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Enhanced peroxynitrite decomposition protects against experimental obliterative bronchiolitis

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Abstract

Obliterative bronchiolitis (OB) affects over half of all survivors following lung or heart–lung transplantation. Respiratory epithelial cell injury, peribronchial inflammation, and proliferation of fibrovascular tissue causing airway occlusion characterize the lesion. While peroxynitrite is known to participate in other models of acute lung injury, its role in the evolution of OB is unclear. Using a rat model of experimental OB, tracheas from Brown–Norway or Lewis rats were transplanted into Lewis recipients. Treated animals received FP-15, a peroxynitrite decomposition catalyst, at 1 mg/kg/day intraperitoneal for 14 days. Luminal obstruction, epithelial loss, and inflammatory infiltrate were examined, as was nitrotyrosine staining by immunohistochemistry in explanted tracheas. By postoperative day 14, control allografts demonstrated marked peribronchial inflammation, near complete loss of respiratory epithelium and extensive intraluminal proliferation of fibrovascular connective tissue, with a mean 83% reduction in airway cross-sectional area. Allograft recipients treated with FP-15 showed reduced nitrotyrosine formation, preservation of respiratory epithelium, limited peribronchial inflammation, and only 14% ($P < .001$) reduction in airway cross-sectional area. Peroxynitrite therefore appears to play a role in the development of obliterative bronchiolitis in rats. The peroxynitrite decomposition catalyst, FP-15, is protective when administered daily and warrants investigation into its potential clinical utility.

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Introduction

Nitric oxide (NO) is a fundamental signaling molecule involved in the regulation of a vast array of physiological functions. However, NO production also results in the elaboration of nitrogen-centered free radicals, specifically peroxynitrite (ONOO⁻), which is a major cytotoxic effector (Beckman, 1996). From a therapeutic viewpoint such a paradoxical fate of NO is troublesome. Whether peroxynitrite production or NO predominates is dependent on both the flux of NO and the surrounding chemical milieu (Liaudet, 2000).

It is convenient to categorize the chemical reactions

of NO as *direct* and *indirect*. Generally, the *direct* effects of NO prevail when its production is minimal and transient and are usually involved in functions that are protective and signaling related. In contrast, *indirect* effects occur under pathophysiological circumstances and reflect a high and sustained flux of NO. These circumstances result in the formation of peroxynitrite and dinitrogen trioxide from the interaction of NO with the superoxide radical and oxygen, respectively. Peroxynitrite is a potent cytotoxin with a vast array of deleterious effects.

Obliterative bronchiolitis (OB) is a progressive and commonly fatal disorder involving small airways that affects more than 60% of long-term recipients of lung and heart–lung transplants. This process represents the major impediment to long-term survival in lung and com-

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bined heart–lung transplantation (Sundaresan, 1995). Clinically, OB is characterized by progressive dyspnea, a nonproductive cough, and reductions in FEV₁ and mid-expiratory flow volumes (Reichenspurner, 1995). Treatment typically consists of intensification of immunosuppressive therapy, which is at best, capable of only slowing the rate of progression. There is neither a specific nor effective therapy to prevent or reverse OB.

Extensive research has demonstrated increased formation of peroxynitrite in tissue from lung transplant recipients with active OB (Mason, 1998, Hansen, 2000, McDermott, 1997). This corresponded with an increase in the inducible form of nitric oxide synthase (iNOS) (Mason, 1998; McDermott, 1997; Gabbay, 2000) in lung tissue and exhaled nitric oxide (Gabbay, 2000) from patients with OB. These findings have been reproduced in animal models of orthotopic allograft airway transplantation (Romanska, 2000). The histopathological features of this experimental OB model largely reproduce the changes in human recipients developing OB after lung allotransplantation (Hertz, 1993). Typically, complete airway obstruction develops by day 28 following implantation of rat airways that are heterotopically transplanted into allogeneic recipients.

The presence of increased levels of peroxynitrite has been conclusively demonstrated in active OB. However its role in the pathogenesis of this disease has received little attention. Aminoguanidine, an inhibitor of iNOS, accelerated OB in rat airways and administration of L-arginine, a substrate for NO production, protected against the development of OB (Kallio, 1997). This suggests that products of iNOS could be protective against OB. However, a distinction between the effects of NO and peroxynitrite was not made in their study. A protective role for peroxynitrite would appear to be contradictory to the notion that it is a potent cytotoxin and expressed in active OB. Therefore, elaboration upon the functional significance of increased iNOS activity in OB would be novel and important.

