Endotoxin and ischemic preconditioning: TNF-α concentration and myocardial infarct development in rabbits

Sergej Belosjorow, Rainer Schulz, Hilmar Dörge, F. Ulrich Schade, and Gerd Heusch. Endotoxin and ischemic preconditioning; TNF-α concentration and myocardial infarct development in rabbits. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2470–H2475, 1999.—Ischemic preconditioning (IP) and prior exposure to lipopolysaccharides (LPS) reduce infarct size (IS) and serum tumor necrosis factor-α (TNF-α) concentration resulting from myocardial ischemia-reperfusion in rats. The decrease in TNF-α might relate to an induced TNF-α inhibitory serum activity (TNF-α-ISA). We analyzed TNF-α and TNF-α-ISA during 30 and 180 min ischemia and reperfusion, respectively, in anesthetized rabbits either untreated (group 1, n = 7), preconditioned (5 and 10 min ischemia and reperfusion, respectively, group 2, n = 9), or exposed to LPS 72 h before ischemia (group 3, n = 9). TNF-α-ISA was assayed by cocultivating LPS-stimulated rabbit blood with serum of groups 1–3 and measuring TNF-α (WEHI assay). With comparable concentration at risk, IS in group 1 was 36.9 ± 11.1 (SD)% and it was reduced to 13.1 ± 11.6% and 17.3 ± 11.3% (both P < 0.05) in groups 2 and 3, respectively. TNF-α was increased during ischemia-reperfusion in group 1 but remained unchanged in rabbits subjected to IP or LPS. TNF-α-ISA was detected during ischemia-reperfusion in group 2 (29% and 38% of maximum inhibition, respectively) and during baseline, ischemia and reperfusion in group 3 (51%, 46%, and 48% of maximum inhibition, respectively) but was absent in group 1. Cardioprotection by IP and LPS is associated with a reduced TNF-α and an induced TNF-α-ISA during ischemia-reperfusion.

Ischemic preconditioning; tumor necrosis factor-α

Previous exposure to nonlethal doses of lipopolysaccharides (LPS) in animals and humans results in a state of hyporesponsiveness to a second LPS challenge (10). This hyporesponsiveness is transient, and the phenomenon has been termed “endotoxin tolerance.” The endotoxin tolerance is biphasic; i.e., characterized by an early and a late phase (26). The early phase develops within several hours after a single LPS injection, reaching an optimum after 4 days. The late phase of tolerance is mediated by specific antibodies against the O-chain of LPS and becomes apparent several days after the first LPS exposure (26).

The early phase of tolerance is associated with protection against myocardial ischemia. Induced tolerance to LPS reduced myocardial infarct size in rats following coronary artery occlusion and reperfusion (5, 29), and such infarct size reduction was associated with a marked reduction of spontaneous activation and adhesion of circulating polymorphonuclear neutrophils during reperfusion (5). Node and colleagues (23) reported that LPS induced ecto-5’-nucleotidase activity and subsequently increased production of adenosine, which then provided cardioprotection. Finally, Rowland and co-workers (25) demonstrated a protective effect against infarction at 72 h following LPS administration in an in vivo rabbit model of regional coronary artery occlusion; such infarct size reduction was associated with enhanced stress protein expression.

Endotoxins can activate macrophages, monocytes, and cardiac myocytes, which then display enhanced cytotoxic activity and synthesis of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) (9, 11, 15, 19). Recently, it has been reported that resident cardiac mast cells might also release TNF-α in response to myocardial ischemia-reperfusion in canine hearts (8). Circulating and cardiac TNF-α levels are increased during myocardial ischemia in experimental animals (13, 14) and in patients with ischemic heart disease (32) and advanced heart failure (3). Elevated cardiac and serum TNF-α concentrations correlated to the depression of myocardial function (2, 13, 30). In isolated, contracting myocytes, an immediate negative inotropic effect was attributed to a TNF-α-induced activation of the neutral sphingomyelinase (24, 31), whereas the delayed negative inotropic effect was related to an expression of the calcium-independent nitric oxide synthase (28).

