NITRIC OXIDE: Biology and Chemistry
Vol. 4, No. 4, pp. 399–411 (2000)
doi:10.1006/niox.2000.0297, available online at http://www.idealibrary.com on IDEAL®

Suppression of Bleomycin-Induced Nitric Oxide Production in Mice by Taurine and Niacin[1]

G. Gurujeyalakshmi, Yingjin Wang, and Shri N. Giri[2]

Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California 95616

Received January 25, 2000, and in revised form April 11, 2000

The effects of taurine (T) and niacin (N) on the influx of inflammatory cells and nitric oxide (NO) levels in bronchoalveolar lavage fluid (BALF) and expression of inducible NO synthase (iNOS) mRNA and iNOS protein in lungs were evaluated in the bleomycin (BL)–mouse model of lung fibrosis. Mice were placed into four groups: saline-instilled (SA) with a control diet (CD) (SA + CD); saline-instilled with TN (1% taurine in water + 2.5% (w/w) niacin in diet) (SA + TN); BL-instilled with CD (BL + CD); and BL-instilled with TN treatment (BL + TN). There was no difference in differential cell counts in BALF between the SA + CD and SA + TN control groups. Intratracheal instillation (IT) of BL (0.1 U/mouse) in mice stimulated an early influx of neutrophils followed by an increase in lymphocytes and macrophages in the BL + CD group. Taurine and niacin treatment significantly reduced the numbers of neutrophils, lymphocytes, and macrophages in the BL + TN group and caused significant reductions in BL-induced increases in the lung hydroxyproline content at 14 and 21 days in the BL + TN group. The mice in the SA + CD and SA + TN control groups had low levels of NO in BALF, whereas mice in the BL + CD group as compared to the SA + CD control group had elevated levels of NO from day 3 through day 21. Taurine and niacin treatment caused significant reductions in BL-induced increases in NO levels in BALF from mice in the BL + TN group at 7, 14, and 21 days as compared to the corresponding BL + CD group. The increases in NO levels in BALF from the BL + CD group were associated with elevated levels of iNOS gene expression and protein in the lungs. RT-PCR analysis of total RNA isolated from the lungs indicated that taurine and niacin treatment suppressed the BL-induced increases in iNOS message and iNOS protein. The ability of taurine and niacin to suppress the BL-induced increased production of NO secondary to decreases in iNOS mRNA and protein appears to be one of the mechanisms for their anti-inflammatory and antifibrotic effects.

Key Words: **lung fibrosis; bleomycin; taurine; niacin; nitric oxide; cytokines; transcription factor NF-κB.**

Idiopathic pulmonary fibrosis (IPF)[3] is a disease of unknown etiology, characterized by alveolar inflammation, an excess infiltration of inflammatory cells in the airways, and proliferation of fibroblasts in the alveolar walls and increased production of extracellular matrix proteins followed by a loss of normal lung architecture. The cellular injury in lung fibrosis is mediated by oxygen radicals produced by infiltrating inflammatory cells. The activated macro-

[1] A preliminary report of this work was presented in part at the International Taurine Symposium Certosa di Pontignano, Siena, Italy, August 4–8, 1999.

[2] To whom correspondence should be addressed. Fax: (530) 752-4698. E-mail: sngiri@ucdavis.edu.

[3] Abbreviations used: IPF, idiopathic pulmonary fibrosis; NO, nitric oxide; BL, bleomycin; T, taurine; N, niacin; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; BALF, bronchoalveolar lavage fluid; PBMC, peripheral blood mononuclear cell; TGF-β, transforming growth factor beta.

1089-8603/00 $35.00

phages have been shown to produce both nitric oxide (NO) and peroxynitrite (1). Peroxynitrite is a potent oxidant produced by the rapid reaction of NO and superoxide (2). Recent evidence suggests that macrophage- and neutrophil-derived nitrogen free radicals may have important roles in cytotoxicity (3).

NO has a wide biological role in modulating physiological and pathological processes involving regulation of blood pressure and neurotransmissions, macrophage toxicity, and neurotoxicity (4). Low concentrations of NO are sufficient, in most cases, to modulate the physiological processes. However, during infection and inflammation, *in vivo* formation of nitric oxide is increased. High concentrations of NO have been shown to cause deamination of deoxynucleotides and bases within the intact DNA *in vitro* and are known to be mutagenic *in vivo* (5). A role for NO in the physiologic processes of pulmonary functions is suggested by findings of the nitric oxide synthase (NOS) activity in lung and the vasodilatory effect of inhaled NO on pulmonary vessels and airways and the ability of the lung epithelial cells to produce factor(s) capable of causing smooth-muscle relaxation. In lungs, NOS has been localized and identified as two different forms (6), i.e., a constitutive form present in endothelial cells and brain (7) and an inducible form found in macrophages (8). Several investigators have demonstrated that macrophages when activated *in vitro* by cytokines, including interferon-γ and tumor necrosis factor, or lipopolysaccharide secrete reactive nitrogen intermediates through an L-arginine-dependent pathway (9–11). These findings suggest a definite role of NO in the pathophysiology of lung diseases. In fact, increased production of peroxynitrite has been shown in acute lung injury and adult respiratory distress syndrome, suggesting a role for this potent oxidant in inflammatory lung diseases (12, 13).

Bleomycin-induced lung fibrosis results from an inflammatory reaction of the lung partially mediated by neutrophils and alveolar macrophages that produce reactive oxygen species (ROS) such as hydroxyl radicals, superoxide ions, and hydrogen peroxide. The ROS are believed to damage the lung parenchyma. In addition, BL itself produces superoxide and hydroxyl radicals by binding to DNA:Fe^{2+} and forming a DNA:Fe^{2+}:BL complex which undergoes redox cycling and generates ROS (14). The ability of BL to generate superoxide radicals *in vivo* enhances the possibility of increased production of peroxynitrite, a potent oxidant known to be involved in lung inflammatory diseases. A variety of stimuli are known to stimulate the overexpression of inducible nitric oxide synthase (iNOS) mRNA, protein, and nitric oxide production in macrophages and neutrophils (9–11). Huot and Hacker (15) reported that macrophages activated *in vivo* by BL secrete nitrite spontaneously and this secretion can be blocked by a substrate-specific analogue of the L-arginine-dependent effector mechanism, N^6-monomethylarginine. Taurine chloramine has been shown to block the induction of NOS at the transcriptional level in the activated RAW 264.7 cell (16) and this is considered to be one of the mechanisms for its protective effect against lung injury.