Until recently there has been difficulty in studying peroxynitrite directly. However, specific peroxynitrite decomposition catalysts have recently been developed (Salvemini, 1998). These agents, generally iron-containing metalloporphyrins, are water soluble, potent, and specific to peroxynitrite decomposition. All of these characteristics heighten the potential of these compounds for *in vivo* investigation and clinical application.

Knowing that peroxynitrite is expressed in human and animal models of OB and that it is involved in a wide variety of pathophysiological processes, we hypothesize that peroxynitrite formation is important in the development of experimental OB. We used a specific peroxynitrite decomposition catalyst to test this hypothesis in an animal model.

Materials and methods

Experimental animals

Inbred male Lewis rats and Brown–Norway rats weighing 250–275 g were obtained from Simonsen Laboratories Inc. (Gilroy, CA). These two strains were chosen since they represented a complete MHC mismatch. Lewis rats served as donors in the isograft experiments and as recipients in the allograft and isograft groups. All animals were cared for in compliance with the “Principles of Laboratory Animal Care” formulated by the Institute of Laboratory Animal Resources and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Experimental surgery

After induction of ketamine anesthesia (200–250 mg/kg) and systemic heparinization (50 units), donor animals were shaved and prepped with betadine. A median sternotomy was performed with extension of the incision into the neck. The trachea and mainstem bronchi were removed, including the first-order branches to the upper and lower lobes. This explanted graft was then placed into cold normal saline.

Under sterile conditions, a dorsal incision was made on anesthetized recipients. A dorsolateral subcutaneous pocket was fashioned and the underlying fascia was fenestrated overlying the flank musculature. Meticulous hemostasis was maintained throughout the dissection. The graft was secured into place with two interrupted 6-0 sutures to prevent migration. The wound was closed with 4-0 absorbable sutures on the deep tissue and nylon on the skin.

The animals were allowed to recover from the surgery and were returned to their cages. The wounds were monitored daily for infection and dehiscence. FP-15, a peroxynitrite decomposition catalyst, was injected intraperitoneally (ip) at a dose of 1 mg/kg/day in treated animals. Isograft and allograft controls received ip saline beginning on the day of the transplant. Each experimental group, including isograft and allograft controls, contained at least four animals. At the time of graft harvest on day 14, the animals were anesthetized with a lethal dose of pentobarbital (50 mg/kg), and explanted grafts were placed into an appropriate preservative solution.

Histologic evaluation

As mentioned, allograft and isograft airways were explanted at 14 days following transplantation and fixed in 10% phosphate-buffered formalin for subsequent sectioning, hematoxylin and eosin (H&E) staining, and examination by light microscopy. The degree of intaluminal and peritracheal inflammation was graded, and the composition

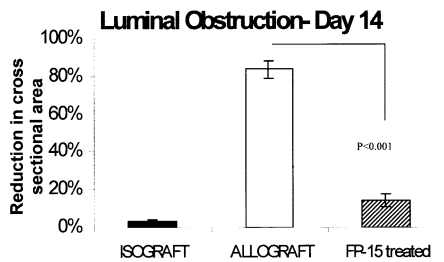


Fig. 1. In animals treated with FP-15 at a dose of 1 mg/kg/day, airway obstruction was significantly reduced by 83% compared to time-matched saline-treated allografts.

of the inflammatory cell infiltrates was noted by operators blind to the treatment status of the animals.

Computerized morphometry: luminal obstruction

Images of the H&E-stained airway cross sections were taken with a high-resolution video camera attached to the microscope. These images were imported to a computer and analyzed using NIH Image software. The percentage of luminal obstruction in transplanted airways was calculated in two steps. First the outline of the inner surface of the cartilage was traced. The line representing the membranous trachea was drawn straight by connecting the two ends of the cartilage. In the second step the cursor was used to trace the inner surface of the actual residual lumen. The cross-sectional area of the actual residual lumen was subtracted from the area contained within the inner circumference of the cartilage. This value was then divided by the area within the cartilage to determine the degree of luminal obstruction. The formula for calculating the percentage of airway obstruction is expressed as follows:

$$\text{Percentage obstruction} = \frac{\text{Airway within cartilage} - \text{Airway of residual lumen}}{\text{Area within the cartilage}} \times 100$$

In normal unmanipulated airways the respiratory epithelium and submucosa lie within the cartilaginous rings. Therefore, using the calculation, normal airways will demonstrate a "baseline airway obstruction" of approximately 3%. Multiple sections ($n > 10$) were taken for each graft (from the middle 1 cm) and the mean calculated percentage of obstruction was determined (Fig. 1).

