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Involvement of heme oxygenase-1 in delayed cardioprotection induced by monophosphoryl lipid A in rats[1]

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KEY WORDS monophosphoryl lipid A; heme oxygenase; carbon monoxide; reperfusion injury

ABSTRACT

AIM: To explore whether the heme oxygenase-1 (HO-1) pathway is involved in the delayed cardioprotection induced by monophosphoryl lipid A (MLA). **METHODS**: Sprague-Dawley rats were pretreated with MLA 24 h before the experiment. Ischemia-reperfusion injury was induced by 60 min coronary artery occlusion followed by 3 h reperfusion. Infarct size, the serum creatine kinase (CK) activity, the serum content of nitric oxide (NO), and expression of HO-1 mRNA and protein in the heart were measured. **RESULTS**: Pretreatment with MLA (500 μg/kg, ip) markedly reduced infarct size and CK release and increased the serum content of NO ($P < 0.01$). The effects of MLA were completely abolished by pretreatment with *L*-nitroarginine methyl ester (*L*-NAME 10 mg/kg, ip), an inhibitor of NO synthase ($P < 0.01$), or Zinc protoporphyrin IX (45 μmol/kg, ip), an inhibitor of HO ($P < 0.01$). MLA caused a significant increase in the expression of HO-1 mRNA and protein, an effect which was not affected by *L*-NAME ($P > 0.05$). **CONCLUSION**: The results suggest that the HO-1/NO pathway is involved in the delayed cardioprotection induced by MLA.

INTRODUCTION

A great number of studies have shown that ischemic preconditioning can provide heart strongly protective effects against ischemia-reperfusion injury, and this phenomenon has been demonstrated in various animal species and in humans[1]. Recent studies have found that substitution of some drugs for ischemic stimulus is also capable of inducing a preconditioning-like protection, termed pharmacological preconditioning[1]. Monophosphoryl lipid A (MLA), a detoxified derivative of endotoxin, was first derived and purified from bacterial lipopolysaccharide (LPS). It has been shown that MLA significantly reduces infarct size in the rat, rabbit, pig, and dog models of ischemia-reperfusion[2,3]. However, the mechanism responsible for the delayed protection induced by MLA has not yet been fully understood. Several lines of studies have suggested that nitric oxide (NO) is involved in the delayed preconditioning afforded by MLA[4-6].

Carbon monoxide (CO) may act as an intercellular messenger molecule and is predominantly produced by heme oxygenase (HO) catalyzing heme degradation. Three isoforms (HO-1, HO-2, and HO-3) have been identified, and the HO-1 isoform is inducible and ubiquitous and also regarded as a heat shock protein (HSP32)[7]. Recently, a compensatory increase in activity of HO-1 has been shown in the liver treated with ischemia-reperfusion or in the endothelial cells treated with hypoxia[8-10], and pretreatment with hemin, an analogue of heme, to induce HO-1 expression significantly reduces the cardiac injury due to ischemia-reperfusion[11]. There is evidence that HO-1 mRNA expression and activity are markedly upregulated by endotoxin[12]. It is of interest that endogenous CO is involved in the cardioprotective effects of NO donors on ischemic-reperfusion injury[13]. It is probable that MLA, a detoxified derivative of endotoxin, induces the generation of CO. In the present study, therefore, we examined whether NO-mediated delayed protection induced by MLA involves the HO-1 pathway.

MATERIALS AND METHODS

Reagents Monophosphoryl lipid A (MLA), Zinc protoporphyrin IX (ZnPP-9), *L*-nitroarginine methyl

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ester (*L*-NAME), triphenyl tetrazolium choride (TTC), and Evans blue were purchased from the Sigma Chemical Co (St Lous, MO, USA). Trizol reagent was obtained from GIBCO BRL (USA). Creatine kinase assay kits and NO assay kits were purchased from Zhongsheng Co (Beijing, China) and Ju-Li (Nanjing, China), respectively. The RT-PCR kits and Western blot kits were obtained from TaRaKa Co (Dalian, China) and Bo Shi-de Co (Wuhan, China), respectively. Polyclonal goat anti-rat antibody was got from Santa Cruz Inc (USA). Primers for PCR were synthesized in Sangon Inc (Shanghai, China). MLA was dissolved in 40 % propylene glycol, 10 % ethanol, and 50 % saline, and ZnPP-9 was dissolved in Na_2CO_3 solution 50 mmol/L.

