Effects of induced tolerance to bacterial lipopolysaccharide on myocardial infarct size in rats

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Abstract

Objectives: Induced tolerance to bacterial lipopolysaccharide (LPS) by pretreatment with sublethal doses of LPS has been shown to reduce the inflammatory response of monocytes, circulating PMNs and PMN adhesion to endothelial cells in response to subsequent stimuli, and also to increase cellular and organ tolerance to stress by other mechanisms. Therefore, we undertook to determine whether or not LPS desensitization is associated with reduced myocardial infarct size at 3 days after reperfusion following coronary occlusion.

Methods: Rats were randomized to either daily intraperitoneal LPS injections to provide LPS tolerance, or to equal volumes of saline (controls). In both groups at day 7 nontransmural infarction was produced by a 45 min coronary occlusion followed by 3 days of reperfusion during which LPS injections were continued. Histologic infarct size was assessed as percent of the left ventricle and as a percent of the risk zone (determined by fluorescent microspheres).

Results: Myocardial infarct size as percent of the left ventricle and of the risk zone were significantly reduced in the LPS-tolerant group (n = 14) compared to control rats (n = 12), the latter being reduced by 37% (33.6 ± 18.4 vs. 54.1 ± 8.6% of the risk zone, \( P < 0.002 \)). The percentages of activated circulating PMN after LPS desensitization and saline pretreatment were not different prior to coronary occlusion (at 7 days), but 3 days after coronary occlusion and reperfusion the percent of activated PMNs in the treated group was markedly reduced compared to controls (2.9 ± 1.6 vs. 11.4 ± 7.2%, respectively, \( P < 0.02 \)).

Conclusions: LPS desensitization in rats for 1 week prior to coronary occlusion inhibited activation of circulating PMNs 3 days after reperfusion following 45 min of coronary occlusion. LPS also is well-known to induce heat stress proteins and may affect other protective mechanisms. These actions are associated with a significant reduction in myocardial infarct size in LPS-tolerant animals compared to untreated controls.

Keywords: Myocardial infarct size; Neutrophils; Neutrophil activation; LPS; Reperfusion; Rat

1. Introduction

Tolerance to cell injury, described for several types of insults, could occur through downregulation of inflammatory responses (such as reduced white blood cell activation) or by increased organ tolerance through induced cellular defense mechanisms (such as increased heat shock proteins). Activation of polymorphonuclear neutrophils (PMN) during ischemia and reperfusion is associated with enhanced PMN adhesiveness to endothelial cells and microvascular plugging [1–3]. These events are followed by the release of proteolytic enzymes with enhanced production of toxic superoxides [4] which, in turn, can contribute to myocardial cell death. Activation of PMN by bacterial lipopolysaccharide (LPS) causes a marked increase of PMN adhesiveness to endothelial cells with release of enzymes and peroxides [5,6]. LPS also interacts with monocytes to stimulate the release of tumor necrosis factor (TNF) [7], which can enhance PMN adherence to endothelial cells and degranulation [8]. In addition, there is evidence that LPS has a stimulating effect on the alternative...
complement pathway to generate C5a which, in turn, can activate PMNs [9]. In experimental hemorrhagic shock, activation of circulating leukocytes has been shown to adversely influence survival [10].

Tolerance to LPS by repeated pretreatment with sublethal doses of LPS has been shown to elevate endogenous glucocorticoid levels and downregulate the TNF response, associated with a marked reduction of spontaneous activation of circulating PMN and their adhesion, with decreased microcirculatory plugging [11,12]. LPS tolerance also has been demonstrated to improve survival after both hemorrhagic and endotoxin shock in the rat and to induce cross tolerance [8,11,13]. Depletion of PMNs has been shown to decrease myocardial ischemic injury [14], and pretreatment with endotoxin in rats 24 h before 20 min of global ischemia in the isolated heart was reported to preserve contractile function 40 min after reperfusion [15]. However, whether or not prior treatment with endotoxin, acting through prevention of PMN activation or by other mechanisms, can beneficially affect myocardial infarct size after ischemia with prolonged reperfusion in vivo has not yet been investigated.

