

Critical Role of Reactive Nitrogen Species in Lung Ischemia-Reperfusion Injury

Babu V. Naidu, FRCS,^a Charles Fraga, MSc,^a Andrew L. Salzman, MD,^b Csaba Szabo, MD,^b Edward D. Verrier, MD,^a and Michael S. Mulligan, MD^a

Background: Peroxynitrite is a potent cytotoxic free radical produced by the reaction of nitric oxide with the superoxide ion produced in conditions of oxidative stress. The purpose of the study was to examine the role of this reactive nitrogen species in lung ischemia-reperfusion injury.

Methods: Left lungs of male Long-Evans rats were rendered ischemic for 90 minutes and reperfused for up to 4 hours. Treated animals received FP-15 (a water-soluble iron containing metalloporphyrin that acts as a peroxynitrite decomposition catalyst). Injury was quantitated in terms of tissue neutrophil accumulation (myeloperoxidase content) and vascular permeability (¹²⁵I bovine serum albumin [BSA] extravasation) and bronchoalveolar lavage cytokine, transcriptional factor and leukocyte content. Separate tissue samples were processed for immunohistology and nuclear protein analysis.

Results: Lung vascular permeability was reduced in treated animals by 61% compared with control animals ($p < 0.005$). The protective effects of enhanced peroxynitrite decomposition correlated with a 72% reduction in tissue myeloperoxidase content ($p < 0.001$) and marked reductions in bronchoalveolar lavage leukocyte accumulation. This correlated positively with the diminished expression of pro-inflammatory chemokines and nuclear transcription factors.

Conclusions: The deleterious effects of lung ischemia-reperfusion injury are in part mediated by the formation of peroxynitrite, as enhanced decomposition of this species is protective in this model. The development of potent water-soluble decomposition catalysts represents a potentially useful therapeutic tool in the prevention of lung ischemia-reperfusion injury after lung transplantation. *J Heart Lung Transplant* 2003; 22:784-793.

Over the past decade, nitric oxide (NO) has emerged as a fundamental signaling molecule critical to the regulation of a vast array of physiologic

functions. However, NO production also results in the elaboration of nitrogen-centered free radicals, specifically peroxynitrite (ONOO⁻), which is a ma-

^aDivision of Cardio-thoracic Surgery, Department of Surgery, University of Washington, Seattle, Washington; and ^bInotek Corporation, Beverly, Massachusetts.

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Poster presented at the meeting of the International Society of Heart and Lung Transplantation, Washington, DC, April 2002. Reprint requests: Michael S. Mulligan, MD, University of Wash-

ington, Box 356310, 1959 NE Pacific Street, Seattle, Washington 98195.

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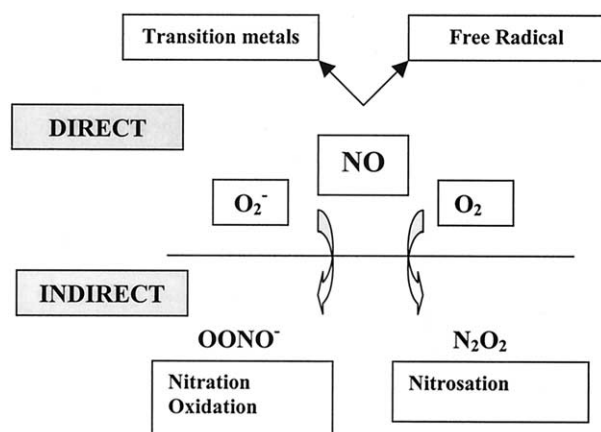


FIGURE 1 The chemistry of nitric oxide (NO). Direct effects prevail with low and brief NO production. Indirect effects occur under high and sustained flux of NO, resulting in the formation of reactive nitrogen intermediates.

major cytotoxic effector.¹ From a therapeutic viewpoint, such a paradoxical fate of NO is troublesome. Whether peroxynitrite production predominates is dependent on both the flux of NO and the surrounding chemical milieu.²

It is convenient to categorize the chemical reactions of NO into direct and indirect. Generally, the direct effects of NO prevail with low and brief NO production and are usually involved in protective and signaling functions encountered under normal physiologic conditions. In contrast, indirect effects occur under high and sustained flux of NO under pathophysiologic circumstances. These indirect effects result in the formation of peroxynitrite and dinitrogen trioxide from the

interaction of NO with the superoxide radical and oxygen, respectively (Figure 1). Peroxynitrite is a potent cytotoxin with a number of deleterious effects (Table I).

Lung ischemia–reperfusion (LIRI) injury occurs in up to 25% of human lung transplant recipients.³ In LIRI, the role of NO and the associated family of derived molecules, collectively termed reactive nitrogen intermediates (RNIs), is unclear. Exogenous administration of NO has been reported by some to be protective in animal models of LIRI.^{4,5} However, administration of NO at the time of reperfusion is associated with an early rise in endothelial dysfunction as characterized by an increase in vascular permeability.⁴ This could relate to the early interaction of nitric oxide with reactive oxygen species, because it is prevented by superoxide free radical scavengers.

The recent development of peroxynitrite decomposition catalysts has produced not only an exciting potential clinical therapeutic option, but also a useful tool in basic science research. These agents, generally iron-containing metalloporphyrins, are water-soluble, potent and specific to peroxynitrite decomposition. All these characteristics increase the attractiveness of these compounds for clinical application. With these tools, peroxynitrite has been implicated in cardiac and splanchnic ischemia–reperfusion injury.^{6–8}

Therefore, knowing that exogenous administration of NO exacerbates the early rise in vascular injury, that this is blocked by superoxide free radical scavengers, and that peroxynitrite forms from the reaction of NO and superoxide, we hypothesized that peroxynitrite formation is critical to the initiation of LIRI.

