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Lipopolysaccharide pre-conditioning is protective in lung ischemia-reperfusion injury

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Abstract

BACKGROUND—The availability of suitable lung donors has remained a significant barrier to lung transplantation. The clinical relevance of an isolated positive Gram stain in potential donor lungs, which occurs in >80%, is unclear. Low doses of lipopolysaccharide (LPS) have been protective in several models of ischemia-reperfusion injury through a pre-conditioning response. We sought to demonstrate that low-dose LPS is protective against subsequent lung ischemia-reperfusion injury.

METHODS—Pathogen-free Long-Evans rats were pre-treated with vehicle or LPS 24 hours before 90 minutes of ischemia and up to 4 hours of reperfusion. Lungs were assessed for vascular permeability, myeloperoxidase content, bronchoalveolar lavage inflammatory cell and cytokine/chemokine content, as well as nuclear translocation of nuclear factor κ B (NF κ B) and activator protein-1 (AP-1), and interleukin-1 receptor-associated kinase-1 (IRAK-1) and stress-activated protein kinase (SAPK) activation.

RESULTS—Compared with positive controls, LPS pre-treatment resulted in reductions in vascular permeability (70%, $p < 0.001$), myeloperoxidase content (93%, $p < 0.001$), bronchoalveolar lavage inflammatory cells (91%, $p < 0.001$), and inflammatory cytokine/chemokine content (cytokine-induced neutrophil chemoattractant, 99%, $p = 0.003$; interleukin-1 β , 72%, $p = 0.0001$; tumor necrosis factor- α , 76%, $p < 0.0001$), NF κ B (86%, $p < 0.001$) and AP-1 (97%, $p < 0.001$) nuclear translocation, and IRAK-1 (87%, $p < 0.001$) and SAPK (80%, $p < 0.001$) phosphorylation.

CONCLUSIONS—Lipopolysaccharide pre-treatment reduced lung injury and inflammatory mediator production after subsequent exposure to ischemia-reperfusion. Understanding the clinical significance of lipopolysaccharide in donor lungs has the potential to expand and clarify donor inclusion criteria.

Introduction

Although survival after transplantation has improved, lung ischemia-reperfusion (IR) injury (LIRI) continues to affect up to 25% of recipients (1). LIRI results in enhanced major histocompatibility complex class II expression, acute graft dysfunction, earlier onset of bronchiolitis obliterans, and increased patient death (2–4). Inflammatory signaling within

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pulmonary cell populations is thought to drive the development of LIRI. The alveolar macrophage secretes tumor necrosis factor- α (TNF- α) within 15 minutes of reperfusion and enhances the responses of other cell types (5). In vivo alveolar macrophage depletion and inhibition studies demonstrated marked protection from LIRI (5).

The availability of suitable donor organs is a significant barrier to lung transplantation. Despite efforts to expand donor criteria, the number of lungs recovered has risen only modestly (6). Because all lungs are procured from ventilated patients, bacterial colonization is common, with up to 80% having tracheobronchial colonization (7). Although overt infection, such as gross purulence within the tracheobronchial tree or an infiltrate on a chest X-ray image is considered a contraindication to donation, the significance of an isolated positive Gram stain remains unclear. Postulated risks of using colonized lungs include higher rates of recipient pneumonia, exacerbation of graft dysfunction, and increased death (8,9).

Intratracheal administration of lipopolysaccharide (LPS) induces acute lung injury when given in sufficient amounts (10). Conversely, several models suggest that lower quantities of LPS may modulate subsequent IRI, and in studies of cardiac, cerebral, and hepatic IR, pre-treatment with low-dose LPS was protective (11–14). This suggests that bacterial colonization and the presence of lower quantities of LPS in donor airways may provide protection against subsequent LIRI through a pre-conditioning response.

LPS is the best-known ligand of the innate immune receptor toll-like receptor 4 (TLR-4). TLR-4 has demonstrated a proinflammatory response in several models of IRI, including LIRI (15–19). In the absence of a pre-conditioning stimulus, TLR-4 activation up-regulates proinflammatory signaling through recruitment of the adaptor protein myeloid differentiation factor 88 (MyD88) (20,21). Downstream interleukin-1 receptor-associated kinase (IRAK-1) and stress-activated protein kinases (SAPK) are phosphorylated, and subsequent nuclear translocation of nuclear factor B (NF κ B) and activator protein-1 (AP-1) initiates transcription and secretion of inflammatory mediators (20,21).