Other mechanisms of tolerance to cell injury that could be induced by LPS or other forms of stress include the induction of stress or heat shock proteins [16-18], which also can be induced by ischemia [19]. An increase in such a cellular protective mechanism, including increased adenosine levels, can lead to a late “second window” of protection, as seen in preconditioning, whether induced by heat stress or ischemia [20-22]. In addition, detrimental organ reaction to injury such as nitric oxide production, which is induced by acute injury and leads to peroxynitrite formation, might be downregulated by LPS tolerance or other mechanisms [12,23,24].

In order to study the potential cardioprotective effect of induced tolerance to bacterial LPS, we have used a model of nontransmural myocardial infarction in the rat involving a 45-min period of coronary occlusion followed by reperfusion. Studies on myocardial infarct size were performed 3 days after reperfusion, when any net beneficial effect due to LPS treatment on the PMN activation, adhesion, and migration cascade, on stress proteins, or on other potential protective mechanisms should be evident.

2. Methods

The animals in this study were handled according to the animal welfare regulations of the American Heart Association and the AAALAC accredited Animal Subjects Committee of the University of California San Diego.

2.1. Experimental design

Male Wistar rats (250-450 g) were randomly assigned to one of two groups to achieve an estimated group size of 15-20 animals. In one of the two groups (LPS-tolerant group), LPS (Escherichia coli 0127:B8, Difco Laboratories, Detroit, MI), which was reconstituted in sterile pyrogen-free saline and filtered through a 0.45 µm pore size filter, was given by daily intraperitoneal injections; in the second (control) group the rats were treated with daily injections of equal volumes of saline. LPS was administered in increasing doses for 4 consecutive days (0.15, 0.30, 0.45 and 0.60 mg/kg). This dose has previously been shown to be effective in rats for preventing PMN activation and improving survival in hemorrhagic and LPS shock [8,11]. The 0.60 mg/kg dose was then continued through day 7 when coronary occlusion with reperfusion was performed in both groups and, since we wished to maintain LPS tolerance during the reperfusion phase, the LPS injections were continued at that dose for the 3-day reperfusion period (total 10 days). Saline injections also were continued for 10 days.

2.2. Animal model

The rats were anesthetized with a mixture of ketamine hydrochloride (100 mg/kg i.p.), xylazine (10 mg/kg i.p.), and morphine sulfate (5 mg/kg i.p.), intubated, placed in the supine position on a table and ventilated using positive pressure (Harvard rodent ventilator, model 683). Additional anesthesia was given as needed. Under a dissecting microscope, a left thoracotomy was performed in the fourth intercostal space and the pericardium was opened. Using a 6-0 Ethibond cardiovascular suture with a small needle (Taper BV-1) the coronary artery, which is intramural, was encircled together with a small band of myocardium between the left atrial appendage and the right ventricular outflow tract. To produce coronary occlusion, the suture was tightened with a snare (polyethylene catheter PE 200) and fixed with a mosquito clamp, a small piece of plastic foam being placed between the tourniquet and the myocardium to minimize myocardial injury. A single bolus of lidocaine (5 mg/kg) was administered intravenously just after occlusion to reduce ventricular arrhythmias. A color change of the myocardium and ECG changes (ST elevation and broadening of the QRS complex) were considered to indicate satisfactory coronary occlusion, which was maintained for 45 min. ECGs were recorded before, 5, 15, 30, and 45 min after coronary occlusion, as well as 5 and 15 min after reperfusion. Reperfusion was performed by removing the clamp and tourniquet and confirmed by visual inspection and from ECG changes. The suture was cut to an approximate length of 5 cm, and left in place for subsequent use. The chest was then closed in layers and the pneumothorax evacuated.

After surgery the animals were caged in proportion to size. Water and standard rat chow were given ad libitum, and the rats were housed in a climate-controlled environment subjected to 12-h light/dark cycles.
2.3. Measurements

The systolic pressure of the tail artery, the heart rate (HR) and the body weight were measured in all animals preoperatively and 3 days after reperfusion. For blood pressure measurements, the rats were placed in small restraining cages according to size, and three consecutive measurements were performed (HICT Life Science Inc., Woodland Hills, CA), recorded on a strip chart recorder, and the systolic pressures averaged.