TABLE I Peroxynitrite: targets and biologic actions

Target	Effect	Biologic consequence
Oxidation		
DNA	DNA strand breakage	Activate PARS
Thiols	Glutathione and Zn finger protein inhibition	Reduced anti-oxidant, transcription factors
Lipids	Peroxydation	Membrane damage
Mitochondria	Inhibits aconitase and complexes I, II and V	Inhibition of cell respiration
Nitration		
MnSOD	Inhibition	Reduced superoxide dismutation
PGI ₂ synthase	Inhibition	Reduced anti-aggregation and vasodilation
Cytoskeleton	Altered structure	Disorganization of cell architecture
Kinase substrates	Altered structure	Disturbed tyrosine kinase signaling

PARS, poly-adenosine diphosphate-ribose synthetase.

MATERIALS AND METHODS

Reagents

All reagents were purchased from Sigma Chemical Co. (St Louis, MO), unless otherwise specified.

Animal Model

Pathogen-free adult male Long-Evans rats (Simonsen Labs, Gilroy, CA), weighing 280 to 320 g, were used for all experiments. All animals received humane care in compliance with the *Guidelines for Care and Use of Laboratory Animals*, published by the National Society for Medical Research and the NIH. Animals were anesthetized with 30 to 35 mg of intraperitoneal pentobarbital, shaved and prepped. A 14-gauge angiocatheter was inserted into the trachea through a mid-line neck incision and secured with a 4-0 braided suture. Animals were then placed on a rodent ventilator (Harvard Apparatus, Inc., Holliston, MA) with an inspired oxygen content of 60%, at a rate of 80 breaths per minute and 2 cmH₂O of positive end-expiratory pressure. Maximal peak pressures were maintained at below 10 cmH₂O. All animals received 0.4 mg of atropine intramuscularly and anesthesia was maintained with inhaled halothane. Dissection was conducted using an operating microscope and a warming blanket was placed underneath the animals throughout the experiment. A left anterolateral thoracotomy in the fifth intercostal space was performed. The left lung was mobilized atraumatically and the inferior pulmonary ligament was divided sharply. Animals then received 50 U of heparin intravenously (IV) in saline (total volume 500 μ l). Five minutes after heparin was administered, the left pulmonary artery, veins and main stem bronchus were occluded with a non-crushing microvascular clamp. During the experiment, the lungs were kept moist with periodic application of topical warm normal saline and the incision was covered to minimize evaporative losses. The period of ischemia was constant at 90 minutes. At the end of the ischemic period the clamp was removed from the hilum and the lung was allowed to ventilate and reperfuse for periods of up to 4 hours. Animals were administered 0.5 ml of warm subcutaneous saline per hour to maintain hydration during the experiment. At the end of the reperfusion period, a mid-line incision from the neck to the pubis was made to allow access to the chest and abdomen. Blood samples were obtained from the inferior vena cava just before killing. The heart-lung block was rapidly excised and pulmonary circulation was flushed through the main pulmonary artery with

20 ml of normal saline. The lungs were separated from the heart and mediastinal tissues and then analyzed as outlined in what follows. Time-matched sham control animals underwent the same procedure, except the microvascular clamp was not applied to the hilum (sham/thoracotomy alone).

Treated animals received 3 mg/kg IV of the peroxyntirite decomposition catalyst (FP-15) 30 minutes before the application of the clamp or 30 minutes into reperfusion.

Lung Permeability Index

To quantitate lung injury secondary to ischemia and reperfusion, a lung permeability index was determined in the following manner. ¹²⁵I-radiolabeled bovine serum albumin (¹²⁵I BSA) was obtained from NEN Life Sciences (Boston, MA). Before use of the ¹²⁵I BSA in vivo, serial dilutions were performed to obtain an activity of 800,000 counts per minute (cpm) per dose. This volume of ¹²⁵I BSA, approximately 2 μ l of the stock solution, was then brought to a final volume of 500 μ l in a 1% BSA/phosphate-buffered saline solution. Five minutes before removal of the hilar clamp, or at an equivalent time in sham animals, the ¹²⁵I BSA preparation was injected intravenously. Immediately before killing, 1 ml of blood was drawn from the inferior vena cava. The heart-lung block was then excised and flushed as described previously. Radioactivity was then quantitated for the left and right lung as well as the inferior vena cava blood using a gammacounter. The permeability index was then expressed as the ratio of the cpm in the left lung to the cpm in 1.0 ml of inferior vena caval blood:

$$\text{Permeability index} = \frac{\text{left lung (cpm)}}{1.0 \text{ ml blood (cpm)}}$$

This ratio corrected for any variation in systemic blood levels of radioactivity and provided a reproducible measure of lung microvascular permeability.

Myeloperoxidase Assay

Tissue myeloperoxidase (MPO) content was used to quantitate neutrophil accumulation in the lungs. After flushing the pulmonary circulation, the lungs were homogenized for 1 minute in a solution of 0.5% hexadecyltrimethylammonium bromide (HTAB) and 5 mmol/liter ethylene-diamine tetraacetic acid (EDTA) in 50 mmol/liter potassium phosphate buffer (pH 6.0). Samples were then sonicated for 40 seconds in 4 10-second bursts. The homogenized tissue was maintained on ice between

all tissue-processing activities. Samples were then centrifuged at 2,300g for 30 minutes at 4°C and the supernatants were recovered. Assay buffer was composed of 0.005% H₂O₂ and 0.167 mol/liter *O*-dianisidine dihydrochloride in 100 mmol/liter potassium phosphate buffer (pH 6.0). Fifty microliters of each sample was mixed with 1.45 ml of assay buffer and the change in absorbance at 460 nm over 1 minute was recorded.