Surgical preparation Male Sprague-Dawley rats, weighing 280 – 320 g were anesthetized with sodium pentobarbital (60 mg/kg, ip), and were incubated and ventilated with a positive pressure ventilator, at a rate of 60 cycles/min and a tidal volume of 1 mL per 100 g body weight. ECG leads were connected to the chest and limbs for continuous monitoring ECG throughout the experiment. A left thoracotomy was performed in the fourth intercostal space and the pericardium was opened to expose the heart. A 4 – 0 silk suture was passed around the left coronary artery and an occlusive snare was placed around it. Occlusion of the coronary artery, by clamping the snare against the surface of the heart, caused an area of epicardial cyanosis with regional hypokinesis and ECG changes. Reperfusion was achieved by releasing the snare and was confirmed by conspicuous hyperaemic blushing of the previously ischemic myocardium and gradual resolution of the changes in the ECG signal. The sham group underwent the same procedure but without clipping of the coronary artery.

Experimental protocols The first series of experiments were divided into 9 groups: Sham group, underwent the surgical procedure but without ischemic insult; ischemia/reperfusion (I/R) group, subjected to 60 min of ischemia followed by 3 h of reperfusion; MLA group, pretreated with MLA (500 μg/kg, ip) 24 h before I/R; vehicle (MLA) group, pretreated with (40 % propylene glycol, 10 % ethanol and 50 % saline) 24 h before I/R; *L*-NAME + MLA group, given *L*-NAME (10 mg/kg, ip) 30 min before treatment with MLA; *L*-NAME group, only treated with *L*-NAME 24 h before I/R; ZnPP-9 + MLA group, given ZnPP-9 (45 μmol/kg, ip) 30 min before treatment with MLA; ZnPP-9 group, only treated with ZnPP-9 24 h before I/R. All rats were subjected to coronary artery occlusion for 1 h and reperfusion for 3 h, except for the sham group.

The second series of experiments were designed to evaluate the effect of MLA on the expression of HO-1 mRNA and protein. The same protocol, without surgical procedure, was used as mentioned above except for administration of ZnPP-9.

Infarct size and risk area At the end of 3 h reperfusion, the blood samples were collected from the carotid artery. The left coronary was reoccluded, and 1 mL Evans blue (1 %) was injected into the left ventricular cavity *in vivo* and allowed to perfuse the nonischemic portions of the heart. The entire heart was weighed, rinsed of excess blue dye, and sliced into 1 mm thick sections from the apex to base. The slices were stained by incubation for 20 min in 1 % triphenyl tetrazolium chloride (TTC) phosphate buffer (37 ℃). After staining, the sections were placed in 10 % formalin for preservation. Sections were traced onto acetate sheets, then scanned into a computer and measured by the imaging analysis software. Infarct and risk area was expressed as cm^2 and the percentage of infarct to risk area was calculated.

Creatine kinase assay At the end of 3 h reperfusion, the serum creatine kinase activity was measured spectrophotometrically.

Serum content of NO measurement At the end of 3 h reperfusion, the serum level of NO was measured indirectly by the content of nitrite and nitrate spectrophotometrically.

RNA preparation and reverse-transcription polymerase chain reaction (RT-PCR) After treatment with drugs for 8, 12, 16, or 24 h, heart tissues were rapidly removed and homogenized in Trizol reagent. Total RNA isolation and semiquantitative RT-PCR were performed according to standard techniques. The following PCR primers were used[14].

HO-1, 5′-TGGCCCACGCATATACCCGCT-3′ (sense)
5′-TTGAGCAGGAAGGCGGTCTTAG-3′ (antisense)
β-actin, 5′-GAGACCTTCAACACCCCAGCC-3′ (sense)
5′-TCGGGGCATCGGAACCGCTCA-3′ (antisense)

The predicted size of the amplified products of HO-1 and β-actin was 244 and 422 base pairs, respectively. The PCR amplification profiles consisted of denaturation at 94 ℃ for 30 s, annealing at 62 ℃ for HO-1 and at 58 ℃ for β-actin for 30 s, and elongation at 72 ℃ for 45 s. The linear exponential phases for HO-1 and β-actin PCR were 30 and 25 cycles, respectively. Equal amounts of corresponding HO-1 and β-actin RT-PCR products were

loaded on 1.7 % agarose gels. Optical densities of ethidium bromide-stained DNA bands were quantitated and results were expressed as HO-1/β-actin ratios.

