Endothelial adhesivity, pulmonary hemodynamics and nitric oxide synthesis in ischemia-reperfusion

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Abstract

Objective: Alterations in nitric oxide synthesis, endothelial adhesivity and pulmonary hemodynamics are investigated in an animal model of lung ischemia-reperfusion. Methods: Two sets of rats, each containing seven animals, were either subjected to unilateral pulmonary ischemia and reperfusion (Study Group) or underwent the same surgical procedure without ischemia (Control Group). Pulmonary artery pressure (PAP), pulmonary blood flow (PBF) trend, NOS-2, intercellular adhesion molecule-1 (ICAM-1), myeloperoxidase (MPO) and cGMP expression of the reperfused lung tissue and, final paO2 were compared between the two groups. Results: ICAM-1 expression was increased (369\(\pm\)114 vs. 115\(\pm\)65; \(P\leq0.02\)), NOS-2 expression and tissue cGMP levels were decreased (377\(\pm\)44 vs. 452\(\pm\)54; \(P=0.03\) and 7.8\(\pm\)3.5 vs. 9.4\(\pm\)2.3 pmol/ml; \(P=0.03\), respectively) and MPO activity was increased (2.7\(\pm\)0.9\(\pm\)3.5\(\pm\)0.8; \(P=0.03\)) in the reperfused lungs. Pulmonary artery pressure was 15\(\pm\)7 mmHg in the Control Group vs. 22\(\pm\)16 mmHg in the Study Group \((P=0.04)\) at the 30th min of reperfusion. Pulmonary blood flow was greater in the Study Group at the beginning of reperfusion (9.5\(\pm\)4.1 vs. 7.1\(\pm\)3.1 ml/min at the 30th min) but considerably reduced thereafter (3.2\(\pm\)1.4 vs. 6.2\(\pm\)2.1 at the 60th minute and 2.9\(\pm\)1.6 vs. 5.8\(\pm\)1.9 at the 120th min). At the end of the experiment, paO2 was 95\(\pm\)30 in the Control Group vs. 71\(\pm\)32 in the Study Group \((P=0.03)\). Conclusions: These data establish that nitric oxide synthesis was suppressed after reperfusion. Pulmonary blood flow was first increased and then reduced. A parallel increase in MPO and ICAM-1 indicated proinflammatory reaction. Decreased tissue cGMP level was consistent with the suppressed NOS-2 production. Organ function was negatively influenced as represented by the decreased oxygenation, probably due to no-reflow phenomenon. \(\copyright\) 2000 Elsevier Science B.V. All rights reserved.

Keywords: Reperfusion; Nitric oxide; Adhesion molecule; Ischemia; Endothelium; Inflammation

1. Introduction

Endothelium subjected to ischemia-reperfusion may maintain cell viability and basic biosynthetic mechanisms to some extent, but displays multiple changes in properties relevant to vascular homeostasis. For example, reduced nitric oxide-cGMP levels in the subsequent reperfusion period are possible mechanisms of vascular dysfunction affecting coagulation, permeability, vasomotor tone and leukocyte adhesivity after reperfusion. Pulmonary circulation and lungs are frequent target organs for reperfusion injury and constitute a good model for the investigation of the effects of ischemia-reperfusion. Because the nitric oxide (NO) pathway modulates pulmonary vascular tone and leukocyte-endothelial interactions, reactive oxygen intermediates may lead to decreased NO (and hence cGMP) levels following pulmonary reperfusion, leading to increased pulmonary vascular resistance and leukostasis.

This study aimed to investigate vascular phenotype modulation in terms of cellular adhesion molecule expression and changes in inducible nitric oxide synthase activity and leukocyte accumulation, as well as the influences on regional hemodynamics, using an in vivo rat lung ischemia-reperfusion model. Today’s clinical cardio-thoracic surgery practice frequently deals with ischemia-reperfusion phenomenon as an important intervening problem with major unfavorable consequences after technically well-managed procedures involving organ procurement and transplantation, protection of brain, heart and spinal cord. Better understanding of basic molecular and cellular mechanisms will serve to keep the physician’s alertness and ability for timely employing necessary measures.