Bronchoalveolar Lavage

Additional animals underwent bronchoalveolar lavage (BAL) at the time of killing. Through an extended median sternotomy, a 14-gauge angiocatheter was placed and the lungs were lavaged individually with 3.0 ml of cold sterile saline. Individual lung BAL analysis was accomplished by clamping the contralateral hilum. At least 80% of the instilled fluid was recovered from each lung. This fluid was centrifuged (1,500g for 8 minutes at 4°C) to pellet the cells. The supernatant was snap frozen for later cytokine analysis after the addition of a protease cocktail inhibitor (leupeptin 1 µg/ml, aprotinin 1 µg/ml, trypsin inhibitor 5 µg/ml, pepstatin A 1 µg/ml). The red blood cells were lysed and the pellet was re-suspended in normal saline. Cells were then counted and a differential performed using a hemacytometer (Hausser Scientific, Reading, PA).

Enzyme-Linked Immunoassay

Sandwich enzyme-linked immunoassays (ELISAs) for MIP-2, CINC, MIP-1 α and RANTES were developed by adding 50 µl of a 10-µg/ml anti-chemokine antibody (Peprotech, Rocky Hills, NJ) in a carbonate-coating buffer solution (pH 9.6) to a 96-well (Dynex) immunoassay plate. The plate was incubated overnight at 4°C and washed with phosphate-buffered saline (PBS) with 0.05% Tween. Bovine serum albumin (1%) in PBS was used to block (30 minutes at 37°C) non-specific binding. Samples and standards were diluted in saline and 50 µl added to each well (1-hour incubation at 37°C). A secondary biotinylated antibody (Peprotech), specific to the epitope being studied (0.5 to 2 µg/ml), was added to each well (1-hour incubation at 37°C). Following a 30-minute incubation with a streptavidin-horseradish-peroxidase conjugate (Pierce, Rockford, IL), the assay was developed by adding *o*-phenylenediamine dihydrochloride substrate. The reaction was stopped by adding 50 µl of 3-mol/liter H₂SO₄. Tumor necrosis factor- α (TNF- α) ELISA was performed according to the manufacturer's guidelines (R&D). The linear sensitivity range of the assays was determined and the assays showed no cross reac-

tivity with one another. Samples and standards were run in triplicate, and well-to-well variation did not exceed 5%.

Electrophoretic Mobility Shift Assay

Additional left lungs were snap frozen at the end of the experimental protocol. These frozen tissue samples were ground to a fine powder and then suspended in 4.0 ml of buffer containing 0.06% Nonidet P-40, 150 mmol/liter NaCl, 10 mmol/liter HEPES, 1 mmol/liter EDTA and 0.5 mmol/liter PMSF. The solution was then homogenized and centrifuged for 15 seconds at 12,000g. The pellet was discarded and the supernatant was cooled to 4°C. The supernatant was then centrifuged again for 15 seconds (12,000g). The resultant pellet was suspended in 40 µl of buffer containing 40 mmol/liter NaCl, 20 mmol/liter HEPES, 0.2 mmol/liter EDTA, 1.2 mmol/liter MgCl₂, 0.5 mmol/liter PMSF, 0.5 mmol/liter DDT, 25% glycerol, 5 µg/ml aprotinin and 5 µg/ml leupeptin at 4°C for 20 minutes. This solution was then centrifuged for 5 minutes, the pellet was discarded, and the supernatant containing the nuclear protein was stored at -70°C. Quantification of nuclear protein was performed using the bichinoic acid assay (BCA).

Ten micrograms of nuclear protein was incubated in a binding reaction with either double-stranded ³²P end-labeled oligonucleotide containing the nuclear factor (NF)- κ B-binding sequence or the AP-1 consensus sequence (Promega, Madison, WI). The binding reaction was carried out at room temperature for 60 minutes and the proteins were resolved on a 6% non-denaturing polyacrylamide gel at 100 V for 1 to 2 hours. The gels were dried and autoradiographed. Triplicate samples for each specimen were analyzed. Densitometry was performed with IMAGE J software (version 1.2) to assess relative signal intensity.

Immunocytochemistry

Whole lung tissue specimens were fixed in formalin and dehydrated, cleared, infiltrated and embedded in paraffin. Serial sections (5 µm) were cut and baked overnight at 50°C. In preparation for immunocytochemistry (ICC), sections were de-waxed and re-hydrated through graded alcohols to a final distilled water wash. Antigen retrieval with steam and target unmasking solution antibody (Vector Laboratories, Burlingame, CA) was performed as recommended by the manufacturer. After the blocking step, the primary anti-nitrotyrosine antibody (5 µg/ml) (Upstate, Waltham, MA) was incubated over-

