Attenuation of lung reperfusion injury after transplantation using an inhibitor of nuclear factor-κB

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Ross, Scott D., Irving L. Kron, James J. Gangemi, Kimberly S. Shockey, Mark Stoler, John A. Kern, Curtis G. Tribble, and Victor E. Laubach. Attenuation of lung reperfusion injury after transplantation using an inhibitor of nuclear factor-κB. Am J Physiol Lung Cell Mol Physiol 279: L528–L536, 2000.—A central role for nuclear factor-κB (NF-κB) in the induction of lung inflammatory injury is emerging. We hypothesized that NF-κB is a critical early regulator of the inflammatory response in lung ischemia-reperfusion injury, and inhibition of NF-κB activation reduces this injury and improves pulmonary graft function. With use of a porcine transplantation model, left lungs were harvested and stored in cold Euro-Collins preservation solution for 6 h before transplantation. Activation of NF-κB occurred 30 min and 1 h after transplant and declined to near baseline levels after 4 h. Pyrrolidine dithiocarbamate (PDTC), a potent inhibitor of NF-κB, given to the lung graft during organ preservation (40 mmol/l) effectively inhibited NF-κB activation and significantly improved lung function. Compared with control lungs 4 h after transplant, PDTC-treated lungs displayed significantly higher oxygenation, lower PCO2, reduced mean pulmonary arterial pressure, and reduced edema and cellular infiltration. These results demonstrate that NF-κB is rapidly activated and is associated with poor pulmonary graft function in transplant reperfusion injury, and targeting of NF-κB may be a promising therapy to reduce this injury and improve lung function.

pyrrolidine dithiocarbamate; ischemia; organ preservation

The transcription factor nuclear factor-κB (NF-κB) regulates the expression of many genes in which early response products are critical for the development of acute inflammation. NF-κB is composed of various homo- or heterodimeric combinations of NF-κB/Rel proteins, and different NF-κB dimers have varying transcriptional activation properties. In unstimulated cells, NF-κB is retained in the cytoplasm and bound to inhibitory proteins of the IκB family including IκB-α. In response to inflammatory stimuli, including tumor necrosis factor-α, interleukin-1β, and reactive oxygen species, IκB-α is phosphorylated by specific kinases and degraded by proteasomes. Degradation of IκB-α releases NF-κB and allows translocation to the nucleus, where NF-κB binds specific promoter elements and induces gene transcription (2, 12). NF-κB acts on target genes for proinflammatory cytokines, chemokines, immunoreceptors, cell adhesion molecules, acute phase proteins, and inducible nitric oxide synthase. The activation of NF-κB therefore leads to a coordinated increase in the transcription of many genes in which products mediate inflammatory responses. In addition, products of the genes that are regulated by NF-κB often cause the activation of NF-κB, which creates a positive feedback loop that may amplify and perpetuate local inflammatory responses (2).

The inflammatory products of NF-κB activation have been demonstrated in several cellular models (22), and a critical role of NF-κB in the inflammatory cascade in vivo is now emerging (3, 5, 13–15). In fact, several studies suggest that the nuclear translocation of NF-κB is a prerequisite for the full development of lung inflammatory injury (14). Ischemia-reperfusion injury is also reported to be influenced by the activation of NF-κB in the heart (5) and liver (23). Reperfusion of ischemic tissues rapidly produces reactive oxygen metabolites, which activate NF-κB and increase its inflammatory products (21), all of which exacerbate tissue reperfusion injury. Whereas these patterns of gene expression can be correlated with tissue damage in models of endotoxin-induced lung injury (3), immune

RECENT ADVANCEMENTS IN LUNG transplantation have led to the increasing use of this life-saving operation for more patients with various end-stage pulmonary diseases. However, despite these improvements, difficulties with maintaining lung graft viability remain, and severe graft dysfunction occurs in 10–20% of lung transplant recipients (17). It has become increasingly evident that reperfusion after ischemia is responsible for the majority of tissue injury in lung transplantation. In addition, recent evidence confirms that lung ischemia-reperfusion injury is a result of the activation of inflammatory mediators in the early phase of reperfusion (7, 8, 18, 19).

