Amelioration of Bleomycin-induced Lung Fibrosis in Hamsters by Dietary Supplementation with Taurine and Niacin: Biochemical Mechanisms

Shri N. Giri,[1] Robert Blaisdell,[1] Robert B. Rucker,[2] Qingjian Wang,[1] and Dallas M. Hyde[3]

[1]Department of Molecular Biosciences, Division of Pharmacology and Toxicology; [2]Department of Nutrition, School of Veterinary Medicine; [3]Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, California

Interstitial pulmonary fibrosis induced by intratracheal instillation of bleomycin (BL) involves an excess production of reactive oxygen species, unavailability of adequate levels of NAD and ATP to repair the injured pulmonary epithelium, and an overexuberant lung collagen reactivity followed by deposition of highly cross-linked mature collagen fibrils resistant to enzymatic degradation. In the present study, we have demonstrated that dietary supplementation with taurine and niacin offered almost complete protection against the lung fibrosis in a multidose BL hamster model. The mechanisms for the protective effect of taurine and niacin are multifaceted. These include the ability of taurine to scavenge HOCl and stabilize the biomembrane; niacin's ability to replenish the BL-induced depletion of NAD and ATP; and the combined effect of taurine and niacin to suppress all aspects of BL-induced increases in the lung collagen reactivity, a hallmark of interstitial pulmonary fibrosis. It was concluded from the data presented at this Conference that the combined treatment with taurine and niacin, which offers a multipronged approach, will have great therapeutic potential in the intervention of the development of chemically induced interstitial lung fibrosis in animals and humans. — Environ Health Perspect 102(Suppl 10):137–148 (1994)

Key words: bleomycin, interstitial lung fibrosis, taurine, niacin

Introduction

Our understanding of pulmonary interaction with the environment has been markedly advanced over the last several years. The lung is strategically located between the external or outside and the internal or systemic environments. Thus, it is continuously exposed to an increasing number of potentially harmful chemicals found in our biosphere as well as to chemicals entering the body following systemic administration. Some of these chemicals disrupt the delicate structure of the lung during the initial phase of acute inflammation followed by an increase in pulmonary connective tissue during the chronic phase leading to interstitial pulmonary fibrosis.

This paper was presented at the Conference on Oxygen Radicals and Lung Injury held 30 August–2 September 1993 in Morgantown, West Virginia.

This research was supported by the National Heart, Lung, and Blood Institute, National Institutes of Health; Grant No. 5R01 HL-27354. The authors thank Mary J. Schiedt and Mary Y. Stoval for their expert technical assistance.

Send correspondence to Dr. Shri N. Giri, Professor, Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, CA 95616. Telephone (916) 752-1059.

Interstitial pulmonary fibrosis is a crippling disease that is characterized by an excessive accumulation of collagen, reduced gas exchange, and reduced lung compliance (*1*). This disease is associated with a high incidence of morbidity and mortality and it may result from exposure to a number of environmental or pharmaceutical agents (*2*). Some of the processes leading to interstitial pulmonary fibrosis from these various agents are understood and some still are ill defined. Regardless of the multifactorial origin, interstitial pulmonary fibrosis is invariably accompanied by an overexuberant repair process that is characterized by an excess number of fibroblasts (*3*), an absolute increase in lung collagen, abnormality in the ultrastructural appearance, and spatial distribution of collagen types (*4–6*).

Bleomycin (BL), an antineoplastic drug, is widely used in the treatment of lymphoma and testicular and squamous cell tumors (*7*) because of its minimal hematopoietic toxicity or immunosuppressive activity (*8*). Unfortunately, BL therapy is complicated by a dose-dependent interstitial pneumonitis that often progresses into interstitial pulmonary fibrosis (*8*), greatly limiting its use on a long-term basis. Thus, the search for effective antifibrotic agents is of a great significance not only for elucidating the mechanisms for BL-induced lung fibrosis, but in preventing the development of lung fibrosis induced by a variety of therapeutic agents.