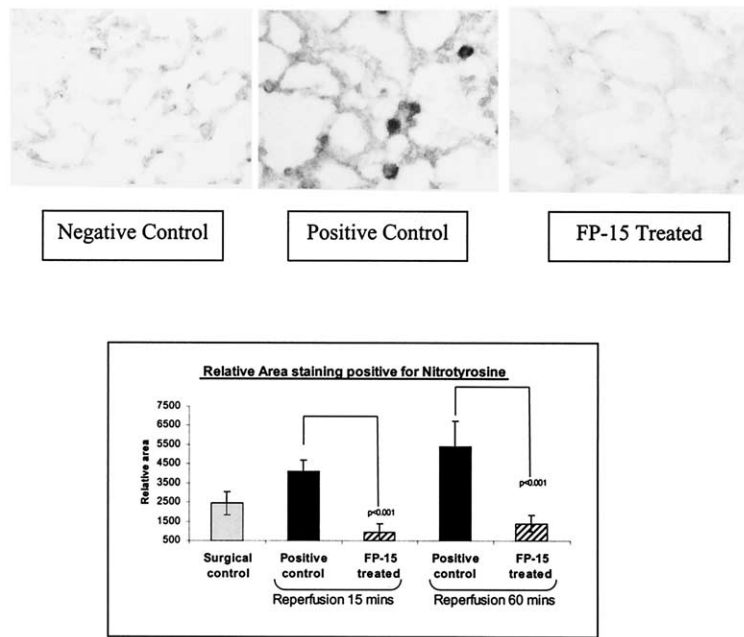


FIGURE 2 Nitrotyrosine staining (“footprint” of peroxynitrite). The panel showing left lung sections demonstrates minimal staining in unmanipulated negative controls and strong brown staining in positive controls undergoing 90 minutes of ischemia followed by 15 minutes of reperfusion. This is predominantly localized to the alveolar macrophage and is markedly reduced by FP-15 treatment. The graph depicts quantification of this reduction at 15 and 60 minutes of reperfusion.

night at 4°C followed by stock anti-rabbit secondary antibody (Vector Laboratories), then in an avidin-biotin complex and finally in diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with methylgreen and then dehydrated, cleared and mounted with permanent mounting media. Stained sections were examined using image analysis software, IMAGE PROPLUS (Media Cybernetics, Silver Spring, MD). Ten random fields were acquired by digital photography per sample at uniform magnification and light intensity. Analysis was automated by calibrating and establishing a threshold for positive staining based on intensity and applying it to all the samples. The values acquired by the software therefore represent a total area per field staining positively.

RESULTS

Administration of FP-15 Reduces Formation of Nitrotyrosine in LIRI

Immunohistochemistry was used to localize formation of peroxynitrite by staining for nitrotyrosine. In these studies, 5 experimental groups were studied: sham controls that underwent a thoracotomy but no surgical intervention, and animals undergoing lung

ischemia and 15 or 60 minutes of reperfusion, in both untreated and animals treated with 3 mg/kg FP-15 ($n = 3$).

Sham controls showed minimal staining for nitrotyrosine. Positive controls at both 15 and 60 minutes of reperfusion stained strongly positive. At 15 minutes of reperfusion, the alveolar macrophage was the predominant site of nitrotyrosine staining (arrows in Figure 2a). At 60 minutes, this pattern was still evident, but in addition there was a more diffuse parenchymal distribution (image not shown). FP-15 pre-treatment significantly reduced the intensity of staining by 76.9% ($p < 0.001$) and 73.9% ($p < 0.001$), as quantified by image analysis software (Figure 2b) at 15 and 60 minutes, respectively. Reduced formation of nitrotyrosine so early in reperfusion strongly suggests that FP-15 was effective in enhancing decomposition of peroxynitrite.

FP-15 Inhibits Nuclear Translocation of NF- κ B and AP-1

Injury in this model is associated with activation of transcription factors.²⁹ To study this, nuclear protein was extracted from lung homogenates from sham controls, injured positive controls that underwent 90

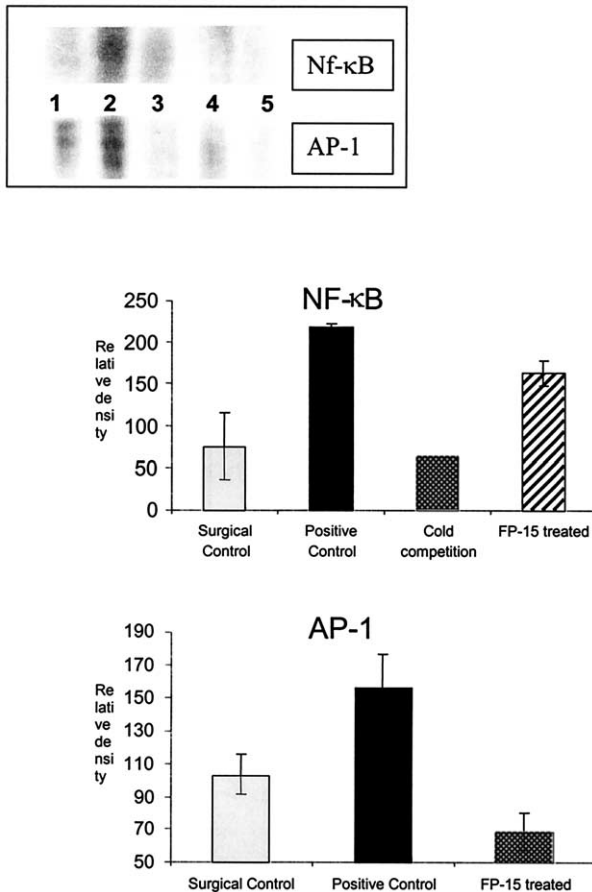


FIGURE 3 EMSA investigating nuclear protein expression of NF-κB and AP-1. The far two left lanes of both gels show a representative unmanipulated negative control lung and a positive control lung that underwent ischemia followed by 15 minutes of reperfusion illustrating activation of both factors. The third through fifth lanes demonstrate marked reductions in nuclear translocation of NF-κB and AP-1 in three separate animals pre-treated with FP-15. This was confirmed by Densitometric analysis using Image J software and is depicted graphically.

