Inhibition of inducible nitric oxide synthase attenuates platelet adhesion in subpleural arterioles caused by lung ischemia-reperfusion in rabbits

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Inhibition of inducible nitric oxide synthase attenuates platelet adhesion in subpleural arterioles caused by lung ischemia-reperfusion in rabbits. J Appl Physiol 99: 2423–2432, 2005. First published July 21, 2005; doi:10.1152/japplphysiol.01302.2004.—Oxidative stress, induced by lung ischemia-reperfusion, leads to platelet and leukocyte activation and may contribute to decreased alveolar perfusion by platelet adhesion to the arteriolar wall. We investigated the hypothesis that ischemia-reperfusion injury increases inducible nitric oxide synthase (iNOS) activity and subsequent generation of reactive nitrogen species with P-selectin-dependent platelet-endothelial interactions and vasoconstriction during lung reperfusion. Subpleural arterioles, labeled platelets, and leukocytes were examined in anesthetized, open-chest rabbits by intravital fluorescence microscopy. Ischemia was caused by reversible occlusion of the right pulmonary artery for 1 or 2 h (1IR and 2IR groups). During 2 h of reperfusion, postschismic platelet rolling and adhesion were independent from leukocyte-arteriolar wall interactions and correlated with pulmonary arteriolar constriction in proportion to the length of ischemia. In rabbits treated with an iNOS inhibitor (1400W) before occlusion (2IR + 1400W group), platelet-arteriolar wall interactions and vasoconstriction were prevented. iNOS expression and activity in ischemic lung tissue were markedly greater than control and also were proportional to ischemia duration. NOS activity, immunochemically detected P-selectin, and nitrotyrosine expression in ischemic lung tissue from animals subjected to ischemia-reperfusion, as well as the plasma level of soluble P-selectin, were significantly higher than in nonischemic lungs and were inhibited by pretreatment with 1400W. These results show that platelet adhesion and arteriolar constriction during early reperfusion in the ventilated lung can result from increased iNOS activity and is highly correlated with reactive nitrogen species and P-selectin expression.

PULMONARY ISCHEMIA-REPERFUSION injury may result from trauma, atherosclerosis, respiratory distress syndrome, pulmonary embolism, and surgical procedures such as cardiopulmonary bypass and lung transplantation. Ischemia-reperfusion induces oxidative stress and is characterized by formation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals (2, 34). Ischemia-reperfusion causes platelet- and leukocyte-endothelial interactions (8, 31) and release of vasoactive substances (35) and proinflammatory cytokines (33). Injury elicited by ischemia is amplified by formation of ROS during ischemia (2, 39) and compounded by incomplete restoration of blood flow due to postschismic vasoconstriction and ventilation-perfusion mismatch (7, 26).

Platelets play an important role in postschismic hypoperfusion in tissues such as intestine, liver, pancreas, brain, and kidney (10, 23, 43). It was recently shown that ischemia-reperfusion of the ventilated lung causes platelet-endothelial interactions in postschismic pulmonary arteries during early reperfusion that correlate with arteriolar vasoconstriction (38). ROS activate platelets and cause upregulation of the adhesion molecule P-selectin, which is expressed on the surface of platelets and endothelial cells (7, 10, 27) and can lead to platelet-endothelial adhesion during pulmonary ischemia-reperfusion (38). Platelet activation and accumulation could mediate ischemia-reperfusion injury through endothelial adhesion and thrombus formation, as well as by interactions with leukocytes (8, 19). Although neutrophil activation has not been found to be necessary for development of early lung reperfusion injury (15), some studies indicate that they have a role in increased permeability of the alveolar capillary membranes and extravascular albumin accumulation during the early phase of lung reperfusion (22).

Overproduction of nitric oxide (NO) via the inducible NO synthase (iNOS) pathway is an important component in the pathogenesis of ischemia-reperfusion injury (1, 24, 42). During ischemia-reperfusion, excess NO production has been attributed to iNOS, which is not stimulated under normal conditions, but can be induced within 2 h of lung reperfusion (29) and results in upregulation of adhesion molecules (11). Normal concentrations of NO act as an inhibitory regulator of P-selectin expression (4), and inhibition of physiological NO production leads to increased platelet and leukocyte rolling and adhesion in various vascular beds (18, 21). In contrast, high and sustained flux of NO under pathophysiological circumstances leads to increased P-selectin expression (4, 29), which may lead to platelet adhesion. When NO reacts with ROS, it forms secondary reactive products such as nitrosionium cation (NO•+), nitroxyl anion (NO•–), and peroxynitrite (ONOO–) (29), which are known as reactive nitrogen species (RNS) (12).