Bleomycin produces pulmonary toxicity that subsequently develops into fibrosis in a wide variety of animal species (*9*). The intratracheal (IT) instillation of BL in the rodent, as a model of pulmonary fibrosis, is frequently employed. A single IT instillation of BL in the rodent produces biochemical changes and histologic lesions that resemble interstitial pulmonary fibrosis as seen in humans (*10*). Consequently, the IT instillation of BL in rats (*11*), mice (*12*), and hamsters (*13*) provides a convenient and reproducible method to expose the lungs to high concentrations of BL in a single dose that is fibrogenic and at the same time devoid of toxicity to other organs. However, the single BL dose model is invariably associated with morbidity and high mortality.

Our laboratory has recently developed a multidose IT instillation BL hamster model of pulmonary fibrosis (*14*). Using

this model, we have investigated the effects of dietary supplementation with taurine and niacin against the BL-induced lung fibrosis in hamsters. In this respect, we have examined the various aspects of lung collagen reactivity including collagen content, prolyl hydroxylase, lysyloxidase, and collagenase activities, and collagen cross-links and poly(ADP-ribose) polymerase activity. Also examined were the protein content and acid phosphatase activity, total and differential cell counts in the bronchoalveolar lavage fluid (BALF), lung volume, and histopathologic features of the lung lesions.

Materials and Methods

Pathogen-free male golden Syrian hamsters weighing 80 to 100 g were purchased from Simonsen, Inc. (Gilroy, CA). Bleomycin sulfate (Blenoxane) was a gift from Bristol Laboratories (Syracuse, NY). Taurine and niacin were obtained from Sigma Chemical Company (St. Louis, MO). Tritiated (498 mCi/mmole) sodium borohydride and [1]^{14}C-acetic anhydride (25 mCi/mmole) were purchased from Amersham (Arlington Heights, IL). [4,5]^{3}H-Lysine (specific activity 95 Ci/mmole) was obtained from New England Nuclear (Boston, MA). Other chemicals were reagent grade or higher purity and obtained from standard commercial sources.

Hamsters were acclimatized for a minimum of 1 week prior to the experiment. The facility provided filtered air, a constant temperature and humidity, and a 12-hr/12-hr light/dark cycle. Animal care was in accordance with NIH guidelines for animal welfare.

Animals were fed *ad libitum* either pulverized Rodent Laboratory Chow 5001 (Purina Mills Inc., St. Louis, MO) or the same pulverized rat chow containing 2.5% taurine and 2.5% niacin (w/w). Animals were fed these diets starting 4 days before the first intratracheal instillation and continuing throughout the course of the experiment. The hamsters were randomly placed into four treatment groups: saline-instilled fed control diet (SA), bleomycin-instilled fed control diet (BL), bleomycin-instilled fed taurine-niacin in diet (TNBL), and saline-instilled fed taurine-niacin in diet (TNSA). The animals were intratracheally instilled with either three consecutive doses of bleomycin sulfate in saline, or saline 1 week apart (2.5, 2.0, and 1.5 U/5 ml/kg) under pentobarbital anesthesia (75–85 mg/kg) as described by Zia et al. (*14*).

The animals were sacrificed 1, 3, 4, and 8 weeks after the instillation of the last dose of saline or BL. The lungs of animals sacrificed at 3 weeks were subjected to BAL and morphologic and morphometric analysis as described in earlier papers (*15,16*). An aliquot of the fluid was apportioned for total and differential cell count (*17*). The remaining BALF was centrifuged at 1500*g* for 20 min at 4°C, and the resulting supernatant was used for the measurement of protein (*18*) and acid phosphatase activity (*19*).

