Lipopolysaccharide triggers late preconditioning against myocardial infarction via inducible nitric oxide synthase

You-Ping Wang\textsuperscript{a,}\textsuperscript{*}, Chubun Sato\textsuperscript{b}, Kazuhiro Mizoguchi\textsuperscript{a}, Yoichi Yamashita\textsuperscript{a}, Masahiro Oe\textsuperscript{a}, Hajime Maeta\textsuperscript{a}

\textsuperscript{a}First Department of Surgery, Kagawa Medical University, 1750-1, Ikenobe, Miki, Kita, Kagawa, 761-0793, Japan
\textsuperscript{b}Department of Molecular Biology, Kagawa Medical University, 1750-1, Ikenobe, Miki, Kita, Kagawa, 761-0793, Japan

Received 19 February 2002; accepted 23 May 2002

Abstract

Objective: The aim of this study was to investigate the role of inducible nitric oxide synthase (iNOS) as a trigger in lipopolysaccharide (LPS)-induced late preconditioning against myocardial infarction. Methods: Rats were pretreated intraperitoneally with two different doses of LPS (0.5 or 2.5 mg/kg) or normal saline (control) 24 h prior to lethal myocardial ischemia. Subsequently, all rats were subjected to a sustained 30-min coronary occlusion followed by 180-min reperfusion. In the second study, total RNA and protein were extracted from myocardium of the control and LPS-treated rats (after 4, 6 and 24 h of treatment) for reverse transcription-polymerase chain reaction and Western blot analysis. Results: In LPS (2.5 mg/kg, but not 0.5 mg/kg)-treated rats receiving no pharmacological intervention, the percentage of myocardial infarct within the area at risk and left ventricle was significantly reduced to 42\% and 24\% (P<0.01) compared with the control group. The cardioprotection was abolished by injection of dexamethasone (4 mg/kg, i.p.) or the selective iNOS inhibitor aminoguanidine (300 mg/kg, s.c.) before LPS treatment. The expression of iNOS mRNA and the iNOS protein significantly increased 4 and 6 h after administration of LPS (2.5 mg/kg), respectively. The increases in iNOS mRNA and protein were eliminated by dexamethasone. But the iNOS mRNA and protein were not detectable 24 h after administration of LPS (2.5 mg/kg). Conclusions: These data provide molecular and pharmacological evidence that LPS-induced late preconditioning against myocardial infarction is triggered by iNOS.

\textsuperscript{*}Corresponding author. Tel.: +81-878-912-186; fax: +81-878-912-187.
E-mail address: wang@kms.ac.jp (Y.-P. Wang).

Time for primary review 29 days.
is involved in the delayed cardioprotection afforded by MLA [13]. However, the cellular mechanism(s) that triggers the protection remains to be clarified.

Several studies have demonstrated that the development of late PC is triggered by the generation of nitric oxide (NO) or reactive oxygen species during the initial ischemic stimulus [6,14]. It is well known that LPS drives iNOS gene expression in various cells, leading to an increase in the release of NO. Therefore, in the present study, we hypothesized that LPS triggers late PC against myocardial infarction via induction of iNOS gene expression. To investigate whether iNOS-induced NO as a possible trigger contributes to the late PC, we examined the effects of dexamethasone (DX, a suppressor of iNOS gene expression) or aminoguanidine (AG, a selective inhibitor of iNOS), given before administration of LPS, on the late PC. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis were used to examine the expression of iNOS mRNA and protein levels in the myocardium.

2. Methods

This study was conducted in accordance with the committee on animals of Kagawa Medical University and conforms with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health; NIH Publication No. 85-23, revised 1996).

2.1. Surgical preparation

Male Sprague–Dawley rats (300–450 g) were used for all studies. All rats were anesthetized with pentobarbital (60 mg/kg, i.p.), and mechanically ventilated with oxygen-supplemented room air. Catheters were placed in the femoral artery and vein to monitor arterial pressure, heart rate (HR), and inject drugs, respectively. Arterial blood gas parameters were maintained within a normal physiological range. The body core temperature was maintained at 36.5–37.5°C using a heating pad throughout the experiment.

