

Nitric oxide-dependent mechanism of anti-ischemic myocardial protection induced by monophosphoryl lipid A¹

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KEY WORDS myocardial reperfusion injury; potassium channels; myocardial infarction; nitric oxide; knockout mice; cytokines; lipopolysaccharides; signal transduction

ABSTRACT

Monophosphoryl Lipid A (MLA) is a detoxified derivative of endotoxin and was first derived and purified from bacterial lipopolysaccharide in 1980s. This pharmacological agent has been studied as a vaccine adjunct, anti-septic, or anti-tumor agent by means of its immunomodulatory properties. In addition, MLA is one of the most well documented protective drugs against cardiac ischemia/reperfusion injury in various animal species. Mechanisms involved with the MLA-induced cardioprotection are still not fully understood. A key role for ATP-sensitive potassium channels and inducible nitric oxide synthase (iNOS) has been proposed. This article provides a brief overview on the updated understanding of MLA-induced cardioprotection and focuses on the new evidence and insights that were brought into the field by a number of new publications during 1998 - 1999. Our recent study in a globally ischemic mouse heart model is particularly highlighted. An obligatory role for nitric oxide (NO) in mediating the delayed

cardioprotective effect induced by MLA via induction of iNOS was double-confirmed by using *S*-methylisothiourea (SMT) — a specific inhibitors of iNOS as well as the iNOS gene knockout mice. A direct association of the MLA-induced infarct size reduction with increased NO production was also demonstrated in this study. Future studies should target on identifying the key type(s) of cytokine and the receptors as well as free radical-activated transcription factors that may be responsible for induction of iNOS and the subsequent anti-ischemic cardioprotection with MLA. Information gathered in the studies on MLA may eventually enhance our understanding in the mechanisms of delayed phase of myocardial preconditioning and its clinical applications.

BACKGROUND OF MONOPHOSPHORYL LIPID A (MLA)

MLA is a relatively non-toxic derivative of endotoxin and was first derived and purified from lipopolysaccharide (LPS) of the gram negative strains in 1980s^[1]. Its chemical structure is shown in Fig 1. It retains several of the immunomodulatory properties of the parent endotoxin molecule with the reduced toxicity. MLA has been shown to induce beneficial immunomodulatory effects such as cytokine production, macrophage stimulation, and a variety of other effects upon both humoral and cell-mediated immunological host defense response^[2-7]. These effects of MLA may be exploited for the use of: 1) inducing the tolerance to endotoxemia which has been observed in both laboratory animals^[2,3] and human subjects^[4]; 2) being an immuno-therapeutic^[8] or immuno-prophylactic^[9]; 3) being an adjuvant for a number of

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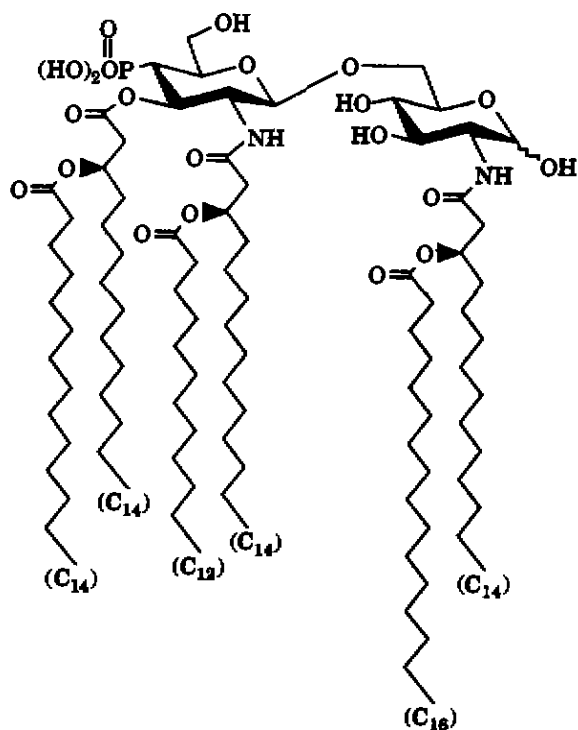
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vaccines^[10]; 4) inducing the delayed protection against cerebral vascular spasm caused by hemorrhage^[11].