Morphologic and Morphometric Analysis

After BAL and thoracotomy, the heart was ligated at the base for isolation of the pulmonary vasculature. The trachea was cannulated, and then both heart and lungs were removed *en bloc* and weighed. The lungs were fixed via the trachea at a pressure of 30 cm of water with a cacodylate-buffered glutaraldehyde-paraformaldehyde fixative (400 mOsm). The cannula was removed, the trachea was tied off, and the lung and heart stored in fixative. Fixed lung volumes were determined by their buoyant weight in saline after dissection of the heart and adjacent mediastinal tissue (*20*). Before embedding the lung tissue, the heart and all nonpulmonary tissue were isolated by blunt dissection and removed. Blocks of tissue were cut from at least two sagittal slabs (2–3 mm thick) from the right cranial, right caudal, and left lung lobes of each lung. Each block was cut with about a 1-cm^2 face. These blocks were dehydrated in a graded series of ethanol and embedded in paraffin. Sections 5 μm thick were cut from the paraffin blocks and stained with hematoxylin and eosin. From selected blocks, adjacent sections were cut and stained with sirus red for specific staining of collagenous fibers (*21*). The volume of parenchymal lesion within the lung was estimated using point counting techniques and a square lattice test system at a final magnification of ×160. All tissue on the slides was counted. Parenchymal lesions were defined as thickening of interalveolar septa due to edematous swelling, inflammatory cells or fibrosis associated with hyperplastic epithelial cells, and some airway inflammatory cells remaining after lung lavage. The volume of parenchymal lesion was calculated from the product of the volume of the lung, the volume of parenchyma per lung, and the volume of lesion per parenchymal volume (*22*).

Preparation of Lung for Biochemical Assays

The lungs of hamsters assigned to various biochemical assays were first perfused *in situ* with cold saline; all lung lobes were removed and frozen in liquid nitrogen. The lungs were stored at –80°C until the assays were performed. Later, the frozen lungs were thawed and homogenized in 0.1M KCl, 0.02M Tris, pH 7.6, with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). The homogenate was mixed thoroughly by repeated inversions and the homogenate volumes (9–10 ml) were recorded. The samples were aliquoted and stored at –80°C, except for the samples for malondialdehyde equivalent and collagen assays that were processed for their measurement on the same day the lungs were homogenated. Enzyme assays were performed on supernatant obtained by centrifugation of the lung homogenate at 12,000*g* for 20 min at 4°C unless specified otherwise.

Determination of Hydroxyproline and Prolyl Hydroxylase Activity. For lung hydroxyproline assay, 1 ml of homogenate was precipitated with 0.25 ml of ice-cold 50% (w/v) TCA and centrifuged and the precipitate was hydrolyzed in 2 ml of 6 N HCl for 18 hr at 110°F. [^{3}H]Hydroxyproline (1×10^{5} dpm) was added to each sample to determine recovery, and the hydroxyproline content was measured by the technique described by Woessner (*23*). The preparation of prolyl hydroxylase substrate (procollagen) and the method for the prolyl hydroxylase assay were essentially as described in our previous paper (*24*). During the reaction, tritium is released in stoichiometric proportion to prolyl hydroxylation and $^{3}H_2O$ is used as a measure of the enzyme activity. The enzyme activity was determined as disintegrations per minute of $^{3}H_2O$ released per total lung per 30 min and it is reported as percent of the control.

Determination of Malondialdehyde Equivalent, and Superoxide Dismutase Activity. Lung malondialdehyde equivalent was estimated from the total amount of thiobarbituric acid-reacting products in unfractionated homogenate by the method of Ohkawa et al. (*25*). SOD activity of the supernatant of lung homogenate was determined from the rate at which it inhibits the autooxidation of epinephrine to adrenochrome, as described by Misra and Fridovich (*26*). The rate of formation of adrenochrome was 0.025 absorbance units/min at 480 nm in a Varian Cary 219 spectrophotometer (Beckman Instruments, Palo Alto, CA). Under these defined conditions, the amount of tissue required to inhibit the rate of formation of adrenochrome by 50% (i.e., rate of 0.0125 absorbance units/min)

was defined to contain 1 unit of SOD activity.