A rat model of myocardial ischemia/reperfusion was used as previously described [15]. Briefly, the chest was opened via left thoracotomy, and a reversible coronary artery snare occluder was placed around the proximal left anterior descending coronary artery. Once pentobarbital-induced instability in the hemodynamics had disappeared, heparin (200 IU, i.v.) was given and a coronary artery was occluded for 30 min followed by 180 min of reperfusion.

2.2. Determination of infarct size

After 180 min of reperfusion, infarcted area (IA) and area at risk (RA) were determined using Evans blue and triphenyltetrazolium chloride as described previously [15]. The volume of RA and the IA volume were calculated using computerized planimetry. The results are expressed as the percentage of the IA to the RA or the total LV, and the percentage of the RA to the total LV.

2.3. Experimental protocol

For infarct size analysis, rats were randomly divided into the following groups (Fig. 1). (1) Control, injected intraperitoneally with normal saline (n=8); (2) LPS (0.5 mg/kg) (n=8); (3) LPS (2.5 mg/kg) (n=8); (4) LPS (2.5 mg/kg)+DX (4 mg/kg×2) (n=8); (5) Control+DX (4 mg/kg×2) (n=7); (6) LPS (2.5 mg/kg)+AG (300 mg/kg×2) (n=8); (7) Control+AG (300 mg/kg×2) (n=7). All groups underwent a sustained 30 min of coronary occlusion followed by 180 min of reperfusion. LPS or normal saline was injected intraperitoneally 24 h before the prolonged ischemia. DX (4 mg/kg, i.p.) or AG (300 mg/kg, s.c.) was given 1 h before and 3 h after injection of LPS.

Myocardial tissue iNOS mRNA and protein were measured in additional groups in which rats did not undergo myocardial ischemia: Control; LPS (0.5 mg/kg); LPS (2.5 mg/kg); LPS (2.5 mg/kg)+DX; LPS (2.5 mg/kg)+AG. For RNA extraction, rats were sacrificed 4 and 24 h after LPS or normal saline treatment. However, for protein measurement, rats were sacrificed 6 and 24 h after LPS or normal saline treatment. Tissues were instantly frozen in liquid nitrogen and stored at −80°C for measurement.

2.4. Drugs

LPS (E. coli, 055:B5), AG and DX were purchased from Sigma (St Louis, MO, USA). LPS and AG were dissolved in normal saline. DX was dissolved in peanut oil. In preliminary experiments, intraperitoneal peanut oil injection was confirmed not to cause any change in myocardial infarction.

2.5. RNA extraction and expression of iNOS mRNA

iNOS mRNA was semiquantitated by RT-PCR method. Total RNA was extracted from tissue by the guanidine thiocyanate method using an Ultraspec RNA isolation system (Biotec Laboratories, TX, USA). The concentration of RNA was measured by a spectrophotometer at a wavelength of 260 nm. An 8-μl aliquot (8 μg) of total RNA was used to synthesize cDNA using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, NJ, USA). The RT mixture (1 μl) was amplified by the hot starting PCR in a 50-μl reaction using Taq DNA polymerase (Amersham Pharmacia Biotech, NJ, USA) for iNOS, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control. The sequences of the primers were as follows: iNOS forward, 5’-ACAAACAAGTGGAGAAAACCC-3’; iNOS reverse, 5’-GACCTGATGGGCCACTGTTAG-3’; GAPDH for-
Fig. 1. Experimental protocol. On day 2, all groups of rats underwent a 30-min coronary occlusion followed by 180-min reperfusion. Twenty-four hours before the coronary occlusion, rats were treated with normal saline (control group) or lipopolysaccharide (LPS). Dexamethasone (DX, 4 mg/kg, i.p.) or aminoguanidine (AG, 300 mg/kg, s.c.) was given 1 h before and 3 h after administration of LPS or normal saline, respectively. Group I, control group; group II, LPS (0.5); group III, LPS (2.5); group IV, LPS (2.5)+DX; group V, Control+DX; group VI, LPS (2.5)+AG; group VII, Control+AG. LPS (0.5), LPS 0.5 mg/kg; LPS (2.5), LPS 2.5 mg/kg.