Indeed, ischemia-induced myocardial contractile dysfunction was abolished by inhibition of TNF-α production or neutralization of circulating and cardiac TNF-α (12). Pretreatment with cycloheximide or dexamethasone abolished the increases in circulating and cardiac TNF-α concentration and preserved myocardial contractile function (22). Prior exposure to LPS also decreased the production of TNF-α in mice, and this decreased production of TNF-α was associated with an increased TNF-α inhibitory plasma activity (27). Recently, a study in isolated, saline-perfused rat hearts demonstrated that ischemic preconditioning and adenosine pretreatment decreased the cardiac TNF-α concentration during reperfusion and improved postischemic myocardial function (20).

From the above findings, we hypothesized that the reduction of TNF-α concentration in LPS-tolerant and in preconditioned hearts plays a role in the development of tolerance against myocardial ischemia and...
that inhibitory components are responsible for the reduced TNF-α concentration during ischemia-reperfusion. We therefore: 1) compared the degree of myocardial protection induced by either ischemic preconditioning or prior exposure to LPS; 2) examined whether or not the serum TNF-α concentration during ischemia-reperfusion is affected in the same way by ischemic preconditioning and prior exposure to LPS; and 3) determined the TNF-α-inhibitory serum activity.

**MATERIALS AND METHODS**

**Surgical preparation**. Chinchilla bastard rabbits (3.0–3.6 kg; mean 3.2 kg) were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg) followed by an intravenous infusion of propofol (6–10 ml/h). Rabbits were then intubated with an endotracheal tube (5-mm internal diameter), placed in the supine position on a table, and ventilated using positive pressure. Ventilation was maintained using a Dräger UV-2 ventilator with 30% oxygen and 70% room air, and the tidal volume was adjusted to maintain arterial PCO2 between 30 and 40 mmHg and PO2 between 120 and 150 mmHg. Body temperature was continuously monitored, and hypothermia was prevented using a heating pad. The left common carotid artery was cannulated with a polyethylene catheter with the tip in the aortic arch for blood pressure measurement and arterial blood withdrawal. Both common jugular veins were cannulated with polyethylene catheters for the administration of saline and drugs. The heart was exposed in a pericardial cradle through a left thoracotomy, and a 4-0 prolene suture was placed around the anterolateral branch of the left circumflex coronary artery, midway between the atrioventricular groove and the apex. The suture was passed through a soft plastic tube to form a snare for coronary artery occlusion. Cyanosis, electrocardiogram changes, and a decrease in aortic pressure were considered to indicate effective coronary artery occlusion. The left atrium was catheterized with a Teflon tubing for the injection of colored microspheres. The animals were heparinized with 2,000 IU heparin sodium. Arterial blood gases were monitored frequently in the initial stages of the preparation until stable and then periodically throughout the study (Radiometer ABL-510, Copenhagen). At the end of each study, the animals were killed with a bolus injection of 10 ml saturated KCl solution.

**Experimental protocol**. Rabbits in group 1 (n = 9) were subjected to 30 min of coronary artery occlusion and 180 min of reperfusion. Rabbits in group 2 (n = 12) were subjected to 5 min of coronary artery occlusion and 10 min of reperfusion before the 30-min coronary artery occlusion and 180-min reperfusion. Rabbits in group 3 (n = 11) were exposed to LPS 72 h before the 30-min coronary artery occlusion and 180-min reperfusion.

Previous exposure to LPS. LPS from Salmonella abortus equi, prepared as triethyl ammonium salt, were a kind gift of Dr. H. Brade, Forschungszentrum Borstel, Germany. LPS were kept in a stock solution of 5 mg/ml in pyrogen-free water and freshly diluted with sterile phosphate-buffered saline before use. The rabbits received a single dose of LPS (10 mg/kg) into a marginal ear vein. This dose was determined from preliminary experiments in which tolerance to LPS was achieved and was similar to the dose of LPS used in a previous study (25). This dose of LPS did not cause signs of illness in most animals, except two, which developed a long-lasting fever and died.