We have consistently demonstrated that the mechanisms for the antifibrotic effects of taurine and niacin in the BL–rodent model of lung fibrosis include, initially, the down-regulation of BL-induced overexpression of TGF-β mRNA followed by suppression of procollagen mRNA and a decreased level of collagen content in the lungs (17, 18). Recently, we found that the anti-inflammatory and antifibrotic effects of the combined treatment with taurine and niacin depend on their ability to inhibit the BL-induced activation of NF-κB and decreased production of proinflammatory and fibrogenic cytokines such as TNF-α, IL-1α, IL-6, and TGF-β (19).

Since a large part of BL-induced lung damage is due to the production of macrophage- and neutrophil-derived ROS, we hypothesized that BL also up-regulates the expression of iNOS mRNA in the lungs, and the reported antifibrotic effects of taurine and niacin may reside in their ability to suppress the production of NO by down-regulating the iNOS mRNA. Therefore, we investigated the expression of iNOS mRNA and protein in lungs and the levels of NO (measured as nitrite) in BALF during BL-induced lung fibrosis in mice with and without the combined treatment with taurine and niacin.

EXPERIMENTAL PROCEDURES

Animal Model

The bleomycin–mouse model of acute lung injury which eventuates into fibrosis has been previously

established in our laboratory and the same model was employed in the present study (20). Briefly, all experiments were carried out in male C57BL/6 mice weighing 25–28 g (Simonsen, Gilroy, CA). Animals were caged in groups of four or five in Animal Resource Services facilities approved by the American Association for the Accreditation of Laboratory Animal Care and allowed to acclimatize for 1 week before starting the study. The mice had access to water and either pulverized Rodent Laboratory Chow 5001 (Purina Mills, St. Louis, MO) or the same pulverized chow containing 2.5% niacin (w/w) and 1% taurine in water. Animals were randomly divided into four experimental groups: saline-instilled (SA) with a control diet (CD) and drinking water (SA + CD); saline-instilled with taurine (T) in drinking water and niacin (N) in diet (SA + TN); BL-instilled with the control diet and drinking water (BL + CD); and BL-instilled with taurine in drinking water and niacin in diet (BL + TN). The animals were fed these diets starting 3 days before intratracheal (IT) instillation and continuing throughout the entire course of the study. After mice were anesthetized with ketamine and xylazine, either 50 μl of sterile isotonic saline or 0.1 U of bleomycin sulfate in 50 μl of saline per mouse was IT instilled.

BALF Collection and Lung Processing

The mice were sacrificed by an overdose of sodium pentobarbital at 1, 3, 5, 7, 14, and 21 days after IT instillation and bronchoalveolar lavage was carried out as previously described (21). Briefly, the lung was lavaged with 1 ml of isotonic sterile saline four times. The recovery of saline ranged from 3.0 to 3.6 ml. Total cells in BALF were counted with a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) and the differential cells from individual mice were determined on Diff-Quik-stained cytospin slides (Shandon, Inc., Pittsburgh, PA) using LeukoStat stain kit (Fisher Scientific, Pittsburgh, PA). After lavage, the lungs were dissected out, freeze clamped, and then stored at −80°C. BALF was centrifuged at 4°C for 10 min at 1500 rpm. The supernatant was gently aspirated and stored at −80°C until used for cytokine assays. In another set, the animals were killed by decapitation and their lungs were quickly removed, freeze clamped, and then dropped in liquid N_2 and stored at −80°C until used for mRNA analysis.

Protein Assay

Total proteins in BALF and proteins extracted from the lung nuclei were determined by the Coomassie blue dye binding assay (Bio-Rad Laboratories, Hercules, CA).

Nitrite Assay

Nitrite in BALF was determined by a spectrophotometric method based on the Griess reaction (22). Prior to analysis, nitrate in the samples was reduced to nitrite using the enzyme nitrate reductase (Boehringer Mannheim Corp., Indianapolis, IN), 0.1 U/ml, in the presence of 50 μM NAPDH, 5 μM flavin adenine dinucleotide (FAD), 6 μg/ml lactate dehydrogenase, and 0.2 mM sodium pyruvate. A total of 150 μl of sample was mixed with 150 μl of Griess' reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylenediamide dihydrochloride in water) and incubated at room temperature for 5 min followed by measuring the absorbance by a plate reader at a wavelength of 550 nm. Nitrite concentrations in the samples were determined from a standard curve generated by different concentrations of sodium nitrite.

Hydroxyproline Content

Collagen content was determined by measuring the hydroxyproline level of the whole lung. The lung was excised, homogenized, and hydrolyzed in 6 N HCl overnight at 110°C. Hydroxyproline content was assessed by the colorimetric method of Woessner (23). Data are expressed as micrograms of hydroxyproline per lung.

RNA Extraction and RT-PCR

Total RNA from the lung was isolated using the RNeasy total RNA extraction protocol (Qiagen, Chatsworth, CA) according to the manufacturer's description. The PCR primers for GAPDH and NOS in message amplification and phenotyping analysis

of cytokine mRNA were obtained from Clontech Laboratories (Palo Alto, CA). The following 5′-primer and 3′-primer sequences were employed: GAPDH, 5′-primer, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, 3′-primer, 5′-CATGTAGGCCATGAGGTCCACCAC-3′; NOS, 5′-primer, 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′, 3′-primer, 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′.

First-strand cDNA synthesis was performed using an Advantage RT-for-PCR kit (Clontech). After completion of the first-strand cDNA synthesis, the samples were diluted 1:5 and the PCR reactions were carried out as described previously (17). Briefly, PCR was performed at 94°C with initial denaturation for 5 min, followed by 30 cycles of amplification at 94 (45 s), 60 (45 s), and 72°C (2 min). Finally, the samples were extended at 72°C for 7 min. The specificity of amplification was checked by assessing whether a fragment of the expected size had been obtained with the positive control. Equal amounts of the PCR-amplified products (10 μl) were run on a 2% agarose ethidium bromide stained gel. The relative intensity of cytokine RT-PCR products was determined with a Bio-Rad image analyzer (Bio-Rad).

Tissue Protein Extraction

Frozen tissue samples were first minced and then homogenized in protein extraction buffer (20 mM Hepes, pH 7.5; 1.5 mM magnesium chloride; 0.2 mM EDTA; 100 mM sodium chloride; 2 mM dithiothreitol; and 0.5 mM PMSF). The homogenized samples were transferred to a microfuge tube, adjusted to a final concentration of 0.4 M sodium chloride, and centrifuged at 9000*g* at 4°C for 30 min. Supernatants were collected and added to an equal volume of protein extraction buffer containing 20% glycerol and 0.4 M sodium chloride (24). Protein concentrations of the tissue extracts were determined by the Bio-Rad reagent (Bio-Rad).