Western blot analysis

The frozen tissue was homogenized at 4 °C in a lysis buffer as described by Tong et al. [17]. Homogenate was centrifuged at 50,000×g for 10 min at 4 °C. The protein concentration of supernatants was determined by BCA Protein Assay Kit (Pierce Chemicals, IL, USA). The cytosolic protein (20 μg) was separated by electrophoresis.
on an 8% sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA, USA). The membrane was incubated overnight at 4 °C in blocking buffer (Tris-buffered saline [TBS] with 5% nonfat dried milk, 2% BSA and 0.1% Tween 20). The blot was incubated with rabbit polyclonal antibody (1:1000) against mouse macrophage iNOS (Santa Cruz Biotechnology) for 90 min at room temperature, and then incubated with a 1:2000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) for 60 min at room temperature. The specific protein (130 kDa for iNOS) was detected by enhanced chemiluminescence (ECL, Amersham) and quantified by the NIH ‘Image’ program. Proteins extracted from spleen of rats treated with LPS (10 mg/kg, i.p.) were used as the positive control for iNOS. The intensity of band was expressed as a percentage compared with positive control (100%).

2.7. Statistical analysis of data

All values are expressed as mean±S.E. Differences in infarct size among groups were compared by one-way ANOVA followed by an unpaired t-test with modified Bonferroni correction. Hemodynamic data among the groups were compared using two-way ANOVA for repeated measurements followed by the unpaired t-test with modified Bonferroni correction. Statistical differences were considered significant if the P-value was <0.05.

3. Results

3.1. Exclusions

No rats died after treatment with LPS. Six of 60 rats entering the procedure of infarct size analysis [two in Control, one in LPS (2.5 mg/kg), one in Control+DX, two in LPS (2.5 mg/kg)+AG] were excluded from data analysis because of ventricular fibrillation or severe hypotension during coronary occlusion and reperfusion. Final numbers in the groups are as follows: Control (n=8), LPS (0.5 mg/kg) (n=8), LPS (2.5 mg/kg) (n=8), LPS (2.5 mg/kg)+DX (n=8), Control+DX (n=7), LPS (2.5 mg/kg)+AG (n=8), Control+AG (n=7).

3.2. Hemodynamics

Table 1 summarizes mean arterial pressure (MAP) and HR in all groups determined at baseline, coronary artery occlusion and reperfusion. There were no differences in the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Changes in systemic hemodynamics during myocardial ischemia/reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAP (mmHg)</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
</tr>
<tr>
<td>LPS(0.5)</td>
<td>8</td>
</tr>
<tr>
<td>LPS(2.5)</td>
<td>8</td>
</tr>
<tr>
<td>LPS(2.5)+DX</td>
<td>8</td>
</tr>
<tr>
<td>Control+DX</td>
<td>7</td>
</tr>
<tr>
<td>LPS(2.5)+AG</td>
<td>8</td>
</tr>
<tr>
<td>Control+AG</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are mean±S.E. MAP, mean arterial blood pressure; HR, heart rate; Control, rats treated with normal saline 24 h before coronary occlusion; LPS(0.5) or LPS(2.5), rats treated with lipopolysaccharide (LPS, 0.5 or 2.5 mg/kg) 24 h before coronary occlusion; Dexamethasone (DX, 4 mg/kg, i.p.) or aminoguanidine (AG, 300 mg/kg, s.c.) was given 1 h before and 3 h after administration of LPS. There was no significant difference between any groups.
baseline values of MAP and HR between any groups. During coronary occlusion and reperfusion, there was a gradual fall in MAP with time. But no significant differences in MAP during coronary occlusion and reperfusion existed between any groups. In all groups, HR remained unchanged throughout the experiment.

