Cytokine mRNA expression in unilateral ischemic-reperfusion rat lung with salt solution supplemented with low-endotoxin or standard bovine serum albumin

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Teng, Shu, Shunichi Kurata, Iyoko Katoh, Gabriela S. Georgieva, Toshihisa Nosaka, Chieko Mitaka, and Takasuke Imai. Cytokine mRNA expression in unilateral ischemic-reperfusion rat lung with salt solution supplemented with low-endotoxin or standard bovine serum albumin. Am J Physiol Lung Cell Mol Physiol 286: L137–L142, 2004; 10.1152/ajplung.00261.2003.—Our aim was to determine whether cytokine mRNA expression is induced by experimental manipulation including artificial perfusate or ischemia-reperfusion (I/R) in an isolated, perfused rat lung model. Constant pulmonary flow [Krebs-Henseleit solution supplemented with low-endotoxin (LE) or standard (ST) bovine serum albumin 4%, 0.04 ml/g body wt] and ventilation were maintained throughout. Right and left pulmonary arteries were isolated, and the left pulmonary artery was occluded for 60 min and then reperfused for 30 min. Analysis of tumor necrosis factor-α, IL-1, IL-6, IL-10, and IFN-γ mRNA expression by RT-PCR and evaluation of vascular permeability by bronchoalveolar lavage (BAL) fluid albumin content were conducted separately in right and left lung. Both LE and ST groups (each 12 rats) showed increases in vascular permeability by I/R (BAL fluid albumin content: 5.53 ± 1.55 vs. 15.63 ± 8.87 and 4.76 ± 2.71 vs. 16.72 ± 4.85 mg/ml BAL fluid/g lung dry wt−1, mean ± SD; right vs. left lung in LE and ST groups, P < 0.05 between right and left). Cytokine mRNA expression was significantly higher in the I/R lung than in the control lung in LE and ST groups, whereas it was higher in the control lung in the ST group (P < 0.05). mRNAs of not only proinflammatory but also anti-inflammatory cytokines were expressed in I/R lung, which are expected to aggravate I/R injury. The reversed pattern of cytokine mRNA expression in the ST group was possibly due to the longer perfusion of control lung with perfusate containing endotoxin, which caused no lung damage without I/R.

ischemia-reperfusion injury; reverse transcriptase-polymerase chain reaction; endotoxin

ISCHEMIA-REPERFUSION (I/R) injury is a severe complication in lung transplantation (23, 33), cardiopulmonary bypass (30, 39), and pulmonary thromboembolism (24). Because ischemia of the lung is not the same as hypoxia or anoxia due to the supply of oxygen from the airway even during complete cessation of pulmonary circulation, the pathophysiology of lung I/R injury has attracted much attention (33). The mechanism underlying lung I/R injury is postulated to involve leukocyte-dependent and -independent pathways (8, 19, 36). Because an isolated, physiological salt solution-perfused lung contains few leukocytes, it is suitable for separating blood cell-dependent and -independent mechanisms. We (20) previously induced acute lung injury in rats by selective one-lung ischemia with continued two-lung ventilation, in which only one lung had edema compared with the other lung not exposed to ischemia. Thus, in the initial phase of lung I/R injury, the injury process is independent of circulating leukocytes (8, 19, 20), although the involvement of leukocytes cannot be completely eliminated (36).

In ischemia of the myocardium, proinflammatory cytokine genes are expressed in myocytes (17). Unlike myocytes under ischemia, ischemic lung is hardly exposed to hypoxia; however, reactive oxygen species are produced during ischemia by incompletely elucidated mechanisms, and the generation of reactive oxygen species is postulated to be the initial event in lung I/R injury (2, 27). Those reactive oxygen species stimulate the induction of proinflammatory cytokine genes (2, 18, 32, 34). The induced proinflammatory cytokines lead to the expression of adhesion molecules in the endothelium, and leukocytes adhering to pulmonary vascular endothelium injure the endothelium, thereby resulting in an increase in permeability (40). However, the participation of leukocytes in the early phase of lung I/R is hardly anticipated in an isolated, perfused model, and therefore the direct toxic effect of tumor necrosis factor (TNF)-α on the endothelium is proposed (19, 25).