Computerized morphometry: loss of epithelium

Using the same computerized imaging program, H&E-stained sections of the transplanted airway were examined. The inner circumference of the original airway was determined and the cursor was used to trace areas of intact normal respiratory epithelium, variant epithelium, and absent or necrotic epithelium. The percentage of the original

circumference was then determined for each of these three possibilities.

Immunohistochemistry for nitrotyrosine

Whole tracheal explants 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 immunohistochemistry (IHC), sections were dewaxed and rehydrated 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. Following the blocking step, the primary antinitrotyrosine antibody (Upstate Waltham, MA) was incubated overnight at 4°C at a concentration of 5 $\mu\text{g}/\text{ml}$. This step was followed by incubation in stock antirabbit secondary antibody (Vector Laboratories), then avidin–biotin complex, and finally diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with methylgreen and then dehydrated, cleared, and mounted with permanent mounting media. Stained sections were examined using the image analysis software Image Pro Plus (Media Cybernetics, Silver Spring, MD).

Statistical analysis

Standard deviation and standard error of the mean were calculated for each group. Comparisons between groups were accomplished using Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

Immunohistochemical staining for nitrotyrosine

Allograft tracheal samples were harvested following 4, 7, and 14 days postimplantation, and immunohistochemistry was used to localize formation of peroxynitrite by staining for nitrotyrosine. On day 4, nitrotyrosine staining was localized to the subepithelial mucosa (Fig. 2A). By day 7, intense staining was evident in mononuclear inflammatory cells and fibroblasts (Fig. 2B). By day 14 this pattern became more intense and diffuse (Fig. 2C). Though nitrotyrosine staining was present in isografts, it was much less intense than in time-matched allografts.

In order to test whether administration of FP-15 was associated with reduced nitrotyrosine formation, FP-15-treated allografts harvested at day 14 were analyzed by immunohistochemistry. FP-15-treated tracheal allografts demonstrated marked reductions in nitrotyrosine staining (Fig. 2D).

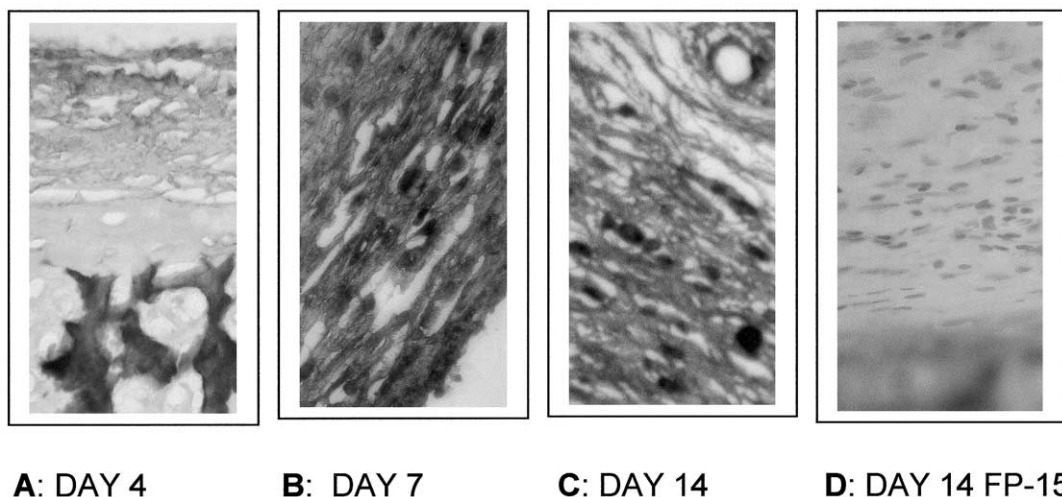


Fig. 2. Immunohistochemical sections of allograft tracheas harvested at days 4, 7, and 14 stained for nitrotyrosine. There is moderate staining evident at day 4 (A), with the intensity increasing dramatically at days 7 (B) and 14 (C). FP-15-treated animals at 1 mg/kg/day at day 14 (D) reveal markedly less staining for nitrotyrosine.

Enhanced decomposition of peroxynitrite correlated with reduced airway obstruction

In isografts harvested at 7 and 14 days, the cross-sectional area of the tracheal lumen was narrowed by < 3%. As mentioned, the thickness of normal submucosa and respiratory epithelium accounted for this minimal but discrete reduction in calculated cross-sectional area. When saline-treated allografts were analyzed 7 days following transplantation the obliterative process was partially developed. Luminal obstruction was $11 \pm 2\%$. In saline treated allografts harvested at day 14 following transplantation, the luminal narrowing was $83.7 \pm 0.5\%$. In contrast, allografts from animals treated with 1 mg/kg/day of FP-15 demonstrated only $14.2 \pm 0.4\%$ reductions in airway cross-sectional area (Fig. 1). This was significantly less than the obstructive changes found in the 14-day saline treated allografts ($P < .001$).