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complex-induced lung inflammation (13), and warm ischemia-reperfusion injury (5, 23), it is unclear what influence NF-κB has on reperfusion injury after ischemic lung storage in a clinical transplant setting. In the current study, we investigated the importance of NF-κB in a survival porcine model of left lung transplantation. We hypothesized that NF-κB is a critical early regulator of the inflammatory response in lung ischemia-reperfusion injury and that inhibition of NF-κB activation reduces this injury and improves pulmonary graft function.

METHODS

Lung harvest. Domestic swine of both sexes (25–30 kg) were randomly assigned to two groups (n = 6 per group). Each animal was anesthetized intramuscularly with xylazine (1 mg/kg), telazol (6 mg/kg), and atropine (0.01 mg/kg). After endotracheal intubation, the pigs were ventilated with a volume-cycled ventilator at a tidal volume of 15 ml/kg, a respiratory rate of 15–20 breaths/min, and an inspiratory oxygen concentration of 1.0. Anesthesia was maintained with isoflurane, and the inferior pulmonary ligament was transected. After pulmonary arterial inflow to exclusively study the newly transplanted left lung. A Fogarty balloon was placed into the right bronchus under bronchoscopic vision, the right PA branches were ligated through the reopened left thoracotomy, and the animal was allowed to stabilize for 30 min. Tidal volume, expiratory minute volume, and airway pressure were displayed on the ventilator (Servo ventilator 900C, Siemens-Elema), and dynamic compliance was then calculated. Mean PA pressure (PAP), pulmonary capillary wedge pressure (PCWP), and cardiac output (CO) were measured. Pulmonary vascular resistance (PVR) was calculated using the equation PVR = [(PAP – PCWP)/CO] × 80. In addition, blood gas measurements were obtained under conditions of isolated transplant ventilation.

Nuclear protein extraction. Nuclear extracts of whole lung tissues were prepared by the method of Deryckere and Gannon (6). Briefly, frozen peripheral lung tissue (0.5 g) was homogenized at 4°C with a Dounce tissue grinder in solution A [0.6% Nonidet P-40, 150 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 4 μg/ml pepstatin A, 4 μg/ml leupeptin, and 4 μg/ml aprotinin]. The homogenate was centrifuged for 1 min at 1,500 rpm, and the remaining supernatant was collected and centrifuged for 5 min at 5,000 rpm. The pelletted nuclei were resuspended at 4°C in 300 μl of solution B (25% glycerol, 20 mM HEPES, pH 7.4, 840 mM NaCl, 1.2 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.4 mM PMSF, 4 μg/ml pepstatin A, 4 μg/ml leupeptin, and 4 μg/ml aprotinin) and incubated on ice for 20 min. Samples were centrifuged, and the supernatant (nuclear protein) was dialyzed at 4°C in solution C (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.2 mM EDTA, and 25% glycerol) and stored at −80°C. Protein concentrations were determined using Coomasie Plus Protein Assay Reagent (Pierce).

Electrophoretic mobility shift assay. Double-stranded consensus oligonucleotide (5′-GTTAGGGGCCCTCCAGGC-3′, Promega) was end labeled with [γ-32P]ATP (6,000 Ci/ mmol; Amersham). Binding reactions containing equal amounts of nuclear protein (5 μg) and 1.5 fmol of labeled oligonucleotide were incubated for 30 min at room temperature in binding solution [10 mM HEPES, pH 7.4, 50 mM KCl, 0.2 mM EDTA, 2.5 mM dithiothreitol, 150 μg/ml poly(dI-dC), and 0.05% Nonidet P-40]. Reaction products were separated in a 6% nondenaturing polyacrylamide gel and analyzed by autoradiography. Autoradiography and quantitation of autoradiographic signals were performed using a PhosphorImager and ImageQuant software (Molecular Dynamics). For supershift analysis, antibodies to NF-κB p65 and p50 subunits were purchased from Santa Cruz Biotechnology.

