Endotoxin Induces a Second Window of Protection in the Rat Heart as Determined by Using p-Nitro-Blue Tetrazolium Staining, Cardiac Troponin T Release, and Histology

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Abstract—Pretreatment of rats with small doses of lipopolysaccharide (LPS), eg, for 24 hours, attenuates the cardiac dysfunction caused by subsequent period of myocardial ischemia. This phenomenon of enhanced tolerance to an ischemic insult has been termed “second window of protection.” Although the cardioprotective effects of LPS were first reported in 1989, it is still unclear whether the observed attenuation by LPS of the ischemia-induced cardiac dysfunction is indeed secondary to the protection of cardiac myocytes against ischemic cell injury and death. This study was designed to investigate the effects of “preconditioning” with LPS on cell injury caused by regional myocardial ischemia and reperfusion in the anesthetized rat. Thirty-five Wistar rats were subjected to 25 minutes occlusion of the left anterior descending coronary artery followed by 2 hours of reperfusion. Hemodynamic parameters were continuously recorded, and at the end of the experiments, infarct size (using p-nitro-blue tetrazolium staining), cardiac troponin T release, and histological markers of cell injury and death were determined. In rats pretreated with a bolus of saline (vehicle for LPS) 2 or 24 hours before left anterior descending coronary artery occlusion and reperfusion, the infarct size was 59±4% (2 hours saline-control, n=6) and 61±3% (24 hours saline-control, n=6), respectively. Pretreatment of animals with a bolus of LPS (1 mg/kg IP) 24 hours before the onset of myocardial ischemia and reperfusion reduced both infarct size (to 18±7%; P<0.05, n=6) as well as histological signs of cell injury. Pretreatment (24 hours, as above) of rats with LPS also reduced the release of cardiac troponin T from 58±13 ng/mL (saline-control) to 16±9 ng/mL. In contrast, pretreatment of rats with LPS (2 hours, as above) did not affect infarct size (56±8%, n=6), cardiac troponin T release, or the histological parameters of cell injury. These data provide the first conclusive evidence that pretreatment of rats with a bolus of LPS 24 hours before intervention reduces the cell injury and death caused by a subsequent period of myocardial ischemia and reperfusion. (Arterioscler Thromb Vasc Biol. 1999;19:2276-2280.)

Key Words: LPS ■ myocardial infarct size ■ myocardial ischemia ■ reperfusion ■ delayed preconditioning

The second window of protection (SWOP) was originally described as enhanced tolerance to myocardial ischemia after exposure of the rabbit1 or canine2 heart to brief periods of myocardial ischemia (ischemic preconditioning, IPC). This SWOP appears to occur at 24 hours after IPC and is maintained for 72 hours. The SWOP against myocardial infarction has been observed in many species, eg, in the rabbit,3 dog,2 pig,4 and rat.5 Several triggers are known to induce a SWOP, eg, brief repetitive cycles of coronary occlusion,1 ventricular rapid pacing,6 stimulation of adenosine A1 receptors,3 and administration of lipopolysaccharide (LPS)7 or monophosphoryl lipid A (MLA).8 Previously, the methods used to investigate SWOP involved the measurement of myocardial infarct size by using triphenyltetrazolium (TTC) macrochemical staining. In addition, SWOP enhances the resistance of the myocardium against the arrhythmias,6,9 the postischemic endothelial dysfunction,10 and the myocardial stunning11,12 caused by a subsequent period of ischemia-reperfusion. It is unclear, however, whether the SWOP caused by IPC (and other stimuli) is indeed due to a protection of myocardial cells against injury or death, as there is one report that demonstrates that SWOP leads to a reduction in infarct size as determined by TTC staining without reducing the histological signs of cell necrosis.13 Here, we used LPS as a trigger for SWOP and report that LPS causes a time-dependent reduction in infarct size in rats subjected to myocardial ischemia-reperfusion. To demonstrate that the observed “cardioprotective” effects of LPS were not an artifact of tetrazolium staining, we also investigated the effects of LPS on cardiac troponin T (cTnT) release and histological signs of tissue injury.
Methods