Blood samples were taken from the tail vein immediately after coronary occlusion as well as from the left ventricle (LV) before termination of the experiment. White blood cell (WBC) counts (UNOPEITE Test 5856, Becton-Dickinson Co., Rutherford, NJ) and differential cell counts (blood smear) were performed. The percentage of circulating activated PMNs was assessed by the nitroblue tetrazolium test (see below) from blood samples obtained from the tail vein before surgery and at the end of the experiment (LV blood sample).

2.4. Nitroblue tetrazolium test (NBT) on circulating PMNs

This test was used to determine the percentage of activated circulating PMNs in a fresh unseparated blood sample. It has been demonstrated that NBT reduction by PMNs, expressed as spontaneous reduction of pale yellow NBT to blue-black formazan crystals, is associated with increased superoxide production [8,25]. Fresh heparinized blood (0.1 ml) was immediately transferred into a clean siliconized glass vial (Sigma, St. Louis, MO) and mixed with an equal amount of 0.1% NBT solution. After incubation at 37°C (Delta Design MK 2300) for 20 min and subsequently at room temperature for another 10 min, the blood—NBT mixture was gently shaken. Coverslip smears were then made and stained with Wright’s stain. Under 100 x objective (NA = 1.4) and 10 x eyepiece, 100 PMNs were counted; those cells exhibiting stippled cytoplasmatic deposits of blue-black formazan or dense clumps of formazan in the cytoplasm were counted as NBT-positive (activated), and expressed as a percentage of the total PMN count.

2.5. Postmortem and histological preparations

Three days after coronary occlusion and reperfusion the experiment was terminated. Rats were intubated and mechanically ventilated under the same anesthesia used previously. The thorax was opened after injection of heparin (1000 units i.p.). A large dose of pentobarbital (150 mg/kg) was then given intraperitoneally to assure deep anesthesia during KCl injection and the coronary artery was reoxygenated using the previously placed ligature; the still contracting left ventricle (LV) was then punctured through the apex with a 25-gauge needle and 3 ml of blood withdrawn for subsequent studies, followed by injection of 125 mg of fluorescent microspheres diluted in 4 ml saline (2-15 μm diameter spheres. Duke Scientific Co.), as described by Hale et al. [26]. The heart was then arrested with a 2–3 ml injection of a saturated KC1 solution through the LV line.

A polyethylene catheter (PE 200) was introduced into the descending aorta. After releasing the coronary ligature the heart was perfused with heparinized saline (5000 units/l) for 2–3 min to wash out blood, and retrograde aortic perfusion of the heart with 10% phosphate-buffered formalin at a constant pressure of 60 mmHg was then performed for 15–20 min. The right atrium was opened to decompress the right ventricle during fixation. To maintain a constant filling pressure throughout the fixation, the LV intracavity pressure was held at 10 mmHg with the LV line attached to a fluid reservoir.

After hardening, the heart was excised and immersed in 10% buffered formalin solution for 24 h. Subsequently, the atria and adhesions were carefully dissected away, and the left and right ventricles were separated and weighed, the interventricular septum being included with the left ventricle.

After 3–4 days in 10% buffered formalin, the whole LV was embedded in paraffin and 10 μm thick transverse serial sections were cut perpendicular to the long axis on a microtome. The first sections up to 0.5 mm above the apex were discarded. Starting at 0.5 mm above the apex two sections were mounted on slides, the next 48 sections were discarded and then the next two subsequent sections were mounted again on slides. Subsequently, two sections were mounted on slides for every 100 sections. Thus, sections were sampled in the first 0.5 mm and every 1 mm thereafter along the long axis from apex to base.

In preliminary studies, it was determined that there was loss of fluorescent microspheres during the H&E staining procedure. Therefore, at each level, one section was stained with hematoxylin–eosin (H&E) and the adjacent section was left unstained. Between 6 and 9 pairs of stained and unstained slides were obtained from each heart. The tissue containing the coronary suture was excluded from the analysis because it showed necrosis of myocardium which was related to the suture itself.