Western blot analysis After treatment with drugs for 8, 12, 16, or 24 h, the total proteins from cardiac tissues in different groups were isolated at the time indicated, homogenized in lysis buffer containing protease inhibitor, and centrifuged at $2500 \times g$ for 10 min (4 ℃). The protein concentration was determined in the supernatant by the Lowry method. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a 12 % acrylamide gel, electroblotted onto nitrocellulose membrane, and probed with primary antibody (polyclonal goat anti-rat HO-1) for 2 h at room temperature (25 ℃). The second antibody (rabbit anti-goat) was incubated at room temperature for 1 h. Detection was performed by using chemiluminescence. Optical densities of bands were scanned and analyzed.

Statistical analysis Data are expressed as $\bar{x} \pm s$. All values were analyzed by using ANOVA and the Newman-Keuls' t test. The significance level was chosen as $P < 0.05$.

RESULTS

Infarct size As shown in Tab 1, there were no changes in heart weight and risk zone among groups ($P > 0.05$), indicating that the size of the risk zone was comparable in all groups. Ischemia-reperfusion caused a (64 ± 9) % necrosis in the area at risk. Pretreatment with MLA caused a dramatic reduction in infarct size ($P < 0.01$), which was abolished by *L*-NAME or ZnPP-9 ($P < 0.01$). The vehicle of MLA, had no effect on infarct size ($P > 0.05$). The vehicle of ZnPP-9 had also no effect on protection afforded by MLA ($P > 0.05$) (Fig 1).

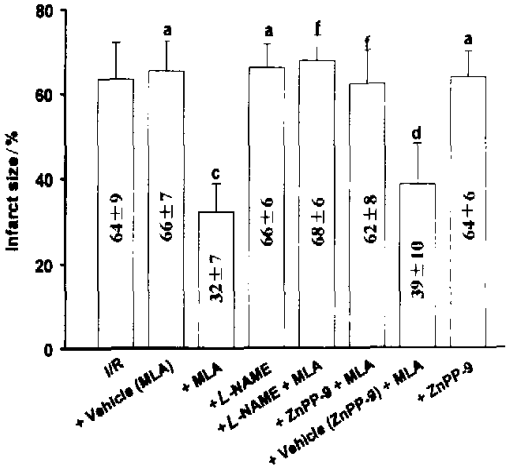

Fig 1. Effect of MLA on myocardial infarct size. I/R: ischemia-reperfusion; MLA: monophosphoryl lipid A (500 μg/kg, ip); *L*-NAME: *L*-nitroarginine methyl ester (10 mg/kg, ip); ZnPP-9: Zinc protoporphyrin IX (45 μmol/kg, ip). $n = 7 - 8$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* I/R. $^dP > 0.05$, $^fP < 0.01$ *vs* MLA.

Creatine kinase release Ischemia-reperfusion caused a significant increase in creatine kinase acitivity. Pretreatment with MLA dramatically reduced creatine kinase release ($P < 0.01$), and the effect was abolished by treatment with *L*-NAME or ZnPP-9 ($P < 0.01$). The vehicle of MLA, as well as *L*-NAME or ZnPP-9 alone had no effect on the release of creatine kinase during reperfusion ($P > 0.05$, Fig 2).

Tab 1. Heart wet weight, area at risk and infarct size of each group. $n = 7 - 8$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* I/R. $^dP > 0.05$, $^fP < 0.01$ *vs* MLA. I/R: ischemia-reperfusion; MLA: monophosphoryl lipid A (500 μg/kg, ip); *L*-NAME: *L*-nitroarginine methyl ester (10 mg/kg, ip); Zinc photoporphyrin IX (45 μmol/kg, ip).