The commonest source of the soluble MLA and its vehicle solvent (40 % propylene glycol and 10 % ethyl alcohol in water) used in the published papers was from the Ribl ImmunoChem Research Inc. (Hamilton MT, USA).



Monophosphoryl lipid A

CARDIOPROTECTIVE EFFECTS OF MLA

A list of sixteen representative studies since 1991 on the MLA-induced cardioprotection is summarized in Tab 1. Knowledge on this drug has been accumulated mainly within the following areas.

1 Forms of the cardioprotection Vast majority of the studies reported a reduction in myocardial infarct size following ischemia/reperfusion in MLA-pretreated rats^[26], rabbits^[15-17,21-23,25], dogs^[13,18-20] and mice^[27]. In addition, a few studies also demonstrated that MLA protected heart against stunning^[12,13,17,24] and/or arrhythmia^[24,26] caused by ischemia/reperfusion. The anti-ischemic cardioprotections were observed not only in healthy anesthetized

animals but also in the conscious rabbits with hypercholesterolemia and atherosclerosis^[25].

2 Temporal profile Almost all published studies listed in Tab 1 had focused on the delayed phase of cardioprotection, ie the protection occurred approximately 24 h following the drug treatment. A more complete time course of myocardial infarct size following a dose of intravenous injection of MLA (35 $\mu\text{g}/\text{kg}$, iv) was recently reported by Elliott^[28] who also showed an "early window" for the anti-infarct protection. These early effects seemed to be short-lived and lasted no more than 30 min after the drug treatment. In contrast, a delayed phase of protection reappeared around 6 h and sustained until 36 - 48 h after the MLA treatment. It is notable that the late window of MLA-induced protection is much better investigated and can be consistently reproduced by numerous laboratories, as compared to the early window.

3 Species-related difference It is interesting that rodents appear to require much higher drug concentration to exert the cardioprotective effects (Tab 1). The cardioprotective doses of MLA were from at least 300 $\mu\text{g}/\text{kg}$ ^[24] to as much as 5000 $\mu\text{g}/\text{kg}$ ^[12,26] for rats, 350 $\mu\text{g}/\text{kg}$ for mice^[27], whereas a small dose of 35 $\mu\text{g}/\text{kg}$ was sufficient to induce protective effects in rabbits and dogs.

MECHANISMS OF CARDIOPROTECTION INDUCED BY MLA

The mechanism of MLA-induced protection is not well understood although several possibilities have been suggested. It is notable that this subject was extensively reviewed by Elliott^[28]. Accordingly, the present article focuses mainly on the new evidence and insights provided by a number of new publications during 1998-1999. Up to date, majority of the previous mechanistic studies had targeted on the following areas:

1 Induction of cytoprotective proteins

One of the plausible explanations for the delayed cardioprotection induced by MLA was that this drug might induce new synthesis of cytoprotective proteins such as antioxidant enzymes and heat shock proteins (HSP). However, all of experimental data accumu-

Tab 1. Representative literatures published during 1991 - 1999 on delayed anti-ischemic cardioprotection induced by monophosphoryl lipid A.