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ONOO\(^-\), the most common form of RNS, has been suggested to be responsible for significant cytotoxic effects and platelet activation (28). Reperfusion injury in muscle tissue (32) and platelet activation in vitro (36) can be prevented by superoxide free radical scavengers. After intestinal ischemia, increased iNOS expression and NO overproduction caused pulmonary damage by nitrosylation of tyrosine residues (40).

In view of the protective and harmful roles that NO could have during ischemia-reperfusion, the emphasis of the present study was to determine whether there is a link between iNOS, RNS, P-selectin, and platelet-endothelial adhesion and microvascular constriction in the intact lung during pulmonary reperfusion. We investigated the hypothesis that ischemia-reperfusion injury increases iNOS activity and subsequent generation of RNS with P-selectin-dependent platelet-endothelial interactions and vasoconstriction during lung reperfusion.

**MATERIALS AND METHODS**

**General.** These experiments, performed acutely on male rabbits (2.2 ± 0.2 kg body wt), were approved by the Institutional Animal Care and Use Committee. Anesthesia was induced with a ketamine-xylazine mixture (1 ml/kg im; containing ketamine at 37.5 mg/ml and xylazine at 5 mg/ml) followed by aliquots of pentobarbital sodium (5 mg/kg) given slowly through an ear vein to gradually reach a loading dose of ~40 mg/kg. A catheter (PE-90) was placed in the left femoral vein for subsequent injections of supplemental doses of pentobarbital sodium (~6–10 mg/kg or 25 mg/h) to maintain an adequate level of anesthesia as defined by absence of an active corneal reflex, whisker twitching, and a pedal reflex to a toe pinch. Spontaneous breathing was suppressed at this level of anesthesia. The trachea was cannulated below the larynx, and the lungs were ventilated with air by a volume-cycled ventilator (model CTP-930, CWE, Ardmore, PA) with 2–3 cmH\(_2\)O positive end-expiratory pressure, tidal volume of ~12–14 ml/kg, and rate of ~25 cycles/min. Tracheal pressure was measured through a sideport of the tracheal catheter, which was attached to a volumetric pressure transducer (model PT-5, Grass, Quincy, MA). Tidal volume and frequency were adjusted as necessary to achieve normal blood gas values during control. A PE-90 catheter was placed in the left femoral artery to monitor arterial blood pressure by a volumetric pressure transducer (model PT-5, Grass, Quincy, MA). Tidal volume and frequency were adjusted as necessary to achieve normal blood gas values during control. A PE-90 catheter was placed in the left femoral artery to monitor arterial blood pressure by a volumetric pressure transducer (model PT-5, Grass, Quincy, MA).

**RNS and platelet adhesion during lung reperfusion.** As we described previously (38), before pulmonary artery occlusion, blood (2.2 ml/kg body wt) was removed from the femoral artery with a syringe containing 3.2% trisodium citrate (10.9 mM final citrate concentration). Platelets were separated and washed twice in calcium-free Tyrode buffer. The suspension was centrifuged, and platelets were resuspended and labeled in 1 ml of Tris-buffered saline solution (TBSS) containing 8 μl of BCECF-AM (Molecular Probes, Eugene, OR). The suspension was incubated for 15 min at 37°C with gentle agitation, and the fluorescently labeled platelets were resuspended in 1.0 ml of TBSS. Before injection into the right jugular vein, the platelets were counted and examined for the presence of aggregates. If aggregation was present, the sample was not used. After the platelets were injected, during observation in vivo, we did not see aggregates.

Labeled platelets in subpleural arterioles were observed by intravital microscopy. A fluorescence filter (450–490 nm) was placed on the light source (100-W mercury arc lamp) with a light-sensitive video camera for frame-by-frame video analysis. Platelets were classified as free-flowing, rolling, or adherent according to their interaction with the arteriolar wall. Free-flowing platelets were those that passed an imaginary line perpendicular to the vessel axis in the area of interest without apparent endothelial interaction. Adherent platelets were defined as those that did not detach from the arteriolar wall for ≥3 s and were quantified as the average number per square millimeter of vessel area over 30 s. Rolling platelets were defined as those that moved along the arteriolar wall at a much slower velocity than those in the center of the vessel and stopped periodically (for <3 s) and were quantified as the number of rolling platelets per square millimeter of vessel area over 30 s. To control for differences in the number of platelets flowing per time, platelet rolling and adherence were normalized by dividing the values by the total number of platelets that could be observed entering the vessel segment during the measurement interval (30 s) and expressed as a percentage. Observations were made in arterioles that were approximately one to two branching orders above the pulmonary capillaries (20), and platelets were counted along relatively straight sections (on average, ~250 μm long) between the visible branches. Internal arteriolar diameter was taken as the average of measurements at the proximal, middle, and distal end of the observed segment, and the area of the vessel segment in the focal plane of the objective was calculated.