TLR-4 knockout mice subjected to IR demonstrated decreased levels of c-Jun N-terminal kinase (JNK) phosphorylation and both NF κ B and AP-1 transactivation (19). TLR-4 has also been shown to initiate an alternative signaling pathway that is MyD88 independent (20,21). It has been proposed that MyD88-independent signaling, through the adaptor proteins TIR-domain-containing adapter-inducing interferon- γ (TRIF) and TRIF-related adaptor molecule (TRAM), increases type I interferon responses and interleukin-10 (IL-10) production, which have been shown to reduce proinflammatory signaling (22).

This study describes the protective effect of low-dose LPS pre-treatment on subsequent LIRI in a warm in situ model. We hypothesize that LPS pre-treatment alters the TLR-4 signaling response to oxidative stress, resulting in a relative decrease in MyD88-dependent signaling and thereby conferring ischemic tolerance.

Methods

The University of Washington Animal Care Committee approved all experimental protocols for this study. Animals received humane care in compliance with the Principles of Laboratory Animal Care established by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals developed by the Institute of Laboratory Animal Resources and published by the National Institute of Health.

Reagents

Purified lipopolysaccharide from *Escherichia coli* 055:B5 and all other reagents were purchased from Sigma-Aldrich Chemical (St. Louis, MO) unless otherwise noted.

Animal model

Pathogen-free Long-Evans rats (weight, 250–275 g; Harlan Sprague-Dawley, Indianapolis, IN) were used for all experiments. A well-established warm in situ, IR model was used as previously described (5,22). In brief, rodents were anesthetized with 120 mg/kg intraperitoneal pentobarbital. A 14gauge angiocatheter was inserted into the trachea through a midline neck incision and connected to a Harvard Rodent Ventilator (Harvard Apparatus, Boston, MA). Ventilator settings were set at an inspired oxygen content of 60%, positive end-expiratory pressure of 2 cm H₂O, and a respiratory rate of 80 breaths/min.

A left thoracotomy was performed and the left lung mobilized atraumatically. Heparin (50 U) was administered through the penile vein, and after 5 minutes of circulation, a non-crushing clamp was placed across the left lung hilum. After 90 minutes, the clamp was removed and the left lung reventilated and reperused for up to 4 hours. A midline laparotomy and sternotomy were then performed. Blood samples were taken from the inferior vena cava, and the animals euthanized by aortic transection. The heart-lung block was excised and the pulmonary circulation flushed with saline (20 ml).

Four experimental groups were studied. Negative control animals (n = 4) did not undergo pre-treatment or surgical manipulation. Positive control animals (n = 6) were pretreated with intratracheal phosphate-buffered saline (PBS) vehicle before undergoing the IR protocol. Two groups received intratracheal LPS pre-treatment before surgical manipulation. The LPS control group (n = 6) was ventilated for up to 4 hours before left lung harvest without undergoing the IR protocol. The LPS-treated group (n = 6) underwent the IR protocol.

LPS administration

Animals were anesthetized with intraperitoneal ketamine (30 mg), the trachea was exposed through a midline neck incision, and a 28-gauge needle was used to inject vehicle or LPS directly into the trachea. To determine the most relevant dose of LPS, preliminary studies were performed using a wide range of doses. A MEDLINE search identified 3 studies evaluating LPS levels in the bronchoalveolar lavage (BAL) fluid of patients (23–25). Assuming average lung volume and weight, we calculated a range of LPS concentrations from 0.64 to 2300 ng/kg in these patients, with infected ventilated patients averaging 1277 ± 734 ng/kg and non-infected ventilated patients averaging 80.4 ± 113.8 ng/kg. We chose a range of concentrations from 8 to 4,000 ng/kg, which included the averages for infected and non-infected ventilated patients from our literature search to evaluate the dose response of intratracheal LPS. In the preliminary studies, rats were pre-treated with 2, 15, 50, or 1,000 ng of LPS in PBS (150 μ l). Vehicle was administered in positive controls in an equivalent volume as that for LPS pre-treated animals. After LPS or vehicle administration, the skin was sutured and the animal was allowed to recover for 24 hours before the IR protocol. In the preliminary studies, lung permeability was assessed after the IR protocol, and the dose yielding the lowest permeability index (15 ng) was used for all subsequent experiments, including LPS control animals.