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2. Materials and methods

2.1. Animal surgery

Two sets of Harlan male Sprague–Dawley rats (Harlan, Indianapolis, IN), each consisted of seven animals (Control and Study Groups), were anesthetized by intraperitoneal injection of xylazine (5 mg/kg) and ketamine (50 mg/kg). After ensuring adequate depth of anesthesia, the animal was fixed in a supine position and connected to a rat ventilator (Harvard Rodent Ventilator, Model 683, Harvard Apparatus, Inc., Holliston, MA) through a tracheostomy stoma created in the neck. Anesthesia was maintained during the experiment with repetitive doses of xylazine and ketamine at 45-min intervals or on return of somatic reflexes. A bilateral clamshell incision was then made to expose the thoracic cavity. The right, left and main pulmonary arteries were located and dissected free from the surrounding adjacent tissues under microscope. Following dissection, heparin was administered (1 mg/kg), a Millar catheter (2F; Millar Instruments, Inc., Houston, TX) was introduced into the main pulmonary artery. After obtaining the blood pressure, this catheter was removed and replaced by a flow probe (Transonics, Ithaca, NY). Then, the left pulmonary artery was temporarily ligated in the Study Group animals. To ensure complete pulmonary ischemia, pulmonary hilus was also ligated with a heavy silk tie, interrupting bronchial circulation. After 1 h of ischemia, the left pulmonary artery was re-opened. A snare was passed around the right pulmonary artery, the pressure catheter was introduced into the main pulmonary artery again and pulmonary artery pressure at the 30th min was obtained. Then it was replaced by the flow probe, and the pulmonary flow was read at the 30th, 60th, and 120th min of reperfusion. On-line hemodynamic monitoring was accomplished using MacLab software and a Macintosh IIci computer (Apple Computer, Cupertino, CA). Total reperfusion time was 2 h and finally, following left ventricle sampling for PaO₂ analysis the left lung was excised and snap frozen in liquid nitrogen. Animals in the Control Group were not subjected to ischemia and reperfusion; otherwise the procedure was the same. Animals in the Control Group were not subjected to ischemia and reperfusion; otherwise the procedure was the same. Animal use complied with the ‘Principles of Laboratory Animal Care’ and the ‘Guide for the Care and Use of Laboratory Animals’ (NIH Publication No. 86-23, revised 1985) and was approved by the Institutional Animal Care and Use Committee.

2.2. Protein source

Tissue samples were homogenized for 30 s at 4°C with a Polytron homogenizer (Kinematica GmbH, Kriens-Luzerne, Switzerland) with ice-cold 20 mM Tris–HCl (pH 7.4) containing 100 mM NaCl, 2 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mg/l leupeptin and 0.7 mg/l pepstatin. Homogenates were shaken at 4°C for 3 h, then centrifuged at 13 000 rev./min for 10 min at 4°C and the supernatant was collected as the source of sample protein.

2.3. Assays

2.3.1. Western blotting

Samples were run in a 7.5% polyacrylamide gel and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked for non-specific binding using 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 12 h at 4°C. The membrane was then incubated in 0.05% BSA and 0.05% Tween 20 in PBS containing goat anti-rat intracellular adhesion molecule-1 (ICAM-1) (Santa Cruz Inc., Santa Cruz, CA) antibody at 1:1000 concentration for 1 h. After subsequent washes, the membrane was incubated with anti-goat IgG-peroxidase conjugate (Sigma Chemical Co.) at 1:2000 concentration in PBS, 0.5% BSA and 0.05% Tween 20 for 45 min. After the subsequent washes, X-ray films were obtained using enhanced chemiluminescence (ECL) detection (Amersham, Piscataway, NJ). The staining intensity of specific bands was quantified by densitometric scanning by a computer software (Molecular Analyst, Bio-Rad Laboratories). Those calculated areas were then statistically compared. The same procedure was repeated for the detection of inducible nitric oxide synthase (NOS-2) using a rabbit-derived anti-NOS-2 (Santa Cruz Biotechnology Inc.) and an anti-rabbit IgG-peroxidase conjugate (Sigma Chemical Co.) at 1:2000 concentration.