2.6. Analysis for white blood cell infiltration

The same transverse sections used for infarct size determination also were graded for inflammation. Two transverse sections from each animal taken near the minor equator of the LV were graded and averaged by a blinded observer to provide a single data set for each animal. For cell type (monocytes, leukocytes, PMNs) at least three representative regions of the infarcted and immediately adjacent areas were visualized, with at least 20 cells counted per region to determine cell type: the cell type was graded on a 0–4 + scale and the values arranged to yield a mean score for an individual heart. The overall degree of
cellular infiltrate in the infarcted and perinfarct areas was graded 0–4+, 0 = no cell infiltrate, 1+ = few widely separated cells, 2+ = scattered clusters, 3+ = extensive infiltration with a few areas of myocytes without cellular infiltrate, 4+ = solid sheets of cells.

2.7. Morphometric analysis for infarct size

Stained sections were analyzed in a blinded manner. Slides were mounted on a scope (Wild M32, Heerbrugg, Switzerland), and the epicardial and endocardial borders of the LV transverse sections were traced on paper at 13.25-fold magnification. The infarcted area was then traced on the same paper; all necrotic regions showing heavy infiltration of inflammatory cells and hemorrhage, and areas containing more than 50% of non-viable myocytes (i.e., loss of striations, severe edema, and appearance of contraction bands, verified by microscopy at 30× power) were included in the infarct zone. The adjacent unstained sections were then viewed while illuminated with ultraviolet light (Model UVL-56, UVP-Inc., San Gabriel, CA) with the image superimposed on the tracing of the stained section. The normally perfused zone, indicated by the illuminated fluorescent microspheres, was then traced on the paper, clearly demarcating the area at risk which was free of microspheres (Fig. 1). Microspheres were never seen within the infarct zone, suggesting the absence of significant collateral development.

The infarcted area, the area at risk, and the normally perfused area of all sections were measured by planimetry (Compensating Polar Planimeter, Keuffel & Esser Co., Germany). The volume of each myocardial cylindrical slice (V_in mm^3) was calculated from the formula:

\[ V_i = \frac{(a + b)}{2} \times \frac{1}{13.25^2} \times \text{thickness} \]

where \(a\) and \(b\) are the planimetered areas of the two sections on each face, 13.25 is the linear magnification factor, and the thickness is 0.5 mm for the first (apical) section and 1.0 mm for all subsequent sections. For all infarct and necrotic volumes the H & E stained sections were used, whereas for the normally perfused volume was determined from the unstained fluorescent sections. Finally the volumes for each zone were summed to comprise the whole heart.

2.8. Data collection

Among the 52 animals randomized, the operative mortality of the surgical procedure with coronary occlusion and reperfusion was 32% (9 control and 8 LPS animals); also, one animal in each group died on the first and one on the second postoperative day. Two hearts were excluded because of the presence of a transmural myocardial infarction, indicating lack of reperfusion. Two hearts were excluded because they showed only a small risk area immediately around the suture (considered to indicate necrosis from the needle tract and tie) without distal infarction, indicating inadequate coronary occlusion. In one heart, the staining of the perfused area with fluorescent microspheres was imperfect and the risk area could not be evaluated reliably. In another heart, subsequently found to be in the LPS-treated group, the risk area was appropriate but little infarction could be identified despite color and ECG changes indicating successful coronary occlusion and reperfusion; this animal was retained in the study. Finally, two hearts in the LPS-tolerant group were not used because the NBT test showed a high degree of PMN activation (∼20%), and the animals were considered not to be LPS-tolerant; however, inclusion of these two animals with the LPS-tolerant group did not change the statistical outcome of the study. Therefore, the final analysis included 26 animals.

2.9. Statistics

Infarct size was expressed both as percent of risk zone and percent of LV volume and risk zone as a percent of LV volume; these variables, the degree and type of cell infiltration, as well as the right ventricular (RV) weights, were compared by t-tests (two-tailed). Infarct and risk zone variables were unequally distributed and also were analyzed by the non-parametric Wilcoxon test. To adjust for possible inequalities between groups in the size of the area at risk (risk zone), infarct size was used as a dependent variable in an analysis of covariance with risk zone as the covariate together with group as independent variable; two separate analyses considered infarct size as a percent of the risk zone and as a percent of left ventricular volume.