In vivo fluorescence labeling of leukocytes and analysis. According to the methods of Kühnele et al. (20), rhodamine 6G dye solution in saline (0.02 g/100 ml) was injected (0.2 ml/kg body wt) into the right jugular vein. Leukocyte flux in the arteriole was studied by counting the number of leukocytes rolling per observation time (30 s). Rolling leukocytes were defined as those moving considerably slower than leukocytes in the center stream and stopping intermittently (for <3 s).

**Topical application of ONOO\(^-\) to subpleurale arterioles.** Sodium peroxynitrite (34 mM) dissolved in 0.3 N NaOH was purchased from Cayman Chemical (Ann Arbor, MI). A 100 μM solution was prepared...
by addition of 29.4 μl of sodium peroxynitrite to 100 ml of saline, and a 100-μl aliquot (1 μM) was immediately applied to the subpleural surface over an arteriole while the lung was held statically inflated. For vehicle controls, 29.4 μl of 0.3 N NaOH was added to 100 ml of saline, and a 100-μl aliquot was applied to the subpleural surface in five animals without ischemia-reperfusion during static inflation. Microvacular observations were made during a control period and after topical application of ONOO− or vehicle.

Administration of 1400W. To inhibit iNOS, 1400W (10 mg/kg) was infused (4.1 ml/h) through the catheter in the right jugular vein for 1 h before a 2-h occlusion.

Western blot analysis for iNOS. After 2 h of reperfusion, the lungs were perfused with 60 ml of ice-cold saline via the main pulmonary artery, removed from the animal, frozen in liquid nitrogen, and stored at −80°C. Lung tissue was homogenized on ice in a buffer containing Tris (25 mM), EDTA (1 mM), EGTA (1 mM), NaF (10 mM), PMSF (100 mM), aprotinin (25 μg/ml), and leupeptin (25 μg/ml). Homogenized lung samples were sonicated on ice and centrifuged at 10,000 g for 15 min. Cell membrane and cytosolic fractions were separated by ultracentrifugation of the supernatant at 100,000 g for 1 h, and the cytosolic fraction was used for Western blot analysis of iNOS. Proteins (100 μg) were separated by SDS-PAGE on a 4–12% gradient denaturing gel and electroblotted onto nitrocellulose membranes. Gel transfer efficiency and equal loading of proteins were verified by Ponceau staining of nitrocellulose membranes. The membrane was blocked for 1 h in 5% nonfat milk in Tris-buffered saline with Tween 20 (0.05%) (TBST) and incubated overnight with a rabbit polyclonal anti-iNOS primary antibody (Upstate, Lake Placid, NY; 1:1,000 dilution). After they were washed with TBST, the nitrocellulose membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technologies, Beverly, MA) and washed with TBST. Proteins were visualized by a standard chemiluminescence method (ECL, Amersham Biosciences). The membrane was probed with a mouse monoclonal anti-β-actin primary antibody (Sigma; 1:20,000 dilution) for verification of equal loading of proteins. The films were scanned by an optical scanner (UMAX Powerlook II) and quantified by measurement of the density of each band and subtraction of the background density using UN-SCAN-IT software (Silk Scientific, Orem, UT). To correct for possible unequal loading, each band’s optical density was normalized by its β-actin density.

NOS activity measurements. The radioactivity of the eluate (citrulline) was counted with a liquid scintillation counter using a NOS activity assay kit (Cayman Chemical). Lung tissue (100 mg) was homogenized in a buffer containing 25 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 1 mM EGTA and centrifuged at 10,000 g for 15 min at 4°C to obtain and preserve cytosolic and membrane fractions. Protein (40 μg) from the supernatant was incubated with a reaction buffer containing 50 mM Tris·HCl (pH 7.4), 6 μM tetrahydrobiopterin, 2 μM flavin adenine dinucleotide, 2 μM flavin adenine mononucleotide, and [14C]arginine (50 μCi/ml) for 1 h. After incubation, the reaction was stopped with a buffer containing 50 mM HEPES (pH 5.5) and 5 mM EDTA. An equilibrated resin that binds unreacted arginine was added to the reaction mixture, and the entire solution was placed into spin cups for separation of arginine and citrulline. Activity was expressed as counts per minute per 40 μg of protein. Positive and negative (no sample) controls were used to check reaction efficiency and control for background readings, respectively. Because the assay cannot distinguish iNOS activity from total NOS activity, this was accomplished by running the samples in the presence and absence of 1400W.