**Hemodynamics**. Maximal aortic pressure and heart rate were recorded on an eight-channel recorder (Gould MK 200A) and stored directly on the hard disk of an AT-type computer. Data were taken under baseline conditions, at 20 min of coronary artery occlusion, and at 60 and 120 min of reperfusion.

**Surgical preparation**. Rabbits in group 4 (n = 11) were subjected to 5 min of coronary artery occlusion and 10 min of reperfusion.

**Materials and Methods**

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- **Heart exposure**: The heart was exposed in a pericardial cradle through a left thoracotomy, and a 4-0 prolene suture was placed around the anterolateral branch of the left circumflex coronary artery, midway between the atrioventricular groove and the apex. The suture was passed through a soft plastic tube to form a snare for coronary artery occlusion. Cyanosis, electrocardiogram changes, and a decrease in aortic pressure were considered to indicate effective coronary artery occlusion. The left atrium was catheterized with a Teflon tubing for the injection of colored microspheres. The animals were heparinized with 2,000 IU heparin sodium.

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- **Rabbits killed**: At the end of each study, the animals were killed with a bolus injection of 10 ml saturated KCl solution.

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ELISA and the WEHI assay. The inhibitory activity was not associated with TNF receptors, corticosteroids, varying numbers of leukocytes, interleukin-10 (IL-10), transforming growth factor-β, or interleukin-4 (18).

Infarct size. At the end of each experiment, the heart was removed and cut from base to apex into five slices of 3- to 4-mm thickness each. The tissue slices were stained in 1.0% triphenyl tetrazolium chloride (TTC, Sigma, Deisenhofen, Germany) and 8% dextran (77,800 mol wt) for 20 min at 37°C. Infarcted tissue indicated by the lack of TTC staining was excised from all slices. The weight of the infarcted tissue was averaged 0.09 ± 0.05 g and was thus comparable to a previous study in anesthetized rabbits (34). The remaining noninfarcted tissue was subsequently cut into small transmural pieces. A reduction in blood flow by at least 90% during coronary artery occlusion was assumed to indicate the area at risk (33). Area at risk was expressed as percentage of the left ventricle, and infarct size was expressed as percentage of the area at risk.

Regional myocardial blood flow. Eight to ten million colored microspheres (15 µm diameter) were injected into the left atrium. Each microsphere injection was followed by a flush of 2 ml of saline. The withdrawal of arterial reference blood samples from the aorta was begun 15 s before injection of the microspheres and continued for 150 s at a rate of 1.5 ml/min (withdrawal pump model 901A, Harvard Apparatus, S. Natick, MA). Regional myocardial blood flow under baseline conditions, during coronary artery occlusion, and at 1 h reperfusion was determined with one of three different colors (yellow, red, or blue). The color spectra of each myocardial and the arterial reference withdrawal sample were measured with a spectrophotometer (model 8452A, Hewlett-Packard, Palo Alto, CA). To obtain color spectra of myocardial tissue samples, TTC staining was removed using ethanol and acetone (9:1, at room temperature for 12 h), followed by digestion of the tissue samples and extraction of the dye (4).

Regional myocardial blood flow per sample, corrected for wet weight, was calculated using the equation: regional myocardial blood flow = reference withdrawal rate × absorbance per sample per absorbance in arterial withdrawal (16).

Statistics. Statistical analysis was performed with SigmaStat software (Jandel Scientific, San Rafael, CA). All data are reported as means ± SD. Changes in systemic hemodynamics, regional myocardial blood flow, serum TNF-α concentration, and TNF-α inhibitory serum activity were compared using two-way analysis of variance. When a significant overall effect was detected, single mean values were compared using Bonferroni's method. Comparisons of area at risk and infarct size were made by one-way analysis of variance. Data were considered statistically significant at a P value < 0.05. Linear regression between serum TNF-α concentration and TNF-α inhibitory serum activity was calculated using SigmaPlot software.

RESULTS

Mortality. Two rabbits of group 1 and three rabbits of group 2 died from ventricular fibrillation during coronary artery occlusion. Eleven rabbits in group 3 received an intravenous endotoxin dose of 10 µg/kg. Nine rabbits subsequently underwent 30 min of coronary artery occlusion and 3 h of reperfusion; the other two rabbits developed a long-lasting fever and died before the experiment.