For the body weight, heart rate, systolic pressure in the tail artery, WBC and differential counts, and NBT test comparisons, an analysis of variance was performed with time (operation vs. 3 days) as a repeated measures factor and control or treatment as a grouping factor. When a significant interaction was detected, the Neuman-Keuls procedure was used to compare groups at each time. Post-hoc tests were performed at the \( P < 0.05 \) level, and this value was considered significant.

3. Results

The body weights, blood pressures, heart rates, and LV and RV weights are summarized in Table 1. Body weight was lower in both groups at 3 days after operation. Ventricular weights were not significantly different between groups at post mortem. The systolic pressures in the tail artery were reduced at 3 days after reperfusion in both groups compared to preoperatively, but the heart rates were not significantly different. There were no statistically significant differences in these variables between LPS-tolerant animals and controls.
Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 12)</th>
<th>LPS-tolerant (n = 14)</th>
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<tr>
<td>B.W. (g)</td>
<td></td>
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<tr>
<td>Preop</td>
<td>373 ± 74.1</td>
<td>338 ± 41.3</td>
</tr>
<tr>
<td>3 days</td>
<td>355 ± 70.8 *</td>
<td>327 ± 41.9 *</td>
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<tr>
<td>HR (beats/min)</td>
<td></td>
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<tr>
<td>Preop</td>
<td>460 ± 49.1</td>
<td>467 ± 30.0</td>
</tr>
<tr>
<td>3 days</td>
<td>482 ± 25.3</td>
<td>475 ± 30.9</td>
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<tr>
<td>SBP (mmHg)</td>
<td></td>
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<tr>
<td>Preop</td>
<td>120 ± 14.0</td>
<td>110 ± 13.6</td>
</tr>
<tr>
<td>3 days</td>
<td>101 ± 12.5 *</td>
<td>98 ± 13.7 *</td>
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B.W., body weight; HR, heart rate; SBP, systolic pressure of the tail artery; LV, left ventricle; RV, right ventricle; Preop, preoperative; 3 days, 3 days after reperfusion; Control, treated with daily saline injections for 10 days; LPS-tolerant, treated with daily LPS injections for 10 days. All values are mean ± s.d. * P < 0.003, Preop vs. 3 days. Differences between groups were not significant.

3.1. Effect of LPS pretreatment on myocardial infarct size

Representative drawings of two LV cross sections from an LPS-tolerant and a control rat 3 days after reperfusion are shown in Fig. 1. LPS-tolerance resulted in a significantly reduced myocardial infarct size expressed as a percent of the risk zone (Fig. 2), a reduction of 37%. Also, there was a highly significant reduction of infarct size expressed as a percent of left ventricular volume in the LPS-treated group (Table 2). Results using the Wilcoxon analysis were closely similar (Table 2). There was a nonsignificant trend for the risk zone as percent of the left ventricular volume (Table 2) to differ between groups (P = 0.077 by t-test; P = 0.15 by Wilcoxon test). However, analysis of covariance controlling for this variable showed significant differences between groups for myocardial infarct size, both as percent of the risk zone (P < 0.01) and as percent of the left ventricular volume (P < 0.01).

3.2. Blood counts (Table 2)

Circulating polymorphonuclear leukocyte (PMN) counts were similar in the two groups at the time of coronary occlusion, and a significant increase (P < 0.0001) in PMN counts occurred in both groups at 3 days after ischemia/reperfusion (Fig. 3). The circulating monocyte/lymphocyte counts were similar in the two groups at control, but there was a decrease of the monocyte/lymphocyte count at 3 days after ischemia/reperfusion in the
Table 2