Western Blot Analysis

Whole-lung tissue was homogenized in lysis buffer (10 mM Hepes, pH 7.9; 150 mM NaCl; 1 mM EDTA; 0.5 mM PMSF; 1 μg/ml leupeptin; 1 μg/ml aprotenin; and 1 μg/ml pepstatin) on ice. Homogenates were centrifuged at 9000*g* at 4°C for 30 min to remove cellular debris. The NOS protein was immunoprecipitated from lung homogenates with agarose conjugates specific for NOS (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentrations were determined as described for nuclear extracts. Total cellular protein (200 μg) was immunoprecipitated with 10 μg of NOS antibody agarose conjugate. Immunoprecipitates were processed according to the manufacturer's instructions. Aliquots of immunoprecipitates were separated on SDS–PAGE gels (4–20% Tris–glycine minigels), transferred to PVDF membranes, and immunoblotted as described previously (25). Nonspecific binding sites were blocked with Tris-buffered saline (100 mM Tris, 0.9% NaCl, pH 7.5), 0.1% Tween 20 (TBS–T), and 5% nonfat dry milk at room temperature for 18 h. Membranes were then incubated in a 1:1000 dilution of a rabbit polyclonal anti-NOS (Santa Cruz Biotechnology) in TBS–T. After four washes in TBS–T, membranes were incubated in a 1:5000 dilution of horseradish peroxidase conjugated anti-rabbit IgG (Santa Cruz Biotechnology). Immunoreactive NOS protein was detected by enhanced chemiluminescence (ECL) and quantitated on a scanning densitometer (Shimadzu Model CS-9301 PC, Columbia, MD).

Statistical Analysis

Treatment-related differences were evaluated using a two-way ANOVA, followed by pairwise comparisons using the Newman–Keuls test. A value of $P \leq 0.05$ was considered to be the minimum level of statistical significance.

RESULTS

Recovery of Total and Differential Cells in BALF

The effects of saline or BL instillation with and without taurine and niacin treatment on total and differential cells in BALF from mice in various groups are summarized in Fig. 1. Both total and the differential cells in BALF from mice in the SA + CD and SA + TN groups were more or less the same at all time points of the study. The number of total cells recovered in BALF from mice in the BL + CD group was gradually increased from day 5 through day 21 and significant increases occurred at 7, 14, and 21

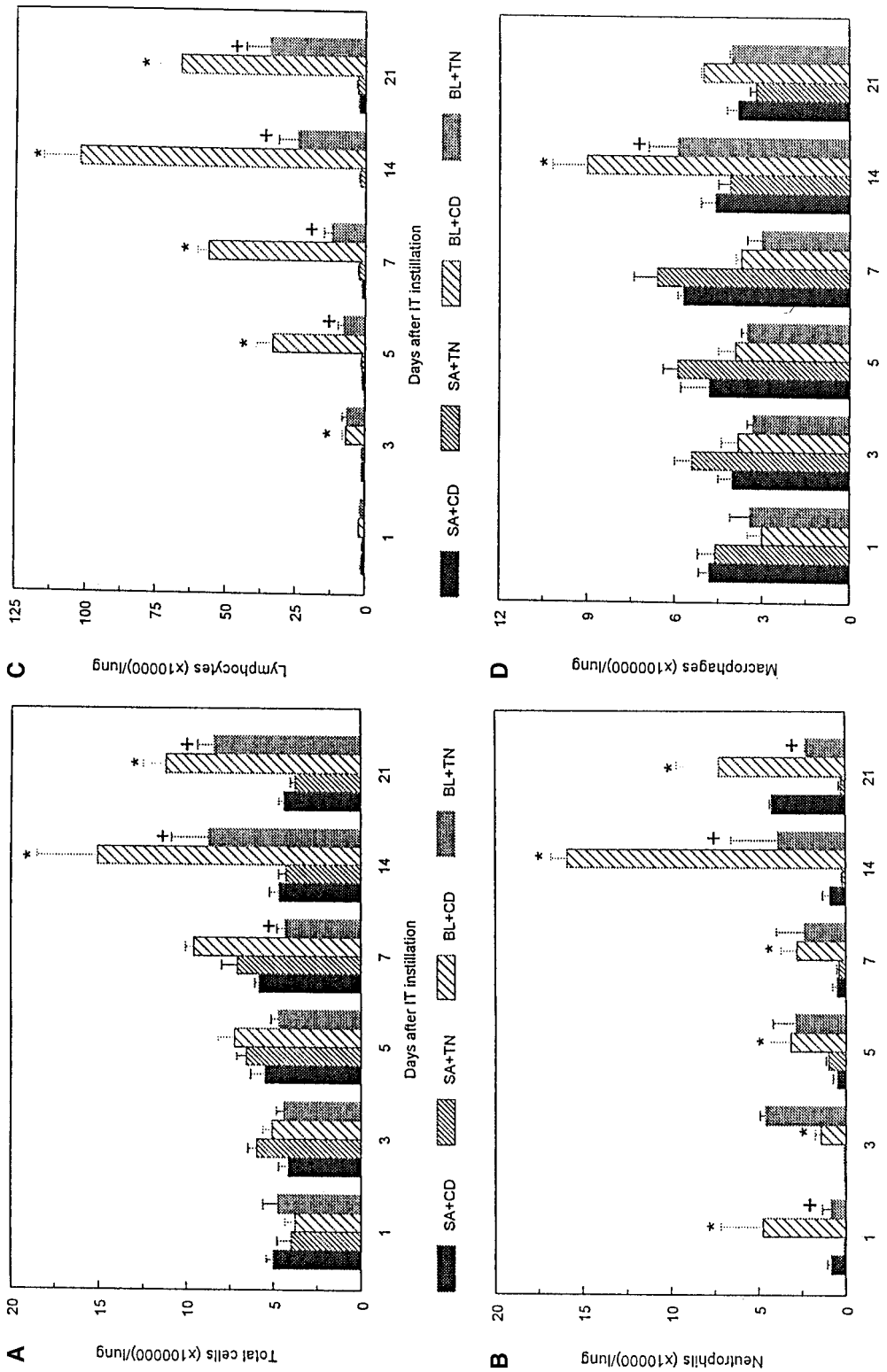


FIG. 1. Effects of intratracheal (IT) instillation of saline or bleomycin (0.1 U/mouse) with and without 1% taurine (T) in drinking water and 2.5% (w/w) niacin (N) in diet on total and differential cell counts in BALF at different times after instillation. See Experimental Procedures for treatment details. Abbreviations: SA + CD, saline + control diet; SA + TN, saline + taurine + niacin; BL + CD, bleomycin + control diet; BL + TN, bleomycin + taurine + niacin. Each value represents the mean ± SE of five mice. (*) Significantly higher ($P < 0.05$) than SA + CD and SA + TN control groups. (+) Significantly lower ($P < 0.05$) than BL + CD groups. (A) Total cells; (B) neutrophils; (C) lymphocytes; (D) macrophages.