Lung permeability index

Changes in lung vascular permeability were assessed as a ratio of ¹²⁵I radiolabeled bovine serum albumin (BSA; NEN Life Sciences, Boston, MA) in the left lung to that of 1 ml of blood as previously described (5,26). The ¹²⁵I-BSA was diluted to obtain an activity of approximately 800,000 counts/min (cpm). A 1% BSA/PBS solution was added to make a

final volume of 500 μ l, which was injected intravenously 5 minutes before the hilar clamp was removed. Immediately before sacrifice, 1 ml of blood was aspirated from the inferior vena cava and then the left lung was harvested as previously described. The CPM of the left lung and blood were quantified in a gamma counter. The permeability index was calculated as follows: Permeability index. left lung (cpm)/1 ml blood (cpm).

Myeloperoxidase assay

Left lung neutrophil accumulation was measured after 4 hours of reperfusion by determining myeloperoxidase (MPO) content, as previously described (5,26,27). Lung specimens were homogenized for 1 minute in homogenate buffer solution, sonicated in three 15-second bursts, and centrifuged at 3,000g for 30 minutes at 4°C. Supernatant (50 μ l) was mixed with assay buffer (1.45 ml) and the change in absorbance at 460-nm wavelength for 1 minute was recorded. Bronchoalveolar lavage and inflammatory

Cell counts

After 4 hours of reperfusion, a clamp was placed across the right hilum and the left lung lavaged with sterile saline (5 ml) through the intratracheal angiocatheter. The recovered lavage fluid was centrifuged at 1,800 rpm for 10 minutes at 4°C. The supernatant was used for cytokine/chemokine analysis, and the pellet resuspended in PBS (10 ml). The resuspended cells (1 ml) were stained with Gill's solution and counted using a hemacytometer (Hausser Scientific, Horsham, PA). Differential cell counts were not performed because previous work found nearly all cells were neutrophils.

Western blot analysis

After 15 minutes of reperfusion, total protein was harvested from left lung samples as previously described.^{5,26} Protein concentrations were quantitated using the bicinchoninic assay (Pierce Biotechnology Inc, Rockford, IL). Protein (60 μ g) was loaded on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoresed at 90 volts for 90 minutes. After transfer to a polyvinylidene difluoride membrane, the membranes were incubated overnight with a phospho-specific p38 antibody, a phospho-specific JNK polyclonal-antibody, or a phospho-specific IRAK-1 antibody (Cell Signaling Technology Inc, Beverly, MA). A horseradish peroxidase (HRP) conjugated secondary antibody was applied for 1 hour and the proteins were visualized using Pierce Supersignal Reagents (Pierce Biotechnology Inc) and autoradiography. Densitometry was performed using Image J 1.2 software (Cybernetics Corp, Silver Spring, MD). Electrophoretic mobility shift assay Nuclear protein was harvested from left lung samples after 15 minutes of reperfusion as previously described.²⁷ Protein concentrations were determined using the bicinchoninic assay. Protein (5 μ g) was incubated with biotin-labeled NF κ B or AP-1 oligonucleotide probe (Panomics Inc, Redwood City, CA) for 20 minutes. Samples were resolved on a 6% SDS-PAGE gel run at 80 volts for 90 minutes. After transfer to a nylon membrane (Amersham, Rockford, IL), membranes were incubated for 15 minutes in a streptavidin-HRP conjugate solution and visualized using Pierce Enhancer Solution (Pierce Biotechnology Inc) and autoradiography. Densitometry was performed as described.