<table>
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<tr>
<th></th>
<th>Control (n = 12)</th>
<th>LPS-tolerant (n = 14)</th>
</tr>
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<tbody>
<tr>
<td>Infarct size as % of risk zone</td>
<td>54.1 ± 8.6</td>
<td>33.6 ± 18.4**</td>
</tr>
<tr>
<td>Infarct size as % of LV</td>
<td>32.0 ± 5.6</td>
<td>18.6 ± 12.7**</td>
</tr>
<tr>
<td>Risk zone as % of LV</td>
<td>59.7 ± 8.3</td>
<td>50.7 ± 15.6</td>
</tr>
<tr>
<td>% activated PMNs Preop</td>
<td>6.9 ± 4.8</td>
<td>4.2 ± 1.5</td>
</tr>
<tr>
<td>% activated PMNs 3 days</td>
<td>11.4 ± 7.2</td>
<td>2.9 ± 1.7*</td>
</tr>
<tr>
<td># activated PMNs Preop</td>
<td>57 ± 36</td>
<td>55 ± 42</td>
</tr>
<tr>
<td># activated PMNs 3 days</td>
<td>348 ± 256</td>
<td>76 ± 58</td>
</tr>
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</table>

Infarct size is expressed as percent of the risk zone and percent of left ventricular (LV) myocardial volume. Risk zone is expressed as percent of the left ventricular (LV) myocardial volume.

* * P < 0.002. By the Wilcoxon test, P values were 0.001 and 0.005 for infarct size as % of risk zone and as % of LV, respectively.

% activated PMNs: percentage of nitroblue tetrazolium positive [NBT(+)] polymorphonuclear neutrophils (PMNs).

# activated PMNs: absolute number of NBT(+)PMNs (cells × 1/1000/mm³).

Preop, preoperatively; 3 days, 3 days after reperfusion; control, with daily saline injections; LPS-tolerant, lipopolysaccharide (LPS) injections.

Fig. 3. Upper panel — polymorphonuclear neutrophil (PMN) counts during coronary occlusion and 3 days after reperfusion in the control group (control) and in the lipopolysaccharide-tolerant group (LPS). There is no statistically significant difference of means between groups, but at 3 days after ischemia and reperfusion a significant increase in PMNs is present in both (P < 0.0001). Lower panel — the monocyte/lymphocyte count in the control group is significantly decreased 3 days after reperfusion (P < 0.05) while values in the LPS group are unchanged.

3.3. Circulating PMN activation (Table 2)

Before surgery, neither the absolute number nor the percent of circulating NBT-positive PMNs was significantly different between the LPS-tolerant and control groups (Table 2). At 3 days after coronary occlusion/reperfusion the absolute number of NBT-positive PMNs in the control animals showed a highly significant six-fold increase, whereas the number of circulating NBT-positive PMNs in LPS-tolerant rats was unchanged at 3 days after surgery and significantly lower than in the control group (Table 2). Also, the percent of activated PMNs was significantly lower in the LPS-tolerant rats than in controls (Table 2). In both groups, linear regression analysis showed no correlations between the percentage or absolute number of circulating NBT-positive PMNs and infarct size expressed as a percent of the risk area.

3.4. White blood cell infiltration (Table 3)

There was no significant difference in the degree of cell infiltration between the two groups, and there were no differences in the cell type. Polymorphonuclear leukocytes were rarely seen in the inflammatory infiltrate in either group (Table 3).

4. Discussion

The findings in this study indicate that induced tolerance to bacterial lipopolysaccharide reduces myocardial infarct size in rats after coronary occlusion/reperfusion. Thus, when severe ischemia is followed by reperfusion, either general nonspecific downregulation of the inflammatory response with reduced PMN activation or induced cardiac tolerance to ischemia, or both, were associated with a reduction of ischemic myocardial damage. Furthermore, the reduction in infarct size was observed at 3 days after reperfusion, a significant observation since interventions dealing with beneficial effects on myocardial infarct size of altering specific steps in inflammation or control group without change in the LPS-tolerant group (Fig. 3).