2.3.2. Myeloperoxidase assay

Myeloperoxidase assay was performed as previously described [1].

2.3.3. Enzyme-linked immunosorbent assay (ELISA)

Because nitric oxide stimulates soluble guanylate cyclase found in multiple tissues (smooth muscle cells, platelets endothelial cells, etc.) to form the cyclic nucleotide cGMP, ELISAs were performed to quantify tissue cGMP levels. Tissue homogenates in PBS were added to 5% trichloroacetic acid and kept on ice for 30 min. Those homogenates were spun at 4000 rev./min for 15 min. Supernatant was collected and washed with ether three times. After storing the homogenates at 20°C for 2 min, cGMP content was measured by an ELISA assay kit (R&D Systems Inc., Minneapolis, MN) which is based on the competitive binding technique in which cGMP present in a sample competes with a fixed amount of alkaline phosphatase-labeled cGMP for sites on a rabbit polyclonal antibody. That antibody was bound to a goat anti-rabbit antibody coated onto the microplate. The absorbance was read at 405 nm using a Biokinetis microplate reader Model EL 340 (Biotek Instruments, Winooski, VT).

2.4. Statistical analysis

All statistics were performed using SPSS statistical soft-
ware (release 6.0, SPSS Inc., Chicago, IL). Means are presented ± standard deviation. A non-parametric test (Mann–Whitney U-test) was used for the comparison of the studied molecules, instead of a parametric one, due to unknown distribution of those molecules. Unpaired t-tests were performed for the pulmonary hemodynamics comparisons between the groups. A P-value equal to or smaller than 0.05 was considered as statistically significant. Also, the multiple comparisons in pulmonary blood flow necessitated the use of one-way ANOVA. In the case that ANOVA indicated a significant P-value (P < 0.05), pairwise comparisons between the groups were made by a post-hoc test (Tukey’s HSD procedure). Again, the significance level was set at P < 0.05.

3. Results

3.1. Hemodynamic parameters

3.1.1. Pulmonary artery pressure

Before the ischemia period, pulmonary artery pressures were 16 ± 5 mmHg and 15 ± 5 mmHg for the Control and Study Groups, respectively (P = 0.3). Thirty minutes after the initiation of reperfusion, mean pulmonary artery pressure was recorded. Mean pulmonary artery pressure was 22 ± 16 mmHg in the Study Group vs. 15 ± 7 mmHg in the Control Group (P = 0.04).

3.1.2. Pulmonary blood flow

Before the ischemia period, pulmonary blood flow rates were 8.2 ± 3.2 ml/min and 8.1 ± 2.2 ml/min for the Control and Study Groups, respectively (P = n.s). Pulmonary blood flow was measured at the 30th, 60th and 120th min of reperfusion. Initially, pulmonary blood flow was greater in the Study Group (9.5 ± 4.1 vs. 7.1 ± 3.1 ml/min at the 30th min; P = 0.02), however, considerably reduced thereafter in comparison to the Control Group (3.2 ± 1.4 vs. 6.2 ± 2.1 ml/min at the 60th min and 2.9 ± 1.6 vs. 5.8 ± 1.9 ml/min at the 120th min).

3.1.3. End-experiment $paO_2$

After a 2-h period of reperfusion, the average $paO_2$ was 71 ± 32 in the Study Group vs. 95 ± 30 in the Control Group (P = 0.03).

3.2. Assays

The results of the assays are presented in Table 1 and Fig. 1.

3.2.1. ICAM-1

The expression of ICAM-1 in rat lung tissue was examined by Western blotting. ICAM-1 expression, quantified as the intensity of a given band on the Western blot was 369 ± 114 in the Study Group vs. 115 ± 65 in the Control Group (P = 0.02).

3.2.2. NOS-2

The expression of the inducible isoform of nitric oxide synthase in the setting of pulmonary ischemia and reperfusion was also studied by Western blotting. NOS-2 expression, determined as the intensity of a given band on the Western blot, was 377 ± 44 in the Study Group vs. 452 ± 54 in the Control Group; P = 0.03.

3.2.3. cGMP content

ELISAs were performed to quantify tissue cGMP levels. Tissue cGMP levels were 7.8 ± 3.5 pmol/ml for the Study Group and 9.4 ± 2.3 pmol/ml for the Control Group (P = 0.03).