Determination of Lung Calcium and Poly(ADP-ribose) Polymerase Activity. For calcium determination, 1 ml of lung homogenate was first deproteinized in a final concentration of 10% (w/v) TCA on ice. After centrifugation, the TCA supernatant was decanted and its volume recorded. The calcium content of the supernatant was determined by inductively coupled plasma (ICP) atomic emission spectrometry (*27*), using a model 3510 ICP spectrometer (Applied Research Laboratories, Sunland, CA). Lung poly(ADP-ribose) polymerase activity was determined by measuring the incorporation of [^{14}C]ADP-ribose for 15 min at 37°C into an acid-insoluble product as reported previously (*28*). The activity of the enzyme is expressed as picomoles of [^{14}C]ADP-ribose incorporated per total lung per 15 min.

Determination of Lung Lysyl Oxidase and Collagenase Activity. At the time of assay, the frozen lungs were weighed and pulverized in foil packages just after immersion in liquid nitrogen. The pulverized lung tissue was then quantitatively transferred to a test tube, allowed to thaw on ice, and a volume of ice-cold saline in ml equal to the weight (in g) of the lung was added. The tissue was then homogenized for 30 sec in three bursts with a Polytron (Brinkman) set to a medium power setting. A 0.5-ml aliquot was removed for collagenase assay and adjusted to 100 mM Tris HCl buffer, pH 7.6, in 15 mM $CaCl_2$ with 10× buffer. A 0.3-ml aliquot was removed from the whole homogenate for hydrolysis in 6 N HCl, and subsequent hydroxyproline determination according to the procedure of Woessner (*23*). The remaining volume of homogenate was used for lysyl oxidase assay.

Lysyl oxidase substrate was prepared according to the procedure of Pinnell and Martin (*29*) as modified by Kagan and Sullivan (*30*). [4,5]^{3}H-Lysine was used to label the chick aorta elastin as substrate for lysyl oxidase. The lung homogenates were adjusted to 16 mM potassium phosphate, 0.15 M NaCl, pH 7.8, with 10× buffer and centrifuged at 24,000*g*. The precipitates were then re-extracted with the same volume of 16 mM phosphate buffer, 0.15 M NaCl. The phosphate buffer extracts were then combined and frozen at –70°C until assayed.

For the assay, the precipitates were then re-extracted 2× with the same volume of 16 mM potassium phosphate, pH 7.8, in 4 M urea as used in the phosphate buffer extractions. The urea supernatant fractions were combined and dialyzed overnight against three changes of 16 mM potassium phosphate, 0.15 M NaCl at 4°C. The protein concentration of the samples was determined by the method of Lowry et al. (*18*). The protein concentration of dialyzed urea extract samples were adjusted to the protein concentration of the lowest sample in the group being assayed. The same assay procedure was followed with the phosphate buffer extracts.

Lysyloxidase assays were done in triplicates except in the few cases where only duplicates were possible. Incubations were conducted with 1×10^6 dpm substrate and 0.9 ml of extract in a total volume of 1 ml at 43°C for 4 hr. The reaction was stopped by adding 100 µl of 100% (w/v) TCA. The tritiated water generated by enzyme activity was vacuum distilled and counted using a liquid scintillation counter (Beckman model LS 5801). For each assay, a 0.9-ml aliquot of pooled leftover enzyme extracts was assayed in the presence of 0.4 mM β-aminoproprionitrile, an inhibitor of lyslyoxidase. Controls without enzyme were run with every assay and subtracted from the enzyme assay values. Activity is expressed as dpm tritiated H_2O per hour per lung.

Collagenase assays were performed according to the procedure of Cawston and Murphy (*31*). Type I collagen was purified from rat tail tendon. The purified collagen was labeled with ^{14}C-acetic anhydride. Collagenase incubations were run for 18 hr at 37°C with 0.1 ml of lung homogenate. Solubilization of the collagen by hamster lung homogenates was demonstrated to be linear over this time period (data not shown). This activity could be abolished with a final concentration of 10 mM EDTA in the assay. Activity is expressed as µg collagen solubilized per hour per lung.