minutes of ischemia followed by 15 minutes of reperfusion, and similar positive controls that were treated 30 minutes before injury with FP-15. This timepoint was chosen because preliminary experiments demonstrated that this was when peak early expression of NF-κB and AP-1 occurred (data not shown). There was an intensely staining band in samples from lungs subjected to ischemia and 15 minutes of reperfusion in animals that underwent gel electrophoresis (Figure 3a). Cold competition with unlabeled binding oligonucleotide abolished this signal, demonstrating that this band as being specific to NF-κB (Figure 3b). Pre-treatment with FP-15 significantly reduced the inten-

sity of this band, demonstrating reduced nuclear translocation of NF-κB (Figure 3b). Similar patterns of expression and reduction associated with FP-15 pre-treatment were demonstrated for AP-1. The reduction in translocation of NF-κB and AP-1 with enhanced peroxynitrite decomposition therefore clearly suggests that peroxynitrite plays a key role in the activation of both these transcription factors in this injury model.

FP-15 Reduces Pro-Inflammatory Chemokine Elaboration in LIRI

To demonstrate whether a reduction in transcription factors is associated with reduced elaboration of inflammatory mediators, the expression of several pro-inflammatory chemokines and cytokines was analyzed in the lavage fluid of injured lungs. MIP-2, CINC, MIP-1α and TNF-α were studied because we have shown that neutralizing antibodies directed against these mediators protects against injury (findings submitted for publication). Three groups were studied, including negative unmanipulated controls; positive controls that underwent 90 minutes of ischemia, followed by 4 hours of reperfusion; and similar positive controls that, in addition, were treated with FP-15. All 4 of these mediators were significantly elevated in the bronchoalveolar fluid (BAL) fluid of injured left lungs (Figure 4). Treatment with FP-15 reduced expression of all 3 chemokines: MIP-2 by 72% ($p < 0.003$); CINC by 60% ($p < 0.03$); and MIP-1α by 89% ($p < 0.05$). However, at 4 hours of reperfusion, expression of TNF-α was unaffected by pre-treatment with FP-15 (Figure 4).

Enhanced Decomposition of Peroxynitrite Reduces Severity of LIRI

To determine whether the reduction in transcription factor activation and inflammatory mediator release were associated with reductions in tissue injury, 2 parameters were studied: vascular permeability (permeability index) and tissue neutrophil accumulation (MPO content and BAL cell count).

Changes in Lung Vascular Permeability (Figure 5a)

Five different groups were generated: Negative controls did not undergo any surgical manipulation. A second group underwent ischemia for 90 minutes, followed by 4 hours of reperfusion. Three additional groups were also subjected to this injury but were also treated as follows: FP-15, 30 minutes before injury; and FP-15, 15 minutes into reperfusion or as group inactive catalyst 30 minutes before reperfusion. A marked increase in permeability was seen in animals that underwent 4 hours of reperfusion after

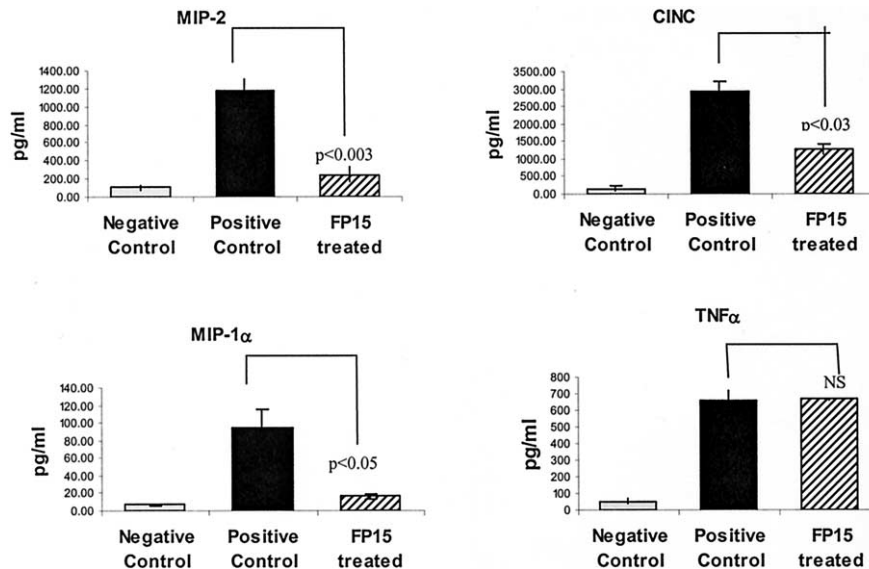


FIGURE 4 ELISA for chemokines MIP- α , CINC, MIP-1 and cytokine TNF α . This panel of graphs demonstrates the marked increases in chemokine and cytokine content in lavage fluid from injured left lungs. There are significant decreases in concentration of all three chemokines but not with TNF α in a lavage fluid from FP-15 pre-treated animals.

90 minutes of ischemia (0.82 ± 0.02) compared with the unmanipulated control groups (0.09 ± 0.01) ($p < 0.001$). Permeability values for positive controls that received inactive catalyst were not significantly different from untreated positive controls. Similarly, there was no significant benefit of FP-15 administered 15 minutes into reperfusion (0.76 ± 0.19). However, FP-15 given before injury markedly reduced vascular permeability by 61% (0.38 ± 0.04) ($p < 0.005$). As expected, only FP-15 given before injury offered protection against injury, because ONOO $^-$ is generated within minutes of reperfusion.