Myocardial Ischemia and Reperfusion in the Rat In Vivo

The technique used to produce coronary artery occlusion is identical to that described previously. Briefly, male Wistar rats (240 to 350 g, Tuck, Rayleigh, Essex, UK) were anesthetized using thiopentone sodium (120 mg/kg IP). The trachea was cannulated and artificial respiration was maintained using a Harvard ventilator with a frequency of 70 strokes/min, a tidal volume of 8 to 10 mL/kg, an inspiratory oxygen concentration of 30%, and a positive end-expiratory pressure of 1 to 2 mm Hg resulting in pCO₂ values of 36 to 44 mm Hg and pO₂ values over 150 mm Hg. Body temperature was maintained at 38±1°C. The right carotid artery was cannulated and connected to a pressure transducer to monitor mean arterial blood pressure (MAP) and heart rate (HR) continuously on a 4-channel Grass 7D polygraph recorder from which pressure rate index (PRI), a relative indicator of myocardial oxygen consumption, was calculated as the product of MAP and HR and expressed in mm Hg/(min · 10¹⁰). The right jugular vein was cannulated for the administration of drugs. The chest was opened by a left-side thoracotomy, the pericardium was incised, and an atrumatic needle and occluder were placed around the left anterior descending coronary artery (LAD). After completion of the surgical procedure, the animals were allowed to stabilize for 30 minutes before LAD ligation, which involved constriction of the occluder (time 0). This was associated with typical hemodynamic changes observed during myocardial ischemia, eg, fall in MAP. After 25 minutes of acute myocardial ischemia, the occluder was released allowing the reperfusion of the previously ischemic myocardium for 2 hours. After reocclusion of the LAD, Evans blue dye (1 mL of 2% wt/vol) was administered IV to determine between ischemic (area at risk) and nonischemic myocardium (area not at risk). Subsequently, the heart was cut into horizontal slices and then into small pieces. The area at risk (AR) was separated from the area not at risk and then incubated with p-nitro-blue tetrazolium (NBT, 0.5 mg/mL, 20 minutes at 37°C) to distinguish between ischemic and infarcted tissue, whereas the area not at risk was incubated with saline. The AR and infarct size were calculated after weighing the respective tissue samples and expressed as % of the AR.

Measurement of the Plasma Levels of Cardiac Troponin T in the Rat

At the end of the experiment, a blood sample (1 mL) was obtained from the carotid cannula and centrifuged to obtain plasma. The plasma supernatants were separated and stored at −20°C until assayed. The concentration of cardiac troponin T was determined using the STAT (Short TurnAround Time) immunoassay (Boehringer Mannheim) using an Elecsys System 2010.

Determination of the Degree of Myocardial Tissue Injury Using Light Microscopy

Biopsies of all sections of the heart (nonischemic, ischemic, and infarcted) were fixed in paraformaldehyde (4% wt/vol), embedded in paraffin, cut into 4-μm section, de-waxed, and stained with hematoxylin-eosin, Fuchsin, and Luxol-Fast-Blue.

Experimental Groups

Rats were randomly allocated into the following groups: (1) Sham operated controls (no occlusion of the LAD, n=3). (2) Injection of saline (1 mL/kg IP, n=6) 2 hours before 25 minutes LAD occlusion and 2 hours reperfusion. (3) Injection of saline (1 mL/kg IP, n=6) 24 hours before 25 minutes LAD occlusion and 2 hours reperfusion. (4) Injection of LPS (1 mg/kg IP, n=6) 24 hours before no occlusion of the LAD. (5) Injection of LPS (1 mg/kg IP, n=6) 2 hours before 25 minutes LAD occlusion and 2 hours reperfusion. (6) Injection of LPS (1 mg/kg IP, n=6) 24 hours before 25 minutes LAD occlusion and 2 hours reperfusion. The n-numbers in the above groups refer to rats, which survived until the end of the experiment. The number of rats that died in the individual groups were as follows: group 3, n=1; group 5, n=1.