Group	Heart wet weight/g	Area at risk/cm^2	Infarct size/cm^2
I/R	1.12 ± 0.16	4.0 ± 0.3	2.5 ± 0.3
+ Vehicle (MLA)	1.08 ± 0.24	3.9 ± 0.5	2.6 ± 0.5^a
+ MLA	1.14 ± 0.19	4.4 ± 0.8	1.4 ± 0.5^c
+ *L*-NAME	1.20 ± 0.03	4.1 ± 0.5	2.8 ± 0.8^a
+ *L*-NAME + MLA	1.19 ± 0.08	3.9 ± 0.8	2.7 ± 0.3^f
+ ZnPP-9 + MLA	1.16 ± 0.19	3.9 ± 0.9	2.4 ± 0.3^f
+ Vehicle (ZnPP-9) + MLA	1.09 ± 0.21	3.9 ± 0.3	1.5 ± 0.5^d
+ ZnPP-9	1.17 ± 0.11	3.9 ± 0.8	2.5 ± 0.8^a

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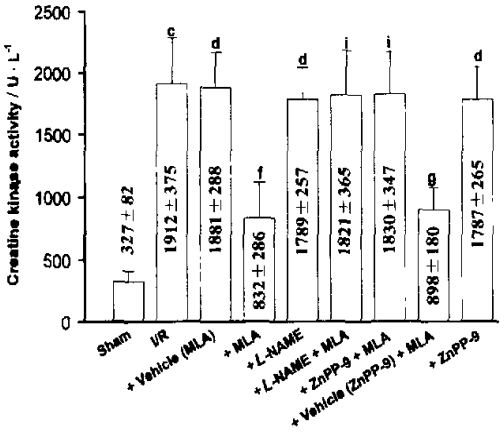

Fig 2. Effect of MLA on creatine kinase activity. I/R: ischemia-reperfusion; MLA: monophosphoryl lipid A (500 μg/kg, ip); *L*-NAME: *L*-nitroarginine methyl ester (10 mg/kg, ip); ZnPP-9: Zinc protoporphyrin IX (45 μmol/kg, ip). $n = 7-8$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* sham. $^dP > 0.05$, $^fP < 0.01$ *vs* I/R. $^gP > 0.05$, $^iP < 0.01$ *vs* MLA.

Serum NO content Serum concentrations of NO in the rats treated with MLA were significantly increased compared with the I/R group ($P > 0.05$). The elevated level of NO by MLA was abolished by treatment with *L*-NAME or ZnPP-9 ($P < 0.01$). *L*-NAME or ZnPP-9 or vehicle of MLA alone had no effect on the release of NO ($P > 0.05$, Fig 3).

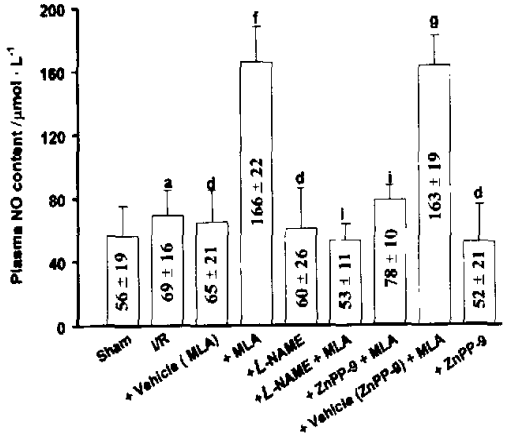

Fig 3. Effects of MLA on serum concentrations of NO. I/R: ischemia-reperfusion; MLA: monophosphoryl lipid A (500 μg/kg, ip); *L*-NAME: *L*-nitroarginine methyl ester (10 mg/kg, ip); ZnPP-9: Zinc protoporphyrin IX. $n = 7-8$. $\bar{x} \pm s$. $^aP > 0.05$ *vs* Sham. $^dP > 0.05$, $^fP < 0.01$ *vs* I/R. $^gP > 0.05$, $^iP < 0.01$ *vs* MLA.

Induction of HO-1 mRNA and protein The expression of HO-1 mRNA was not detectable in the control group. However, HO-1 mRNA was expressed in the hearts pretreated with MLA for 8, 12, or 16 h, but was not detectable again at 24 h (Fig 4). The increased expression level of HO-1 mRNA induced by MLA was unaltered by *L*-NAME ($P > 0.05$) (Fig 4). The vehicle of MLA or *L*-NAME alone had no effect on the expression of HO-1 (data not shown).