Myeloperoxidase Activity (Figure 5b)

Myeloperoxidase activity was measured in lungs from unmanipulated controls, positive controls (90 minutes of ischemia followed by 4 hours of reperfusion) and positive controls pre-treated with FP-15 at 30 minutes before injury. As a measure of tissue neutrophil content, the change in absorbance at 460 nm over 1 minute in unmanipulated controls was 0.04 ± 0.01 , and in positive controls was 0.44 ± 0.05 . FP-15-treated animals demonstrated a 72% reduction (0.16 ± 0.021) ($p < .001$) in tissue neutrophil sequestration.

BAL Analysis (Figure 5c)

BAL analysis was undertaken to evaluate leukocyte content in the distal airways and alveolar compart-

ment. Leukocyte counts were recorded in the same 3 groups. A statistically significant increase in BAL leukocyte content was seen between negative and positive controls (0.85 ± 0.16 to $21.8 \pm 2.1 \times 10^{-6}$ cells) ($p < 0.001$). The predominant cell type at the end of the reperfusion period was the neutrophil (98%). As seen with tissue neutrophil accumulation, bronchoalveolar cell count was reduced in FP-15-treated animals by 72% ($6.6 \pm 1.6 \times 10^{-6}$ cells) ($p < 0.001$).

DISCUSSION

The deleterious effects of lung ischemia-reperfusion injury are in part mediated by the formation of peroxynitrite. The enhanced decomposition of this species is not only protective against injury but reduces the transcription of pro-inflammatory chemokines. Although the apparent lack of effect on TNF- α secretion may seem surprising, a number of reasons may account for this. Initially, the source of peroxynitrite is localized to the alveolar macrophage (AM). We and others have demonstrated that the AM is the central cell in coordinating reperfusion injury.^{9,10} We have also shown that macrophages are the early source of TNF- α in this model. It is therefore highly probable that this early secretion of TNF- α is affected, but the late secretion from other resident cells, and also migrated leukocytes, is not susceptible.

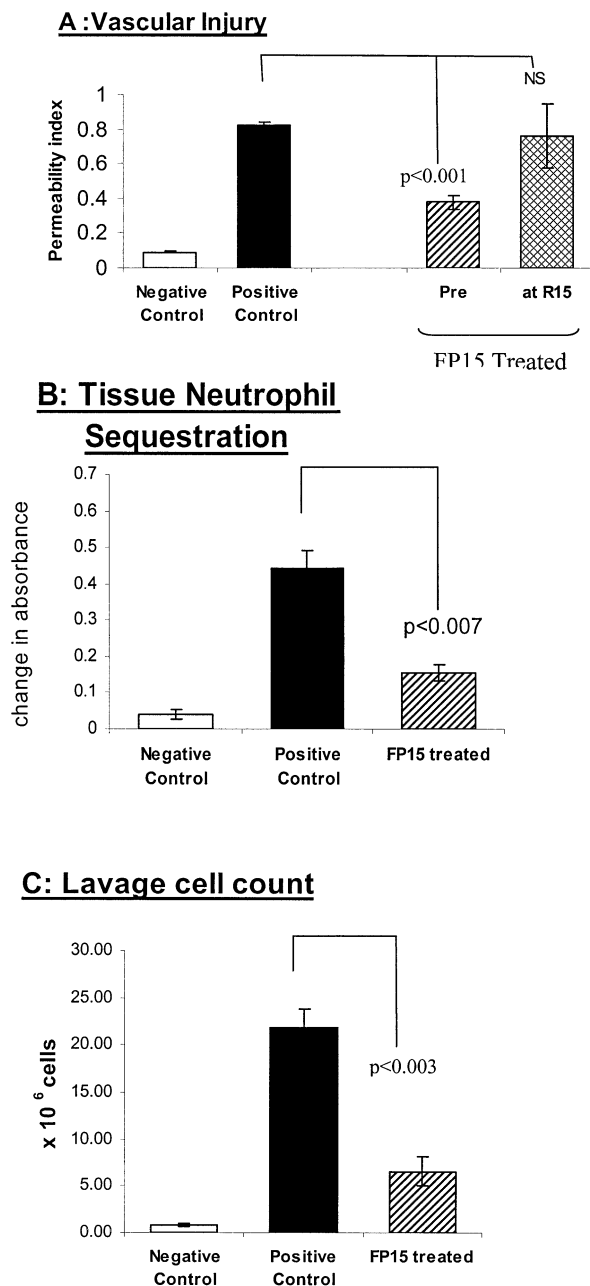


FIGURE 5 A. Left lung permeability in control and treated animals. Lung permeability index is defined as the counts per minute in the left lung divided by the counts per minute in one milliliter of inferior vena cava blood. There is a seven-fold increase in lung vascular permeability in animals that undergo ischemia and reperfusion when compared to unmanipulated animals ($p < 0.001$). In animals receiving FP-15, thirty minutes into reperfusion there is no significant difference compared to injured positive controls. However, when FP-15 is administered 15 minutes prior to injury, there is a marked reduction in vascular injury (ANOVA $p < 0.001$ $n = 4$). B. Myeloperoxidase content. MPO is

Peroxynitrite forms as a result of the reaction of superoxide (generated early in reperfusion) and nitric oxide produced by nitric oxide synthase (NOS). The changes in expression and activity of NOS in this injury have been reported to be both up- and downregulated.¹¹⁻¹³ This paradox may be explained in part by the differences in the type of animal model employed, namely ex vivo isolated perfused lung, in vivo warm ischemic hilar clamp or single-lung isortransplant.^{8,14,15} In addition, differences may arise due in part to the type of perfusate used and the pH changes associated with the use of NOS inhibitory drugs. The administration of co-factors of NOS (tetrahydrobiopterin) has been associated with reduced lung injury, and some investigators have suggested that this is in part due to enhanced NO production.¹⁶ Ultimately, the important determinant of NO's protective or injurious effects likely relates to the balance of products elaborated.