Nitrate/nitrite measurements. For plasma nitrate + nitrite (total NOx) measurements, blood samples (1.5 ml) were taken from the left atrium in syringes with sodium citrate (10.9 mM) and centrifuged at 2,500 g for 5 min. The supernatant was drawn off with a pipette and then subjected to ultrafiltration using a 30-kDa molecular weight cut-off filter (Millipore). The resulting ultrafiltrate was used for colorimetric detection of NOx concentration (Nitrate/Nitrite Assay kit, Cayman Chemical). The nitrate standards and each sample were added to individual wells on 96-well plates. Enzyme cofactor mixture (10 μl) and nitrate reductase (10 μl) were added to each well, and the plate was incubated at room temperature for 3 h. Griess reagents were added to each well for conversion of nitrate + nitrite to an azo dye.
compound that could be read photometrically at 540 nm. Sample NOx concentrations (µM) were based on the optical density of the azo compound and determined as [nitrate + nitrite (µM)] = [absorbance at 540 nm – y-intercept (standard curve)/slope (standard curve)] * (200 µl/80 µl) and expressed as µM nitrate + nitrite/mg protein.

**Plasma soluble P-selectin concentration.** Plasma samples were taken during baseline and after 2 h of reperfusion. Soluble P-selectin (sP-selectin) was quantitatively analyzed by using the ZyQuik sP-selectin enzyme-linked immunosorbent sandwich assay (ELISA) kit (Zymed Laboratories, San Francisco, CA) and SpectraMax M2 analyzer (Molecular Devices, Sunnyvale, CA) according to the manufacturers’ instructions. The data were expressed as sP-selectin concentration in plasma (ng/ml).

**Fluorescence immunohistochemistry for lung tissue P-selectin and nitrotyrosine expression analysis.** Sections (10 µm) were cut from snap-frozen tissue blocks, air dried for 2 h, and fixed at room temperature (20–25°C) in acetone for 10 min. After complete dehydration and removal of the media around tissue, slides were washed in 0.1 M Tris-buffered saline (TBS) for 5 min and blocked with 10% donkey serum in 0.1 M TBS + 0.3% Triton X-100 + 0.5% BSA solution for 1 h. Primary antibodies (mouse anti-P-selectin CD62P or anti-nitrotyrosine IgG1 isotype, 1:200) in 0.1 M TBS + 0.3% Triton X-100 + 0.5% BSA + 5% serum were applied for 24 h at 4°C, and the slides were rinsed for 10 min in 0.1 M TBS. After they were washed in 0.1 M TBS (3 times for 10 min each), slides were covered with fluorescently labeled secondary antibodies in 0.1 M TBS + 0.3% Triton X-100 + 5% serum and incubated in the dark at room temperature for 1 h. The slides were washed again as described above and covered using a Prolong Antifade kit (Molecular Probes). A negative control was performed by replacement of specific antibodies with nonspecific IgG1 antibodies. The expression was detected on a fluorescent microscope (Nikon Eclipse E800) with a ×10/0.30 Plan Fluor objective and an RT Color Spot camera (Diagnostic Instruments, Sterling Heights, MI). Five fields from every tissue section were randomly selected using a UV-2A filter followed by an FITC-HYQ filter. Quantitative analysis was performed using Image Pro (Media Cybernetics, Silver Spring, MD). Data are expressed in area (µm², square pixels) of marker and averaged for each group.

**Data analysis.** Arterial blood pressure, heart rate, and tracheal pressure were recorded continuously, and measurements were obtained during microvascular observations. The duration of each measurement period was ~30 s. In each animal, we examined one to three arterioles and as many major branches of these vessels as possible in the area of interest. Platelet determinations were averaged for each observation so that each animal counted equally in the analysis. Arterioles were observed during a baseline period, after treatment with 1400W, during pulmonary artery occlusion, and at 0.5, 1.0, and 2.0 h during reperfusion of the ischemic lung. Values are means ± SE. Differences between observation periods and between groups were compared by one-way ANOVA with repeated measurements and multiple comparisons (with consideration given to within-sample variability of observations). Correlation coefficient and linear regression analysis were used to determine the relation between two variables. P < 0.05 was used to indicate statistical significance.

