Pharmacological preconditioning with monophosphoryl lipid A improves post ischemic diastolic function and modifies TNF-alpha synthesis

Ram Sharonya,*, Inna Frolkisa, Dvir Froylicha, Stephan M. Wildhirtb, Itzhak Shapiroa, Bruno Reicharta, Nahum Neshera, Gideon Uretzkya

*Department of Cardiothoracic Surgery, Tel-Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, 6 Witzman St., Tel Aviv, 64239, Israel
bDepartment of Cardiac Surgery, Ludwig-Maxillians University, Munich, Germany

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1. Introduction

Ischemic preconditioning was described initially as the multiple brief ischemic episodes that protect the organ (heart, brain, skeletal muscle) from a subsequent sustained ischemic insult. Preconditioning of the heart has a broad spectrum of potential clinical applications. It preserves myocardial and renal function, as assessed by biochemical markers in patients undergoing coronary artery bypass grafting (CABG) [1]. Favorable effects of preconditioning on stroke volume index and cardiac troponin I release have also been observed in patients undergoing off-pump CABG [2].

Two types of cardiac preconditioning have been defined: the early, short-lived wave of protection phase and the delayed type, which is also called the second window of protection. The early phase of preconditioning occurs immediately and lasts up to a few hours and is caused by the activation of G-coupled membrane receptors and downstream kinases. The delayed phase of preconditioning develops hours after the insult, remains effective for several days, and requires de novo protein synthesis [3]. It is believed that several molecules, generated during the preconditioning stimulation act in autocrine and/or paracrine fashion as triggers of cellular adaptation. These include adenosine, opioid, nitric oxide, C-reactive protein, reactive oxygen species and bradykinine [4,5]. Several pharmacological agents have been applied for the induction of delayed preconditioning. One of them, monophosphoryl lipid A (MLA), a derivative of lipopolysaccharides (LPS)
(Corixa, Hamilton, MT), is free of major endotoxic properties and was shown to be safe, well tolerated, and having a significant cardioprotective effect in an experimental model in terms of reducing infarct size, improving contractile dysfunction, and attenuating ventricular arrhythmias [6]. Injections of MLA adjuvant have been shown to be well tolerated in patients, and have modulated immunological response [7]. Taking together the safety of this drug and its cardioprotective properties, MLA has the potential for being suitable for pretreatment against myocardial injury.

Tumor necrosis factor-alpha (TNF-α), an autocrine cytokine, has been shown to be released from the ischemic heart and to directly correlate with the degree of myocardial dysfunction and cellular necrosis in an ischemia and reperfusion protocol [8]. It also has been shown that early ischemic preconditioning significantly decreases postischemic TNF-α content [9] and that pretreatment with MLA improves contractility while reducing TNF-α content [10]. Several endogenous mediators of MLA cardioprotective effect have been suggested [10,11].

However, no study has yet demonstrated the effect of pharmacologically delayed preconditioning on left ventricular (LV) diastolic function, on TNF-α messenger Ribonucleic Acid (mRNA) expression, and concomitantly the protein content of TNF-α in the effluent and myocardial tissue in a model of normothermic ischemia and reperfusion. We hypothesized that in a model of isolated heart undergoing normothermic ischemia and reperfusion, MLA-induced delayed pharmacological preconditioning plays a protective role on diastolic cardiac function in addition to its known beneficial effect on systolic function, and these changes are associated with modification of mRNA TNF-α expression, and TNF-α protein synthesis.

2. Materials and methods

Adult male Sprague-Dawley rats (300-350 g) were studied in this experiment. All animals received humane care in compliance with the European Convention on Animal Care. The study was approved by the Institutional Ethics Committee.

