not known if K<sub>ATP</sub> channel activation plays a role in delayed preconditioning, the importance of this potassium channel in ‘first window’ ischemic preconditioning is well established [13,18,33]. We therefore undertook an examination of the potential role of K<sub>ATP</sub> channel modulation in the cardioprotective effects of MLA in this rabbit model of regional in situ cardiac ischemia/reperfusion injury.

Monophosphoryl lipid A, as is also well known with endotoxin, can be expected at certain dose levels to induce the elaboration of various cytokines, including tumor necrosis factor, into the systemic circulation following in vivo administration [35]. In fact, tumor necrosis factor has been detected in serum following administration of certain doses of MLA to mice or humans [34,36]. Tumor necrosis factor has been reported in the literature at high doses to be cardioprotective in rat and rabbit models of cardiac ischemia/reperfusion injury [32,37]. Protection is associated with induction of antioxidant enzymes such as MnSOD, which appears to be a compensatory mechanism to reduce the inherent sensitivity of various cells to the oxidative cytotoxicity of tumor necrosis factor [38]. Therefore, an enzyme-linked immunosorbent assay which allowed detection and quantification of mouse tumor necrosis factor was developed. This assay was used to detect increases in rabbit serum tumor necrosis factor concentration in response to administration of cardioprotective and higher (mildly pyrogenic) doses of MLA (35 or 120 μg/kg, respectively). S. abortus equi endotoxin was administered to rabbits which did not undergo surgery as a positive control for tumor necrosis factor detection using our enzyme-linked immunosorbent assay.

2. Methods

2.1. General animal surgical preparation

Male New Zealand White rabbits (1.8–2.5 kg) were used in this study. The experiment conforms with the Guide for the Care and Use of Laboratory Animals pub-
lished by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1985). The experimental protocol was also approved by the Institutional Animal Care and Use Committee.

Rabbits were fasted overnight and anesthetized by intramuscular injection of ketamine (100 mg/kg)/xylazine (20 mg/kg)/atropine (80 μg/kg) combination. One carotid artery was cannulated to measure arterial pressure, while the other carotid artery was used to implant a catheter in the left ventricle for monitoring ventricular function. Rabbits were ventilated with room air supplemented with 100% oxygen. Body temperature was maintained at 37°C by using a water-circulating heating pad. A left thoracotomy was performed at the fourth intercostal space. The pericardium was incised, and an anterolateral branch of the circumflex coronary was identified. A silk suture on a curved taper needle was passed underneath the coronary artery at approximately midway between the apex and the base of the heart, a location at which the vessel is routinely observable on the epicardium surface of the heart. The ends of the suture were passed through a button and a short plastic tube to form a snare. Thirty minutes of myocardial regional ischemia was induced by pulling the ends of the suture and tightening the snare using a hemostat. Occlusion was confirmed by the development of epicardial cyanosis (blanching) and electrocardiographic (ECG) changes. Three hours of reperfusion following occlusion was accomplished by release of the snare and confirmed by blushing of the previously ischemic myocardium and reversal of ECG changes.

Lead II ECG, arterial and left ventricular pressure, as well as dp/dt were monitored throughout the experiment. The hemodynamic parameters at certain time points were collected and summarized using the digital acquisition analysis system, PO-NE-MAH (Scientific Systems Integration, Mansfield, MA). Arterial blood pH, Po2, and PCO2 were measured at selected intervals by AVL® Compact 1 Blood Gas Analyzer and maintained within a normal physiological range (pH 7.30–7.45, Po2 90–120 mmHg, PCO2 30–45 mmHg) by adjusting the respiratory rate and oxygen flow. Rabbit blood glucose concentrations were measured by ACCU-Chek® Monitoring Systems (Boehringer Mannheim Corporation, Indianapolis, IN) before occlusion and at selected time points during glibenclamide blocking experiments.