Enhanced decomposition of peroxynitrite is associated with epithelial preservation

In isografts, there was near complete preservation of luminal epithelium at 14 days. At 7 days there was some alteration in epithelial morphology (i.e., epithelial cells were flattened or cuboidal) but by 14 days epithelial cells assumed their normal morphology, with $97 \pm 2\%$ of the epithelium normal respiratory type (ciliated columnar cells). Saline-treated allografts at day 7 had already lost $40 \pm 4\%$ of the epithelium lining the airways; of the remaining epithelium, the majority were flattened or otherwise atypical cells. In 14-day saline treated allografts virtually all of the epithelium was lost and what minimal amount remained was atypical. However, 14-day allografts treated with FP-15 were found to have $38 \pm 2\%$ ($P < .03$) of their epithelium

remaining (Fig. 3). Although three-quarters of the remaining epithelium was atypical, approximately one-quarter of the luminal surface was still covered with normal respiratory epithelium.

Histology

Isografts examined at 7 and 14 days following implantation demonstrated excellent epithelial preservation, and few inflammatory cells were present (Fig. 4A). Day 7 allografts, which had lost a significant portion of their epithelial lining, had lymphocytes and macrophages accumulate in the submucosa and surrounding tissues. Fourteen days following transplantation, saline-treated allografts demonstrated nearly complete loss of respiratory epithelium and dense peritracheal inflammation was evident, characterized by the presence of mononuclear cells. Airway lumens were largely obscured by fibrovascular connective tissue and scattered accumulations of inflammatory cells (Fig. 4B). In contrast,

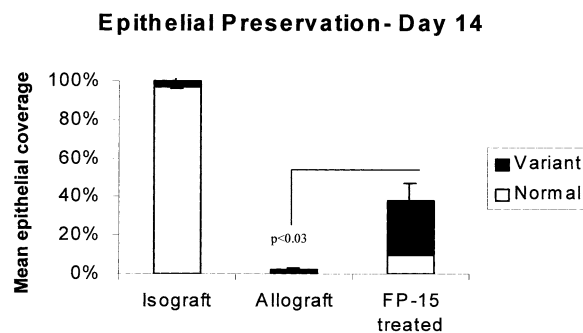


Fig. 3. In animals treated with FP-15 at a dose of 1 mg/kg/day, epithelial loss was significantly reduced by 61% compared to time-matched saline-treated allografts.

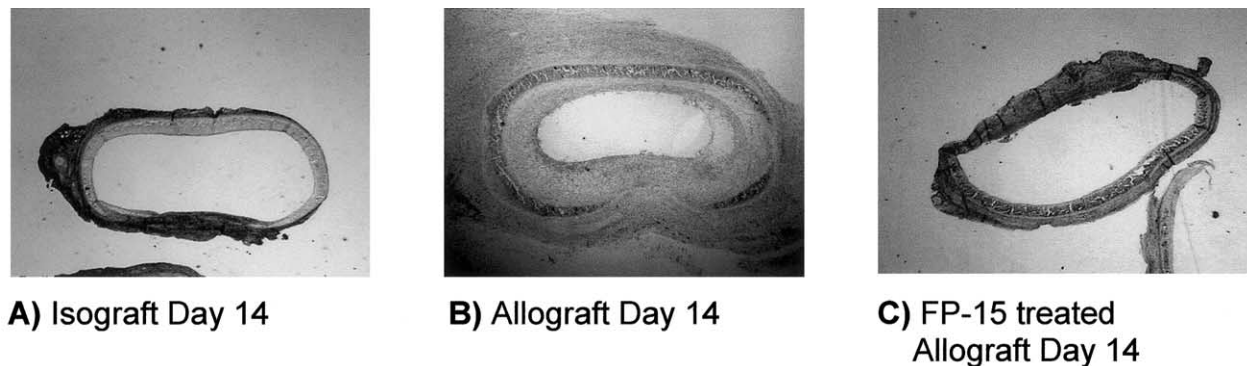


Fig. 4. Representative sections of H&E-stained tracheas harvested on day 14 (20X) (A) Isograft: There is preservation of epithelium and minimal luminal obstruction present. (B) Allograft: By day 14, there is complete loss of respiratory epithelium and dense peritracheal inflammation. Furthermore, fibrovascular connective tissue and scattered accumulations of inflammatory cells fill the lumen. (C) FP-15 treated allograft: FP-15-treated animals retained some epithelial lining and had a modest degree of airway obstruction and reduced accumulation of inflammatory cells.