2.1. Experimental protocol

The animals were randomly assigned to two groups. The study group (MLA, n=10) received intravenous MLA (350 μg/kg, dissolved in propylene glycol 40%, ethyl alcohol 10% and water) [11] and the control group (n=9) was treated by vehicle. Twenty-four hours later, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (30 mg/kg). Their hearts were rapidly excised, immersed in ice-cold saline, and mounted on a cannula of a modified Langendorff perfusion apparatus. Retrograde aortic perfusion was initiated at a perfusion pressure of 90 mmHg with an oxygenated modified Krebs-Henseleit (KH) buffer solution: NaCl 118 mmol/L; KCl 4.7 mmol/L; CaCl2 2.0 mmol/L; MgSO4 7H2O 1.2 mmol/L; KH2PO4 1.2 mmol/L; glucose 11.1 mmol/L; and NaHCO3 25 mmol/L. The perfusate was continuously bubbled with 95% O2 and 5% CO2, maintaining a pH of 7.4-7.5, at 37 °C. Cardiac temperature was measured by a thermistor implanted in the right ventricular wall and carefully maintained at 37 °C by wrapping a water jacket around the perfusate reservoir and the isolated heart. A water-filled latex balloon was inserted into the left ventricular (LV) cavity via a small left atrial incision and connected to a pressure transducer (Biometrix, Breda, Netherlands). The balloon was adjusted to a mean LV end-diastolic pressure of 2-4 mmHg. After 15 min of stabilization, the hearts underwent a 35-min period of global ischemia at 37 °C followed by 40 min of reperfusion. In addition to the animal that completed the ischemia-reperfusion protocol, six animals from each group underwent the same experimental protocol up to the end of ischemia, then the hearts were assayed for TNF-α mRNA and immunohistochemistry.

LV peak systolic-developed pressure (LVDP), end diastolic pressure (EDP), the first derivative of LV pressure rise (+dP/dt_max) and fall (-dP/dt_min), and coronary flow were calculated by measuring the effluent per minute after 15 min of stabilization (baseline) and at 5, 10, 20, 30 and 40 min of reperfusion.

2.2. TNF-α analysis

2.2.1. TNF-α protein content

Effluent samples from the coronary sinus for TNF-α measurement were drawn at the end of stabilization, at baseline, and at 5 and 40 min of reperfusion and were then immediately stored at −70 °C until assayed. TNF-α activity was measured using the commercially available ELISA kit (Cytoscreen TM rat kit TNF-α, Biosource, Camarillo, CA, USA). The lower limit of detection was 4 pg/ml.

2.2.2. RNA isolation and semi-quantitative RT-PCR analysis

Hearts were assayed for LV TNF-α mRNA expression at baseline, immediately after ischemia and at the termination of reperfusion. The LV myocardium was excised, frozen in liquid nitrogen and stored at −70 °C for subsequent mRNA determination. Total RNA was extracted from myocardial samples using the guanidinium thiocyanate method. RNA pellets were kept at −20 °C with 75% ethanol until assay. Dried sediments were dissolved in sterile RNase-free water and quantitated spectrophotometrically at λ = 260 nm. Two μg of total RNA were subjected to reverse transcription reaction (RT) in 20 μl using a commercial RT system (Promega Corporation, Madison, WI, USA). After completion of the reaction, 5 μl of this reaction mixture was used for TNF-α complementary deoxyribonucleic acid (cDNA) PCR amplification, and 5 μl of a 1:10-diluted reaction mixture was used for glyceraldehyde-phosphate dehydrogenase (GAPDH) cDNA amplification. Our PCR negative control contained H2O instead of cDNA, and the cDNA negative control contained H2O instead of RNA. The primer sequences, annealing temperature and PCR product size are summarized in Table 1. A mini cyclerTM (MJ Research Inc., Waltham, MA, USA) was used for PCR amplification and reverse transcription reaction. The cDNA was amplified after determining the optimal number of amplification cycles within the exponential amplification phase and the amplification products were proportional to the sample input.
The PCR products (10 μl) were separated in 1.8% agarose gel, stained with ethidium-bromide, visualized by ultraviolet irradiation and photographed with Polaroid film (Kodak Co., New Haven, CT, USA). The film was adapted to evaluate band densities using the Fujifilm Thermal Imaging System (Model FTJ-500, Fuji Photo Film Co., Ltd, Osaka, Japan), and the TINA program package (Raytest Isotope Messgerate, Gmbh, Staubenhardt, Germany). The intensities of the bands were expressed in arbitrary densitometry units. All TNF-α band intensities were normalized by their respective GAPDH values. Each PCR reaction was performed at least twice, and seven hearts were used for each experimental group.