2.2. Experimental design

In the infarction study, animals were randomly assigned to 7 groups. Dose–response evaluation of monophosphoryl lipid A (MLA) cardioprotection was initiated as the first level of investigation. Four groups of animals were pretreated with intravenous injection of MLA at either 35, 10, 5.0 μg/kg and 2.5 μg/kg in a standard injection volume 24 h before surgery. The fifth group received an equivalent volume of injection vehicle. MLA was formulated in 40% propylene glycol, 10% ethanol and 60% Water for Injection, USP. The sixth and seventh groups were used to test if cardiac protection by MLA is mediated by opening KATP channels. In appropriate test groups the KATP channel blocker, glibenclamide, was given 30 min before occlusion at 300 μg/kg via intravenous injection to rabbits pretreated with either 35 μg/kg MLA or MLA injection vehicle.

Three additional groups of rabbits (n = 4) were treated with a non-pyrogenic yet cardioprotective dose and a pyrogenic dose of MLA (35 and 120 μg/kg, respectively) or injection vehicle. Blood was collected immediately before treatment, 1, 2, 3 and 24 h after treatment from an ear vein. Serum was separated and used to measure the pyrogenic and potentially cardioprotective cytokine tumor necrosis factor as described below. As a positive control for the tumor necrosis factor assay, 2 additional rabbits received 17.5 μg/kg (i.v.) of Salmonella abortus equi endotoxin (Ribi ImmunoChem Research, Inc.), and blood was collected before and 1 h after endotoxin injection.

2.3. Infarct size determination

At the end of 3 h reperfusion the same coronary artery was reoccluded. Three milliliters of a 50% W/V solution of Unisperse® blue dye (DuPont, Wilmington, DE) were injected into the left ventricle to allow delineation of non-ischemic area of the heart. The heart was excised and frozen at −20°C for 35 min and cut into 2 mm thick transverse slices from apex to the position of the suture. The slices were then stained by incubation at 37°C for 20 min in 1% triphenyltetrazolium chloride which was made in pH 8.5 phosphate buffer. The area of infarct (the pale color tissue within risk area), area of risk (area unstained by the blue dye), and total left ventricle area were measured by computer morphometry using Optimas imaging software (Optimas Corp., Bothell, WA). Risk area is expressed as percentage of left ventricle, and infarct area is calculated as percentage of area at risk.

2.4. Tumor necrosis factor assay

Rabbit serum tumor necrosis factor was measured by an enzyme-linked immunosorbent assay method developed in our lab using purified goat antirabbit tumor necrosis factor polyclonal antibody and biotin-conjugated secondary antibody from Pharmingen (San Diego, CA). Rabbit tumor necrosis factor isolated and purified from endotoxin-stimulated rabbit peritoneal exudate macrophages (PharMingen) was used as a standard in the assay. Serum collected from all test rabbits before endotoxin or MLA challenge was pooled and served as normal rabbit serum for blanks and additionally for making standard dilutions in the assay. Assay sensitivity was determined to be between 100 and 200 pg/ml. The absorbance for each rabbit baseline serum sample on the multiwell assay plate was normalized as 0 pg/ml tumor necrosis factor. Serum cytokine concentrations following endotoxin or MLA dos-
ing were calculated from the differences in absorbance between the baseline and post-treatment serum samples.

2.5. Statistics

All values are expressed as mean ± s.e.m. ANOVA for repeated measures across time, followed by Duncan’s multiple range test, were used to define any significant differences among groups for hemodynamics, blood gas parameters and glucose concentrations. Differences among groups in risk area and infarct size were identified by one-way analysis of variance and Duncan test. A P-value of < 0.05 was considered statistically significant.