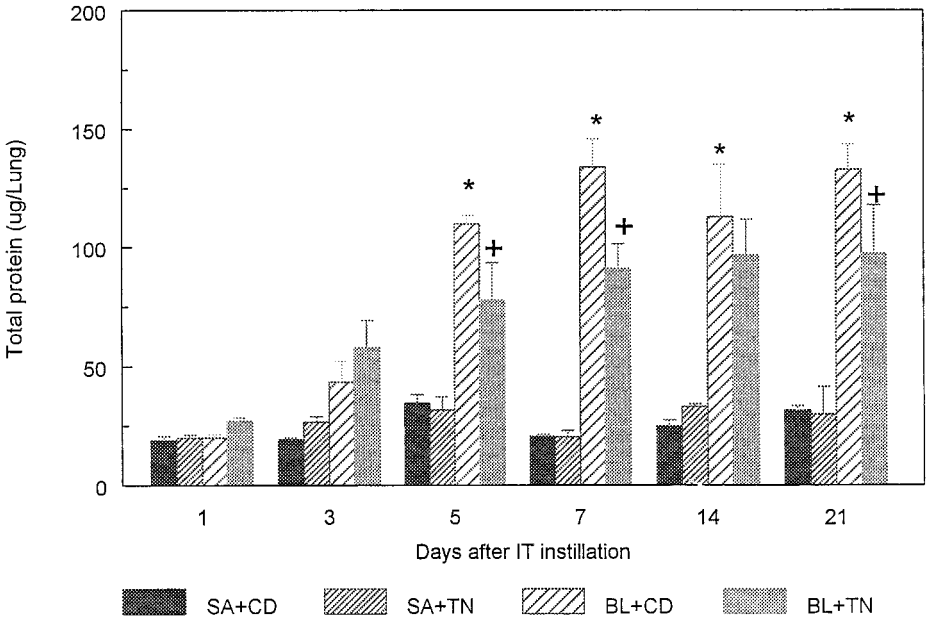


FIG. 2. Effects of IT instillation of saline or bleomycin with and without taurine + niacin on the total protein content of BALF supernatant at different time points. See Experimental Procedures for treatment details and legend to Fig. 1 for explanation of abbreviations. Each value represents the mean ± SE of five animals. (*) Significantly higher ($P < 0.05$) than all other groups at the corresponding times. (+) Significantly lower than the BL + CD group at the corresponding time points.

days as compared to the corresponding SA + CD control group. Treatment with taurine and niacin caused significant reductions in the total cells in the BL + TN group at 14 and 21 days as compared to the BL + CD group at the corresponding times (Fig. 1A). The differential cell count revealed that the increased number of total cells recovered in BALF from the BL + CD group was due to an increased influx of neutrophils, macrophages, and lymphocytes. However, the pattern of these increases with respect to time varied from cell to cell type after BL instillation. For example, a predominance of neutrophils in the BL + CD group occurred on day 1, peaked on day 14, and remained significantly elevated on day 21 as compared to the SA + CD control group at the corresponding time points (Fig. 1B). Taurine and niacin treatment in the BL + TN group significantly reduced the influx of neutrophils in BALF at 1, 14, and 21 days as compared to the BL + CD group at the corresponding time points (Fig. 1B). IT instillation of BL also increased the influx of lymphocytes in BALF from mice in the BL + CD group beginning on day 3 through day 21. However, treatment with taurine and niacin in the BL + TN group caused significant reductions in the lymphocyte influx at 5, 7, 14, and 21 days as compared to the corresponding BL + CD group (Fig. 1C). The changes in the number of macrophages following BL instillation in the BL + CD group were not as remarkable as changes in neutrophils and lymphocytes. The macrophages in the BL + CD group were increased by 2-fold at 14 days as compared to the SA + CD control group, and treatment with taurine and niacin significantly reduced the number of macrophages in the BL + TN group as compared to the BL + CD group at this time point (Fig. 1D).

Total Protein in BALF

The total protein content in the BALF supernatant was measured as an index of pulmonary vascular permeability in response to IT instillation of BL with and without the combined treatment with taurine and niacin (21). The protein levels in the SA + CD and SA + TN groups were almost the same through the entire period of the study (Fig. 2). Intratracheal instillation of BL caused significant increases in the protein levels of BALF from mice in the BL + CD group starting at day 5 and continuing through day 21 as compared to the SA + CD control

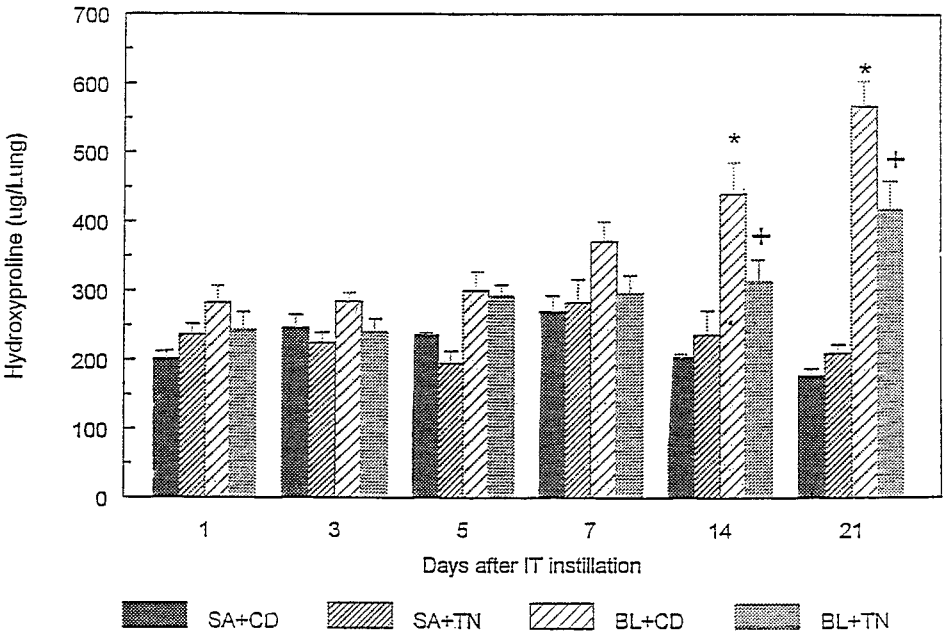


FIG. 3. Effects of IT instillation of saline or bleomycin with and without taurine + niacin on hydroxyproline content of the lung at different time points. See Experimental Procedures for treatment details and legend to Fig. 1 for explanation of abbreviations. Each value represents the mean ± SE of five animals. (*) Significantly higher ($P < 0.05$) than all other groups at the corresponding times. (+) Significantly lower than the BL + CD group at the corresponding time points.

group at the corresponding times. The combined treatment with taurine and niacin significantly reduced the BL-induced increases in protein levels of BALF from mice in the BL + TN group at 5, 7, and 21 days as compared to the BL + CD group at the corresponding time points (Fig. 2).