3.2.4. Myeloperoxidase activity

MPO activity was significantly increased in the reperfused lungs in comparison to the Control Group (2.7 ± 0.9 vs. 3.5 ± 0.8; P = 0.03), indicating the enhanced inflammatory milieu in the reperfused tissue.

4. Discussion

Considerable insights into the cellular and molecular

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Table 1

<table>
<thead>
<tr>
<th></th>
<th>paO$_2$</th>
<th>PAP initial</th>
<th>PAP 30 min</th>
<th>PBF initial</th>
<th>PBF 30 min</th>
<th>PBF 60 min</th>
<th>PBF 120 min</th>
<th>MPO activity</th>
<th>ICAM-1 intensity</th>
<th>NOS-2 intensity</th>
<th>cGMP (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group (n = 7)</td>
<td>95 ± 30</td>
<td>16 ± 5</td>
<td>15 ± 7</td>
<td>8.2 ± 3.2</td>
<td>7.1 ± 3.1</td>
<td>6.2 ± 2.1</td>
<td>5.8 ± 1.9</td>
<td>2.7 ± 0.9</td>
<td>115 ± 65</td>
<td>452 ± 54</td>
<td>9.4 ± 2.3</td>
</tr>
<tr>
<td>Study Group</td>
<td>71 ± 32</td>
<td>15 ± 5</td>
<td>22 ± 16</td>
<td>8.1 ± 2.2</td>
<td>9.5 ± 4.1</td>
<td>3.2 ± 1.4</td>
<td>2.9 ± 1.6</td>
<td>3.5 ± 0.8</td>
<td>369 ± 114</td>
<td>377 ± 44</td>
<td>7.8 ± 3.5</td>
</tr>
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</table>

| P-value* | 0.03 | n.s | 0.03 | N.S. | * | * | 0.03 | 0.02 | 0.03 | 0.03 |

|   |   |   |   |   |   |   |   |   |   |   |

* Data compared using unpaired t-test. Each P-value indicates statistical significance of difference between the values of the upper and lower rows in the same column.

b Significant difference by one-way ANOVA and following Tukey’s HSD post-hoc test.
mechanisms of pulmonary ischemia-reperfusion injury have occurred since the publication of the first reviews on lung preservation. Recent evidence indicates that the endothelium plays an essential role in regulating the dynamic interaction between pulmonary vasodilatation and vasoconstriction and is a major target during ischemia-reperfusion injury [2]. In addition, the nitric oxide dependent mechanisms generated by the endothelium and the role of many different types of circulating cells including neutrophil leukocytes, monocytes, mast cells [3,4] and lymphocytes, as well as pulmonary artery endothelial cells, alveolar macrophages, pulmonary alveolar type-II cells are increasingly being recognized as important contributors in pulmonary ischemia-reperfusion injury [5]. Further research on cellular mechanisms of endothelial perturbation in ischemia-reperfusion is likely to provide insights ultimately applicable to daily clinical practice and may lead to substantial achievements in terms of improving results following cardio-thoracic surgery or organ transplantation.

In the current study, we investigated the effects of reperfusion on several prototypical factors in a rat lung model. Since neutrophil leukocytes play a major role in reperfusion injury, ICAM-1 expression indicating leukocyte sequestration and myeloperoxidase activity reflecting leukocyte activation were compared. Endothelial response to reperfusion was assessed by comparing NOS-2 expression and tissue cGMP levels. Hemodynamic parameters such as pulmonary artery pressure and pulmonary artery blood flow were also measured. Impacts on organ function was assessed by oxygenation capability of the lung tissue at the end of the reperfusion.

Pulmonary ischemia followed by reperfusion results in microvascular pulmonary occlusion by platelet and leukocyte aggregates, pulmonary endothelial injury and pulmonary parenchymal neutrophil sequestration. Leukocyte adhesion to vascular endothelium is a critical step during the early stages of inflammation, and is mediated by the interaction of adhesion receptors expressed on the surfaces of both endothelial cells and leukocytes. ICAM-1, expressed on endothelial cells, mediates leukocyte binding to endothelial cells through interactions with its integrin counter receptors on leukocytes, such as CD 11/18. Circulating leukocytes adhere to vessel wall using those molecules, leave the blood stream and, enter the tissue. After having been activated, they release toxic substances which may cause a considerable amount of damage to the adjacent tissues [6].