3. Results

3.1. Hemodynamics, blood pH and gases

Hemodynamic data from all the groups are summarized in Table 1. There were no statistically significant differences at baseline among various hemodynamic indices between treatment groups. Heart rate, mean arterial blood pressure and left ventricular developed pressure were in general somewhat higher in the 10 μg/kg MLA treatment group compared with vehicle control at baseline and through the reperfusion time points. It is not clear that this was a treatment-related observation. Statistical difference was found in left ventricular developed pressure at the end of reperfusion and in heart rate at various post-baseline timepoints in the 10 μg/kg MLA-pretreated rabbits when compared with controls, but the lack of a dose–response relationship across MLA dose levels suggests that this observation occurred as a result of random chance rather than as a result of a true drug effect. Heart rate was slightly decreased during reperfusion in all groups, but only vehicle-control-treated animals showed a statistically significant decrease by the end of reperfusion as compared with mean baseline value. Mean arterial blood pressure was lower at the end of ischemia and during reperfusion when compared with baseline in most of the groups. There was an appreciable drop in left ventricular developed pressure at the end of ischemia and during reperfusion.
pressure and positive rate of contraction (dp/dt) after ischemia or reperfusion in all the groups across time. Administration of glibenclamide with or without 35 μg/kg MLA was not associated with pronounced differences in various hemodynamic indices when compared with the treatment group receiving 35 μg/kg MLA alone. In summary, the few significant differences in hemodynamics observed among groups did not clearly appear to be treatment-related. Reversal of ECG changes was noted for all treatment groups upon reperfusion with the exception of 3 rabbits, one vehicle control, one MLA + glibenclamide and one vehicle control + glibenclamide where the physiographic tracings were difficult to interpret (these animals were included in the database). Blushing of the myocardium was noted upon reperfusion in all animals tested. These observations suggest to us that reperfusion was successfully achieved in the animals enrolled in the study.

Mean values ± s.e.m. for blood gases and pH in 3 representative groups, vehicle control, 35 μg/kg MLA and MLA + glibenclamide, are listed in Table 2. There were no differences in blood pH or dissolved gases between groups across time.

3.2. Myocardial infarction: dose–response and glibenclamide blockade

The effects of pretreatment with different doses of MLA, administration of glibenclamide alone or in combination with 35 μg/kg MLA pretreatment on infarct size and area at risk in left ventricle are summarized in Figs. 1 and 2, respectively (data represented as mean value ± s.e.m.). Left ventricular weight did not significantly differ between treatment groups (data not shown). No treatment-related differences were observed in area at risk. Pretreatment with MLA 24 h pre-ischemia at dose levels of 10 μg/kg (n = 7) and 35 μg/kg (n = 7) significantly reduced infarct size (6.2 ± 3.0 and 7.5 ± 3.0%, respectively) versus 21.0 ± 3.6% (n = 9) in control. Rabbits pretreated with 5 μg/kg MLA (n = 7) displayed a trend towards smaller infarct size (13.4 ± 2.3%) as compared to controls; however, the difference (36%) was not statistically significant. Animals which received 2.5 μg/kg MLA (n = 7) 1 day before surgery displayed an infarct size (18.3 ± 8.1%) similar to controls. Glibenclamide, when given at 300

![Diagram](https://example.com/diagram.png)
Table 3

<table>
<thead>
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<th>Treatment</th>
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<th>Time after injection</th>
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<tr>
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MLA and LPS were administered through car vein. Baseline blood samples were obtained immediately before injection and TNF-α levels at baseline were normalized as zero for all rabbits. UDL = under detectable level; ND = not determined.

clamidine administered to vehicle-control-pretreated animals (n = 7) did not alter infarct size (17.8 ± 3.5%) in comparison with vehicle-control-treated rabbits (21.0 ± 3.6%).

3.3. Serum tumor necrosis factor levels

Tumor necrosis factor levels were quantitated in rabbits intravenously administered MLA or vehicle injection just prior to administration of test material and at 1, 2, 3 and 24 h post-administration (Table 3). Among vehicle-treated...
rabbis, 2 of 4 animals had slightly higher serum tumor necrosis factor concentrations (400–1000 pg/ml) at 1 and 2 h after injection. None of the rabbits treated with a cardioprotective dose of MLA (35 μg/kg) had tumor necrosis factor levels that were above the sensitivity of the assay. Consequently, we also administered intravenously to a separate group of 4 animals a higher dose of MLA (120 μg/kg) known by the investigators to induce fever in rabbits with the idea that elaboration of the pyrogenic cytokine tumor necrosis factor might be responsible for the fever observed at this higher MLA dose. Only 1 of 4 animals treated with 120 μg/kg MLA developed an elevated serum tumor necrosis factor concentration following dosing (800 pg/ml at 2 h post-injection). In contrast, however, the 2 rabbits which received endotoxin displayed serum tumor necrosis factor concentrations of 11 000 and 99 000 pg/ml 1 h after dosing, confirming the assay capable of detecting rabbit tumor necrosis factor secreted. These data indicate that a cardioprotective MLA dose of 35 μg/kg and an even higher pyrogenic MLA dose (120 μg/kg) do not reliably induce detectable tumor necrosis factor release into the bloodstream in comparison with vehicle-treated controls within the limits of detection for this assay (100–200 pg/ml). Sporadic and dose-unrelated detection of low tumor necrosis factor levels in the vehicle- or MLA-treated groups (2 of 4 and 2 of 8 animals, respectively) may have been a consequence of the stress caused by repetitive animal handling during blood collection. In contrast, endotoxin at a dose capable of causing lethargy in rabbits (17.5 μg/kg) resulted in the elaboration of high levels of tumor necrosis factor into serum at 1 h post-dose, serving as a good positive control for the experiment.