3.3. Myocardial infarct size

Table 2 summarizes the infarct size data. The volume of LV or RA was not significantly different in the seven groups. Fig. 2A,B shows the effect of LPS on myocardial infarct size expressed as the percentage of the RA or LV. In the control group, the infarct size/LV was 69±4%, and the infarct size/LV was 30±2%. Pretreatment with 2.5 mg/kg LPS 24 h pre-ischemia significantly reduced the infarct size/LV to 42±4% and the infarct size/LV to 24±2% compared with the control group (P<0.01), but pretreatment with 0.5 mg/kg LPS did not reduce the infarct size. Pretreatment with DX or AG alone affected neither the infarct size/LV (73±4 and 70±4%, respectively) nor the infarct size/LV (32±3 and 33±2%, respectively). However, pretreatment with DX or AG completely abolished the cardioprotection induced by LPS (2.5 mg/kg). The RA/LV was identical in all groups compared with the control group (Fig. 2C).

3.4. Effect of LPS on expression of iNOS mRNA

A representative ethidium bromide-stained gel of RT-PCR products is shown in Fig. 3A. The intensity of GAPDH bands (950 bp) was not significantly different in all groups. In contrast, the intensity of iNOS bands (642 bp) increased significantly 4 h after administration of LPS (2.5 mg/kg) compared with the control group. The increase in the expression of iNOS mRNA was attenuated by DX, but not by AG. The expression of iNOS mRNA was not found 24 h after administration of LPS (2.5 mg/kg) (data not shown). A summary of the relative levels of iNOS corresponding to GAPDH is presented in Fig. 3B.

4. Discussion

In the present study, we demonstrated that administration of LPS produces a late PC against myocardial infarction in rats. This is associated with increases in the levels of iNOS mRNA and protein. This cardioprotection was attenuated by pretreatment with AG, and was completely abolished by DX. These findings strongly suggest that the activation of iNOS induced by LPS is necessary to trigger the development of the cardioprotection observed 24 h later when the heart is subjected to a lethal ischemic insult. The abrogation of protection against myocardial infarction cannot be attributed to an inherent deleterious effect of DX or AG pretreatment, because administration of DX or AG 24 h before the lethal ischemic insult had no influence on infarct size. The specificity of the RT-PCR reactions was verified by cloning and sequencing the 642-bp product amplified by the iNOS primers. The results of these experiments indicate that the products exhibited 100% homology with published sequences.

3.5. Effect of LPS on induction of iNOS protein

A representative Western blot analysis of iNOS protein is illustrated in Fig. 4. No induction of iNOS protein was detectable in the control group and LPS (0.5 mg/kg) group. Six-hour treatment with LPS (2.5 mg/kg) markedly increased the induction of iNOS protein to 18±3.5% (P<0.05) compared with the control group. The induction of iNOS protein was significantly reduced by DX, but not by AG. The induction of iNOS protein was not found 24 h after administration of LPS (2.5 mg/kg) (data not shown).
Fig. 3. Effects of dexamethasone (DX) and aminoguanidine (AG) on the expression of inducible nitric oxide synthase (iNOS) mRNA induced by lipopolysaccharide (LPS) in rat myocardium. Rats were treated with normal saline (control group) or LPS (0.5 or 2.5 mg/kg) for 4 h. LPS (2.5 mg/kg)-treated rats were pretreated with or without DX or AG. Tissue samples of positive control were obtained from the spleen of rats treated with LPS (10 mg/kg) for 4 h. (A) Representative RT-PCR photograph for iNOS and GAPDH mRNA expression. (B) Densitometric analysis for iNOS mRNA signals. Each iNOS mRNA signal was normalized to the GAPDH signal from the same sample. Values are mean±S.E. of 5–6 experiments. *P<0.05 versus control group; **P<0.05 versus LPS (2.5 mg/kg) group.
In iNOS activity only in ischemic myocardium and can be blocked by administration of AG before lethal ischemic insult. Moreover, Xi et al. [10,11] recently showed that MLA reduced infarct size in mice after global ischemia–reperfusion and that anti-infarct protection was absent in the iNOS gene knockout mice. Taken together, these results suggest that MLA-induced late PC is mediated by iNOS. However, the signaling mechanisms responsible for the upregulation of iNOS activity rapidly during the lethal ischemic insult remain incompletely understood.