Authors	Species	Experimental model	Drug dose	Type & duration of ischemia	Forms of protection
1 Nelson <i>et al</i> (1991)	Rat	Isolated heart "Langendorff"	5000 $\mu\text{g}/\text{kg}$, ip	Global 25 min	Post-ischemic function \uparrow
2 Yao <i>et al</i> (1993)	Dog	<i>In vivo</i>	30, 100 $\mu\text{g}/\text{kg}$, iv	Regional 30 min	Infarct size \downarrow
3 Maulik <i>et al</i> (1995)	Rat	Isolated heart "Working"	500 $\mu\text{g}/\text{kg}$, ip	Global 30 min	Post-ischemic function \uparrow
4 Yoshida <i>et al</i> (1996)	Rabbit	<i>In vivo</i>	35 $\mu\text{g}/\text{kg}$, iv	Regional 30 min	Infarct size \downarrow Post-ischemic function \downarrow
5 Baxter <i>et al</i> (1996)	Rabbit	<i>In vivo</i> / Isolated heart	35 $\mu\text{g}/\text{kg}$, iv	Regional/30 min Global/20 min	Infarct size \downarrow (regional) Infarct size \rightarrow (global)
6 Zhao <i>et al</i> (1996)	Rabbit	<i>In vivo</i>	35 $\mu\text{g}/\text{kg}$, iv	Regional 90 min	Post-ischemic function \uparrow Infarct size \rightarrow
7 Przyklenk <i>et al</i> (1996)	Dog	<i>In vivo</i>	35 $\mu\text{g}/\text{kg}$, iv	Regional 60 min	Infarct size \downarrow
8 Mei <i>et al</i> (1996)	Dog	<i>In vivo</i>	35 $\mu\text{g}/\text{kg}$, iv	Regional 60 min	Infarct size \downarrow
9 Zhao <i>et al</i> (1997)	Rabbit	<i>In vivo</i>	35 $\mu\text{g}/\text{kg}$, iv	Regional 30 min	Infarct size \downarrow
10 Przyklenk <i>et al</i> (1997)	Dog	<i>In vivo</i>	35 $\mu\text{g}/\text{kg}$, iv	Regional 20 min	N/A
11 Elliott <i>et al</i> (1997)	Rabbit	<i>In vivo</i>	35 $\mu\text{g}/\text{kg}$, iv	Regional 45 min	Infarct size \downarrow
12 Janin <i>et al</i> (1998)	Rabbit	<i>In vivo</i>	35 $\mu\text{g}/\text{kg}$, iv	Regional 35 min	Infarct size \downarrow
13 Tosaki <i>et al</i> (1998)	Rat	Isolated heart "Working"	300, 450 $\mu\text{g}/\text{kg}$, ip	Global 30 min	Arrhythmia \downarrow Post-ischemic function \uparrow
14 Szilvassy <i>et al</i> (1998)	Rabbit	<i>In vivo</i> / Conscious	10, 35 $\mu\text{g}/\text{kg}$, iv	Pacing-induced 30 min	ST-elevation \downarrow
15 Song <i>et al</i> (1998)	Rat	<i>In vivo</i>	5000 $\mu\text{g}/\text{kg}$, ip	Regional 30 min	Infarct size \downarrow Arrhythmia \downarrow
16 Xi <i>et al</i> (1999)	Mouse	Isolated heart "Langendorff"	35, 350 $\mu\text{g}/\text{kg}$, ip	Global 20 min	Infarct size \downarrow (350 $\mu\text{g}/\text{kg}$) Post-ischemic function \rightarrow

lated up to today showed very little supportive evidences to this hypothesis. For example, Nelson *et al* found an association of post-ischemic functional improvement with a significant increase of catalase enzyme activity in the rat hearts^[12]. However, the drug dose used by these authors was very high (5000 $\mu\text{g}/\text{kg}$, ip). In contrast, Yao *et al* found only a marginal increase in myocardial catalase activities in the MLA-treated dogs as compared to the control group^[13]. In addition, despite LPS pretreatment was shown to induce HSP70i expression in rat myocardium that is associated with the delayed cardioprotection^[29], cardioprotective dose of MLA did not result in a concomitant induction of this protein in rabbit heart^[15,16]. These evidences indicated neither

antioxidants nor HSP were responsible for the remarkable cardioprotection afforded by MLA.