4. Discussion

In this rabbit model of in situ regional ischemia and reperfusion, monophosphoryl lipid A formulated in a 10% ethanol 40% propylene glycol parenteral formulation suitable for intravenous use in humans significantly reduced infarct size expressed as a percent of left ventricle at risk (> 60%) at dose levels of 10 and 35 μg/kg, with dose levels of 2.5 and 5.0 μg/kg being ineffective. Area at risk was not affected by treatment with MLA. Previous human experience with intravenously administered MLA suggests that doses of at least 20 μg/kg may be safely administered to humans, with mild flu-like symptoms being the initial reported side-effect [34]. In our laboratory cardioprotective doses of 35 μg/kg MLA did not induce a measurable increase in body temperature in the rabbit. The finding that significant infarct reduction can likely be achieved in the rabbit at doses of 10 μg/kg lends some support to the idea that administration of MLA to humans at below the maximum tolerated dose may prove cardioprotective against ischemia/reperfusion injury.

Dose–response work with MLA in a canine infarct model conducted by G. Gross et al. also indicates that doses of 10 and 35 μg/kg are cardioprotective (≥ 50% reduction in infarct size), while protection falls off at 3 μg/kg (27% reduction of infarct size), corroborating findings in this rabbit model (unpublished observations).

Attempts to detect tumor necrosis factor in serum of rabbits treated with cardioprotective doses of MLA (35 μg/kg) failed to document the elaboration of this cytokine in response to drug administration within the appropriate window of time following drug administration (first 3 h post-dose). Even higher doses of MLA (120 μg/kg) did not result in release of appreciable cytokine into serum, while in contrast intravenous administration of endotoxin at dosing causing a symptomatic response (lethargy) (17.5 μg/kg) was associated with detection of high serum concentrations of tumor necrosis factor 1 h post-dose. It is not apparent from these studies that induction of serum tumor necrosis factor could potentially play a role in the cardioprotective activity of MLA in the rabbit.

Attempts to date by other investigators to correlate generation of a stress response (heat shock protein induction) or systemic inflammatory response (catalase or superoxide dismutase induction, neutrophil infiltration) in myocardium at low (10–100 μg/kg) cardioprotective doses of MLA have failed to do so [24,25,27]. Protection from cardiac ischemia/reperfusion injury with MLA, therefore, appears at this time not to be associated with induction of a sublethal oxidative stress as a means of protecting from a subsequent ischemia/reperfusion-associated oxidative stress as has been previously suggested from cardioprotection studies utilizing high-dose tumor necrosis factor (10 μg in rabbits or rats), endotoxin (500 μg/kg in rats) and even high-dose MLA (5000 μg/kg in rats) [30,32,37,39].

Administration of a single intravenous dose of glibenclamide (300 μg/kg) to rabbits 30 min prior to ischemia appears to very effectively block the ability of pretreatment with MLA 24 h prior to ischemia to limit infarct size. In contrast, administration of 300 μg/kg glibenclamide to MLA vehicle-treated control rabbits had no effect in and of itself on infarct size at this dose level. Measurement of blood glucose in MLA or vehicle-pretreated rabbits which were subsequently treated with a single dose of glibenclamide indicated that significant 'hypoglycemia' (defined as ≤ 80 mg/dl or ≥ 50% decrease from baseline) was not observed in MLA + glibenclamide-treated animals in comparison with animals receiving MLA alone, although 3 of 7 rabbits receiving vehicle pretreatment followed by glibenclamide 30 min prior to ischemia developed some degree of 'hypoglycemia' (Table 4). Hypoglycemic animals were administered 5–10 ml/dose of 5% W/V dextrose in Water for Injection to adjust blood glucose into 'normal' range.