It has been suggested that the injurious effects of NO leading to tissue injury are secondary to peroxynitrite production. However, no direct evidence has been published linking peroxynitrite to the development of LIRI. A suggested role for peroxynitrite in an isolated perfused model was indirectly suggested.¹⁷ However, this model is certainly not physiologic. The present study is the first to demonstrate protection against LIRI with enhanced decomposition of peroxynitrite.

Peroxynitrite's diverse injurious effects may be direct or indirect. Lipid peroxidation is initiated directly by peroxynitrite extracting a hydrogen atom from polyunsaturated fatty acids, resulting in the formation of lipid hydroperoxy radicals. These compounds then further propagate free radical reactions. Peroxynitrite irreversibly inhibits mito-

measured as the change in absorbance at 460 nm over one minute. The increase in MPO content at 4 hours of reperfusion was statistically different from unmanipulated controls ($p < 0.002$). Tissue neutrophil accumulation was decreased in FP-15 animals. C. Bronchoalveolar lavage cell count increased significantly in those animals undergoing ischemia followed by reperfusion compared to unmanipulated controls ($p < 0.005$). The predominant cell type in the unmanipulated animals was the alveolar macrophage and the majority of the cells in the reperfused lungs were neutrophils. A significant decrease in alveolar leukocyte sequestration was also noted in animals receiving FP-15.

chondrial respiratory enzyme complexes (1,2,5- and cis-aconitase). In addition, peroxynitrite also impairs cellular function by inducing DNA damage and activating the nuclear enzyme, poly-ADP-ribose synthetase (PARS). PARS covalently attaches ADP-ribose to various nuclear proteases and rapidly depletes the cellular NAD stores, slowing glycolysis, electron transport and ATP formation, resulting in further cellular dysfunction. Nuclear transcription factors once ribosylated by PARS bind more avidly to their sequence-specific DNA targets, potentiating the pro-inflammatory signal.¹⁸ Inhibitors of PARS reduce tissue injury in models of splanchnic and myocardial reperfusion.^{19,20} Peroxynitrite may also indirectly potentiate injury by reacting with glutathione and thus disabling important protective anti-oxidant mechanisms.

The biologic marker of nitrosative stress used in this study was the formation of the stable product of tyrosine nitration. Nitrotyrosine may also be formed by the reaction of nitrite with the enzyme myeloperoxidase (MPO) or its product, HOCl.²¹ A substantial rise in MPO will occur only after the first hour of reperfusion, associated with significant tissue neutrophil sequestration. By studying nitrotyrosine formation at earlier timepoints, this possible confounding factor was minimized. Increasingly, it is being appreciated that tyrosine nitrosylation is not a random process, but occurs on distinct proteins. Pathophysiologic roles for nitrated proteins have been suggested in human kidney rejection,²² amyotrophic lateral sclerosis,²³ Parkinson's disease²⁴ and cytoskeletal changes in the endothelium after inflammatory stimuli.²⁵ Even more interest has focused on the reversible *s*-nitrosylation of cysteine residues, which have been proposed to affect a number of redox-sensitive signaling pathways; for example, p21 *ras*, MAP kinases,²⁶ and the inhibition of protein phosphatases.²⁷ Furthermore, cysteine nitration of the transcription factor NF- κ B, and thus its activation, may contribute to the pathologic effects of peroxynitrite in the regulation of pro-inflammatory cytokines and chemokine expression.²⁸ In this study we have shown that FP-15 pre-treatment results in a reduction in NF- κ B activation early in reperfusion, suggesting that peroxynitrite may indeed act directly on transcription factors *in vivo*. Furthermore, we have previously demonstrated, using the same model, that calcineurin inhibition of NF- κ B is highly protective against injury.²⁹ This finding clearly suggests that another potential mechanism of protection with enhanced decomposition of peroxynitrite is mediated in part by transcriptional modulation.

The major decay pathway for peroxynitrite is isomerization to the harmless nitrate ion. Certain water-soluble iron (III) porphyrins are highly active in catalyzing this isomerization, probably by the production of an oxo iron (IV) intermediate.³⁰ In this study we have demonstrated the utility of a peroxynitrite decomposition catalyst, FP-15, in a rat model of warm ischemia of the lung. The clinical use of this class of compound to reduce reperfusion injury after lung transplantation holds promise.