Hydroxyproline Content of Lungs

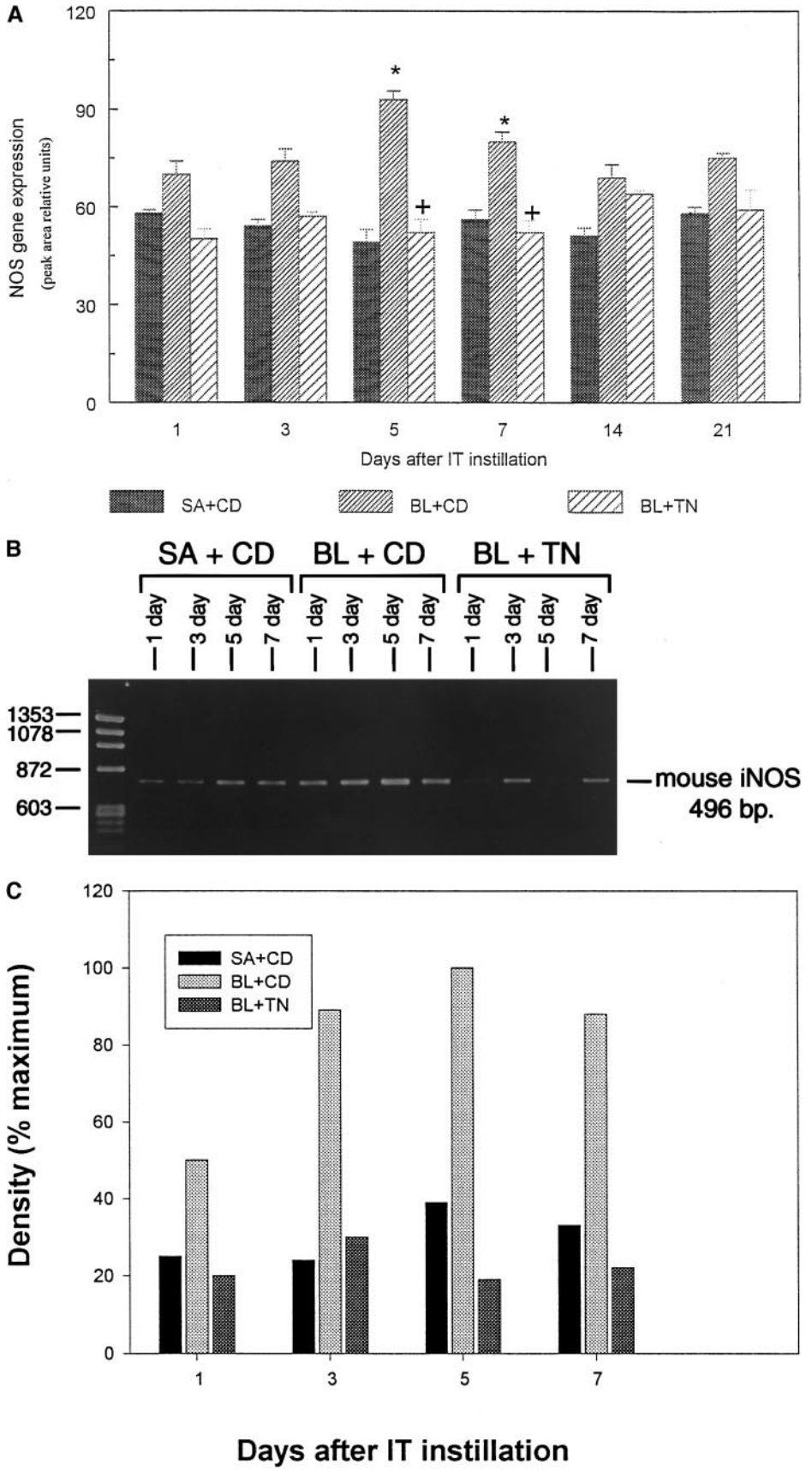
The lung levels of hydroxyproline in whole-lung homogenates were measured as an index of collagen content, a hallmark of lung fibrosis, in various treatment groups. Intratracheal instillation of BL significantly increased the lung levels of hydroxyproline of mice in the BL + CD group at 14 and 21 days as compared with mice in the corresponding SA + CD and SA + TN control groups (Fig. 3). The combined treatment with taurine and niacin significantly attenuated the BL-induced increases in the lung hydroxyproline levels of mice in the BL + TN group at both time points (Fig. 3).

Expression of iNOS mRNA in the Lungs

NOS catalyzes the production of nitric oxide from L-arginine. It is known that NO plays a critical role in a variety of physiological and pathological conditions of the lung. In this part of the study, we examined the kinetics of iNOS mRNA expression in the lungs of mice following IT instillation of BL with and without taurine and niacin treatment. The expression of iNOS mRNA was significantly increased by BL instillation in lungs of mice in the BL + CD group at 5 and 7 days as compared to the SA + CD control group at the corresponding time points (Fig. 4A). Although the combined treatment with taurine and niacin down-regulated the BL-induced overexpression of iNOS mRNA in the BL + TN group at all time points, significant down-regulations occurred only at 5 and 7 days (Fig. 4A). A representative ethidium bromide stained agarose gel showing amplified iNOS PCR product after 30 cycles of PCR for various groups is shown in Fig. 4B and the corresponding densitometric values of the stained agarose gel are shown in Fig. 4C.

Levels of iNOS Protein in Lung

We measured the levels of iNOS protein in lungs from mice in the SA + CD, BL + CD, and BL + TN


A
NOS gene expression
(peak area relative units)
120
90
60
30
0
*
*
+
+
1
3
5
7
14
21
Days after IT instillation
SA+CD
BL+CD
BL+TN
B
SA + CD
BL + CD
BL + TN
1 day
3 day
5 day
7 day
1353
1078
872
603
mouse iNOS
496 bp.
C
Density (% maximum)
120
100
80
60
40
20
0
SA+CD
BL+CD
BL+TN
1
3
5
7
Days after IT instillation

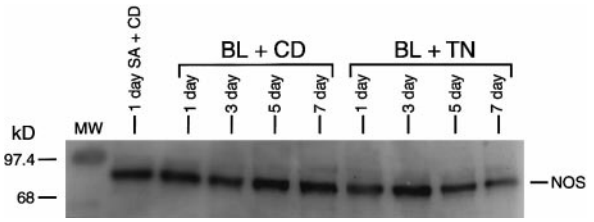


FIG. 5. Effects of IT instillation of saline or bleomycin with and without taurine + niacin on lung iNOS protein expression at different time points. See Experimental Procedures for treatment details and legend to Fig. 1 for explanation of abbreviations. Western blot analysis showing time-dependent effect of taurine + niacin on BL-induced increased iNOS protein expression. These gel data are representative of three independent experiments.

groups by Western blot analysis. The lung homogenates from mice in the SA + CD control group had an appreciable level of iNOS protein (Fig. 5). However, IT instillation of BL caused marked increases in the levels of this protein in the BL + CD group at 5 and 7 days and treatment with taurine and niacin attenuated these increases in the BL + TN group at both time points (Fig. 5).