Determination of Lung Collagen Cross-links. Sufficient ice-cold saline was added to a known amount of pulverized lung tissue to make a 1:1 ratio of tissue to saline. The tissue was then homogenized for 30 sec in three bursts with a Polytron that was set at a medium power setting. A 0.3-ml aliquot was removed and placed in a 1.5-ml conical centrifuge tube. The samples were centrifuged for 5 min at 16,000*g* and the supernatants discarded. The precipitates were washed in 1 ml 0.1 M sodium phosphate, pH 7.2, by centrifugation three times, and resuspended in 1.0 ml of the same buffer. The supernatant wash fractions were analyzed for hydroxyproline in a trial experiment and none was detected (data not shown).

Reduction was carried out similarly to the procedure of Fukae and Mechanic (*32*). Six microliters of tritiated sodium borohydride with specific activity of 142 mCi/mole (diluted from the original stock) dissolved in dimethylformamide (5 mg/ml) was added to each sample in three aliquots 5 min apart and allowed to incubate an additional 45 min after the last addition. The reaction was stopped by adding 0.3 ml of glacial acetic acid. The unincorporated radioactivity was separated from the collagen by centrifugation at 16,000*g* and discarding the supernatant fraction five times. The pellet was transferred to a hydrolysis tube containing 1 ml of 6 N HCl and purged with nitrogen. Hydrolysis was carried out at 110°C for 24 hr. The HCl was evaporated by heating at 100°C under nitrogen using a heating block. The samples were then resuspended in 0.3 ml water and filtered using Rainin 0.45 µm syringe filters. Hydroxyproline, dihydroxylysinonoroleucine (DHLNL) and hydroxypyridinium (OHP) were analyzed on the same samples. Hydroxyproline content per lung was determined using the procedure of Woessner (*23*).

Determination of DHLNL content was performed according to the procedure of Reiser and Last (*33*). Briefly, a sample containing 5 µg of hydroxyproline was injected onto a Perkin Elmer Series 4 HPLC running in isocratic mode with 0.1 M sodium phosphate buffer, pH 2.84, in 22% *n*-propanol, and 0.3% SDS at a flow rate of 0.8 ml/min. An ultrasphere C18 column (0.4[d]×25 cm) (Beckman, Sunnyvale, CA) was used for the separation. Postcolumn derivatization was carried out with a Perkin Elmer Series 10 pump with 0.08% *o*-phthalaldehyde (w/v) (Pierce Chemicals, Rockford, IL), 0.1% β-mercaptoethanol (v/v) in 0.3 M sodium borate buffer, pH 10.4, with a flow rate of 0.5 ml/min. Fluorescence was monitored using a Shimadzu model RF-535 fluorescence detector equipped with a 12-µl flow cell. The excitation and emission wavelengths were 360 and 455 nm, respectively. A Perkin Elmer LCI 100 computing integrator was used to record the signal. Radioactivity was monitored by collecting fractions at 0.5-min intervals. Tritium content was determined by using a Beckman model 5801 liquid scintillation counter with a preset tritium channel.

The identity of the DHLNL was determined by its elution position just after

arginine on the HPLC profile, as detected by orthophthalaldehyde, of hydrolyzed bovine achilles tendon and hydrolyzed hamster lung. This peak identified as DHLNL was radiolabeled when the sample was reduced with tritiated sodium borohydride. The putative DHLNL peak was present in bovine Achilles tendon and not present in rat tail tendon as described by Eyre et al. (*34*). The radioactivity of the DHLNL peak was converted to mmoles of DHLNL using the procedure of Gallop et al. (*35*) as modified by Reiser et al. (*36*).

The OHP content of the samples was determined by the method of Eyre et al. (*34*). An aliquot of each sample containing 1.5 μg of OHP was injected with a manual injector onto the Perkin Elmer Series 4 HPLC system. The flow rate was 1.0 ml/min with 0.01 M heptafluorobutyric acid in 16% acetonitrile as the eluent. OHP was separated using a (0.25[d]×10 cm) Perkin Elmer Series 4 C18 HPLC column. Fluorescence was detected by a Shimadzu model RF535 fluorescence detector with excitation and emission wavelengths of 294 and 395 nm, respectively. The signal was recorded using the Perkin Elmer LCI 100 Computing Integrator. The area under the OHP peak was determined by cutting out the peak and weighing it.