Table 3

<table>
<thead>
<tr>
<th>Cell infiltrate in the infarct and periinfarct zones</th>
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<tr>
<td>Cell PMN Monocytes Lymphocytes</td>
</tr>
<tr>
<td>Infiltrate (0−4+) (0−4+) (0−4+)</td>
</tr>
<tr>
<td>Control (n = 11) 2.3 ± 1.3 0.3 ± 0.4 1.9 ± 1.3 1.5 ± 1.3</td>
</tr>
<tr>
<td>LPS-tolerant (n = 13) 2.5 ± 1.1 0.5 ± 0.9 2.2 ± 0.7 1.5 ± 0.8</td>
</tr>
<tr>
<td>P value NS NS NS NS</td>
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<td>Mean ± s.d. PMN = polymorphonuclear leukocytes</td>
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other mechanisms often have been examined after only a few hours of reperfusion [14, 27, 28]; some investigations have shown beneficial effects of superoxide dismutase, for example, at 3 h after reperfusion with negative results at 3 days [29, 30], although others have described negative results at both 3 h and 2–4 days [31]. The response of PMN infiltration in the ischemic/infarcted zone to coronary occlusion (30 min) and reperfusion in rats shows marked infiltration at 5.5 h and 24 h (myeloperoxidase activity), but by 48–96 h after coronary occlusion this PMN accumulation has largely disappeared and is replaced with mononuclear cells [32, 33]. This change appears to indicate a shift from an acute inflammatory reaction to one of tissue repair with early scar formation [3, 34]. Therefore, the present study was undertaken to determine whether or not sustained LPS tolerance would have a beneficial effect on the extent of myocardial injury at about 72 h (3 days) after reperfusion. We found a downregulation of PMN activation associated with reduced infarct size, but we do not have data on the possible induction of stress proteins or other protective mechanisms.

4.1. Potential leukocyte-mediated mechanisms

PMNs appear to be key participants in the development of injury in the heart when coronary occlusion is followed by reperfusion [1]. In the inflammatory process initiated by myocardial ischemia and reperfusion, neutrophils may contribute to determining the final extent of myocardial damage, since neutrophil depletion has been shown to decrease the amount of myocardial injury when evaluated early after reperfusion [14]. Neutrophils respond to a wide range of activators and chemotactic factors including platelet-activating factor (PAF), complement fragment C5a, LPS, tumor necrosis factor alpha (TNF-α), and leukotrienes (LTB₄) [35–37]. The activation of neutrophils is associated with induction of PMN adherence to activated endothelial cells, associated with microvascular entrapment [1, 2]. After firm adhesion of neutrophils to the endothelium, they migrate out of the bloodstream into the surrounding tissue following a chemotactic gradient where, by the production of oxygen free radicals and the release of proteolytic enzymes [34], the activation and degranulation of neutrophils can cause destruction of viable cells in the surrounding tissue. Although neutrophil depletion generally has produced protection from ischemia and reperfusion injury, the mechanisms used to reduce neutrophil counts could have had other effects, and the reperfusion periods have been less than 24 h [14]; therefore, we sought to test whether a less specific mechanism of reducing inflammation would result in a sustained reduction in infarct size following ischemia and reperfusion.

The induction of tolerance to bacterial lipopolysaccharide (LPS) is associated with a number of effects including reduction of PMN activation and adhesion to endothelium, downregulation of TNF and IL6 activity production by macrophages [7–9, 11, 38], elevation of endogenous corticosteroids and attenuation of nitric oxide induction [12]. Thus, several steps in the neutrophil activation, adhesion, and migration cascade might be affected by induced tolerance to bacterial lipopolysaccharide (LPS). After transient myocardial ischemia and reperfusion, the activation of TNF could be important for the activation of PMNs and PMN/endothelial cell interactions, since TNF-induced activation of cell adhesion molecules increases PMN adherence to endothelial cells [35, 36] and TNF also primes and activates PMNs to increase superoxide production and to degranulate [8, 9]. In our experiment we found that 3 days after reperfusion both the percent and the absolute number of NBT-positive PMNs were markedly downregulated in the LPS-tolerant group compared to the control group, whereas the total number of circulating PMNs was similar in treated and untreated groups consistent with previous studies on LPS tolerance showing persistence of leukocyte-tosis despite a return of cytokines to baseline levels [8, 39, 40]. The downregulation of PMN activation late after reperfusion by LPS desensitization indicates a low degree of PMN adhesiveness to the endothelium, which might relate to elevation of the glucocorticoid level [12] or to reduced TNF activity [7, 9, 10, 38]. We did not measure TNF-α in these experiments, since other experiments have shown no detectable plasma TNF activity during or after a 3-h period of hemorrhage shock [11] and detection of differences with this approach seemed unlikely. The near absence of residual PMNs in the myocardium at 3 days after infarction in the rat was expected since, as mentioned earlier, previous studies have shown that this phase of the inflammatory response is over by 24–48 h [33]. The type of inflammatory cell infiltrate in the infarct zone at 3 days was not different between groups, although the differences between infiltrating cell types were small and might not have been detected by our semi-quantitative grading method.