Western blot analysis. Frozen peripheral lung samples (0.5 g) were homogenized in 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 4 μg/ml pepstatin A, 4 μg/ml leupeptin, and 0.1% 2-mercaptoethanol and centrifuged at 4°C at 5,000 rpm. Protein concentrations were determined using Coomasie Plus Protein Assay Reagent (Pierce). Western analysis of IκB-α was performed using the
PhosphoPlus IkB-α Antibody Kit (New England Biolabs). Briefly, samples (75 μg) were separated in a denaturing 7.5% polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was incubated with primary IkB-α antibody (1:1,000, rabbit polyclonal IgG) overnight at 4°C, washed, and incubated with horseradish-conjugated secondary antibody (1:2,000) for 1 h. Proteins were detected by incubating the membrane with LumiGLO as instructed. Quantitation of autoradiographic signals was performed using the IS-1000 digital imaging system (Alpha Innotech).

Histological assessment. After harvest, representative blocks from the resected lungs were processed through Formalin fixation and routine paraffin embedding. Five-micrometer sections were stained with hematoxylin and eosin. Randomly coded sections from control vs. treated transplanted lungs as well as normal lungs directly harvested without any transplantation were submitted to a pulmonary pathologist for an assessment of "lung injury," if any, and if present a relative severity of injury. After a preliminary survey of the slides, lung injury was defined as the presence of monotomous interstitial cellularity within pulmonary parenchyma and/or extension of the cellular infiltrates into the alveolar spaces. The cells most commonly represented in these infiltrates were almost always segmented neutrophils with some additional macrophage infiltrates. Variable pulmonary edema in the form of intra-alveolar proteinaceous material was also assessed. These histological features were the basis for ranking the slides into three conceptual groups: normal, i.e., having no evidence of any increased cellular infiltrates or edema; mildly involved, i.e., having patchy or focal areas of abnormality involving less than 25% of the sampled histological area; and a third group that exhibited diffuse involvement (greater than 30% of the lung area involved), with denser interstitial infiltrates, more prominent intra-alveolar cellular infiltrates, and more prominent alveolar edema.

Wet-to-dry weights. For wet-to-dry weight ratios, parenchymal samples of whole lung tissue were blotted and weighed. After baking in a vacuum oven for 24 h at 50°C, tissues were then weighed to obtain dry weights. Tissues were again weighed another 24 h later to verify that complete dehydration occurred. Data were calculated as wet weight divided by dry weight and used as an indicator of pulmonary edema as illustrated by an increase in the wet-to-dry weight ratio.

Lung myeloperoxidase. Myeloperoxidase (MPO) assay was performed on lung samples to quantify neutrophil sequestration. Tissue (0.5 g) was placed in 5 ml of 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM potassium phosphate solution (pH 7.4) and disrupted by homogenizing at 4°C. The solution was centrifuged at 15,000 g for 15 min at 4°C, and the supernatant was discarded. The pellet was resuspended in 2 ml of 0.5% HTAB in 50 mM potassium phosphate solution (pH 6.0) and homogenized. Tissue was disrupted further by sonication and then underwent three freeze-thaw cycles. The solution was then centrifuged at 15,000 g for 15 min at 4°C. Aliquots (0.1 ml) of supernatant were added to 0.3 ml of assay buffer (0.1 mg/ml o-dianisidine, 0.7% H₂O₂, and 50 mM potassium phosphate, pH 6.0), and absorbance at 460 nm was measured over 2 min at room temperature. MPO activity is expressed as change in absorbance per milligram of wet lung weight per minute (ΔA460·mg⁻¹·min⁻¹).