Results

The Cardioprotective Effects of LPS In Vivo

The mean values for the AR were similar in all animal groups studied and ranged from 46±2% to 56±1% (P>0.05, data not shown). In rats pretreated with saline (2 or 24 hours before ischemia), occlusion of the LAD (for 25 minutes) followed by reperfusion (for 2 hours) resulted in an infarct size of 59±4% (n=6) or 61±3% (n=6) of the AR, respectively. When compared with vehicle, administration of 1 mg/kg LPS 2 hours before coronary artery ligation did not cause a reduction in infarct size (56±8%, n=6), (P>0.05, Figure 1). When compared with vehicle, bolus injection of LPS (24 hours before ischemia) caused a significant reduction in infarct size of approximately 65% (P<0.05, Figure 1). Sham operation alone did not result in a significant degree of infarction in any of the animal groups studied (<3% of the AR).

Hemodynamic Effects of LPS in Rats Subjected to Myocardial Ischemia and Reperfusion

Hemodynamic data, eg, MAP (mm Hg), HR (bpm), and PRI [mm Hg/(min · 10¹⁰)] measured during the course of the experiments were similar in all groups studied (P>0.05, data not shown). In sham-operated rats (no LAD occlusion), injection of vehicle (saline) did not cause any significant effects on MAP, HR, or PRI. In rats subjected to LAD occlusion and reperfusion that received either saline or LPS,
The purpose of this study was to examine the effects of pretreatment with LPS, a well known trigger of the second window of protection, in a model of regional myocardial ischemia and reperfusion in the rat on (1) myocardial infarct size, (2) cTnT release, and (3) histological markers of tissue injury. In 1989 Brown and colleagues demonstrated for the first time that pretreatment of rats with LPS 24 hours before ischemia improved myocardial function in isolated, perfused hearts subjected to global myocardial ischemia and reperfusion. In the same study, hearts isolated from rats that were pretreated with LPS 1 hour before ischemia did not show enhanced resistance to ischemia-reperfusion injury. The authors suggested that SWOP produced by LPS was associated with and possibly due to an upregulation of catalase activity. Interestingly, compounds with less toxicity than LPS, such as MLA, also produce a significant reduction in myocardial infarct size in dogs. The methods used to investigate the SWOP triggered by LPS or MLA involved the measurement of myocardial infarct size by using TTC macrochemical staining. In addition, pretreatment of animals with LPS or MLA also reduced the number of ischemia-reperfusion arrhythmias and the degree of myocardial stunning caused by ischemia-reperfusion. In this study, the reduction in infarct size caused by pretreatment of rats with LPS 24 hours before ischemia was determined by staining of the viable myocardium (within the AR) with the formazan dye NBT. This reduction in infarct size (as determined by NBT) is due to a reduction in myocardial tissue necrosis, as LPS also attenuated the increase in the plasma levels of cTnT caused by regional myocardial ischemia and reperfusion. There is good evidence that a rise in the plasma levels of cTnT is specific for myocardial tissue injury. Unlike plasma levels of creatine phosphokinase or lactate dehydrogenase, which are elevated in open-chest models of myocardial infarction due to the surgical procedure (Zacharowski and Tiemermann, unpublished data, 1998), the thoracotomy used here did not result in any detectable rise in the plasma levels of cTnT. Thus these data strongly support the conclusion that LPS does cause a significant reduction in myocardial infarct size. In addition, we have demonstrated for the first time that pretreatment of rats with LPS (1 mg/kg 24 hours before ischemia) does not lead to myocardial necrosis by itself, as determined by measurement of cTnT concentrations in the plasma. Previously, we have demonstrated that there is a significant increase in the levels of cTnT in the plasma within 3 hours after regional myocardial ischemia and reperfusion in the rat. In cases of acute myocardial infarction in humans, cTnT levels in serum rise approximately 3 to 4 hours after the occurrence of cardiac symptoms and can remain elevated for up to 14 days.