An isolated, salt solution-perfused lung is preferred to eliminate humoral and nonintrinsic nervous effects (14) as well as blood cell participation; however, to maintain the colloid osmotic pressure of the perfusate, bovine serum albumin or other macromolecules [pentastarch (6, 7), Ficol (8), or dextran (26)] are usually supplemented. Bethmann et al. (3) demonstrated that compared with a buffer supplemented with low-endotoxin albumin (concentration 4%), a buffer supplemented with standard bovine serum albumin caused the release of large amounts of TNF-α into the perfusate in isolated, perfused rat lung because of the endotoxin contained in the standard bovine serum albumin. Some previously reported studies that used isolated, perfused lung were carried out with a perfusate supplemented with standard bovine serum albumin (18, 20, 25, 36). To our knowledge, except for the reports by Bethmann et al. (3) and Held et al. (16), the effect of bovine serum albumin on cytokine mRNA expression has not been mentioned. Moreover, the influence of endotoxin contained in bovine serum...
albumin on the expression of cytokine mRNAs other than TNF-α mRNA has not been investigated in detail.

Our objective was to elucidate the differences in cytokine mRNA expression in I/R injury between a perfusate supplemented with low-endotoxin albumin and one supplemented with standard bovine serum albumin and how such differences are related to I/R injury. We present the hypothesis that mRNAs of proinflammatory cytokines are expressed in I/R in isolated, perfused rat lung independently of the type of perfusate used because the expected stimulus of the I/R is sufficient to overcome the effect of trace amounts of endotoxin contained in standard bovine serum albumin. Our unilateral ischemic lung model is suitable for differentiating the effect of experimental procedures from that of I/R because cytokine mRNA expression may be easily induced by experimental stimuli.

MATERIALS AND METHODS

This experiment was approved by the institutional animal use and care committee of Tokyo Medical and Dental University.

Isolated, perfused rat lung preparation. Male Sprague-Dawley rats (specific pathogen-free, body wt 271 ± 15 g, mean ± SD; purchased from Sankyo Labo-Service, Tokyo, Japan) were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). Because the isolated, perfused rat lung preparation was reported previously (20), only an outline is provided here. After tracheostomy, airway pressure (2.5 cmH2O) was applied to prevent their collapse to prevent pulmonary microembolization by particulate contaminants. Albumin, Sigma, St. Louis, MO) was also added at 4 g/dl. Before use, KH2PO4, 5.5 glucose, and 3.2 CaCl2. Two types of commercially fed constant-temperature Plexiglas chamber were injected into the right ventricle. Polyethylene cannulas were placed in the pulmonary artery (PE-200) and the left atrium (PE-320). Perfusion via the pulmonary artery cannula was started immediately after placement of the left atrial cannula. A peristaltic pump (Persta MF pump AP-7000, Atto, Tokyo, Japan) provided a constant flow rate of 0.04 ml·g body wt−1·min−1. Perfusion was restored within a few minutes after the interruption of the animal’s own circulation. The trachea, lungs, and heart were then freed from the chest cavity and transferred to a humidified constant-temperature Plexiglas chamber maintained at 37°C ± 1°C. The perfusate consisted of (in mmol/l) 22.6 NaHCO3, 119.0 NaCl, 50.0 sucrose, 4.7 KCl, 1.17 MgSO4, 1.18 KH2PO4, 5.5 glucose, and 3.2 CaCl2. Two types of commercially available bovine serum albumin (low-endotoxin grade and fraction V albumin, Sigma, St. Louis, MO) were also added at 4 g/dl. Before use, the perfusate was passed through a filter of 0.2-μm pore size to prevent pulmonary microembolization by particulate contaminants. After the lungs were excised from the body, continuous positive airway pressure (2.5 cmH2O) was applied to prevent their collapse and the tidal volume was adjusted to obtain the peak airway pressure of 5 cmH2O above positive end-expiratory pressure throughout the experiment. Pulmonary perfusion pressure was measured at the main pulmonary artery with a Statham Gould P23Gb pressure transducer (Gould, Oxnard, CA), and mean pressure was obtained electronically. When a homogeneous white appearance with no signs of hemostasis, edema, or atelectasis was observed throughout 30-min basal perfusion, the isolated lung preparation was used for each experiment.