Hemodynamics and regional myocardial blood flow. Heart rate and maximal aortic blood pressure did not change throughout the protocol and were not different among groups (Table 1).

Regional myocardial blood flow in normal myocardium was constant throughout the protocol and was comparable among groups (Table 2). The blood flow in the area at risk was similar among all groups under baseline conditions and decreased to the same extent during coronary artery occlusion. During reperfusion, blood flow in the area at risk was not different among groups.

Infarct size. After 30-min coronary artery occlusion and 180-min reperfusion, 36.9 ± 11.1% of the area at risk was infarcted in group 1. Infarct size as significantly reduced to 13.1 ± 11.6% by ischemic preconditioning in group 2 (P < 0.05 vs. group 1). Prior exposure to LPS resulted in a significant reduction of infarct size to 17.3 ± 11.3% in group 3 (P < 0.05 vs. group 1; Fig. 1).

Table 1. Systemic hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>IP</th>
<th>Rep 1 h</th>
<th>Rep 2 h</th>
<th>Occl 20</th>
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</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Group 1</td>
<td>216 ± 38</td>
<td>227 ± 29</td>
<td>228 ± 21</td>
<td>222 ± 44</td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>216 ± 27</td>
<td>76 ± 10</td>
<td>66 ± 9</td>
<td>74 ± 8</td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>200 ± 22</td>
<td>82 ± 7</td>
<td>73 ± 14</td>
<td>74 ± 11</td>
<td></td>
</tr>
<tr>
<td>Maximal aortic pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>82 ± 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>82 ± 12</td>
<td>71 ± 17</td>
<td>68 ± 13</td>
<td>67 ± 8</td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>80 ± 7</td>
<td>76 ± 10</td>
<td>66 ± 9</td>
<td>74 ± 8</td>
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</tbody>
</table>

Table 2. Regional myocardial blood flow

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Occl 20</th>
<th>Rep 1 h</th>
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</thead>
<tbody>
<tr>
<td>Normal zone</td>
<td></td>
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<tr>
<td>Group 1</td>
<td>2.56 ± 0.75</td>
<td>2.77 ± 0.91</td>
<td>2.24 ± 0.88</td>
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<tr>
<td>Group 2</td>
<td>2.12 ± 0.62</td>
<td>2.01 ± 1.01</td>
<td>2.04 ± 0.73</td>
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<tr>
<td>Group 3</td>
<td>2.24 ± 0.92</td>
<td>2.21 ± 1.36</td>
<td>3.27 ± 1.04</td>
</tr>
<tr>
<td>Area at risk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>2.13 ± 1.08</td>
<td>0.90 ± 0.05*</td>
<td>2.46 ± 1.11</td>
</tr>
<tr>
<td>Group 2</td>
<td>2.31 ± 1.24</td>
<td>0.06 ± 0.02*</td>
<td>1.92 ± 0.72</td>
</tr>
<tr>
<td>Group 3</td>
<td>2.64 ± 0.98</td>
<td>0.13 ± 0.05*</td>
<td>3.27 ± 1.33</td>
</tr>
</tbody>
</table>

Data are means ± SD, n = 7 in group 1, n = 9 in groups 2 and 3, respectively. *P < 0.05 vs. baseline.
The area at risk was similar among groups 1–3, averaging 22 ± 8%, 23 ± 9%, and 22 ± 13% of the left ventricle, respectively.

Serum TNF-α concentration. Serum TNF-α concentration was increased during coronary artery occlusion, and this increase was maintained throughout reperfusion in group 1 (Fig. 2). In group 2, serum TNF-α concentration was increased during the initial period of ischemia-reperfusion. During the sustained period of coronary artery occlusion and the subsequent reperfusion period, serum TNF-α concentration was, however, not different from that at baseline. In group 3, serum TNF-α concentration was decreased already under baseline conditions compared with groups 1 and 2, and it did not increase during coronary artery occlusion and reperfusion.