It is interesting to speculate on the prospective mechanisms by which LPS causes a significant reduction in the degree of necrosis as demonstrated in this study. Recently the problems involved in using the tetrazolium staining have been discussed. Miki et al demonstrated a SWOP (infarct size reduction) using TTC, but these findings could not be confirmed using light microscopy. The same group also proposed that exogenously administered SOD may cause false-positive staining with tetrazolium and have suggested that endogenous upregulation of SOD caused by preconditioning may account for an artifactual limitation of myocardial injury. However, these results could not be confirmed by other groups. Clearly, in both intervention studies, and in

**Figure 2.** cTnT release in a model of myocardial ischemia (25 minutes) and reperfusion (2 hours) of the LAD in the anesthetized rat. Different groups of animals were pretreated at different time points before experimental intervention with saline (2 hours, 2-con, n=6), saline (24 hours, 24-con, n=6), LPS (2 hours, 2-LPS, 1 mg/kg IP, n=6), or LPS (24 hours, 24-LPS, 1 mg/kg IP, n=6). In addition, sham operated rats (no LAD occlusion and reperfusion) were studied: sham-con (24 hours pretreatment with saline, n=3) and sham-LPS (24 hours pretreatment with LPS, n=6). *P<0.05 when compared with control.

Mean values for MAP and PRI fell throughout the experiment (P>0.05, data not shown).

**Effects of LPS on Plasma Levels of Cardiac Troponin T in the Rat**

In rats that were subjected to the surgical procedure, but not to LAD occlusion (sham-operation), there was no significant increase in the plasma levels of the cardiac-specific marker cTnT (Figure 2). Pretreatment of rats with saline (2 or 24 hours) followed by ischemia-reperfusion of the LAD resulted in a significant increase in the plasma levels of cTnT (Figure 2). When compared with vehicle, pretreatment with LPS 24 hours before ischemia caused a significant reduction in the release of cTnT (P<0.05, Figure 2). LPS pretreatment 2 hours before ischemia did not reduce the cTnT release (P>0.05, Figure 2).

**Effects of LPS on Histological Signs of Tissue Injury in the Myocardium of the Rat**

Histological evaluation by light microscopy of biopsies of control hearts subjected to regional ischemia and reperfusion demonstrated the occurrence of complete coagulation necrosis. Cytoplasm of myocytes was deeply eosinophilic, which demonstrates a strong staining with Fuchsin as well as deep eosinophilia. Cytoplasm of myocytes was deeply eosinophilic, which demonstrated the occurrence of complete coagulation necrosis. In the same study, hearts isolated from rats that were subjected to regional myocardial ischemia and reperfusion did not show any detectable rise in the plasma levels of cTnT caused by regional myocardial ischemia and reperfusion. There is good evidence that a rise in the plasma levels of cTnT is specific for myocardial tissue injury. Unlike plasma levels of creatine phosphokinase or lactate dehydrogenase, which are elevated in open-chest models of myocardial infarction due to the surgical procedure (Zacharowski and Tiemermann, unpublished data, 1998), the thoracotomy used here did not result in any detectable rise in the plasma levels of cTnT. Therefore, these data strongly support the conclusion that LPS does cause a significant reduction in myocardial infarct size. In addition, we have demonstrated for the first time that pretreatment of rats with LPS (1 mg/kg 24 hours before ischemia) does not lead to myocardial necrosis by itself, as determined by measurement of cTnT concentrations in the plasma. Previously, we have demonstrated that there is a significant increase in the levels of cTnT in the plasma within 3 hours after regional myocardial ischemia and reperfusion in the rat. In cases of acute myocardial infarction in humans, cTnT levels in serum rise approximately 3 to 4 hours after the occurrence of cardiac symptoms and can remain elevated for up to 14 days.

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all groups studied, there were no differences in body weight, heart weight, AR, or hemodynamic parameters such as mean MAP or HR, suggesting that the beneficial effects of LPS pretreatment (24 hours before ischemia) were not related to differences in the amount of myocardial tissue sampled nor to changes in myocardial oxygen demand. Therefore, we have also used histology as a tool to determine the degree of cell injury in the myocardium of rats, which were subjected to LAD occlusion and reperfusion. LPS caused a convincing reduction in myocardial infarct size and demonstrates the existence of SWOP.

Thus this study demonstrates, for the first time, that pretreatment with LPS 24 hours before ischemia reduces infarct size, cTnT release, and the signs of histological tissue injury in rats subjected to myocardial ischemia-reperfusion. These results imply that LPS induces a SWOP without interfering with the staining procedure. The mechanism(s) underlying in the cardioprotective effects afforded by LPS warrants further investigation.

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