2 Involvement of adenosine and bradykinin Most of the studies on the subject of adenosine and MLA-induced protection had been done by Przyklenk and co-workers in a canine model^[18,20]. They found the disparate effects of MLA-pretreatment and ischemic preconditioning on the myocardial adenosine level and activity of an adenosine-regulating enzyme-5'-nucleotidase (5'-NT)^[20]. There was no correlation between 5'-NT activity and infarct reduction. In fact, during both of the preconditioning approaches, the myocardial 5'-NT activity and adenosine level need not to be elevated beyond the control level to achieve the infarct reduction. Taken

together, an adenosine-dependent mechanism for the MLA-induced cardioprotection is unlikely the primary determinant.

A recent study of Mazenot *et al.*^[30] demonstrated that, in contrast to LPS, pretreatment of MLA (100 $\mu\text{g}/\text{kg}$, iv) in rabbits did not induce bradykinin B₁ receptor synthesis and its associated hypotensive response to (des-Arg⁹)-bradykinin 24 h later. Therefore, MLA-induced delayed cardioprotection seemed not related to a B₁ receptor-dependent pathway.

3 Opening ATP-sensitive potassium (K_{ATP}) channels Opening of K_{ATP} channel appears to be protective due to 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^[31]. Decreased intracellular calcium overload may reduce ischemic injury and lead to better myocyte preservation. There is mounting evidence supporting the involvement of K_{ATP} channels in the mechanism of ischemic preconditioning^[32] and pharmacological protection with MLA^[19,22,23].

Although opening of K_{ATP} channel is strongly believed to be the most important end-effector of MLA-induced cardioprotection, the key intermediate factors of the signal transduction cascade remain to be hypothetical. It was recently proposed that phosphorylation caused by activation of the possible receptor-coupled tyrosine kinase and in turn protein kinase C may be the common mediators to further activate the nuclear factor kappa B and upregulation of NO production through induction of iNOS^[28]. It is notable that until today the role of tyrosine kinase and protein kinase in MLA-induced cardioprotection has not yet been proved by any published experimental data. However, evidences for a critical role of iNOS have emerged during the time period of 1998–1999. In the following paragraphs we discuss these new evidence and insights in more details.

NEW EVIDENCE OF AN iNOS-DEPENDENT MECHANISM FOR MLA-INDUCED CARDIO-PROTECTION

Recent studies have shown the ability of MLA to induce iNOS either *in vitro* or *in vivo*^[33,34]. It was

Zhao *et al.*^[21] who first demonstrated that delayed cardioprotection with MLA can be abolished by aminoguanidine — an inhibitor of inducible nitric oxide synthase (iNOS). However, some non-specific effects of the pharmacologic inhibitors can not be ruled out. Since nitric oxide (NO) is produced from L-arginine through chemical reaction which is catalyzed by at least three major isoforms of NOS, ie iNOS (inducible), eNOS (endothelial), and nNOS (neuronal) ad^[35], 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. Accordingly, in one of our recent studies^[27], we pretreated adult male ICR mice and the B6.129 homozygous (-/-) iNOS knockout mice with MLA. The ischemia/reperfusion protocol was carried out in our well-established Langendorff isolated perfused mouse heart model^[36,37]. The protocol consists of 30 min of stabilization, 20 min no-flow global ischemia, and 30 min reperfusion. Parameters of cardiac function as well as myocardial infarct size were assessed in this mouse model. In addition, the NO oxidation products (nitrite and nitrate) accumulated in the heart tissue samples were also measured as index of total NO production, using a nitric oxide analyzer (SIEVERS, Model 280NOA). In this investigation, we observed that the cardioprotection was markedly abolished by SMT — a potent and selective inhibitor of iNOS^[38]. 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. Furthermore, the complete lack of MLA-induced protection in the iNOS knockout mice suggests an obligatory role of this isoform of NOS in the protective process. We also found that the cardioprotective dose of MLA markedly increased NO production following ischemia/reperfusion but not in the non-ischemic hearts, suggesting that iNOS was functional only in the ischemic hearts^[27]. Similar observation was also previously reported in MLA-treated rabbits^[21]. This suggests that post-translational modifications of the iNOS enzyme are required before it is able of generating NO^[39]. It is possible that ischemia may activate certain kinases (such as protein kinase C and tyrosine kinase) or inhibit phosphatases

that may promote phosphorylation-dependent activation of the inactive iNOS induced by MLA within certain time period after the drug administration.