Enzyme-linked immunosorbent assay

BAL cytokine-induced neutrophil chemoattractant (CINC), IL-1 β , and TNF- α . concentrations were analyzed using enzyme-linked immunosorbent assay, as previously described (R&D Systems, Minneapolis, MN) (5,26,27). Briefly, 50 μ l of 10 mg/ml anti-chemokine antibody (Peprotech, Rocky Hills, NJ) was added to a carbonate-coating buffer solution, plated, and incubated overnight. The plate was washed with PBS containing 0.05% Tween (PBST) and incubated with a 1% BSA/PBS solution for 1 hour. Samples and

standards were diluted 1:1 in PBS, and 100 μ l was added to each well and incubated for 2 hours. The wells were washed in PBST and 0.5 to 2 mg/ml of secondary biotinylated antibody (Peprotech) was added to each well and incubated for 2 hours. Wells were washed with PBST and incubated for 20 minutes with a streptavidin-HRP conjugate (Pierce). The assay was developed by adding o-phenylenediamine dihydrochloride substrate and analyzed on a spectrophotometer. Samples and standards were run in triplicate, and well-to-well variation did not exceed 5%.

Statistical analysis

All data are presented as mean values $\pm$ standard error of the mean (SEM). Statistical differences between groups were assessed using the 2-tailed t-test (Excel 2002, Microsoft Corp, Redmond, WA) with post hoc Bonferroni adjustment for multiple comparisons. Statistical significance was defined as values of $p < 0.05$.

Results

The dose-dependent response to LPS pre-treatment, as measured by the lung permeability index, is demonstrated in Figure 1. Although protection from lung injury was seen across the spectrum of LPS doses studied, the 15- and 50-ng doses were associated with the greatest protection from lung injury (lung permeability index 0.245 ± 0.033 and 0.264 ± 0.005 , respectively). We then used 15 ng as the LPS pretreatment dose for the experiments because it represented the lowest dose with the greatest protection from lung injury. Markers of left lung injury, including the permeability index, MPO content, and BAL cell counts are summarized in Table 1. BAL cytokine and chemokine content can be found in Table 2.

Permeability index

Positive controls demonstrated a near 10-fold increase in lung vascular permeability compared with negative controls ($p < 0.001$). LPS pre-treatment before IR led to a 70% reduction in the permeability index vs positive controls ($p < 0.001$). LPS controls did not manifest an increase in lung vascular permeability compared with negative controls ($p = 0.267$), indicating that the dose of LPS was not injurious.

MPO assay

Positive controls exhibited a 40-fold increase in MPO content compared with negative controls ($p < 0.001$). LPS controls did not manifest an increase in MPO content vs negative controls ($p < 0.804$). LPS-treated animals demonstrated a 93% reduction in MPO content compared with positive controls ($p < 0.001$).

BAL inflammatory cell counts

The BAL fluid of positive controls demonstrated a nearly 30-fold increase in inflammatory cells compared with negative controls ($p < 0.001$). BAL fluid in LPS controls had equivalent quantities of inflammatory cells compared with negative controls ($p = 0.890$). The BAL fluid in LPS-treated animals demonstrated more than a 90% reduction in inflammatory cells compared with positive controls ($p < 0.001$).

BAL cytokine/chemokine content

CINC, IL-1 β , and TNF- α content within the left lung BAL fluid was significantly increased in positive controls compared with negative controls (>100-, >15-, and >12-fold increase, respectively; $p < 0.0001$). The BAL fluid of LPS controls had equivalent quantities of CINC, IL-1 β , and TNF- α compared with negative controls ($p = 0.9$, $p = 0.92$, $p = 0.9$, respectively). The BAL fluid of LPS-treated animals demonstrated significantly reduced CINC, IL-1 β , and

TNF- α content compared with positive controls (99%, $p = 0.003$; 72%, $p < 0.0001$; and 76%, $p < 0.0001$; respectively).

MyD88-dependent downstream signaling

Positive controls demonstrated up-regulated IRAK-1 activation compared with negative controls and LPS controls (Figures 2 and 3). IRAK-1 activation was reduced by 87%, as assessed by relative optical densitometry (OD; $p < 0.001$) in LPS-treated rats compared with positive controls (Figures 2 and 3). Similarly, p38 and JNK (SAPK) phosphorylation were up-regulated in positive controls compared with negative controls and LPS controls (Figures 4 and 5). LPS-treated animals demonstrated significant reductions in JNK (80%, $p < 0.001$) and p38 (80%, $p < 0.001$) phosphorylation compared with positive controls, as assessed by relative OD (Figures 4 and 5).