The identity of the OHP was determined by purifying a standard from bovine achilles tendon according to the procedure of Eyre et al. (*34*). The fluorescence spectrum of this compound in dilute acid matched the published spectrum for OHP (*37*). Also the compound was destroyed by overnight exposure to ultraviolet light as reported by Koob et al (*38*). The putative OHP was absent in hydrolyzed samples of rat tail tendon as had been previously reported (*34*). The molar fluorescence yield of OHP was determined using the method of Eyre et al. (*34*).

Statistical Analysis of Data

The data are expressed on a per lung basis, which avoids the artificial lowering of the values in bleomycin-treated animals due to presence of proteins of extrapulmonary origin (*39*). However, lung DHLNL and OHP levels were expressed as mmoles cross-link per mole of collagen. Lung collagen content is known to increase with intratracheal instillation of bleomycin (*4*). Expression of the data on the basis of mole cross-link per mole of collagen avoids an increase in lung cross-link content as a result of an overall increase in the total collagen content in the lungs of bleomycin-treated hamsters.

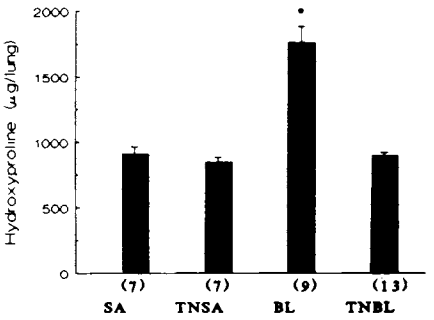

Figure 1. Effects of combined treatment with taurine and niacin on BL-induced increases in the lung hydroxyproline content of hamsters. Hamsters were fed taurine (2.5%) and niacin (2.5%) in the pulverized diet and instilled with BL intratracheally (IT) (2.5, 2.0, and 1.5 units/5 ml/kg bw) in three consecutive doses at weekly intervals. Twenty days after the last IT instillation, hamsters were killed and their lungs processed for the hydroxyproline assay as described in "Materials and Methods". SA, saline control; TNSA, taurine + niacin + saline; BL, bleomycin alone; TNBL, taurine + niacin + bleomycin. The number of animals in each group is shown in parenthesis below each bar and treatment groups are indicated along the X-axis and explained in Materials and Methods. Values are means ± SE. *Significantly higher ($p<0.05$) than all other groups.

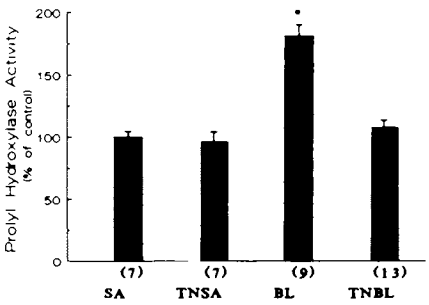

Figure 2. Effects of combined treatment with taurine and niacin on BL-induced increases in the prolyl hydroxylase activity of hamster lungs. See legend to Figure 1 for explanation of abbreviations and experimental details. The lungs were processed for the prolyl hydroxylase assay as described in "Materials and Methods". The saline control activities of this enzyme averaged 112,000 ± 5250 dpm/30 min/lung. The number of animals in each group is shown in parentheses below each bar and treatment groups are indicated along the X-axis and explained in Materials and Methods. Values are means ± SE. *Significantly higher ($p<0.05$) than all other groups.

All values are reported as the mean ± one standard error (SE) and analyzed by one-way analysis of variance and Fisher's least significant difference test. A value of $p \leq 0.05$ was considered significant.

Results

Lung Hydroxyproline Content and Prolyl Hydroxylase Activity

Lung hydroxyproline content in various groups of hamsters at 20 days after the last IT instillation is shown in Figure 1. Bleomycin significantly elevated the lung hydroxyproline level to 194% of the saline controls. Combined treatment with taurine and niacin blocked the BL-induced increases in lung hydroxyproline content. Bleomycin alone increased the lung prolyl hydroxylase activity to 181% of the saline controls (Figure 2). Likewise, combined treatment with taurine and niacin inhibited the BL-induced increases in lung prolyl hydroxylase activity to the levels that were near controls.