Statistical analysis. Statistical analysis was performed using analysis of variance on SPSS software (SPSS, Chicago, IL), and Student’s t-tests were used to compare the two groups at various time points. Significant differences were determined using Tukey’s honestly significant difference test. The data are expressed as the means ± SE. For quantitation of electrophoretic mobility shift assay (EMSA) gels, statistical analysis was performed on the raw data from the PhosphorImager. Inasmuch as we present an increase of NF-κB DNA binding activity as a multiple increase, we do not include the means ± SE of the raw data in RESULTS.

RESULTS

Time course of NF-κB DNA binding activity during lung reperfusion after transplantation. The time course of NF-κB activation (more precisely DNA binding activity) during lung reperfusion was established by EMSA of nuclear extracts from whole lung obtained at various time points after initiation of reperfusion following transplantation. A low level of NF-κB DNA binding activity was detected in the nuclei from normal lungs that were not exposed to ischemia or reperfusion (Fig. 1A, lanes 1–2) and this was not altered after cold ischemic storage alone (Fig. 1A, lanes 3–4). Nuclear activity of NF-κB increased dramatically with the onset of reperfusion, reaching maximal levels by 30 min and maintaining high activation at 1 h (Fig. 1A, lanes 5–6 and 7–8, respectively). NF-κB activity was eight-fold higher after 30 min of reperfusion and sixfold higher after 1 h compared with normal or ischemic lung (P < 0.01 for both time points vs. normal lungs). After 4 and 20 h of reperfusion, NF-κB DNA binding activity approached baseline levels. Two representative samples from each group for each time point are shown, and a small degree of variability between animals was observed.

The specificity of the NF-κB DNA binding complex was confirmed using EMSA analysis (Fig. 1B). Nuclear extracts from ischemic-reperfused lungs incubated only with 32P-labeled NF-κB consensus oligonucleotide probe showed the typical binding complex (Fig. 1B, lanes 7–8) vs. normal lung (lanes 1–2).
In pig lung tissue, the banding pattern of NF-κB included three different complexes. During reperfusion after ischemic storage in a transplant setting, preferential upregulation of the fastest moving NF-κB complex was noted at 30 and 60 min (Fig. 1A, open arrowhead). The composition of the NF-κB complex activated by ischemia-reperfusion was determined by supershift assays conducted using antibodies reactive with the p50 or p65 subunit of NF-κB (Fig. 2). Addition of the anti-p65 antibody to the binding reaction resulted in elimination of the fastest moving NF-κB complex (as well as the minor, slowest moving complex) and led to the appearance of a supershifted band, indicating that this complex was composed entirely of p65 protein. There was no supershift observed with the addition of p50 antibody alone.

Inhibition of NF-κB nuclear activity by PDTC. After demonstrating that in vivo reperfusion following lung transplantation induces early NF-κB DNA binding activity, we next tested whether PDTC, an antioxidant and potent inhibitor of NF-κB activation (20), could suppress this activation. Ischemia-reperfusion caused a significant increase in NF-κB DNA binding activity at 30 and 60 min after the onset of reperfusion (Fig. 3, lanes 5–6 and 9–10, respectively). PDTC, which was administered to the transplanted lung only during organ preservation (see METHODS), dramatically inhibited NF-κB activation during reperfusion (Fig. 3, lanes 7–8 and 11–12). In transplanted lungs that received PDTC-enhanced Euro-Collins preservation solution, the nuclear activation of NF-κB was reduced by 75% at 30 min and 68% at 60 min of reperfusion compared with normal lungs. PDTC did not alter NF-κB binding activity at other time points.