Endothelium subjected to oxygen deprivation maintains cell viability and basic biosynthetic mechanisms, but displays multiple changes in properties relevant to vascular homeostasis, including suppression of the anticoagulant cofactor thrombomodulin, decreased barrier function, and generation of proinflammatory cytokines. Diminished intracellular cAMP during the period of hypoxia and lowered nitric oxide/cGMP in the subsequent reperfusion period are proposed as fundamental mechanisms driving vascular dysfunction impacting on coagulation, permeability, vaso-constriction, and leukocyte adhesivity [7].

Endogenously produced oxides of nitrogen appear to play important roles in tissue and organ homeostasis. Endogenous production of nitric oxide, which can be altered in response to various stimuli, maintains pulmonary vascular homeostatic properties, modulates vascular tone, suppresses oxynradical cascades, cell adhesion, microvascular permeability, platelet aggregation and other aspects of inflammation [8]. Nitric oxide is produced in endothelial cells by nitric oxide synthase, a calcium- and calmodulin-dependent enzyme [9]. NO-related vascular response uses cGMP as second messenger. The effects of nitric oxide are mediated by activation of guanylate cyclase, resulting in the formation of cyclic guanosine monophosphate (GMP) [10], which in turn reacts with cGMP-dependent protein kinase and causes a cascade of changes in protein phosphorylation, including dephosphorylation of myosin light chain, leading to cell relaxation. Therefore, a reduction in cGMP may cause endothelial cell contraction and increase the size of interendothelial junctions, resulting in a leaky endothelial barrier. Reduced levels of nitric oxide synthesis also leads to superoxide accumulation, which could directly cause an increase in endothelial permeability and the release of various mast cell-derived chemical agents, including PAF and histamine [11]. Nitric oxide also decreases the adhesive interaction between neutrophils and endothelial cells and limits damaging effects of neutrophils on tissues.

In the present study, we demonstrated a substantial increase in ICAM-1 expression which indicated a pro-inflammatory milieu and enhanced leukocyte adhesion following reperfusion. Consistently, myeloperoxidase activity, an indication for leukocyte activation, was also found.
significantly elevated. On the other hand, endothelial response appeared as reduced expression of inducible form of nitric oxide synthase (NOS-2). This was consistent with the parallel decrease in tissue cGMP levels. A transient increase in pulmonary blood flow was observed at the beginning of reperfusion, however, followed by a sustained decrease after this initial period. One may speculate that this initial increase in pulmonary blood flow may be due to adenosine accumulation during the ischemic phase. However, there should also have been a predisposition to increased vascular tone due to decreased NOS-2 expression and cGMP levels. The washout of adenosine with the establishment of reperfusion might have left this predisposition unopposed. Subsequent decrease in pulmonary blood flow may be due to vascular resistance increase, and no-reflow phenomenon caused by endothelial cell swelling, capillary collapse, platelet aggregation and sludging. This phenomenon of posts ischemic hypoperfusion, seen as a result of reperfusion injury after an initial hyperemic period has also been demonstrated in other organ models [12]. The mechanism for this finding may be in part a failure of endothelial NO production during periods of posts ischemic reperfusion, such that exogenously administered NO may correct the defect in pulmonary vascular relaxation and normalizes flow. More specific studies focused on this particular aspect of reperfusion are needed to clarify the exact underlying reasons. Nevertheless, the total effect of all the abovementioned changes was organ dysfunction reflected by the decreased paO2 in the samples taken at the end of the experiment.

It is imperative that the work from the experimental laboratory be translated into clinical reality via prospective, randomized trials. Techniques to improve the preservation of both pulmonary endothelial and epithelial cells should be investigated clinically, because a combination of treatment modalities will likely prove necessary to minimize ischemia-reperfusion injury which frequently complicates cardio-thoracic surgical procedures and to improve patient outcomes after lung transplantation.

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References