**Experimental protocol.** The effects of pulmonary ischemia-reperfusion on platelet-endothelium interactions were examined in pulmonary arterioles during reperfusion of the ischemic lung after 1 h of occlusion (1IR, 16 arterioles in 5 animals), 2 h of occlusion (2IR, 14 arterioles in 5 animals), and 1400W treatment followed by 2 h of reperfusion (2IR + 1400W, 15 arterioles in 5 animals). Animals without occlusion (sham, n = 3) or with short occlusions (5 min, n = 3) served as controls. Subpleural arterioles in the right lung were identified during a control period and observed over 30-s periods during occlusion and reperfusion. After release of the occlusion, arterioles were examined at 0.5, 1.0, and 2.0 h of reperfusion. Fluorescently labeled platelets and rhodamine dye (leukocyte behavior studies) were usually injected ~2–10 min before release of the occlusion, and the lung was examined to confirm the absence of platelets in the pulmonary circulation. Each animal received its own labeled platelets, prepared from a blood sample taken before pulmonary artery occlusion. In six other animals, blood samples for plasma NOx were taken from the left atrium before the 2-h occlusion and during reperfusion.

**RESULTS**

For all groups (21 animals), mean arterial blood pressure and heart rate averaged 76 ± 2 mmHg and 193 ± 3 beats/min.

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**Table 1. Labeled platelets rolling and adhered during reperfusion after 2 h of ischemia**

<table>
<thead>
<tr>
<th>Reperfusion Time</th>
<th>0.5 h (n = 14)</th>
<th>1.0 h (n = 14)</th>
<th>2.0 h (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rolling</td>
<td>Adhered</td>
<td>Rolling</td>
<td>Adhered</td>
</tr>
<tr>
<td>Platelets/mm²</td>
<td>72 ± 12</td>
<td>68 ± 10</td>
<td>67 ± 9</td>
</tr>
<tr>
<td>Platelets/mm²⁻²plates flowing⁻¹</td>
<td>873 ± 162</td>
<td>729 ± 93</td>
<td>602 ± 73</td>
</tr>
<tr>
<td>Platelets/platelets flowing × 100, %</td>
<td>12.1 ± 2.2</td>
<td>10.7 ± 1.3</td>
<td>8.9 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of arterioles in 5 animals. Platelets flowing, number of labeled platelets that entered the vessel during 30-s observation period. Numbers of flowing, rolling, or adhered platelets at 0.5, 1.0, and 2.0 h of reperfusion were not significantly different.
respectively, before occlusion. After 2 h of ischemia, at 0.5, 1.0, and 2.0 h of reperfusion, blood pressure (72 ± 1, 74 ± 2, and 76 ± 2 mmHg, respectively) and heart rate (184 ± 3, 171 ± 5, and 170 ± 5 beats/min, respectively) were not significantly different from preocclusion values. Arterial PO₂, PCO₂, and pH averaged 192 ± 12 Torr, 43 ± 1 Torr, and 7.40 ± 0.01, respectively, before occlusion and were also not significantly different during reperfusion (227 ± 11 Torr, 48 ± 2 Torr, and 7.41 ± 0.01, respectively). Injection of labeled platelets and rhodamine, 1400W infusion, or blood sampling did not significantly affect blood pressure, heart rate, or arterial blood gases.

In controls (6 animals), where labeled platelet behavior was examined, in 10 arterioles with an average diameter of 47 ± 3 μm, no significant platelet rolling or adhesion was found (Fig. 1, B and C). In five animals, ischemia was induced by reversible occlusion of the right pulmonary artery for 1 h (1IR group), and 16 arterioles (average baseline diameter = 52 ± 2 μm before occlusion) were examined. During reperfusion, diameter was decreased at 0.5 h (34 ± 3 μm, \( P < 0.05 \)) but soon began to recover. At 1.0 and 2.0 h of reperfusion, diameter (45 ± 5 and 48 ± 4 μm, respectively) was not significantly different from baseline (Fig. 1A). In these arterioles, 15.0 ± 3.2%, 13.1 ± 4.0%, and 8.3 ± 2.2% of the flowing labeled platelets were rolling at 0.5, 1.0, and 2.0 h of reperfusion, but no significant platelet adhesion was observed (Fig. 1C). Significant platelet rolling occurred at all observation times and was less at 2 h than at earlier times (Fig. 1B). The number of rolling platelets was similar at 0.5 and 1.0 h but was significantly less at 2.0 h (Fig. 1B).