2.2.3. Immunostaining of myocardial TNF-α

Immunohistochemical studies of myocardial tissue were performed in the control and MLA groups at the end of reperfusion (40 min). Excised left ventricles were fixed in 10% neutral buffered formalin (Z-fix; Anatech, Ltd, Battle Creek, MI, USA) at room temperature for 18-24 h. The tissue was then dehydrated, embedded in paraffin, and sectioned (5-μm thickness) in a standard manner. Immunohistochemistry was performed using a streptavidin-biotin immunoperoxidase method according to the manufacturer’s instructions (Zymed Lab. Inc., San Francisco, CA, USA). All reagents were prepared with a diluent consisting of Tris buffer pH 7.6 supplemented with 0.2% Tween 20 and 1% BSA. All sections were first blocked with normal goat serum for 10 min at room temperature. The slides were incubated with two concentrations (1:100 or 1:250) of primary polyclonal rabbit anti-rat TNF-α antibodies (R & D System, Minneapolis, MN, USA). The sections were then rinsed in Tris buffer and incubated for 10 min at room temperature with a biotin-conjugated goat anti-rabbit secondary antibody. Negative controls were stained either with normal nonimmune rabbit serum (1:100 dilution) instead of the primary antibody (n=4) or with secondary antibody alone (1:100 dilution, n=4). After blocking the endogenous peroxidase activity with methanol and hydrogen peroxide, the slides were incubated with streptavidin-peroxidase complex. Diaminobenzidine was used as a chromogen to visualize the presence and distribution of TNF-α. The sections were then rinsed in Tris buffer counterstained in hematoxylin, dehydrated, cleared, and mounted using a synthetic mounting medium.

2.3. Statistical analysis

Repeat measurement analysis of variance (rmANOVA) followed by multiple comparisons using Fisher’s protected least-significant difference and unpaired Student’s t-tests, as appropriate by SPSS 11.0 (Chicago, IL) was performed. Significance was established at a P level of <0.05. Results are expressed as mean ± SEM.

3. Results

3.1. Hemodynamic changes

All animals survived the 24 h between injection and harvesting of the hearts, and no signs of shivering, irritability or apathy were observed. At baseline, there were no significant differences in LVDP, dP/dt_max, dP/dt_min, end diastolic pressure (EDP), or coronary flow between the control and MLA groups.

3.1.1. Left ventricular performance

The LVDP, dP/dt_max, and dP/dt_min measurements of the isolated hearts during the study are presented in Fig. 1. During reperfusion, a progressive and significant deterioration in LVDP, dP/dt_max, dP/dt_min, and EDP (F=30.88, P<0.001, F=224.75, P<0.001, F=46.38, P<0.001, F=36.42, P=0.01, respectively) was noted in the control group. Pretreatment with MLA significantly attenuated these reductions (estimated mean difference LVDP=26 mmHg, F=15.8, P<0.01; estimated mean difference dP/dt_max=930.0 mmHg/s, F=8.9, P<0.01; estimated mean difference dP/dt_min=1050 mmHg/s, F=18.6, P<0.01). Subgroup time-point analysis in the MLA group revealed significantly higher LVDP at 5, 20, 30 and 40 min and significantly higher dP/dt_max and dP/dt_min at 20, 30 and 40 min of reperfusion. The effects of ischemia-reperfusion on EDP at the end of reperfusion were attenuated in hearts randomized to pharmacological preconditioning compared with the control group (12±3 mmHg vs 24±5 mmHg, P=0.01).

3.1.2. Coronary flow

There was a progressive and significant reduction in coronary flow (from 15.8±1.4 ml/min at baseline to 11.0±1.3 ml/min at the end of reperfusion, P=0.03) in the control group during reperfusion (Fig. 1D). In contrast, pretreatment with MLA attenuated the reduction in coronary flow from 18.7±2.0 ml/min at baseline to 17.9±1.3 ml/min at 40 min of reperfusion (P>0.05, ns) during reperfusion. A significant difference between groups was observed (estimated mean difference in coronary flow=6.98 ml/min, F=7.37, P=0.03).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Specification of the primer sets used to analyze mRNA expression</th>
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<tr>
<td>Primer set</td>
<td>Number of cycles</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Sense</td>
</tr>
<tr>
<td>Antisense</td>
<td>GGACTCGTGTAGTCTAAGT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
</tr>
<tr>
<td>Antisense</td>
<td>GTAGCATTCAATTTGCTGAT</td>
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</table>
3.2. TNF-α mRNA expression