Nuclear translocation of NF κ B and AP-1

Positive controls exhibited marked NF κ B and AP-1 transactivation compared with negative controls and LPS controls (Figures 6, 7, and 8). LPS-treated animals demonstrated reduced nuclear translocation of NF κ B by 86% ($p < 0.001$) and AP-1 by 97% ($p < 0.001$) compared with positive controls, as assessed by relative OD (Figures 6, 7, and 8).

Discussion

This work describes a functionally relevant, protective effect of LPS pre-treatment on LIRI in an animal model. Low doses of LPS introduced into the airway led to significant reductions in lung endothelial cell dysfunction, leukocyte infiltration, proinflammatory kinase activation, and proinflammatory mediator production after subsequent exposure to oxidative stress.

The ability of LPS pre-treatment to induce both a proinflammatory response when given in sufficient amounts and a protective effect when low doses are used may be related to differential TLR-4 adaptor protein recruitment and their subsequent downstream signaling pathways. Specifically, LPS-induced TLR-4 proinflammatory signaling (as induced with naïve IR) occurs when MyD88 dimerizes with Tollinterleukin 1 receptor domain-containing adapter protein (TIRAP), leading to downstream proinflammatory mediator production. Alternatively, low-dose LPS pre-conditioning may favor recruitment and dimerization of the adaptor proteins TRIF and TRAM, which leads to the protective type 1 interferon responses and IL-10 production, and as a result and as our data indicate, yields an overall reduction in MyD88-dependent signaling. How these adaptor proteins and their downstream differential signaling responses are involved in LPS pre-conditioning warrants further investigation.

Given the dose-dependent differential response to LPS, the dose of LPS is critically important and portends important clinical relevance. Preliminary studies using LPS concentrations equivalent to those seen in BAL effluent from ventilated patients determined the lowest and most effective dose of LPS resulting in protection from LIRI. In addition, animals that received LPS but did not undergo IR (LPS controls) were not significantly different from negative controls for any of the outcomes evaluated, indicating that the dose of LPS used was not injurious to the lung. Furthermore, using a clinically relevant dose, specifically, a dose found within the BAL effluent of ventilated patients without evidence of overt infection, has implications for the translatability of these findings.

The protective effect of low-dose LPS pre-conditioning could expand our current understanding and utilization of lung donors, thereby reducing recipient wait list times. Tracheobronchial bacterial colonization is common among potential lung donors given the prevalence and duration of mechanical ventilation in this population. Differentiating the

clinical significance of an isolated positive Gram stain or a BAL containing LPS in low concentrations vs overt tracheobronchial infection has the potential to significantly affect donor utilization criteria. Specifically, the protective effects brought about by LPS pre-conditioning may indicate that donors with low concentrations of LPS should not only be considered for donation but that these lungs also may yield protection from subsequent LIRI. In addition, targeted, specific pre-conditioning of TLR-4 to down-regulate MyD88-dependent signaling in donor lungs before procurement could potentially decrease primary graft dysfunction and related morbidities.

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Figure 1.

Lipopolysaccharide (LPS) pre-treatment dose response of the lung permeability index is shown after ischemia-reperfusion. Pre-treatment with LPS before ischemia-reperfusion yielded protection from lung injury across the spectrum of LPS doses studied compared with positive controls (PC), but lung permeability remained elevated above that seen in negative controls (NC). A LPS pre-treatment dose of 15 ng was the lowest dose associated with the greatest protection from lung injury.

Figure 2.

Western blot analysis is shown for phosphorylated interleukin-1 receptor-associated kinase-1 (IRAK-1). Positive controls exhibited significantly more IRAK-1 phosphorylation (lane 2) than unmanipulated negative controls (lane 1) or lipopolysaccharide (LPS) controls (lane 5). LPS-treated animals demonstrated significantly reduced IRAK-1 phosphorylation (lanes 3, 4) compared with positive controls (p. 0.001) after undergoing 90 minutes of ischemia, followed by 15 minutes of reperfusion.