Selective perfusion to one lung. After the isolated lung was suspended in the humidified constant-temperature chamber and stable perfusion pressure was obtained with a flow rate of 0.04 ml·g body wt−1·min−1, the left and right pulmonary arteries were isolated from the surrounding tissues and a ligation thread was passed around each of them. In this procedure, selective perfusion to either lung was realized by occluding the other pulmonary artery (22). After a stable baseline condition for 30 min was obtained, the left lung perfusion was occluded by suspending a weight on the occluding thread for the left lung. With two-lung ventilation, left lung-selective ischemia was maintained for 1 h, which was previously confirmed to elicit I/R injury (20). The suspending weight on the thread for the left lung was then removed, and reperfusion was started. After completion of the 30-min reperfusion, lung perfusion pressure was determined by occluding the perfusion for the other lung.

Lung vascular permeability assessment. To quantify injury to the lung as a result of I/R, lung vascular permeability was assessed by measuring protein content in the bronchoalveolar lavage (BAL) fluid. After completion of the experiment, the right and left lungs were harvested, weighed separately. The airway cannula was inserted into the right or left main bronchus and ligated to prevent loss of BAL fluid. Lavage was performed by introduction of physiological saline (2.5 ml) into the airway cannula with three installations and withdrawals. The combined recovered fluid was consistently 6 ml for each right or left lung. The lavage fluid was centrifuged at 15,000 rpm at 4°C for 20 min to remove cells, and the supernatant was assayed for protein content with a Bio-Rad protein assay system (Bio-Rad Laboratories) with bovine serum albumin as standard. Protein content in the lavage fluid was calculated as milligrams of albumin per milliliter of BAL fluid per gram of lung dry weight. From the rats not used for BAL, wet-to-dry ratio was measured by desiccating the lung at 80°C until a constant weight was obtained.

RNA extraction. Total RNA was extracted from the lungs by the acid-phenol method with an RNA extraction kit (RNAwiz, Ambion, Austin, TX). Briefly, lung tissue samples (~0.05 g) were cut into small pieces and homogenized in 0.5 ml of RNAwiz solution with a Polytron tissue homogenizer (Brinkman, Mississauga, ON, Canada) for 1 min at medium speed. The homogenized samples were then incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. After 0.1 ml of chloroform was added to the tubes, the tubes were shaken vigorously by hand for 15 s and then incubated at room temperature for 10 min. The samples were centrifuged at 12,000 g for 15 min at 4°C. A 0.25-ml portion of the aqueous phase (RNA remainder) was collected and transferred into a fresh tube. Diethyl pyrocatechol (0.25 ml, Sigma)-treated, distilled (RNase-free) water and isopropanol (0.5 ml) were then added to the tube, followed by thorough mixing at room temperature for 10 min and centrifugation at 12,000 g for 15 min at 4°C to precipitate the RNA. The supernatant was removed, and the RNA pellet was washed three times with 1 ml of 75% (vol/vol) ethanol. After the RNA pellet was air dried for 5 min, 50 μl of RNase-free water was added and the RNA pellet was dissolved completely for cDNA synthesis.