Change in lung content of IκB-α. The protein level of IκB-α in lung tissue during reperfusion was measured by Western analysis to determine whether the activation of NF-κB was the result of the degradation of its inhibitory protein IκB-α. In whole lungs, IκB-α protein levels showed early evidence of reduction during reperfusion after ischemic storage (Fig. 4). After 30 min of reperfusion (Fig. 4, lanes 3–4), when NF-κB activation was maximal, the level of IκB-α protein was reduced 70% from that of normal, unoperated lungs (Fig. 4, lanes 1–2). The inhibitory protein remained low throughout the 4-h reperfusion period. Thus during the course of the lung inflammatory response, there was loss of IκB-α protein as assessed by Western analysis. Whereas the addition of PDTC to the preservation solution inhibited the activation of NF-κB (see Fig. 3), PDTC did not alter the protein expression of IκB-α compared with control transplants (Fig. 4, lanes 3–4). IκB-α levels in the PDTC group were reduced at 30 min (Fig. 4, lanes 3–4) and 1 h (Fig. 4, lanes 5–6) of reperfusion by 72 and 78%, respectively, from normal levels.

Fig. 2. Supershift analysis of NF-κB components. Nuclear extracts from 2 different lungs after 6 h of ischemia and 30 min of reperfusion are shown (lanes 1–4 and 5–8). These were incubated with (+) and without (−) antibodies to the NF-κB proteins p50, p65, or both before addition of labeled NF-κB oligonucleotide and subjected to EMSA analysis. Solid arrowhead, supershift of 2 p65 complexes (open arrowheads). No supershift was observed with the p50 antibody.

Fig. 3. Effect of pyrrolidine dithiocarbamate (PDTC) on NF-κB activation following lung ischemia and reperfusion. Nuclear extracts from whole lung tissues were subjected to EMSA analysis as indicated in METHODS. Two representative samples per group are shown for each time point. PDTC, presence (+) or absence (−) of PDTC in the Euro-Collins preservation solution. Arrowheads, 3 different NF-κB complexes observed in these tissues.

Fig. 4. Expression of IκB-α after lung ischemia and reperfusion. Whole lung extracts were analyzed for IκB-α protein by Western blot. Two samples per group are shown for each time point. Arrowheads, location of protein molecular size markers.
Improvement in pulmonary graft function with PDTC. We determined the influence of NF-κB and the effects of inhibition of its activity on lung graft function following transplantation. Four hours after the initiation of reperfusion, we isolated and studied the physiology of control transplant lungs and transplant lungs that had received PDTC (Fig. 5). Transplanted lungs that were treated with PDTC displayed a significant improvement in arterial oxygenation and ventilation. The arterial Po2 of the PDTC lungs was significantly higher than that of control lungs at 4 h of reperfusion (334.7 ± 34.3 vs. 88.4 ± 7.9 mmHg, P < 0.001; Fig. 5A), and the PCO2 of PDTC lungs was significantly lower than that of control lungs (49.7 ± 10.4 vs. 96.9 ± 10.4 mmHg, P = 0.005; Fig. 5B). At the conclusion of the 4-h reperfusion period, PDTC significantly lowered the mean PAP to where the mean PAP was 42.5 ± 3.2 mmHg in PDTC lungs and 55.2 ± 3.2 mmHg in control lungs (P = 0.02; Fig. 5C). In addition, the dynamic airway compliance of the PDTC-treated lungs tended to be higher than that of control lungs (7.2 ± 0.8 vs. 5.6 ± 0.9 ml/cmH2O, P = 0.10), although this was not found to be significant (Fig. 5D).

Histology. As described in METHODS, histological evaluation of lung injury was assessed in control transplant lungs after 4 h of reperfusion and compared with normal lungs and with transplanted lungs treated with PDTC during preservation. The pulmonary pathologist was informed only of the existence of three animal groups and had otherwise no knowledge of any of the other results reported herein. In a masked fashion, slides were then categorized into three groups, and the results are as follows: five of six lungs displaying the most diffuse involvement of abnormality and infiltration were correctly identified as control transplant lungs, four of six lungs displaying mildly involved abnormality were correctly identified as PDTC-treated transplanted lungs, and five of six lungs displaying no evidence of abnormality were correctly identified as normal lungs. One PDTC-treated lung was misidentified as normal, one PDCT-treated lung was misidentified as control transplant, one normal lung was misidentified as PDTC treated, and one control transplant was misidentified as PDTC treated. Critically, no normal lungs were misidentified as control transplant and vice versa.