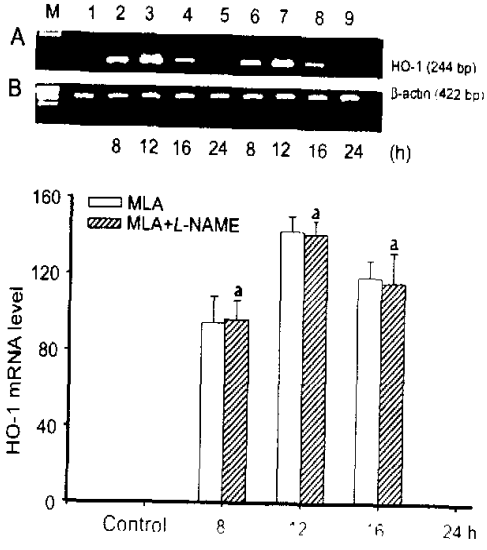

Fig 4. Effect of MLA (500 μg/kg, ip) on the expression of HO-1 mRNA in the presence or absence of *L*-NAME (10 mg/kg, ip) in heart. (A) RT-PCR products of HO-1, M: PUC 19 DNA/*Msp* I (*Hpa*II) marker; 1: control; 2-5: MLA and 6-9: MLA + *L*-NAME. (B) RT-PCR products of β-actin. (C) the results of densitometric scanning for DNA bands of each group at each time point. $n = 6$. $\bar{x} \pm s$. $^aP > 0.05$ *vs* MLA at each corresponding time point.

The expression of HO-1 protein was also not detectable in the control group. Similarly, HO-1 protein was expressed after pretreatment with MLA for 8, 12, 16, or 24 h, but HO-1 protein expression at 24 h was markedly decreased compared with that of 16 h ($P < 0.01$, Fig 5). The expression of HO-1 protein induced by MLA was not affected by pretreatment with *L*-NAME ($P > 0.05$, Fig 5).

DISCUSSION

There is substantial evidence to suggest that endogenous NO plays an important role in the mediation

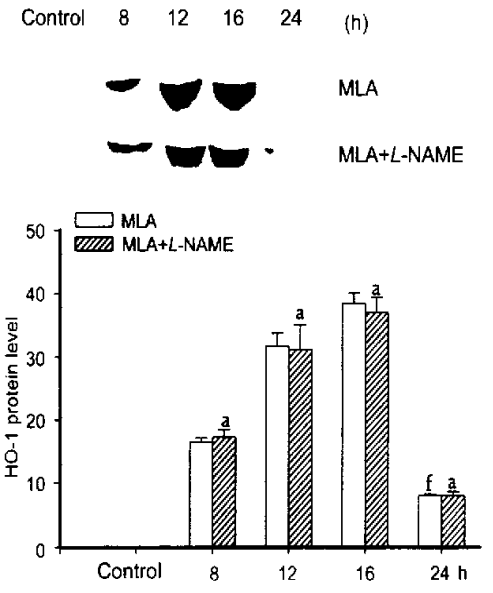

Fig 5. Effect of MLA on the expression of HO-1 protein in the presence or absence of *L*-NAME in heart. MLA: monophosphoryl lipid A (500 μg/kg, ip); *L*-NAME: *L*-nitroarginine methyl ester (10 mg/kg, ip). The results of densitometric scanning for bands of each group at each time point are shown in the bar graph. $n=6$. $\bar{x}\pm s$. $^aP>0.05$ *vs* MLA at each corresponding time point. $^fP<0.01$ *vs* MLA at 16 h.

of delayed protection induced by ischemic or pharmacological pre-conditioning[2-5]. In the present study, we confirmed previous observations that the delayed protection afforded by MLA was abolished by *L*-NAME, an inhibitor of NO synthase. Others have found that the beneficial effect of MLA is absent in the inducible NO synthase (iNOS) knockout mice[15]. Taken together, these studies indicate that endogenous NO is an important mediator in the delayed protection afforded by MLA.

A major new finding in this study is that the delayed cardioprotection afforded by MLA also involves HO-1 activity. It is known that HO catalyzes heme degradation into CO and bilirubin which has antioxidant properties, and the inducible HO isoform (HO-1) is regulated by multiple factors. There is evidence that ischemia-reperfusion strongly increases HO-1 activity[8,9], while downregulation of HO-1 mRNA and the reduction in HO activity facilitates ischemia-reperfusion-induced ventricular fibrillation[9]. HO-1 activity is also upregulated by hypoxia *in vivo* or *in vitro*[16,17], whereas cardiomyocytes have a maladaptive response to hypoxia in HO-1-deficient mice[18]. Others have also shown that in HO-1-deficient mice, administration of endotoxin exhibits the increased mortality, and hepatic and renal dysfunction[19]. These results suggest that induction of HO-1 mRNA and protein expression by a variety of injurious factors may be a compensatory response.