Malondialdehyde Equivalent and SOD Activity

Lung malondialdehyde equivalent (MDAE) was elevated significantly by three doses of BL instillation (Table 1). The SOD activity in the lungs of these animals was found to be stimulated by BL treatment (Table 1). Treatment with taurine + niacin significantly diminished the BL-induced increases in the lung MDAE, although its level in the TNBL group was still significantly higher than those of the SA and TNSA groups (Table 1). As shown in Table 1, taurine + niacin treatment prevented the BL-induced increases in lung SOD activity.

Lung Calcium Content and Poly(ADP-ribose) Polymerase Activity

Intratracheal instillation of BL significantly increased lung calcium content (Table 1). BL-induced increases in lung calcium were decreased by the treatment with taurine and niacin. Lung calcium content in TNBL animals was not significantly different from that of the SA or TNSA groups (Table 1). BL administration significantly elevated poly(ADP-ribose) polymerase activity in hamster lungs compared to the control groups (SA and TNSA) (Figure 3). Taurine and niacin treatment significantly attenuated the increase in poly(ADP-ribose) polymerase activity in the BL-treated animals in TNBL groups. There was no significant difference in the activity of this enzyme among the hamsters in the SA, TNSA, and TNBL groups.

Protein Content, Acid Phosphatase Activity, and Cell Counts in BAL Fluid (BALF)

The protein content and acid phosphatase activity in the BALF supernatant of varying groups are shown in Table 1. Intratracheal

Table 1. Effects of combined treatment with taurine and niacin on bleomycin-induced increases in various biochemical and morphometric parameters in hamster lungs.[a]

Parameters	SA	n	TNSA	n	BL	n	TNBL	n
Malondialdehyde equivalent, nmole/lung	109.4 ± 7.0	7	109.5 ± 8.9	7	211.1 ± 10.5[b]	9	146.4 ± 8.1[c]	13
Superoxide dismutase, U/lung	400.5 ± 13.8	7	360.3 ± 12.2	7	582.2 ± 22.5[b]	9	385.6 ± 7.1	13
BALF supernatant protein, μg/lung)	1520 ± 95	5	1602 ± 113	5	5043 ± 619[b]	6	3249 ± 298[c]	6
BALF acid phosphatase, nmole/hr/lung	235.3 ± 36.1	5	296.8 ± 66.7	5	710.7 ± 141.2[b]	6	380.4 ± 115.9	6
Lung total calcium, μg/lung	33.9 ± 1.3	6	31.7 ± 0.8	6	63.7 ± 4.1[b]	9	38.2 ± 1.6	13
Lung volume, cm^3	6.72 ± 0.3[b]	5	5.7 ± 0.3	5	4.7 ± 0.2[d]	6	5.6 ± 0.1	6
Volume of lesions, cm^3	0	5	0	5	0.92 ± 0.14[b]	6	0.13 ± 0.02	6

[a]Hamsters were fed taurine (2.5%) and niacin (2.5%) in the pulverized diet and given bleomycin IT (2.5, 2.0, and 1.5 U/5 ml/kg/bw) in three consecutive doses at weekly intervals. Twenty days after the last IT instillation, hamsters were killed and lung and bronchoalveolar lavage fluid (BALF) processed for various biochemical and morphometric analysis as described in Materials and Methods. Legend for Figure 1 explains abbreviations and experimental details. Values are expressed as mean ± SE. *n* is the number of animals in each group. [b]Significantly higher ($p < 0.05$) than all other corresponding groups. [c]Significantly lower ($p < 0.05$) than the corresponding BL group, but higher ($p < 0.05$) than corresponding SA and TNSA groups. [d]Significantly lower ($p < 0.05$) than all other corresponding groups.