Levels of Nitric Oxide in BALF

The nitric oxide levels (measured as nitrite) in BALF were significantly increased in the BL + CD group by 2-, 2.3-, 2.1-, 2.3-, 2.5-, and 3.3-fold 1, 3, 5, 7, 14, and 21 days as compared to the SA + CD control group at the corresponding time points, respectively (Fig. 6). The combined treatment with taurine and niacin significantly decreased the BL-induced increases in the levels of nitric oxide in the BL + TN group by 36, 64, 40, and 48% at 5, 7, 14, and 21 days, respectively (Fig. 6).

DISCUSSION

The inflammatory event, which generally precedes the interstitial lung fibrosis, is one of the major contributing factors in fibrosing lung diseases, including cryptogenic fibrosing alveolitis and fibrosing alveolitis (26). Exhaled levels of nitric oxide in active fibrosing alveolitis (27), asthma (28), and bronchiectasis (29) reflect a heightened degree of activity of the inflammatory cells present in the airways. The airway epithelial and endothelial cells in the lung are known to produce nitric oxide (15). The elevated levels of nitric oxide in the lung diseases are most likely due to activation of the inducible form of nitric oxide synthase (iNOS). We tested two hypotheses in the present study: (1) the increased levels of nitric oxide in BALF occur during the course of BL-induced lung fibrosis due to activation of the iNOS in the lungs and (2) the anti-inflammatory and antifibrotic effects of the combined treatment with taurine and niacin as reported in the BL–rodent model (17, 18) are partly attributable to their ability to suppress the production of nitric oxide by inhibiting the induction of iNOS in the lungs.

The data reported in this study demonstrate that IT instillation of BL significantly increased the influx of inflammatory cells and this was associated with the increased levels of NO in BALF from mice in the BL + CD group as compared to mice in the SA + CD and SA + TN control groups. The increased levels of NO in BALF, though not at all time points, were coupled with an overexpression of iNOS mRNA and iNOS protein in lungs of mice in the BL + CD group as compared to mice in the control groups. It is interesting that taurine and niacin treatment suppressed the BL-induced increases in the influx of inflammatory cells and the levels of NO in BALF from mice in the BL + TN group. In addition, this treatment also down-regulated the BL-induced overexpression of iNOS mRNA and iNOS protein in the lungs of mice in the BL + TN group. Intratracheal instillation of BL in mice stimulates

FIG. 4. Effects of IT instillation of saline or bleomycin with and without taurine + niacin on the steady-state levels of iNOS mRNA transcripts in lungs at different time points. See Experimental Procedures for treatment details and legend to Fig. 1 for explanation of abbreviations. (A) Total RNA was isolated from lungs and RT-PCR was performed with primers specific for iNOS and the housekeeping gene GAPDH. Each value represents the mean ± SE of five animals. (*) Significantly higher ($P < 0.05$) than all other groups at the corresponding times. (+) Significantly lower than the BL + CD group at the corresponding times. (B) A representative ethidium bromide stained agarose gel showing amplified iNOS PCR product after 30 cycles of PCR. (C) Densitometric values of the corresponding iNOS PCR products shown in B.

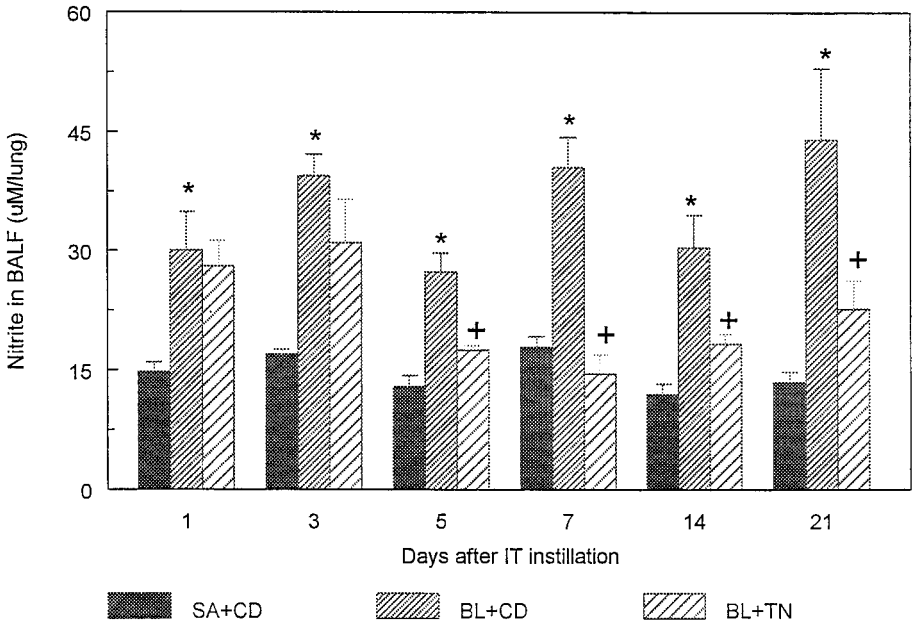


FIG. 6. Effects of IT instillation of saline or bleomycin with and without taurine + niacin on the NO levels of BALF at different time points. See Experimental Procedures for treatment details and legend to Fig. 1 for explanation of abbreviations. Each value represents the mean ± SE of five animals. (*) Significantly higher ($P < 0.05$) than all other groups at the corresponding times. (+) Significantly lower than the BL + CD group at the corresponding times.

an early influx of neutrophils into the lungs followed by an increase in the number of lavageable lymphocytes and alveolar macrophages. The macrophages are primed to release an excess amount of proinflammatory mediators, chemokines and growth factors which seem to be involved in the BL-induced lung fibrosis (30, 31). The results of the present study demonstrate that taurine and niacin treatment suppressed the BL-induced increased influx of inflammatory cells, including neutrophils, lymphocytes, and macrophages, and this may have contributed to their beneficial effects against BL-induced lung inflammation and fibrosis.