TNF-α inhibitory serum activity. TNF-α concentration in the supernatant of LPS-stimulated blood mixed with serum of rabbits in group 1 was was almost zero throughout the experimental protocol (Fig. 3). In group

DISCUSSION

The results of the present study confirm that both ischemic preconditioning and induced tolerance to bacterial LPS reduce myocardial infarct size in rabbits. This cardioprotection by ischemic preconditioning and induced tolerance to bacterial LPS was associated with a reduced serum TNF-α concentration and an increased TNF-α inhibitory serum activity.

In the present study, the area at risk averaged ~22% of the left ventricle in all groups. After 30 min of coronary artery occlusion, 37% of the area at risk was infarcted in the control group, and infarct size was reduced to 13% by ischemic preconditioning. Similar infarct sizes following 30 min of coronary artery occlusion without (39%) and with ischemic preconditioning (8%) have been described in a prior study in anesthetized rabbits (17).

Previous studies have demonstrated an increase in circulating and cardiac TNF-α concentrations during myocardial ischemia in experimental animals and in patients (13, 32). A similar increase in serum TNF-α concentration was found in the control group during the sustained ischemia and in the preconditioned group during the initial cycle of 5 min ischemia and 10 min reperfusion in the present study.

In two previous studies, LPS increased the recovery of contractile function during postischemic reperfusion (1, 21). Also, infarct size was reduced at 72 h following LPS administration in an in vivo rabbit model (25). Our
results of infarct size reduction by LPS confirm this previous finding. The mechanism of cardioprotection by prior exposure to LPS is not fully understood. Prior exposure to LPS and ischemic preconditioning were both associated with a decrease in the serum TNF-α concentration during sustained ischemia in the present study. However, a causal role of reduced TNF-α concentration in the reduction of infarct size has not been established so far.

Ischemic preconditioning has previously been reported to attenuate the reperfusion-induced increase in cardiac TNF-α concentration in an isolated saline-perfused rat heart after 20 min of global ischemia (20). Whether the inhibition of cardiac myocytes and/or cardiac resident macrophages were responsible for the reduced TNF-α concentration observed in this study remains unknown. Blood-derived factors (neutrophils/ blood-derived cytokines), however, could be ruled out. In our study, the alterations in the serum TNF-α concentration could relate to blood-borne macrophages and monocytes but may additionally involve cardiac myocytes, cardiac resident macrophages, and mast cells. One potential explanation for the decline in the serum TNF-α concentration with ischemic preconditioning and LPS relates to an increased serum inhibitory activity to TNF-α, as previously described (27). Indeed, TNF-α inhibitory serum activity was induced in rabbits exposed to ischemic preconditioning and LPS. The TNF-α inhibitory serum activities and serum TNF-α concentrations were found to be inversely correlated, although this does not establish a cause-effect relation, and the observed changes in TNF-α may represent just an epiphenomenon. Whether a similar inhibitory activity to TNF-α is also operative in the myocardium itself remains unknown at present.

The nature of the TNF-α inhibitory serum activity also remains unknown at present but is neither related to LPS-antibodies nor soluble TNF receptors (see Materials and Methods). It also appears unlikely that the TNF-α inhibitory serum activity is related to tumor necrosis factor-β (TNF-β) or IL-10, because in mouse peritoneal macrophages exposed to LPS, production of TNF-β and IL-10 was less than that in macrophages from untreated mice (7). Recently, it has been reported that the TNF-α inhibitory serum activity present in LPS-tolerant mice was not dialyzable but sensitive to heat and protease treatment (27). Also, the increased TNF-α inhibitory serum activity most likely does not depend on increased protein biosynthesis because it was observed within minutes in the ischemic preconditioning protocol. More likely posttranslational modification, i.e., cleavage or phosphorylation of a certain protein, might be responsible for the development of the TNF-α inhibitory serum activity.

The future identification of the nature of TNF-α inhibitory serum activity is expected to provide new insight into the protection against ischemia-reperfusion injury afforded by ischemic preconditioning and early exposure to LPS.

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