NO is critical in the signal transduction in biological systems^[40] including in the ischemic myocardium^[41]. NO is one of the key modulators of vascular smooth muscle tone and its biological action can be cardioprotective against ischemia/reperfusion injury through coronary vasodilatation and reduction in myocardial oxygen consumption via cGMP-dependent as well as cGMP-independent mechanisms^[40-42]. More recently, NO has been appreciated as the possible key trigger and mediator for the ischemic preconditioning^[43]. NO itself or through the second messenger cGMP may also modulate K_{ATP} channels. The cGMP dependent protein kinases may be capable of phosphorylating K_{ATP} channels and priming the channel to offer cardioprotection^[31,42]. The studies that we discussed above on the MLA-induced late cardioprotection further support the concept that opening of K_{ATP} channels may be the ultimate end-effector for the drug-induced protection which is primarily mediated by NO.

UNSOLVED PROBLEMS AND FUTURE DIRECTIONS

On the other hand, the role of constitutive forms of NOS (cNOS) in MLA-induced protection is still unclear. There is a good possibility that MLA may also activate cNOS which may serve as the triggers of the signal transduction cascade that causes the above-discussed secondary induction of iNOS. For example, we observed a consistent improvement in pre-ischemic coronary flow in all MLA-treated groups. Blocking iNOS with SMT did not reverse the improvement of coronary flow in MLA-treated mice. These data suggest that MLA may have been improving vascular endothelial function independent of iNOS enzyme. In addition, despite a great deal of evidence supporting iNOS as the obligatory mediator and K_{ATP} channels as the possible end-effector, the upstream signal transduction including initial triggering mechanism remains unknown. The role of free radicals and/or cytokines acting on any type of membrane receptors and/or other intracellular pathways in the underlying mechanisms has not yet been confirmed by any

published experimental data. Further investigations in the murine model should be necessary in elucidating: 1) the potential role of eNOS or nNOS in MLA-induced cardioprotection; 2) the key types of cytokines and their receptors that may be responsible in activation of iNOS; 3) the potential role of PKC and tyrosine kinases in the signal transduction cascade; and 4) other pharmacological agents which could induce late preconditioning via similar signal transduction pathways as MLA. Most recently, we and another research group in the US have observed that RC552 — a synthetic compound that mimic the chemical structure of MLA can also induce delayed cardioprotection in mice^[44] and dogs^[45] with even less side-effects than MLA.

SUMMARY

A detoxified derivative of bacterial endotoxin, MLA is able to induce late cardioprotection against myocardial infarction in the ischemic/reperfused hearts in a dose-dependent manner. This anti-necrotic effect is associated with enhanced NO production in the ischemic tissue and can be blocked by selective inhibition of iNOS and is completely absent in iNOS knockout mice. The direct evidences obtained from our recent studies in a mouse model suggest an obligatory role of iNOS in mediating the delayed cardioprotection by MLA. In addition, it seems that the upregulation of NO production may open the mitochondrial and/or sarcolemmal K_{ATP} channels which appear to be the end-effectors of the drug-induced cardioprotection. The detailed triggering mechanism by MLA to initiate the signal transduction cascade remains unknown.

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单磷酸酯 A 抗心肌缺血保护的一氧化氮依赖机制¹

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关键词 心肌再灌注损伤; 钾通道; 心肌梗死; 一氧化氮; 敲除小鼠; 细胞因子类; 脂多糖类; 信号传递

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