Semiquantitative RT-PCR. Semiquantitative RT-PCR was carried out with a one-step RT-PCR kit (SuperScript One Step RT-PCR with Platinum Taq, Invitrogen). The cDNA synthesis and preamplification step was performed by one cycle of 50°C for 30 min and 94°C for 2 min with the purified cDNAs described in RNA extraction. The synthesized cDNAs were amplified by ~35 cycles of PCR (denaturation at 94°C, annealing at 55°C, and extension at 70°C), and for the final extension one cycle of 70°C for 5 min was carried out with a PC707 thermal cycler (Program TEMP Control system, ASTEC, Fukuoka, Japan). The PCR products were electrophoresed on 3.2% (3.0% for IL-1β) agarose gel with ethidium bromide staining for visualization. The gels were photographed, and the bands were quantified with NIH Image version 1.5 software for Macintosh computers and standardized to the housekeeping gene GAPDH to represent the level of gene expression. The primers for rat cytokines were synthesized based on rat cDNA databases for TNF-α (10), IL-1β (11), IL-6 (5), IL-10 (12, 15), IFN-γ (38), and GAPDH (41). The sequences of oligonucleotide primers used for PCR amplification of cytokine mRNAs and predicted cDNA size were as follows: TNF-α: 3′ primer 5′-CAT CTG CTA GTA CCA CCA CTA-3′ and 5′ primer 5′-TGA GCA CAG AGC AGA TGG CAC-3′; IL-1β: 3′ primer 5′-GGG TTC CAT GGA GAA GTA AAC-3′ and 5′ primer 5′-CAC TCT TCA AGC AGA GCA CAG-3′ (80 bp); IL-6: 3′ primer 5′-GAG AGC ATT GGA AGT TGG GG-3′ and 5′ primer 5′-CTT CCA GCC AGT TGC.
RESULTS

CTT CT-3′ (496 bp); IL-10: 3′ primer 5′-AAA CTC ATT CAT GGC CTT GTA-3′ and 5′ primer 5′-TGC CTT CAG TCA AGT GAA GAC T-3′ (346 bp); IFN-γ: 3′ primer 5′-GCT GGT GAA TCA CTC TGA TG-3′ and 5′ primer 5′-GCC AAG GCA CAC TGA TTG AA-3′ (390 bp); GAPDH: 3′ primer 5′-GCC TTC TCC ATG GTG AA-3′ and 5′ primer 5′-GTT CGG TGT GAA CAG ATT TG-3′ (309 bp). Under optimized PCR conditions, all data were collected without saturation or missing bands. The background optical density reading for each band was subtracted locally. Each assay was conducted at least twice to ensure reproducibility.

Experimental groups. A total of 24 rats were used for the experiments with perfusates supplemented with low-endotoxin albumin (12 rats) or standard bovine serum albumin (12 rats). In six rats of each group, three small pieces of tissue (each ~50 mg) from different parts of the right and left lungs were obtained for mRNA analysis and the rest of the lungs were used for determining wet-to-dry ratio. Another six rats in each group were used for the BAL procedure.

Endotoxin in perfusates. Endotoxin activates a coagulation cascade in Limulus amebocyte lysate and results in gel formation (21). With this reaction, the kinetic turbidimetric assay using endotoxin single test Wako and Toximeter MT-358 (Wako Pure Chemical Industries, Osaka, Japan) can measure endotoxin at a lower detection limit of 0.5 pg/ml. Endotoxin contents in Krebs-Henseleit solutions without albumin, supplemented with low-endotoxin albumin, and supplemented with standard bovine serum albumin were measured according to the manufacturer’s instructions (Wako Pure Chemical Industries).

Statistics. All results are expressed as means ± SD. Comparisons were made with the Wilcoxon rank-sum test or the Mann-Whitney U-test as appropriate. Significance was determined when P < 0.05 was obtained.

RESULTS

Endotoxin contents in the perfusates were 13.2 ± 5.0 pg/ml in Krebs-Henseleit solution not supplemented with albumin, 36.0 ± 3.6 pg/ml in that supplemented with low-endotoxin albumin, and 480.2 ± 76.9 pg/ml in that supplemented with standard bovine albumin.

Perfusion pressure was stable during the control period (30 min before ischemia), and there was no difference between low-endotoxin and standard bovine serum albumin perfusates (3.50 ± 0.36 mmHg in the former vs. 3.41 ± 0.36 mmHg in the latter). When the right or left pulmonary artery was occluded, perfusion pressure was more markedly increased in the left lung than in the right lung (Table 1). After ischemia, both lung perfusion pressures were the same as before ischemia (3.73 ± 0.37 mmHg in low-endotoxin perfusate and 3.52 ± 0.28 mmHg in standard bovine serum albumin perfusate); however, the ischemia-exposed left lung showed a greater increase in perfusion pressure than the lung in the preischemic period (Table 1). The degree of increase in perfusion pressure after ischemia was the same between low-endotoxin and standard bovine serum albumin perfusates (Table 1).