In five animals, ischemia was induced by reversible occlusion of the right pulmonary artery for 2 h (2IR group), and 14 arterioles (average baseline diameter = 52 ± 2 μm before occlusion) were examined. During reperfusion, diameter was decreased at 0.5 h (34 ± 3 μm, \( P < 0.05 \)) but soon began to recover. At 1.0 and 2.0 h of reperfusion, diameter (45 ± 5 and 48 ± 4 μm, respectively) was not significantly different from baseline (Fig. 1A). In these arterioles, 15.0 ± 3.2%, 13.1 ± 4.0%, and 8.3 ± 2.2% of the flowing labeled platelets were rolling at 0.5, 1.0, and 2.0 h of reperfusion, but no significant platelet adhesion was observed (Fig. 1C). Significant platelet rolling occurred at all observation times and was less at 2 h than at earlier times (Fig. 1B). The number of rolling platelets was similar at 0.5 and 1.0 h but was significantly less at 2.0 h (Fig. 1B).
arterioles (average baseline diameter = 54 ± 4 μm) were examined. In contrast to the 1IR group, diameters were significantly (P < 0.05) decreased at 0.5, 1.0, and 2.0 h of reperfusion (30 ± 3, 34 ± 4, and 38 ± 3 μm, respectively) and the amount of constriction was greater (Fig. 1A). Approximately twice as many platelets were observed rolling along the arteriolar walls at 0.5, 1.0, and 2.0 h of reperfusion than in the 1IR group (Fig. 1B). In addition, labeled platelets were adhering at 0.5, 1.0, and 2.0 h of reperfusion (Fig. 1C, Table 1). Although the number of rolling or adhering platelets tended to decrease during the course of reperfusion, the decrease was not significant (Fig. 1, B and C). In three rabbits from the 2IR group, leukocytes were also labeled (in addition to platelets) and were observed during the reperfusion times. Significantly more leukocytes were rolling than in controls (Fig. 2) and typically were rolling at different sites and at a slower velocity (Fig. 3). However, leukocyte adhesion was not observed at 2 h of reperfusion.

In the 2IR + 1400W group (n = 5), the numbers of rolling and adhering platelets were significantly decreased at 0.5, 1.0, and 2.0 h of reperfusion (Fig. 1, B and C) and postischemic vasoconstriction was prevented (Fig. 1A). In contrast, in three of these rabbits where leukocytes were also labeled, the number of rolling leukocytes was not significantly different from the three rabbits in the 2IR group without 1400W treatment (Fig. 2).

After 2 h of ischemia, total NOx in plasma samples from the left atrium was increased from 79 ± 14 μmol/mg protein before occlusion to 86 ± 15 μmol/mg protein (n = 6, P < 0.05) during early (5–15 min) reperfusion. In three of four experiments, where we also obtained samples after 2 h of reperfusion, plasma NOx was also elevated (87–110 μmol/mg protein).

Western blotting of lung tissues from rabbits subjected to 2 h of ischemia and 2 h of reperfusion showed a significant increase in iNOS expression compared with control animals (Fig. 4A). Radioimmunoassay of lung tissue indicated that ischemia-reperfusion increased total NOS activity compared with control that was prevented by pretreatment with 1400W (Fig. 4B). In lung tissue from animals subjected to 2 or 3 h of ischemia followed by 2 h of reperfusion, NOS activity increased compared with control. This increase was prevented in tissue samples from animals treated with 1400W (Fig. 4B).

Immunohistochemical examination of lung tissue after 2 h of reperfusion from the 2IR group showed a significant increase in nitrotyrosine expression compared with control and 1IR groups (Fig. 5, B and C, see Fig. 7A). There was no difference in nitrotyrosine expression between nonischemic (left) lungs and reperfused lungs from animals treated with 1400W (see Fig. 7A). To verify that RNS can cause platelet adhesion, OONO− (RNS) was applied topically to subpleural arterioles. In three animals without ischemia-reperfusion, application of 100 μl (1 μM) of OONO− caused platelet rolling and adhesion along arteriolar walls and was accompanied by microvascular constriction (see Fig. 9).

Immunohistochemistry for P-selectin in serial lung tissue sections from the frozen blocks that were used to detect nitrotyrosine showed a significant increase in P-selectin expression in the 2IR group compared with the control group (Fig. 6B). P-selectin expression in nonischemic lungs was not increased (Fig. 7B). Similarly, sP-selectin concentration in plasma samples was significantly increased in animals subjected to 2 h of ischemia and reperfusion compared with controls (Fig. 7C). Nitrotyrosine and P-selectin expression in lung tissue, as well as sP-selectin in plasma, were significantly attenuated in the 2IR + 1400W group (Figs. 5D, 6C, and 7C). The levels of nitrotyrosine or P-selectin expres-

![Fig. 5. Immunofluorescence staining of nitrotyrosine as a marker of reactive nitrogen species in lung tissue subjected to 2IR and 2IR + 1400W groups. Fluorescence (green) shows positive nitrotyrosine immunostaining. A: negative control; B: nonischemic (control) lung; C: 2IR; D: 2IR + 1400W. Greater nitrotyrosine immunostaining in 2IR group (C) was prevented by 1400W (D).](J Appl Physiol • VOL 99 • DECEMBER 2005 • www.jap.org)
sion were highly correlated with the length of ischemia \((r = 0.97\) and 0.85, respectively; Fig. 8, A and B). Moreover, when the levels of tissue P-selectin were plotted against RNS levels, the linear correlation was also high \((r = 0.89;\) Fig. 8C).