Representative slides from each group are shown in Fig. 6. Histopathological specimens of the control transplant lungs after 4 h of reperfusion showed clear evidence of diffuse abnormality, with dense interstitial infiltrates and edema formation (Fig. 6, B and E). In the transplanted lungs that were treated with PDTC during preservation, the abnormality and cellular infiltration was only mild (Fig. 6, C and F) and closely resembled that of normal lung tissue (Fig. 6, A and D).

Pulmonary edema. As an indicator of pulmonary edema, wet-to-dry weight ratios were calculated. As shown in Fig. 7, wet-to-dry weight ratios were significantly increased in control transplant lungs after 4 h of reperfusion compared with those in pretransplanted normal lungs (P < 0.01). These wet-to-dry weight ratios were significantly reduced in transplanted lungs that were treated with PDTC during preservation (P < 0.01, Fig. 7).

Lung MPO activity. We utilized the MPO biochemical assay to measure tissue neutrophil infiltration over the course of in vivo reperfusion and its correlation with NF-κB activation. Tissue MPO activity in control transplant lungs was significantly elevated after 30 min of reperfusion compared with that in normal lung tissue and remained significantly elevated throughout the 4 h of reperfusion (10.8 ± 1.8 ΔA460·mg−1·min−1 after 30 min, P = 0.006, and 15.5 ± 1.4 ΔA460·mg−1·min−1 after 4 h, P < 0.001 vs. 4.3 ± 1.5 ΔA460·mg−1·min−1 for normal lung). However, the administration of PDTC to the transplanted lungs did not alter the tissue MPO activities compared with those in the control lungs (i.e., the PDTC-treated lungs displayed a similar elevation in MPO activity as did the control, untreated lungs). The MPO activity in the PDTC-treated group was 13.2 ± 2.3 ΔA460·mg−1·min−1 after 30 min (P = 0.46 vs. control lungs at 30 min), and 14.3 ± 2.2 ΔA460·mg−1·min−1 after 4 h of reperfusion (P = 0.65 vs. control lungs at 4 h).
DISCUSSION

During the past few years, many of the cellular and molecular events mediating lung ischemia-reperfusion injury have been clarified (17, 18). The inflammatory cascade has emerged as a critical mechanism of lung reperfusion injury, but current therapeutic interventions that target this pathway have yet to demonstrate clear clinical benefit. This is likely due to the fact that these therapies mainly focus on inhibition of specific distal components of the inflammatory response; however, inflammation is initiated and amplified by many redundant and overlapping molecular pathways. Recently, NF-κB has emerged as a rapid response transcription factor that plays a central role in the induction of lung inflammatory injury (2, 12). NF-κB is an amplifying and perpetuating mechanism that can exaggerate the disease-specific inflammatory process through the coordinated activation of many inflammatory genes. The role of NF-κB in the transcriptional activation of key inflammatory genes has been studied in numerous cell lines, and recent studies have demonstrated the in vivo regulatory role of NF-κB in models of endotoxin-induced lung injury (3), immune complex-induced lung inflammation (13), systemic inflammation (11, 15), and warm ischemia-reperfusion injury (5).