Albumin content in BAL fluid was significantly increased in the I/R left lung, and this increase was consistent regardless of the type of albumin used (Table 1). Wet-to-dry ratio, another indicator of vascular permeability, was increased significantly in the I/R left lung regardless of the type of albumin used (Table 1).

Cytokine mRNA expression was different between the two types of perfusates. When low-endotoxin albumin was used, cytokine mRNA expression was significantly higher in the left lung than in the right lung (Fig. 1, Table 2). In contrast, when standard bovine serum albumin was used, mRNA expression for all cytokines except IL-10 was significantly higher in the control right lung than in the I/R left lung (Fig. 2, Table 2).

Table 1. Perfusion pressure, albumin content in BAL fluid, and wet-dry ratio in isolated, perfused rat lungs

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<tr>
<th></th>
<th>Low-Endotoxin Albumin</th>
<th>Standard Bovine Serum Albumin</th>
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<tr>
<td></td>
<td>Right</td>
<td>Left</td>
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<tr>
<td>Perfusion pressure, mmHg</td>
<td></td>
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<tr>
<td>Before ischemia</td>
<td>4.04±0.41</td>
<td>5.02±0.50</td>
</tr>
<tr>
<td>After ischemia</td>
<td>4.31±0.47</td>
<td>5.64±0.53*</td>
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<tr>
<td>Albumin content in BAL fluid, mg/ml BAL fluid *</td>
<td>5.53±1.55</td>
<td>15.63±8.87†</td>
</tr>
<tr>
<td>Wet-to-dry ratio</td>
<td>5.53±0.31</td>
<td>6.11±0.76‡</td>
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Values are means ± SD for right and left lung perfused with Krebs-Henseleit solution supplemented with low-endotoxin or standard bovine serum albumin. Perfusion pressure was measured before and after 60-min ischemia. BAL, bronchoalveolar lavage. *P < 0.05 vs. before ischemia; †P < 0.05, ‡P < 0.01 vs. right lung by Wilcoxon signed-rank test.
cytokine mRNA in ischemic-reperfused rat lung

Table 2. mRNA expression of cytokines relative to that of GAPDH in isolated, perfused rat lungs exposed to 60-min ischemia and 30-min reperfusion

<table>
<thead>
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<th>Low-Endotoxin Albumin</th>
<th>Standard Bovine Serum Albumin</th>
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<tr>
<td></td>
<td>Right</td>
<td>Left</td>
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<tr>
<td>TNF-α</td>
<td>0.301 ± 0.473</td>
<td>0.965 ± 1.335*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.204 ± 0.909</td>
<td>1.534 ± 2.462*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.024 ± 1.496</td>
<td>2.778 ± 5.344*</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.006 ± 0.611</td>
<td>1.459 ± 0.878*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.239 ± 0.281</td>
<td>1.114 ± 1.56*</td>
</tr>
</tbody>
</table>

Values (in densitometry units) are means ± SD for right and left lung perfused with Krebs-Henseleit solution supplemented with low-endotoxin or standard bovine serum albumin. TNF, tumor necrosis factor. *P < 0.05 vs. right lung by Wilcoxon signed-rank test.

DISCUSSION

I/R injury in isolated, perfused rat lungs with perfusates containing low-endotoxin albumin and standard bovine serum albumin was of the same extent when evaluated in terms of BAL fluid protein content, wet-to-dry ratio, and perfusion pressure increase. However, cytokine mRNA expression levels were different between the two groups. When the perfusate containing low-endotoxin albumin was used, the ischemic lung expressed higher levels of cytokine mRNAs than the control right lung. In contrast, when the perfusate containing standard bovine serum albumin was used, the ischemic lung showed significantly lower mRNA expression levels for all cytokines except IL-10 than the control right lung.