14-day allografts from animals that received FP-15 retained much of their respiratory epithelium and had only modest degrees of airway obstruction and of inflammatory cell accumulations (Fig. 4C).

Discussion

No direct evidence has previously been published linking peroxynitrite to the development of OB. A role for peroxynitrite in patients with active OB is suggested from the finding of elevated levels of exhaled NO from human lung transplant recipients, as well as by increased iNOS and nitrotyrosine formation in transplanted lung tissue samples (Mason, 1998; Hansen, 2000; McDermott, 1997).

Peroxyntirite impairs cellular function by inducing DNA damage, with subsequent activation of the nuclear enzyme polyADP-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 (Scovassi, 1999). These changes, if unchecked, eventually result in apoptosis. The biological 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 found in neutrophils (Ischiropoulos, 1998). Hence, acute inflammatory events associated with significant neutrophil sequestration may confound interpretation of the immunohistochemistry results. Though we acknowledge this limitation, one still cannot rule out that peroxynitrite is selectively formed in active OB and that it is associated with apoptosis via a PARS-dependent mechanism.

Nitrotyrosine is used as a “footprint” of peroxynitrite activity. This tyrosine nitrosylation is not a random process but occurs on distinct proteins. Pathophysiological roles for nitrated proteins have been suggested in chronic human

kidney allograft rejection (MacMillan-Crow, 1996). Biopsies from patients with kidney transplants exhibiting chronic rejection exhibit an inactivation of manganese superoxide dismutase by nitrosylation, and this may make the graft more susceptible to injury (MacMillan-Crow, 1996). Even more interest has focused on the reversible *S*-nitrosylation of cysteine residues, possibly affecting a number of redox-sensitive signaling pathways like p21 ras and MAP kinases (Lander, 1996) and the inhibition of protein phosphatases (Caselli, 1994). Alterations in these important signaling pathways may modulate the immune response detrimentally.

The distribution of nitrotyrosine staining described in the present study is similar to that described previously (Romanska, 2000). This localization of nitrotyrosine is in agreement with the current concept of “response to injury” in the pathogenesis of OB. Briefly, the immune mediated injury of the epithelium leads to the migration and proliferation of pulmonary mesenchymal cells and the proliferation and release of cytokines from T-lymphocytes and macrophages. Progressive connective tissue deposition eventually results in luminal obstruction. The localization of nitrotyrosine initially to the subepithelium and then to immune and mesenchymal cells follows this pattern of the disease, lending support to the notion that it plays a role in the development of OB.

The present study is the first to demonstrate clearly that enhanced decomposition of peroxynitrite is protective against OB. In a prior study utilizing an inhibitor of iNOS, aminoguanidine, the progression to OB was hastened (Kallio, 1997). Rat tracheal allografts 10 days after transplantation revealed a significantly higher degree of obstruction in aminoguanidine treated animals. Additionally, treatment with L-arginine (a precursor for NO production) protected against the development of OB. Unfortunately, in this study they did not quantify nitrotyrosine formation, so the effects of these interventions on peroxynitrite production are unknown. This point is illustrated in a separate study exam-

ining both iNOS and peroxynitrite in an OB model (Romanska, 2000). Though high levels of iNOS are expressed in both isografts and allografts, only in allografts is nitrotyrosine persistent, thereby refuting the notion that iNOS and peroxynitrite activity are directly proportional to one another in experimental OB. Furthermore, the addition of L-arginine, which is a precursor for NO production, protected against the development of OB. However, L-arginine is a substrate for both iNOS and eNOS. eNOS is the constitutive form of the enzyme that is associated with *protective* functions. eNOS is known in this model to be impaired, and perhaps enhancement of its production affords a degree of protection (Gabbay, 2000). Ultimately, the important determinant of protective or injurious effects likely relates to the balance of NOS products elaborated (NO vs peroxynitrite) and their intracellular location.

We have demonstrated the utility of a peroxynitrite decomposition catalyst, FP-15, in reducing the development of experimental OB in a rat model. The ability of this class of compounds to inhibit experimental OB affords greater opportunity to study the molecular mechanisms involved in this disease and suggests an area for potential therapeutic development for patients receiving lung or heart–lung transplantation.

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