In the present study, we have demonstrated for the first time that NF-κB is activated in vivo in a clinical lung transplant setting of cold ischemia and reperfusion. After 6 h of ischemic organ storage without reperfusion, the level of NF-κB nuclear activation is not increased from that of normal lung tissue (see Fig. 1A). Shortly after initiation of reperfusion, NF-κB activity increases dramatically and peaks in our model at 30 min and maintains high activity at 1 h of reperfusion. This pattern clearly indicates that the activation of NF-κB depends entirely on events occurring during the early phases of reperfusion, a finding also suggested in models of warm ischemia-reperfusion injury (5). Because whole tissues were used in our study, we cannot speculate at this time what cell type or types are involved in the upregulation of NF-κB. It has been shown that many cell types are capable of upregulating NF-κB, and further studies will be needed to address this question. Reperfusion of ischemic tissue has been shown to be associated with a rapid burst of reactive oxygen metabolites. These free radicals have been
shown previously to activate NF-κB in a number of experimental systems (21) and likely contribute to the early rise in NF-κB activity seen in our lung transplant model. We observed the preferential increase in the fastest migrating NF-κB complex during reperfusion and have shown by supershift analysis that this complex is composed of p65 NF-κB protein, most likely in a homodimeric form. Homodimers of p65 protein comprise a specific subset of NF-κB complexes and have been shown to be a transcriptionally active complex for several inflammatory genes such as intercellular adhesion molecule-1, interleukin-8, and tissue factor (1). Of the other two (slower migrating) NF-κB complexes observed in pig lung by EMSA (Fig. 1), the slowest migrating complex also appeared to be supershifted by the p65 antibody (Fig. 2). Thus this complex may also be composed of NF-κB p65 protein but in a different configuration than the smallest migrating complex. The intermediate migrating complex did not supershift with antibody to either p50 or p65 and thus may be composed of a different NF-κB protein such as p52 (NF-κB2), Rel B, or C-Rel. Alternatively, it is possible that the p50 antibody that we used may not be immunoreactive to pig antigen.

The increase in NF-κB activity is associated with poor graft function in our study of lung transplants. After the rise in NF-κB in the control transplant lungs, these lung grafts exhibit poor oxygenation, poor ventilation (PCO2), elevated mean PAP, and reduced airway compliance. On histopathological specimens, the lungs demonstrate alveolar and interstitial edema. In addition, a dramatic increase in tissue neutrophil infiltration is evident in histological specimens. Several studies have described the upregulation of adhesion molecule expression (1) and cytokine-induced neutrophil chemoattractant mRNA expression (3) associated with NF-κB activation, all of which may contribute to the neutrophilic inflammation associated with NF-κB.

To further clarify the connection between NF-κB and in vivo lung reperfusion injury, we treated the lung graft with PDTC, a potent inhibitor of NF-κB activation, during organ preservation. A number of studies have demonstrated the inhibition of NF-κB with PDTC in vitro (16, 20), and recent reports have justified its use in vivo (10, 11, 15). However, treatment of a donor organ with PDTC at the time of harvest and ischemic preservation before transplantation have yet to be described. We have shown that this strategy inhibited the activation of NF-κB after lung transplant and reperfusion (see Fig. 3). At the peak of NF-κB activity 30 min after reperfusion, PDTC reduced this level by 75%. The inhibition of NF-κB nuclear activity improved the physiology of the transplanted lung, with the PDTC-treated lungs demonstrating significantly higher levels of PO2, lower PCO2, and decreased mean PAP, whereas there was a trend toward improved airway compliance. In addition, histological analysis of the PDTC-treated lungs closely resembled that of normal lungs, whereas the nontreated lungs displayed high levels of cellular infiltration and edema. Our results indicate that NF-κB is critically important in lung reperfusion injury after transplantation and is an important regulator of neutrophilic infiltration.

The dithiocarbamates, such as PDTC, represent a class of antioxidants reported to be potent inhibitors of NF-κB and are capable of inhibiting the inflammatory process associated with the activation of NF-κB. The most effective inhibitor of NF-κB appears to be the pyrrolidine derivative of dithiocarbamate (PDTC) as a result of its ability to traverse the cell membrane and its prolonged stability in solution at physiological pH (24). PDTC, as well as other antioxidants, may inhibit NF-κB by suppressing the production of intracellular reactive oxygen species. Another chemical property of PDTC is its chelating activity for heavy metals, which is the reason that the diethyl derivative is used for the treatment of heavy metal poisoning. Because nonthiol metal chelators such as o-phenanthroline and desferrioxamine can also inhibit NF-κB activation, it is possible that the inhibitory effect of PDTC relies on both of its properties (20). Thus PDTC may have other cellular effects as yet undefined, but it is well established that inhibition of NF-κB is a major mechanism of the anti-inflammatory actions of PDTC.