Under normal physiological conditions, there is almost no expression of proinflammatory cytokine mRNAs in the lung; however, experimental manipulation may induce their expression (7, 9). Accordingly, we intended to detect cytokine mRNAs with the RT-PCR technique in lung exposed to I/R and control lung within an animal subjected to the same experimental procedure. In this model, cytokine mRNA expression levels were higher in the control right lung than in the I/R lung in the standard bovine serum albumin group, suggesting that the endotoxin contained in the standard bovine serum albumin (over 13 times more endotoxin than in the low-endotoxin albumin perfusate) may have provided a stronger stimulus to the control right lung because the right lung was perfused more extensively during left lung ischemia. As regards cytokine mRNA expression, the stimulus by endotoxin to the lung seems to be a strong one (4). In contrast, in the low-endotoxin albumin group, the I/R left lung exhibited higher cytokine mRNA expression levels than the control right lung. From those results, we found that I/R induced mRNA expression in the I/R lung not only for proinflammatory cytokines but also for the anti-inflammatory cytokine IL-10, which diminishes inflammatory response. Interestingly, in the standard bovine serum albumin group, only the mRNA expression of IL-10 did not show a significant increase in the control right lung, suggesting that I/R strongly stimulated the IL-10 mRNA expression in the left lung.

It has been suggested that on I/R of lung, proinflammatory cytokine expression is induced in the lung, thereby affecting the I/R injury (32). The expression of TNF-α was increased in the early phase (within 30 min) of reperfusion after ischemia (45 min) in isolated, buffer-perfused rat lung (25) as well as in situ rat lung ischemia for 90 min (9). When antibody to TNF-α was administered intravenously or intraperitoneally to rats before lung ischemia, the increase in lung vascular permeability due to I/R was largely ameliorated (9, 25). Furthermore, I/R injury in isolated lungs that were perfused with physiological saline or University of Wisconsin solution was ameliorated by the addition of monoclonal rat anti-TNF-α antibody (6). Those reports suggested that TNF-α results in an increase in vascular permeability via a mechanism independent of neutrophils on the endothelium in I/R injury.

Khimenko et al. (25) showed that TNF-α (50,000 U) introduced into the perfusate in isolated, perfused rat lung models produced no endothelial damage; however, exogenous TNF-α significantly aggravated the I/R endothelial injury. TNF-α itself seems not to be deleterious to pulmonary endothelium not exposed to I/R (25). This may be the reason why the control right lung perfused with standard bovine serum albumin did not show any damage although it expressed more cytokine mRNAs than the I/R left lung.
We evaluated the mRNA expression of TNF-α, IL-1β, IL-6, IL-10, and IFN-γ because TNF-α, IL-1β, IL-6, and IFN-γ are well-known central proinflammatory cytokines and IL-10 is one of the major anti-inflammatory cytokines in lung (37). To our knowledge, there is no report of whether IL-10 is induced in lung I/R injury; on the other hand, the only report of increased mRNA of IL-1β, IL-6, and IFN-γ by lung I/R is that of Chiang et al. (7). We measured not the absolute cytokine mRNA expression levels but the cytokine mRNA expression levels in the I/R lung and compared them with the levels in the control lung in rat. In this way, we could eliminate the artifactual effect on cytokine mRNA expression by experimental manipulation, and thus we verified cytokine mRNA expression by lung I/R. Increased cytokine mRNA transcription does not always translate into an increase in the amount of secreted cytokine (1, 7, 29). Because we did not measure cytokine proteins, the translation of genes was not confirmed. However, circulating cytokine concentration may not be indicative of local cytokine biosynthesis or activity (28) and the measurement of mRNA at the tissue level might be a better indicator of cytokine biosynthesis than the measurement of circulating cytokine concentrations (1). Correlation between transcription and translation of cytokine in lung I/R must be further investigated. Those cytokines exerted their actions on resident macrophages and other cells in lung tissue (13, 35) and possibly amplified the I/R injury.

I/R led to the expression of TNF-α, IL-1β, IL-6, IL-10, and IFN-γ mRNAs in the isolated, perfused rat lung model, and the expressed mRNAs are expected to aggregate I/R injury. Standard bovine serum albumin, which is widely used in perfusion experiments, affects cytokine mRNA expression possibly because of the endotoxin it contains. Therefore, the results obtained in perfusion experiments that use standard bovine serum albumin should be evaluated cautiously.

GRANTS

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