Glibenclamide, a member of the sulfonylurea class of oral hypoglycemic agents, is known to block the ATP-sensitive potassium channel in myocytes [21] and presumably...
Cardioprotection is calcium-independent nitric oxide synthesis. Studies conducted by Mei et al. in a dog infarct model corroborate that K\textsubscript{ATP} channel may, as well, be important in MLA cardioprotection in the canine [44]. Measurement of monophasic action potential in this dog model suggests that MLA-pretreated dogs show a pronounced enhancement of shortening in action potential in the first 5 min of ischemia, an electrophysiologic response which has been associated with priming of K\textsubscript{ATP} channels for enhanced opening in response to ischemia. In the above described canine study, the K\textsubscript{ATP} channel antagonist, glibenclamide, blocked MLA cardioprotection, as evaluated by infarct size determination, as well as the drug’s effect on monophasic action potential duration.

It remains to be definitely shown that MLA modulates K\textsubscript{ATP} channel either directly or indirectly, although a direct effect is less likely considering that pretreatment is required for cardioprotection. Experiments using patch clamp technique and MLA-treated primary myocyte cultures, which are presently underway, should address this issue. Important questions which remain unanswered include how MLA exerts cardioprotection through the K\textsubscript{ATP} channel. MLA may directly lead to phosphorylation and priming of K\textsubscript{ATP} through kinase activation or alternatively modulate the channel through the elaboration of a secondary factor which acts as a channel agonist.

Candidates for secondary mediators of MLA’s effect include bradykinin and adrenomedullin [19,45,46]. Possibly the best candidate for a secondary mediator of MLA’s cardioprotection is calcium-independent nitric oxide synthase. It has recently been reported that bradykinin and nitric oxide may be important mediators of ischemic preconditioning. Pharmacologic blockade using a bradykinin antagonist [47,48] or the nitric oxide synthase inhibitor, L-NAME [49] blocks the ability of transient ischemia to reduce infarct size in the rabbit. Downey et al. propose that bradykinin-mediated cardioprotection may be coupled to protein kinase C (PKC) activation as he has shown that ‘PKC inhibitors’ block said protection [47]. Wall et al. propose that ultimately the bradykinin–nitric oxide pathway of ischemic preconditioning protection is mediated through ATP-sensitive potassium channels as illustrated by the fact that glibenclamide blocks the ability of bradykinin to limit infarct size in the rabbit [19]. Additionally, single channel recording techniques in myocytes suggest that nitric oxide increases open state probability of K\textsubscript{ATP} channel [50]. It has also been proposed that delayed preconditioning as induced by lipid A materials and rapid cardiac pacing can be the result of inducible nitric oxide synthase (iNOS) upregulation [51].

Three publications have recently looked at the ability of endotoxin to directly or indirectly induce iNOS in myocytes [52–54]. Shindo et al. report that in 3-day-old primary cultures of neonatal rat cardiomyocytes in vitro exposure to endotoxin (1 μg/ml) can induce intracellular iNOS and cGMP [53]. McKenna confirms this effect in primary rat cardiomyocyte cultures (apparently used immediately post-isolation) using 100 ng/ml endotoxin [54]. Luss et al. reported that in vivo administration of endotoxin to rats resulted in pronounced induction of iNOS in myocardial biopsies [52]. Future studies with iNOS inhibitors in this rabbit model combined with efforts to document induction of iNOS mRNA and/or protein in rabbit myocardium and in isolated rabbit cardiomyocytes will evaluate the possible role of iNOS induction and, therefore, presumably nitric oxide signaling through K\textsubscript{ATP} channel in the cardioprotective activity of MLA.

Monophosphoryl lipid A appears to be a novel means of pharmacologically inducing delayed myocardial preconditioning, increasing the tissues’ ability to withstand injury from ischemia/reperfusion events. Studies currently underway in patients undergoing coronary artery bypass grafting should help to define the potential of MLA as a clinically useful cardioprotective agent.

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