We were unable to correlate MPO activity with the decreased neutrophil infiltration (inflammation) observed histologically. Histological evaluation by a pulmonary pathologist clearly indicated significant interstitial cellularity within pulmonary parenchyma and/or extension of the cellular infiltrates into the alveolar spaces of transplanted lungs. The cells most commonly represented in these infiltrates were almost always segmented neutrophils, with some additional macrophage infiltrates. These infiltrates were greatly reduced in histological specimens from PDTC-treated transplanted lungs, but the MPO activities were not reduced. The MPO assay gives no information about the location or extent of neutrophil sequestration, and it is possible that neutrophils may accumulate in non-alveolar regions of the lung such as the airways. A more plausible explanation is that our MPO activity is based on wet weight of the lung samples (see METHODS), and we have shown that significant edema occurs in the control lungs (Fig. 7), which would result in blunted MPO activities in these samples. In addition, the MPO assay can be greatly affected by the presence of whole blood (hemoglobin) in tissues, and the lungs were not perfused with saline to wash out blood remaining in the pulmonary circulation. We do not question the histological evaluation of neutrophil infiltration in our samples, but the lack of correlation with MPO activity indicates that a more rigorous application of MPO studies is required for accurate quantitation of neutrophil infiltration by MPO activity.

The activation of NF-κB involves removal of the inhibitory subunit 1κB-α from a latent cytoplasmic complex. Various stimuli first phosphorylate and then degrade 1κB-α, allowing free NF-κB to translocate to the nucleus where it binds to specific promoter sequences and initiates inflammatory gene transcription (9). Several 1κB proteins have been identified, but the degradation of 1κB-α has shown the highest correlation
with the activation of NF-kB (25). Our findings in a lung transplant model support a role for IκB-α as an inhibitor of NF-κB because activation of NF-κB during lung reperfusion injury was accompanied by the reduction of IκB-α protein soon after the initiation of reperfusion. Interestingly, the inhibition of NF-κB by PDTC does not appear to be regulated by IκB-α. In our study, PDTC inhibited NF-κB activation but not by stabilizing IκB-α. Whereas some studies have suggested that the mechanism of PDTC is through the blockade of phosphorylation of IκB-α (25), our in vivo findings indicate that the inhibition of NF-κB by PDTC does not occur through the preservation of IκB-α. Instead, the mechanisms for the effects of PDTC on NF-κB may involve inhibition of binding of the transcription factor to DNA, as demonstrated by others (4, 16), rather than an effect on the activation process.

There is now abundant evidence showing that lung reperfusion injury after transplantation is largely due to the activation and upregulation of mediators of the inflammatory cascade, and the central role that NF-κB plays in the induction of lung inflammatory injury is now emerging. We have shown for the first time that NF-κB is rapidly activated and is associated with poor pulmonary graft function in transplant reperfusion injury. PDTC given to the lung graft at the time of organ preservation effectively inhibited the activation of NF-κB and significantly improved lung function. One important question remaining is whether PDTC treatment and subsequent NF-κB inactivation have any long-term effect on organ survival and ultimate function. Although this question cannot be addressed by these studies, it is reasonable to speculate that there are positive long-term effects of NF-κB inhibition on graft function. It has become increasingly evident that reperfusion after ischemia is responsible for the majority of tissue injury in lung transplantation, and those lungs that might otherwise undergo severe graft dysfunction following transplant might be prevented from doing so by intervention with NF-κB inhibitors such as PDTC. Whereas the mechanisms of action of PDTC continue to be investigated and new inhibitors of NF-κB continue to be developed, we believe that a therapeutic strategy directed at the inhibition of NF-κB activation within the transplanted lung may prove effective in reducing lung ischemia-reperfusion injury.

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REFERENCES