Recently, it has been reported that some drugs are also able to increase HO-1 activity. Angiotensin Ⅱ upregulates cardiac HO-1 expression which may represent an adaptive response to angiotensin Ⅱ-induced cardiac damages[20], and pretreatment with FK409, an NO releaser or hemin, an analogue of heme to induce HO-1 expression significantly attenuates ischemia-reperfusion injury[21,11]. In the present study, MLA increased the expression of HO-1 mRNA and protein, and the delayed protection afforded by MLA was abolished by ZnPP-9, an inhibitor of HO. The present results confirm previous observations that some drugs upregulate the activity of HO and provide evidence that drugs can induce a delayed protection against ischemia-reperfusion injury.

As mentioned above, both NO and HO-1 are involved in the delayed protection induced by MLA. It is possible that there exists interactions of NO and CO. It has been shown that CO, another putative cellular messenger, mainly regulates cardiovascular function via sGC-cGMP pathway. Previous studies have shown that CO is involved in the cardioprotection afforded by *L*-arginine, a substance of NO synthase[13]. Others have reported that NO itself and its donors can induce HO-1 mRNA and protein expression in multiple tissues or cultured cells[21-24]. Furthermore, there is evidence that CO directly stimulates the release of NO[25,26]. Thus, it is likely that two messengers, CO and NO, converge or exhibit reciprocal feedback regulation in delayed protection induced by MLA. The present study revealed that the release of endogenous NO stimulated by MLA was inhibited by ZnPP-9, while the expression of HO-1 mRNA and protein induced by MLA was not affected by pretreatment with *L*-NAME. These findings suggest that the HO-1/CO pathway may be involved in the modulation of MLA-induced NO generation. However, the precise mechanism responsible for interactions of NO with CO needs to be further investigated.

The mechanisms responsible for the protection mediated by NO are also unclear. It has been reported that the K_{ATP} channel may be involved in MLA-induced cardioprotection in rabbits and dogs[1]. However, several lines of studies have indicated that the K_{ATP} channel does not participate in the cardioprotection provided by ischemic preconditioning in the rat[27,28].

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We and others have shown that endogenous calcitonin gene-related peptide (CGRP) mediates the early and delayed cardioprotection induced by ischemic preconditioning in the rat[29-32]. Our recent study showed that NO donor nitroglycerin-induced preconditioning-like protection was also related to stimulation of CGRP release[33].

It is probable that the delayed protection afforded by MLA, which increases NO production, may involve endogenous CGRP release.

In summary, the present study suggests that delayed cardioprotection induced by MLA involves the HO-1/NO pathway.

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血红素氧合酶-1 介导单磷酰酯 A 诱导的心脏延迟保护[1]

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关键词 单磷酰酯 A；血红素氧合酶；一氧化碳；再灌注损伤

目的：研究血红素氧合酶-1 (HO-1)是否参与单磷酰酯 A 诱导的心脏延迟保护作用. **方法**：在体大鼠心脏缺血-再灌损伤模型，缺血前 24 小时应用单磷酰酯 A (500 μg/kg, ip)诱导心脏延迟保护，观察单磷酰酯 A 对心肌梗死面积、肌酸激酶(CK)活性以及血清一氧化氮(NO)浓度的影响，并检测心脏 HO-1 mRNA 和蛋白的表达. **结果**：单磷酰酯 A 明显缩小心梗面积，减少 CK 释放以及显著升高血清 NO 浓度 ($P<0.01$). 这些作用可被预先给予一氧化氮合酶抑制剂 *L*-亚硝基精氨酸甲酯(*L*-NAME, 10 mg/kg, ip)和血红素氧合酶抑制剂锌原卟啉 IX (ZnPP-9, 45 μmol/kg, ip)所取消($P<0.01$)；单磷酰酯 A 明显增加心脏 HO-1 mRNA 和蛋白的表达，这一作用不被 *L*-NAME 所影响($P>0.05$). **结论**：HO-1/NO 途径介导单磷酰酯 A 诱导的延迟心脏保护.

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