We have demonstrated in an earlier study that the mRNAs for IL-1α, IL-6, TNF-α, and TGF-β are generally up-regulated in the lungs. Consequently, we found increased levels of these cytokines in BALF following IT instillation of a fibrogenic dose of BL in mice in BL + CD groups (19). The levels of these inflammatory cell derived cytokines—IL-1α, IL-6, TNF-α, and TGF-β—were significantly decreased by taurine and niacin treatment in BL + TN groups (19). It appears that the suppression in the influx of inflammatory cells goes hand in hand with decreased levels of inflammatory cell derived cytokines and NO in BALF as reported earlier (19) and found in the present study. It is not known how the combined treatment with taurine and niacin suppresses the BL-induced increased influx of inflammatory cells and increased levels of NO in BALF. The increased production of NO is most likely due to increased *de novo* synthesis of iNOS as reflected by increased steady-state levels of iNOS mRNA in the lungs following IT instillation of BL in the BL + CD group. This suggests that one of the mechanisms for BL-induced lung fibrosis is an excess production of NO. An excess production of NO is thought to play a key role in other models of fibrosis such as the hepatic fibrosis produced by the bile duct ligation in the rat (32). Furthermore, the findings reported in this study are also in agreement with the findings of other studies that show that BL, when incubated with peripheral blood mononuclear cells (PBMC), caused a significant stimulation of NO production in the culture medium. The supernatant (conditioned medium) resulting from the culture medium stimulated the production of NO by the 3T3 fibroblasts and triggered an overexpression of iNOS mRNA in these cells (33). The production of NO by 3T3 fibroblasts in the presence of the conditioned medium

was blocked by antibodies against IL-β, TNF-α, and iNOS inhibitor. This suggests the release of these cytokines in response to BL treatment of PBMC and the ability of BL, IL, and TNF-α to stimulate the production of NO (33). Therefore, our earlier findings that treatment with taurine and niacin suppressed the BL-induced increased levels of IL-α, TNF-α, IL-6, and TGF-β in BALF (19) will explain the decreased levels of NO in BALF from mice in the BL + TN group as found in the present study.

The increase in iNOS mRNA expression in the BL + CD group may be dependent on increased NF-κB binding since the expression of iNOS mRNA is largely regulated by transcriptional activation. The upstream portion of the murine iNOS gene contains sequences that bind to the transcription factor NF-κB, and this is essential for the transcription of murine iNOS mRNA (34, 35). We have previously demonstrated that NF-κB binding was significantly increased in the lungs of BL-instilled mice in the BL + CD group and treatment with taurine and niacin blocked the BL-induced NF-κB activation and decreased the release of fibrogenic cytokines, known to stimulate the production of NO, in the BL + TN group (19). Therefore, it is likely that the binding of NF-κB to the lung nuclei of mice in the BL + CD group is critical in the transcription of iNOS mRNA and supports the involvement of NF-κB in the BL-induced increased levels of NO in BALF. This hypothesis is further supported by the finding of Xie *et al.* (35) that NF-κB activation is important for transcription of NO II gene in murine macrophages. It is conceivable that taurine and niacin may prevent the binding of the essential transcription factor NF-κB to the iNOS promoter, which in turn leads to decreased transcription of the iNOS gene. Thus, the ability of the combined treatment with taurine and niacin to suppress the BL-induced overexpression of iNOS message may constitute one of the possible mechanisms for their antifibrotic effects as demonstrated in the present study. It is not known whether taurine and niacin administered alone will produce a similar effect. However, it should be noted that these two compounds were found to produce antifibrotic effects independently in the BL–hamster model of lung fibrosis (36, 37).

In the present study, we used the whole lung from various treatment groups for iNOS mRNA study since the size of mice did not allow us to isolate and culture an adequate number of macrophages from the lungs for gene expression study. However, it should be noted that the transcription of iNOS mRNA in an epithelial cell line was also dependent on NF-κB activation and it was induced by oxidative mechanisms (38). The same molecular events were noticed in macrophages, Kupffer cells, hepatocytes, and fibroblasts after exposure to endotoxin and/or cytokines which produce large amounts of NO as a result of induction of iNOS (8, 33, 39, 40). Alternatively, our data also suggest that the increased levels of nitric oxide released by activated macrophages are mediated by BL-induced release of inflammatory cytokines, which are known to be potent inducers of nitric oxide synthase in macrophages (4). This is consistent with the finding of other investigators that BL stimulates the synthesis of mRNAs for a variety of cytokines in lungs (18, 30, 31).

ACKNOWLEDGMENT

This research was supported by National Heart, Lung, and Blood Institute Grant R01-56262-02.

REFERENCES

1. Barnes, P. J., and Belvisi, M. G. (1994). Nitric oxide and lung disease. *Thorax* **48,** 1034–1043.
2. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implication for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87,** 1620–1624.
3. Connor, E. M., and Grisham, M. B. (1996). Inflammation, free radicals and antioxidants. *Nutrition* **12,** 274–277.
4. Moncada, S., Palmer, R., and Higgs, E. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43,** 109–142.
5. Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuri, R. K., Misra, M., *et al.* (1991). DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **254,** 1001–1003.
6. Kobsik, L., Bredt, D. S., Lowenstein, C. J., Drazen, J., Gaston, B., Sugarbaker, D., and Stamler, J. (1993). Nitric oxide synthase in human and rat lung: Immunocytochemical and histochemical localization. *Am. J. Respir. Cell Mol. Biol.* **9,** 371–377.
7. Sessa, W. C., Harrison, J. K., Luthin, D. R., Pollock, J. S., and Lynch, K. R. (1993). Genomic analysis and expression pat-

terns reveal distinct genes for endothelial and brain nitric oxide synthase. *Hypertension* **21,** 934–938.