Figure 3. Relative optical densitometry of Western blot analysis is shown for phosphorylated interleukin-1 receptor-associated kinase-1 (IRAK-1). IRAK-1 phosphorylation was reduced by 87% in lipopolysaccharide-treated animals compared with positive controls as measured by relative optical densitometry.

Figure 4.

Western Blot analysis for phosphorylated c-Jun N-terminal kinase (JNK) and p38. Positive controls exhibit significantly more p38 and JNK phosphorylation (lane 3) than unmanipulated negative controls (lane 1) or lipopolysaccharide (LPS) controls (lane 2). LPS-treated animals demonstrate significantly reduced JNK phosphorylation and p38 phosphorylation (lane 4) compared with positive controls ($p < 0.001$, $p < 0.001$, respectively) after undergoing 90 minutes of ischemia, followed by 15 minutes of reperfusion.

Figure 5.

Relative optical densitometry of Western blot analysis is shown for phosphorylated c-Jun N-terminal kinase (JNK) and p38. JNK and p38 phosphorylation were both reduced by 80% in lipopolysaccharide-treated animals compared with positive controls as measured by relative optical densitometry.

Figure 6.

Results of electromobility shift assay are shown for nuclear factor κ B (NF κ B). Positive controls demonstrated marked nuclear translocation of NF κ B (lanes 3, 4, and 5) compared with negative controls (lane 1) and lipopolysaccharide (LPS) controls (lane 2). LPS-treated animals demonstrate significantly reduced nuclear translocation of NF κ B (lanes 6, 7, 8, and 9) compared with positive controls ($p < 0.001$) after undergoing 90 minutes of ischemia, followed by 15 minutes of reperfusion.

Figure 7.

Electromobility shift assay is shown for activator protein-1 (AP-1). Positive controls demonstrated marked nuclear translocation of AP-1 (lanes 3, 4, and 5) compared with negative controls (lane 1) and lipopolysaccharide (LPS) controls (lane 2). LPS-treated animals demonstrated significantly reduced nuclear translocation of AP-1 (lanes 6, 7, and 8) compared with positive controls ($p < 0.001$) after undergoing 90 minutes of ischemia, followed by 15 minutes of reperfusion.

Figure 8. Relative optical densitometry of electromobility shift assay showed that nuclear translocation of factor κB (NFκB) and activator protein-1 (AP-1) were reduced by 86% and 97%, respectively, in lipopolysaccharide-treated rats compared with positive controls.

Markers of Lung Injury

Table 1

Variable ^a	Controls			Treated LPS (n = 6)	p-value ^b
	Negative (n = 4)	LPS (n = 6)	Positive (n = 6)		
Permeability index	0.09 ± 0.005	0.12 ± 0.017	0.86 ± 0.014	0.25 ± 0.02	< 0.001
Myeloperoxidase content (change in absorbance)	0.021 ± 0.007	0.028 ± 0.009	0.85 ± 0.029	0.055 ± 0.015	< 0.001
BAL cell count, ×10 ⁶	6 ± 0.7	5.5 ± 0.3	179 ± 3.5	15.5 ± 2.3	< 0.001

BAL, bronchoalveolar lavage; LPS, lipopolysaccharide.

^aValues are expressed as mean ± standard error of the mean.

^bThe values of p compare positive controls with LPS-treated animals, and negative controls with LPS controls, respectively.

Table 2

Bronchoalveolar Lavage Cytokine and Chemokine Content

Variable ^a	Controls			Treated LPS (n = 6)	p-value ^b	
	Negative (n = 4)	LPS (n = 6)	Positive (n = 6)			
CINC	27.5 ± 4.4	23.5 ± 0.8	3697.9 ± 608.4	20.9 ± 3.7	0.003	0.9
Interleukin-1β	39 ± 1.3	41 ± 1.4	587 ± 11.5	160 ± 5.0	<0.0001	0.92
TNF-α	22 ± 0.7	26 ± 0.6	274 ± 16.2	65 ± 1.9	<0.0001	0.9

CINC, cytokine-induced neutrophil chemoattractant; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

^aValues for the variables are in pg/ml and are expressed as mean ± standard error of mean.

^bThe values of p values compare positive controls with LPS-treated animals, and negative controls with LPS controls, respectively.