**DISCUSSION**

The present results show that postischemic platelet activation and subsequent platelet-endothelial interactions in pulmonary arterioles of the intact ventilated lung are directly proportional to the duration of pulmonary ischemia and can occur independently from leukocyte adhesion. The absence of significant platelet rolling and adhesion during control conditions confirms our previous observation (38). After 1 h of occlusion, during reperfusion, platelet rolling was significant. Platelet adhesion was not increased, and although arteriolar diameters decreased during the first 0.5 h of reperfusion, they returned to baseline by 1 h of reperfusion. However, after 2 h of ischemia, rolling increased to a greater extent and platelet adhesion was accompanied by arteriolar constriction that corresponded with the level of platelet adhesion at 1 and 2 h of reperfusion. Although increased platelet-arteriolar interactions corresponded with a greater decrease in arteriolar diameter, in the present investigation we did not address the role of platelets in vasoconstriction. In addition, during reperfusion, the decrease in arteriolar diameter is not mediated by platelet interactions only (38).

It appears that some threshold level of platelet rolling must be reached before platelet adhesion occurs, and different mechanisms for rolling and adhesion could be involved. This is consistent with results of others who found that rolling triggers upregulation of integrins, which mediate firm adhesion of platelets (3). The numbers of rolling and adhering platelets were expressed as a number per area of vessel and normalized with flow (Table 1). It is unlikely that increased platelet rolling was due only to decreased arteriolar diameter and, subsequently, a reduced number of rolling platelets, because rolling remained significantly elevated when arteriolar diameter was no longer significantly different from control (Fig. 1). Furthermore, in the 2IR group, the number of labeled platelets that entered the vessel were similar during the observation periods (Table 1). According to our observations, leukocyte rolling was a separate process from platelet-endothelial interactions, and leukocyte adhesion did not occur at 2 h of reperfusion following 2 h of ischemia.

These results confirm the finding that pulmonary ischemia-reperfusion causes platelet rolling and adhesion in subpleural arterioles, which are associated with arteriolar constriction (38), and are consistent with the work of others showing that lung ischemia-reperfusion injury consists of an early (first 2 h of reperfusion) leukocyte-independent phase (15). The lack of significant platelet activation in the control group agrees with what others have reported with regard to platelet kinetics in pulmonary microcirculation in vivo (14). This result is consistent with the finding that platelets do not normally interact with the endothelium, unless there is some level of activation or injury (6). To our knowledge, this relation between length of pulmonary ischemia, platelet adhesion, and postischemic arteriolar vasoconstriction has not been reported and indicates that

![Fig. 6. Immunofluorescence staining of P-selectin in 2IR and 2IR + 1400W groups. Fluorescence (green) shows positive P-selectin immunostaining (arrows). A: nonischemic (control) lung; B: 2IR; C: 2IR + 1400W. Note greater P-selectin immunostaining in 2IR (B) than in control (A) and 2IR + 1400W (C).](image-url)
the level of platelet adhesion is in proportion to the degree of injury and/or products produced in postischemic tissue.

Pretreatment with an iNOS inhibitor (1400W) significantly decreased platelet rolling and adhesion (Fig. 1, B and C) and vasoconstriction (Fig. 1A) but had no significant effect on early leukocyte rolling and adhesion (Fig. 2). These results suggest that platelet activation during early reperfusion in the ventilated lung is a more significant factor than leukocyte activation in contributing to postischemic arteriolar constriction and de-

Fig. 7. Effect of pulmonary ischemia on area of nitrotyrosine marker expression (A), area of P-selectin marker expression (B), and concentration of plasma (soluble) P-selectin (C) in lung tissue after ischemia-reperfusion. Only right lungs were ischemic. Values are means ± SE; n, number of animals; px², square pixels. *P < 0.05 vs. 1IR. *Significantly different from left lung (P < 0.05).