REFERENCES

1. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 1996;271:C1424-37.
2. Liaudet L, Soriano FG, Szabo C. Biology of nitric oxide signaling. *Crit Care Med* 2000;28(suppl 4):N37-52.
3. Meyer BF, Lynch J, Trulcock EP, Guthrie TJ, Cooper JD, Patterson GA. Lung transplantation: a decade of experience. *Ann Surg* 1999;230:362-7.
4. Eppinger MJ, Ward PA, Jones ML, Bolling SF, Deeb GM. Disparate effects of nitric oxide on lung ischemia-reperfusion injury. *Ann Thorac Surg* 1995;60:1169-75.
5. Okabayashi K, Triantafyllou AN, Yamashita M, Aoe M, DeMeester SR, Cooper JD, Patterson GA. Inhaled nitric oxide improves lung allograft function after prolonged storage. *J Thorac Cardiovasc Surg* 1996;112:293-9.
6. Cuzzocrea S, Zingarelli B, Costantino G, et al. Beneficial effects of 3-aminobenzamide, an inhibitor of poly(ADP-ribose) synthetase in a rat model of splanchnic artery occlusion and reperfusion. *Br J Pharmacol* 1997;121:1065-74.
7. Thiemeermann C, Bowes J, Myint FP, et al. Inhibition of the activity of poly(ADP ribose) synthetase reduces ischemia-reperfusion injury in the heart and skeletal muscle. *Proc Natl Acad Sci USA* 1997;94:679-83.
8. Zingarelli B, Cuzzocrea S, Zsengeller Z, et al. Protection against myocardial ischemia and reperfusion injury by 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthetase. *Cardiovasc Res* 1997;36:205-15.
9. Fiser SM, Tribble CG, Long SM, Kaza AK, Cope JT, Laubach VE, Kern JA, Kron IL. Lung transplant reperfusion injury involves pulmonary macrophages and circulating leukocytes in a biphasic response. *J Thorac Cardiovasc Surg* 2001;121:1069-75.
10. Naidu B, Krishnadasan B, Byrne K, Fraga C, Thomas B, Verrier ED, Mulligan MS. Essential Role of the alveolar macrophage in lung ischemia reperfusion injury. Abstract presented at the Society of University Surgeons, Honolulu, Hawaii, February 2002.
11. Vural KM, Oz MC. Endothelial adhesivity, pulmonary hemodynamics and nitric oxide synthesis in ischemia-reperfusion. *Eur J Cardiothorac Surg* 2000;18:348-52.
12. Liu M, Tremblay L, Cassivi SD, Bai XH, Mourgion E, Pierre AF, Slutsky AS, Post M, Keshavjee S. Alterations of nitric oxide synthase expression and activity during rat lung transplantation. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L1071-81.
13. Lu YT, Liu SF, Mitchell JA, Malik AB, Hellewell PG, Evans TW. The role of endogenous nitric oxide in modulating

- ischemia–reperfusion injury in the isolated, blood-perfused rat lung. *Am J Respir Crit Care Med* 1998;157:273–7.
14. Kantrow SP, Huang YC, Whorton AR, Grayck EN, Knight JM, Millington DS, Piantadosi CA. Hypoxia inhibits nitric oxide synthesis in isolated rabbit lung. *Am J Physiol* 1997;272:L1167–73.
 15. Moore TM, Khimenko PL, Wilson PS, Taylor AE. Role of nitric oxide in lung ischemia and reperfusion injury. *Am J Physiol* 1996;271:H1970–7.
 16. Schmid RA, Hillinger S, Walter R, Zollinger A, Stammberger U, Speich R, Schaffner A, Weder W, Schoedon G. The nitric oxide synthase cofactor tetrahydrobiopterin reduces allograft ischemia–reperfusion injury after lung transplantation. *J Thorac Cardiovasc Surg* 1999;118:726–32.
 17. Ischiropoulos H, al-Mehdi AB, Fisher AB. Reactive species in ischemic rat lung injury: contribution of peroxynitrite. *Am J Physiol* 1995;269:L158–64.
 18. Chang WJ, Alvarez-Gonzalez R. The sequence-specific DNA binding of NF-kappa B is reversibly regulated by the auto-modification reaction of poly (ADP-ribose) polymerase 1. *J Biol Chem* 2001;14:276:47664–70.
 19. Cuzzocrea S, Zingarelli B, Costantino G, Szabo A, Salzman AL, Caputi AP, Szabo C. Beneficial effects of 3-aminobenzamide, an inhibitor of poly(ADP-ribose)synthetase in a rat model of splanchnic artery occlusion and reperfusion. *Br J Pharmacol* 1997;121:1065–74.
 20. Zingarelli B, Cuzzocrea S, Zsengeller Z, Salzman AL, Szabo C. Protection against myocardial ischemia and reperfusion injury by 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthetase. *Cardiovasc Res* 1997;36:205–15.
 21. Ischiropoulos H. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys* 1998;56:1–11.
 22. MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS, Thompson JA. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc Natl Acad Sci USA* 1996;93:11853–8.
 23. Crow JP, Sampson JB, Zhuang Y, Thompson JA, Beckman JS. Decreased zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration byperoxynitrite. *J Neurochem* 1997;69:1936–44.
 24. Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J, Ischiropoulos H. Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc Natl Acad Sci USA* 1998;95:7659–63.
 25. Ferro TJ, Gertzberg N, Selden L, Neumann P, Johnson A. Endothelial barrier dysfunction and p42 oxidation induced by TNF-alpha are mediated by nitric oxide. *Am J Physiol* 1997;272:L979–88.
 26. Lander HM, Jacovina AT, Davis RJ, Tauras JM. Differential activation of mitogen-activated protein kinases by nitric oxide-related species. *J Biol Chem* 1996;271:19705–9.
 27. Caselli A, Camici G, Manao G, Moneti G, Pazzagli L, Cappugi G, Ramponi G. Nitric oxide causes inactivation of the low molecular weight phosphotyrosine protein phosphatase. *J Biol Chem* 1994;269:24878–82.
 28. Matata BM, Galinanes M. Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor-kappa B DNA binding activity. *J Biol Chem* 2002;277:2330–5.
 29. Krishnadasan B, Naidu B, Farr A, Rosengart M, Barnes A, Verrier E, Mulligan MS. Protective effects of calcineurin inhibitors in lung ischemia reperfusion injury. *J Thorac Cardiovasc Surg* (in press).
 30. Salvemini D, Wang ZQ, Stern MK, Currie MG, Misko TP. Peroxynitrite decomposition catalysts: therapeutics for peroxynitrite-mediated pathology. *Proc Natl Acad Sci USA* 1998;95:2659–63.