In the present study, there was a temporal dissociation between the iNOS gene expression and the LPS-induced cardioprotection. The increase in iNOS mRNA and protein expression preceded the myocardial protection and had returned to undetectable levels by the time the lethal ischemic insult was induced. These results suggest that iNOS acts as a trigger of protective mechanisms responsible for the LPS-induced late PC. It has been well demonstrated that iNOS is induced by LPS, culminating in increased activity of iNOS 4–6 h after administration of LPS [25,26]. Increasing evidence suggests that nitric oxide synthase (NOS) may produce \(^{•}\)O\(_2\) rather than NO under
conditions in which the local tissue levels of l-arginine or tetrahydrobiopterin drop [27,28]. Beckman and colleagues [29] have shown that NO and 'O_2' react rapidly to form the peroxynitrite anion (ONOO'), which then decomposes to generate the hydroxyl radical ('OH) or some other potent oxidant. It is now becoming clear that regulation of iNOS expression is governed predominantly by the transcription factor NF-κB [30,31]. NO and reactive oxygen species are known to activate this transcription factor, which then promotes transcription of the iNOS gene [32,33]. Furthermore, Connelly et al. [23] showed that NO has an explicit biphasic effect on the activity of NF-κB, which depends on the amount of NO. A low dose of NO promotes the expression of iNOS via potentiation of NF-κB activity. However, a high dose of NO inhibits the expression of iNOS. In conjunction with previous studies [23], we therefore propose that LPS-induced NO, which activates a NF-κB-mediated signal transduction cascade via ONOO' and 'OH, may benefit the expression of iNOS induced by ischemia during the lethal ischemic insult. Further studies will be necessary to determine whether LPS-induced late PC is triggered by NO itself or by one of its reactive byproducts.

DX is the synthetic glucocorticoid. Glucocorticoids inhibit the expression of immunomodulatory genes, including iNOS [34]. In the present study, the expression of iNOS mRNA and protein was attenuated by DX. This is in agreement with previous studies, which demonstrated that DX mediated iNOS repression occurred at the level of transcription [35,36]. The transcription factor NF-κB is required for LPS induction of the murine iNOS promoter [37]. Two major mechanisms appear to be at work in DX repression of NF-κB. The first involves the upregulation of IκBα by DX, resulting in a reduction of the amount of NF-κB that translocates to the nucleus [38]. The second mechanism involves cross-coupling and physical association between the activated glucocorticoid receptor and NF-κB, leading to the inhibition of DNA transactivation [39].

AG, a relatively selective inhibitor of iNOS, has been shown to have an IC_{50} of 160.0 μmol/l for the constitutive isoform of NOS (cNOS) versus 5.4 μmol/l for iNOS [40,41]. There is growing evidence showing that AG has no effect on arterial blood pressure and infarct size in the ischemia–reperfusion model at the dose we used [15,42]. However, the inhibition of cNOS by non-selective NOS inhibitors produces a significant rise in arterial blood pressure and affects myocardial infarct size [43]. Taken together, these results suggest that AG is more selective for iNOS than cNOS at the dose we used. In addition, AG has been described to have some effects other than NOS inhibition. Among them, an inhibitory effect on the antioxidant enzyme catalase is the most important [44]. But the inhibitory effect by AG might promote the LPS-induced late PC since reactive oxygen species have been proposed as a trigger of late PC.

In summary, the present study provides significant new information with respect to the cellular mechanism(s) by which LPS induces the late PC against myocardial infarction. In conjunction with previous studies [9–11], our data support a distinctive role of iNOS in the late PC produced by LPS, with iNOS serving as not only the trigger on day 1 but also the mediator 24 h after administration of LPS. We propose that LPS activates iNOS before lethal ischemia, with the increased biosynthesis of NO that may activate the downstream signaling pathway including various transcriptional factors, permitting rapid activation of the iNOS during the lethal ischemic insult and giving the cardioprotection.

References


[29] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620–1624.