Fig. 8. Scatter diagram of lung P-selectin, and lung nitrotyrosine expression after 2 h of reperfusion in animals subjected to no ischemia or 5 min, 1 h, or 2 h of lung ischemia. Correlation coefficient (r) was obtained by linear regression; n, number of animals. A and B: correlation of nitrotyrosine and tissue P-selectin expression, respectively, with duration of ischemia. C: correlation of P-selectin expression with nitrotyrosine expression after 2 h of reperfusion.
increased alveolar perfusion. However, our results do not exclude previous observations (19) that platelet-endothelial adhesion can also involve leukocyte interactions.

Western blotting of postischemic lung tissue showed increased iNOS expression in our model of ischemia-reperfusion.Pathophysiological conditions such as hemorrhage, sepsis, acute respiratory distress syndrome, and ischemia-reperfusion are associated with increased iNOS expression (17). Intestinal ischemia-reperfusion in rats caused increased iNOS expression, NO production, and nitrotyrosine formation, which appeared to be involved in mediating lung injury (42). In the present study, treatment with 1400W prevented the increase in total NO activity, indicating that iNOS activity was a likely source of NO overproduction during ischemia-reperfusion. Macrophages, neutrophils, platelets, and endothelium produce iNOS (16) and are a possible cellular source. The increase in plasma NO that we measured in blood from the left atrium during reperfusion that is in the range reported for rats with chronic hypoxia (41) provides additional evidence for increased NO production during ischemia-reperfusion.

NO reacts with oxygen radicals, which lead to formation of ONOO$^-$ (29); this is especially relevant in the high-oxygen environment of the ventilated, but not perfused, lung. In our experiments, nitrotyrosine levels in lung tissue subjected to ischemia-reperfusion were correlated with the length of ischemia. OONO$^-$ is a selective oxidant that reacts with most biological molecules and modifies tyrosine in proteins to create nitrotyrosine, an RNS footprint, which has been detected in vivo in major pathological conditions, including ischemia-reperfusion (5, 30). The present finding that topical application of RNS (OONO$^-$) to the subpleural arterioles led to platelet adhesion to the arteriolar wall (Fig. 9) indicates that RNS could cause platelet activation. Increased iNOS activity has been suggested to have a role in platelet activation in several vascular beds via RNS formation and a P-selectin-mediated process (4, 13, 24). Thus, in the case of blood returning to the ischemic but ventilated lung, it is likely that overproduction of NO via iNOS leads to RNS formation and subsequent platelet activation through a P-selectin-dependent mechanism.

There was a consistent correlation between RNS formation and tissue P-selectin expression (Fig. 8) in the lung tissue. This expression most likely was from the endothelium, because the lungs were flushed with saline before they were harvested. We also found that the level of plasma P-selectin, which represents platelet P-selectin expression (25), increased during reperfusion. When iNOS activity was blocked by 1400W, nitrotyrosine formation was as low as in the control group. Moreover, pretreatment with 1400W led to a decrease in lung tissue and plasma expression of P-selectin. Our previous study (38) suggested that the platelet-endothelial interactions in arterioles of the reperfused rabbit lung were mediated, at least in part, by platelet P-selectin. The present data indicate that P-selectin could be involved in platelet-endothelial interactions but do not allow distinction between the contribution of platelet and endothelial P-selectin expression in this process. Moreover, we have not determined whether the endothelial P-selectin was expressed in arterioles or venules. Although leukocyte-endothelial interactions also involve selectin-dependent mechanisms and are implicated in ischemia-reperfusion-induced microvascular dysfunction (9), leukocytes did not appear to be required for the platelet adhesion observed in the present study (Fig. 3).

In summary, the results of the present experiments demonstrate that, during pulmonary ischemia-reperfusion in the intact ventilated lung, platelets roll and adhere along pulmonary arterioles in proportion to the duration of ischemia. The mechanism of those postischemic platelet-endothelial interactions appears to involve excess production of NO via iNOS and formation of RNS leading to expression of P-selectin. During lung reperfusion, platelet activation and adhesion in arterioles are associated with vasoconstriction, which would reduce alveolar perfusion. Correlation between the duration of lung ischemia, postischemic arteriolar vasoconstriction, and RNS and P-selectin strongly suggests that NO overexpression by iNOS may induce formation of RNS and increase platelet and endothelial P-selectin expression during lung ischemia-reperfusion (Figs. 7 and 8). Increased platelet-endothelium interactions may result from endothelial dysfunction as well as platelet activation. Other studies are needed to determine the contribution of endothelial and platelet P-selectin and other adhesion molecules to this process. Our study shows that, during reperfusion of the ischemic lung, inhibition of platelet-endothelium interactions by prevention of RNS formation could help improve alveolar blood flow.
GRANTS

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REFERENCES