8. Sherman, M., Aberhard, E. E., Wong, V. Z., Griscavge, J. M., and Ignarro, L. J. (1993). Pyrrolidine dithiocarbonate inhibits induction of rat nitric oxide synthase in rat alveolar macrophages. *Biochem. Biophys. Res. Commun.* **191,** 1301–1308.
9. Hibbs, J. B., Taintor, R. R., Vavrin, Z., and Racchlin, E. M. (1988). Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157,** 87–94.
10. Ding, A., Nathan, C., and Stuehr, D. (1988). Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* **141,** 2407–2412.
11. Stuher, D. J., and Nathan, C. F. (1989). Nitric oxide: A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med.* **169,** 1543–1555.
12. Kooy, N. W., Royall, J. A., Ye, Y. Z., Kelly, D. R., and Beckman, J. S. (1995). Evidence for *in vivo* peroxynitrite production in human acute lung injury. *Am. J. Respir. Crit. Care Med.* **151,** 1250–1254.
13. Haddad, I., Pataki, G., Hu, P., Galliani, C., Beckman, J. S., and Matalon, S. (1994). Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *J. Clin. Invest.* **94,** 2407–2413.
14. Caspary, W. J., Lanzo, D. A., and Niziak, C. (1982). Effect of DNA on the production of reduced oxygen by bleomycin and iron. *Biochemistry* **21,** 334–338.
15. Huot, A. E., and Hacker, M. P. (1990). Role of reactive nitrogen intermediate production in alveolar macrophage-mediated cytostatic activity induced by bleomycin lung damage in rats. *Cancer Res.* **50,** 7863–7866.
16. Park, E., Quinn, M., Wright, C., and Schuller-Levis, G. (1993). Taurine chloramine inhibits the synthesis of nitric oxide and the release of tumor necrosis factor in activated RAW 264.7 cells. *J. Leukoc. Biol.* **54,** 119–124.
17. Gurujeyalakshmi, G., Iyer, S. N., Hollinger, M. A., and Giri, S. N. (1996). Procollagen gene expression is down-regulated by taurine and niacin at the transcriptional level in the bleomycin hamster model of lung fibrosis. *J. Pharmacol. Exp. Ther.* **277,** 1152–1157.
18. Gurujeyalakshmi, G., Hollinger, M. A., and Giri, S. N. (1998). Regulation of transforming growth factor-β_1 mRNA expression by taurine and niacin in the bleomycin hamster model of lung fibrosis. *Am. J. Respir. Cell Mol. Biol.* **18,** 334–342.
19. Gurujeyalakshmi, G., Wang, Y., and Giri, S. N. (2000). Taurine and niacin block the lung injury by down-regulating the bleomycin induced activation of transcription factor NF-κB in mice. *J. Pharmacol. Exp. Ther.* **293,** 82–90.
20. Giri, S. N., Hyde, D. M., and Marfino, B. J., Jr. (1986). Ameliorating effect of murine interferon gamma on bleomycin-induced lung collagen fibrosis in mice. *Biochem. Med. Metab. Biol.* **36,** 194–196.
21. Giri, S. N., Hollinger, M. A., and Schiedt, M. J. (1981). The effect of paraquat and superoxide dismutase on pulmonary vascular permeability and edema in mice. *Arch. Environ. Health* **36,** 149–154.
22. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Rannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and (^{15}N)nitrate in biological fluids. *Anal. Biochem.* **126,** 131–138.
23. Woessner, J. F., Jr. (1961). The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Anal. Biochem.* **93,** 440–444.
24. Choi, A. M. K., Sylvester, S., Otterbein, L., and Holbrook, N. J. (1995). Molecular responses to hyperoxia *in vivo:* Relationship to increased tolerance in aged rats. *Am. J. Respir. Cell Mol. Biol.* **13,** 74–82.
25. Gurujeyalakshmi, G., Hollinger, M. A., and Giri, S. N. (1999). Pirfenidone inhibits PDGF isoforms in the bleomycin hamster model of lung fibrosis at the translational level. *Am. J. Physiol. Lung Cell Mol. Physiol.* **276**(20), L311–L318.
26. Salch, D., Barnes, P. J., and Giad, A. (1997). Increased production of potent oxidant peroxy nitrite in the lungs of patients with idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **155,** 1763–1769.
27. Paredi, P., Kharitonov, S. A., Loukides, S., Pentelidi, S., Bois, R. M., and Barnes, P. J. (1999). Exhaled nitric oxide is increased in active fibrosing alveolitis. *Chest* **115,** 1352–1356.
28. Alving, K., Weitzburg, E., and Lundburg, J. M. (1995). Increased amount of nitric oxide in exhaled air of asthmatics. *Eur. Respir. J.* **6,** 1368–1370.
29. Kharitonov, S. A., Wells, A. U., O'Conner, B. J., Cole, P. J., Hensell, D. M., Logan-Sinclair, R. B., and Barnes, P. J. (1995). Elevated levels of exhaled nitric oxide in bronchiectasis. *Am. J. Respir. Crit. Care Med.* **151,** 1889–1893.
30. Phan, S. H., and Kunkel, S. L. (1992). Lung cytokine production in bleomycin-induced pulmonary fibrosis. *Exp. Lung Res.* **18,** 29–43.
31. Scheule, R. K., Perkins, R. C., Hamilton, R., and Holian, A. (1992). Bleomycin stimulation of cytokine secretion by the human alveolar macrophages. *Am. J. Physiol.* **262,** L386–L391.
32. Mayoral, P., Criado, M., Hidalgo, F., Flores, O., Arvelo, M. A., Eleno, N., Sanchez-Rodriguez, A., Lopez-Nova, J. M., and Esteller, A. (1990). Effects of chronic nitric oxide activation or inhibition on early hepatic fibrosis in rats with bile duct ligation. *Clin. Sci.* **96,** 297–305.
33. Yamamoto, T., Katayama, I., and Nishioka, K. (1999). Nitrite production in mouse 3T3 fibroblasts by bleomycin-stimulated peripheral blood mononuclear cell factors. *Clin. Exp. Rheumatol.* **17,** 343–346.

34. Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russel, S. W., and Murphy, W. J. (1993). Macrophage nitric oxide synthase gene: Two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* **90,** 9730–9734.
35. Xie, Q. W., Kashiwabra, Y., and Nathan, C. (1994). Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem*. **116,** 2545–2546.
36. Wang, Q., Giri, S. N., Hyde, D. M., and Nakashima, J. M. (1989). Effect of taurine on bleomycin-induced lung fibrosis in hamsters. *Proc. Soc. Exp. Biol. Med*. **190,** 330–338.
37. Wang, Q., Giri, S. N., Hyde, D. M., Nakashima, J. M., and Javadi, I. (1990). Niacin attenuates bleomycin-induced lung fibrosis in the hamster. *J. Biochem. Toxicol.* **5,** 13–22.
38. Adcock, I. M., Brown, C. R., Kwon, O., and Barnes, B. J. (1994). Oxidative stress induces NF-κB DNA binding and inducible NOS mRNA in human epithelial cells. *Biochem. Biophys. Res. Commun*. **199,** 1518–1524.
39. Geller, D. A., Nusseler, A. K., Di, S. M., Lowenstein, C. J., Shapiro, R. A., Wang, S. C., Simmons, R. L., and Billiar, T. R. (1993). Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc. Natl. Acad. Sci. USA* **90,** 522–526.
40. Ikeda, K., Kubo, S., Hirohashi, K., Kinoshita, H., Kenada, K., Kawada, N., Sato, E. F., and Inoue, M. (1996). Mechanisms that regulate the nitric oxide production by lipopolysaccharide-stimulated rat Kupffer cells. *Physiol. Chem. Phys. Med. NMR* **28,** 239–253.