The baseline levels of TNF-α mRNA expression (Fig. 2) were higher in the MLA than in the control group (1.3 ± 0.1 vs 0.5 ± 0.03, \( P < 0.01 \)) but remained essentially constant after ischemia and reperfusion (1.3 ± 0.1 and 1.4 ± 0.03, respectively, \( P = \text{NS} \)), while a further TNF-α mRNA increase was clearly observed in the control group during ischemia and reperfusion (1.0 ± 0.1 and 1.4 ± 0.1, respectively, \( P < 0.01 \)). At the end of reperfusion, TNF-α mRNA expression was similar between groups.

3.3. Post-ischemic myocardial TNF-α release

Table 2 demonstrates TNF-α protein content detected in the coronary effluent throughout the study. The TNF-α myocardial effluent was not detectable in either the control or MLA groups at baseline. After the ischemic period, however, the level of TNF-α protein was significantly increased only in the control group and underwent an additional rise at the end of reperfusion, while no TNF-α was detectable in the MLA group at any time point.

3.4. Myocardial immunostaining of TNF-α

Marked TNF-α immunostaining was observed only in the control group at 40 min of reperfusion (Fig. 3A). The TNF-α staining was primarily localized in cardiac myocytes and to a lesser degree in the endothelium of myocardial vessels. No TNF-α staining was evident in the MLA group (Fig. 3B). Incubation with either normal non-immune rabbit serum or
with secondary antibody alone (two groups of negative controls) failed to demonstrate any TNF-α immunostaining (data not shown).

4. Discussion

This study shows that in an animal model of prolonged non-cardioplegic normothermic ischemia, pharmacologically delayed preconditioning with MLA given parenterally 24 hours before ischemia and reperfusion, significantly improved post-ischemic diastolic and systolic cardiac function, normalized coronary flow, and prevents TNF-α production during ischemia and reperfusion, through aborting the translation phase of tumor necrosis factor-α synthesis. The current study is first to show attenuation in diastolic function following delayed preconditioning, and an upregulation in TNF-α mRNA expression following MLA treatment where no TNF-α protein production has been detected.

MLA has been evaluated as a prophylactic agent for septic shock in humans and has been administered safely in clinical trials [7,12,13]. MLA has also been widely shown to convey cardioprotective activity in preclinical models of ischemia-reperfusion injury. The combination of this cardioprotective action and safety in humans provided a rationale for the development of MLA or other LPS derivates as cardioprotective agents for clinical use and corroborated the importance of studying its mechanisms of action.

TNF-α is a proinflammatory polypeptide cytokine characterized by a potent negative inotropic effect. Our group previously reported that TNF-α is released from the isolated heart undergoing ischemia and reperfusion and that this cytokine production was directly correlated with the degree of myocardial dysfunction [8]. Moreover, administration of monoclonal antibodies to TNF-α attenuated postischemic myocardial injury. Therefore, TNF-α is not only a marker of myocardial injury but plays a role in post-ischemia myocardial dysfunction.

LPS depresses cardiac contractility, induces TNF-α synthesis by circulating monocytes, tissue-residential macrophages and cardiac myocytes [14]. Our results strengthen previous reports in models of hypothermic crystalloid cardioplegic ischemia demonstrating that preconditioning with MLA inhibits TNF-α production and plays a protective role on systolic cardiac function [10].

Several recent animal studies and one clinical trial showed the benefit of pharmacological preconditioning on diastolic function but none of these studies used delayed preconditioning [15,16]. The current study is the first to demonstrate the benefit of pharmacologically delayed preconditioning on diastolic function.

MLA-induced delayed cardioprotection initially appeared to be mediated through ATP-sensitive potassium sarcolemmal channels, heat-shock proteins and antioxidant enzymes [17]. Later, it has been shown that MLA preconditioning is associated with an increase in inducible nitric oxide synthase (iNOS) activity [11]. Moreover, nitric oxide (NO) plays a dual role in the pathophysiology of the late phase of preconditioning, acting initially as the trigger and subsequently as the mediator of this adaptive response [18].