The low level of PMN activation in LPS-tolerant animals has been found to be associated with lack of a plasma factor [8] which has been partially identified [41] and which causes PMN activation in control animals [8, 41], as well as by glucocorticoid-induced downregulating [12]. Plasma from tolerant animals also appears to contain LPS-induced components which inhibit activation factors produced by LPS challenge in plasma from non-tolerant rats [42]. The downregulation of O₂⁻ formation observed by study of PMN in the tolerant animals [8] might include other cells of the cardiovascular system as well, such as monocytes and platelets. The leukocytes of LPS-tolerant animals show no signs of receptor downregulation, since they can be readily activated in vitro; however, the low level of PMN activation in these animals is associated with reduced superoxide formation, as well as with impaired adhesion to nylon fibers in vitro and to endothelium in vivo [11].
4.2. Other potential mechanisms

Rat hearts exhibit minimal or no coronary collateral circulation, and after 1–1.5 h of coronary occlusion myocardial infarction is over 90% complete, with no significant tissue salvage upon reperfusion [43]. Thus, our model of 45 min of occlusion with reperfusion in the rat may resemble the setting in some patients who have few or no coronary collaterals, in which thrombolysis is effective only when accomplished within 1 or 2 h of coronary occlusion [43]. In the rat model it is clear that a number of myocytes are irreversibly damaged by a 45-min period of coronary occlusion with reperfusion, but whether the LPS pretreatment had an effect before, during or after reperfusion cannot be ascertained from the present experiments.

Although we postulate that inhibition of adverse events early following reperfusion (when PMN influx is high [33]) due to diminished migration of circulating PMNs may be a significant factor in the observed reduction of infarct size at 3 days, our experiments do not provide direct evidence for this hypothesis or for other mechanisms by which LPS desensitization may have acted. It is possible that an effect of LPS could have occurred prior to coronary occlusion by altering cytokine responses [39], during the temporary coronary occlusion, as well as after reperfusion through reduced PMN endothelial adhesion and/or migration, perhaps secondary to the altered plasma factors discussed above, leading to reduced oxidative damage by infiltrating PMNs. In addition, a variety of protective mechanisms which may be induced by a temporary coronary occlusion could provide a late or second window of protection [21] which could have been favorably affected by prior LPS treatment. Tolerance to LPS is known to diminish induction of NO synthase upon subsequent rechallenge [12]. Nitric oxide production during ischemia or reperfusion is potentially injurious through formation of the highly toxic peroxynitrite upon combination with superoxide [23,44,45]. However, there is some conflicting evidence about the effect of NO on myocardial infarct size [46,47].

The induction of stress or heat shock proteins has been shown to provide a delayed "preconditioning-like" effect which is dependent on protein synthesis. Exposure to LPS is known to induce these stress proteins [17,18]. Such late enhancement of cellular resistance to ischemia (usually studied by inducing a second period of occlusion after prior preconditioning is produced by several temporary coronary occlusions) has also been suggested to relate to adenosine-receptor activation [20], and in other experiments to be associated with activation of protein kinase C [48], which can activate a number of nuclear transcription factors. In the experiments with endotoxin-treated isolated hearts, cited earlier, the endogenous antioxidant protein catalase was induced [15]. Any of these mechanisms, or perhaps several acting in concert, might be influenced by LPS pretreatment and no firm conclusions about which is primarily responsible for the observed decrease of infarct size can be reached from the present study.

We conclude that LPS treatment is associated with reduced myocardial damage after coronary occlusion followed by a prolonged period of reperfusion. Continuing study of the detailed mechanisms of myocardial injury is warranted in the search for interventions which can reduce the morbidity and mortality due to myocardial infarction.

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