Nitric oxide plays an important role in preconditioning and is one possible source of reactive oxygen species. Moreover, TNF-α is known to be up-regulated by oxidative stress [19]. Taking together the capability of MLA to express TNF-α, the importance of NO in preconditioning, the relationship between NO and the TNF-α pathways, and finally, the protective effect of TNF-α in certain conditions [20], it should not be surprising that TNF-α plays a role in preconditioning, and explain the rationale for utilizing MLA as an agent for delayed pharmacological preconditioning.

Delayed preconditioning induces regulatory modifications of gene expression and synthesis of new proteins that promote cell repair and protection against ischemia-reperfusion [21].

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>End of Ischemia (pg/min)</th>
<th>40 min of Reperfusion (pg/min)</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td>Below detectable</td>
<td>79 ± 30</td>
<td>200 ± 22*</td>
</tr>
<tr>
<td><strong>MLA</strong></td>
<td>Below detectable</td>
<td>Bellow detectable levels</td>
<td>Bellow detectable levels</td>
</tr>
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Data are presented as mean value ± SEM.*P < 0.05. MLA = Monophosphoryl lipid A.
The observed increased baseline TNF-α mRNA expression 24 h following pharmacological preconditioning, but without further increase following ischemia and reperfusion, and the absence of any TNF-α protein in the effluent may result from post-transcriptional regulation (such as mRNA instability), translational suppression or, alternatively, be related to post-translational events. A previous study supported the hypothesis of TNF-α post-transcriptional regulation by suppression of translation efficiency [22]. Moreover, it was suggested that TNF-α mRNA rapid turnover does not constitute a regulatory mechanism of TNF-α biosynthesis in macrophages upon stimulation of lipopolysaccharides [23]. Taken together, our observed results of reduction in TNF-α protein expression may be related to down-regulation of the translational stage rather than a post-translational process. Time-course experiments examining TNF-α mRNA and protein expression at different intervals will clarify any apparent discordance between transcription and protein expression following MLA administration. This will be performed in a further study. Hence, the current study, using MLA preconditioning, shows that down-regulation of cardio-depressant protein synthesis (TNF-α) may be an additional mechanism of pharmacological preconditioning and myocardial protection.

Pharmacological preconditioning has a broad spectrum of potential applications. Administration of cardioprotective drugs prior to scheduled myocardial insult (CABG, PCI) or imminent myocardial injury (unstable angina, major surgery in patient with known coronary artery disease) could potentially acquire better resistance to myocardial injury and minimize ischemic damage. Finally, in light of the reported benefits of pre-infarction angina on the CK-determined infarction size in those patients experiencing angina within 24 h of the onset of infarction [24], pharmacological preconditioning, given hours before the insult, represents an attractive conceptual treatment strategy. It should be noted that controversy exists about the benefit of preconditioning among patients undergoing on-pump CABG, and in patients undergoing off-pump CABG [2, 25].

The current study protocol includes a non-cardioplegic normothermic ischemic model which is more demanding than a post-translational process. Time-course experiments examining TNF-α mRNA and protein expression at different intervals will clarify any apparent discordance between transcription and protein expression following MLA administration. This will be performed in a further study. Hence, the current study, using MLA preconditioning, shows that down-regulation of cardio-depressant protein synthesis (TNF-α) may be an additional mechanism of pharmacological preconditioning and myocardial protection.

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The current study protocol includes a non-cardioplegic normothermic ischemic model which is more demanding than the usual clinical practice of myocardial preservation by cardioplegia and some degree of hypothermia. Therefore, direct clinical implication of our findings cannot be applied. In light of previous studies that have characterized the mediators of MLA preconditioning [11, 17] the current study focused on TNF-α transcription and protein synthesis pathway. Administration of anti-TNF-α could clarify the role of TNF-α in MLA-induced preconditioning, but would prevent precise measurements of endogenous TNF-α levels.

In conclusion, preconditioning by MLA renders the heart more tolerant to ischemia-reperfusion in terms of LV diastolic and systolic function. MLA treatment up-regulates TNF-α mRNA but prevents further TNF-α mRNA increase during ischemia and reperfusion, and abolishes myocardial TNF-α protein production through aborting the translation phase of TNF-α synthesis. Therefore, pre-treatment with MLA suggests a new mechanism for myocardial preconditioning. We believe that this study contributes to the development of cardioprotective drugs that might be used hours prior to myocardial insult in order to minimize myocardial morbidity.

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