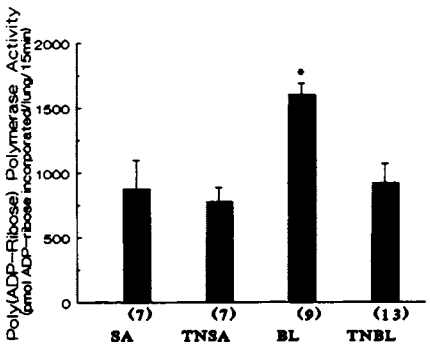

Figure 3. Effects of combined treatment with taurine and niacin on BL-induced increases in poly(ADP-ribose) polymerase activity of hamster lungs. Legend for Figure 1 explains abbreviations and experimental details. The lungs were processed for the poly(ADP-ribose) polymerase assay as described in Materials and Methods. The number of animals in each group is shown in parentheses below each bar and treatment groups are indicated along the X-axis and explained in Materials and Methods. Values are means ± SE. *Significantly higher ($p < 0.05$) than all other groups.

instillations of BL significantly increased the protein content to 332% of the SA group, and acid phosphatase activity to 302% of the SA group. However, combined treatment with taurine and niacin significantly decreased the BL-induced increases of BALF supernatant protein to 214% of the SA group, and acid phosphatase activity to 162% of the SA group. Although the protein content of TNBL was still significantly higher than that of SA and TNSA groups, there were no significant differences in BALF supernatant acid phosphatase activity among SA, TNSA, and TNBL groups (Table 1).

The number of total cells and the absolute number of differential cells in the BALF of the four treatment groups after intratracheal instillation of saline or BL are summarized in Table 2. As compared with the SA group, the numbers of total cells, neutrophils, macrophages, and lymphocytes in the BL group were increased by 3.1-, 245-, 2.5-, and 8.0-fold. In contrast, as compared with the SA group, the numbers of total cells, neutrophils, macrophages, and lymphocytes in the TNBL group were increased by only 2.1-, 30.3-, 2.0-, and 4.1-fold. The total numbers of neutrophils, monocytes, macrophages, and lymphocytes recovered in the BALF of TNBL groups were not significantly higher than those of the SA and TNSA groups in most cases. Taurine and niacin significantly suppressed the BL-induced increases of neutrophils in the airways to such an extent that there were no significant differences in the number of BALF neutrophils among the hamsters in the SA, TNSA, and TNBL groups. There were remarkable increases in the BALF eosinophils in the BL groups and fewer BALF eosinophils in the TNBL groups than in the BL groups, but the differences in the numbers of BALF eosinophils among all 4 groups were not statistically significant (Table 2).

Morphology and Morphometry

The biochemical and cell data in the BALF correlated well with morphologic and morphometric observations. Normal pulmonary parenchymal tissue was observed in SA and TNSA hamsters with normal lung volume and essentially no lesions. In contrast, hamsters that received BL alone had decreased lung volume associated with a patchy alveolitis and multifocal interstitial fibrosis that comprised about 20% of the total parenchyma (Table 1, Figure 4*A*). The lungs of hamsters in the BL group had thickened interalveolar septa and inflammatory cells in adjacent airspaces. The focal lung lesions were randomly distributed throughout pulmonary acini. In half of the hamsters, foci of fibrotic consolidation were observed. In diffuse lesions, alveolar airspaces were often obliterated by organized connective tissue. These regions stained heavily with the sirus red stain. These fibrotic regions contained abundant fibroblasts and numerous inflammatory cells, and were usually adjacent to necrotic epithelial cells. Even thick septa in BL-treated hamster lungs contained more inflammatory cells and necrotic epithelial cells than thick septa in the lungs of TNBL hamsters. Hamsters in the TNBL groups

Table 2. Total and differential cell counts in bronchoalveolar lavage fluid intratracheal instillation of bleomycin or saline with or without combined treatment with taurine and niacin.[a]

Treatment group (*n*)	Total cells, $\times 10^{-6}$	Neutrophils, $\times 10^{-4}$	Monocytes, $\times 10^{-4}$	Macrophage, $\times 10^{-6}$	Lymphocytes, $\times 10^{-5}$	Eosinophils, $\times 10^{-4}$
SA (5)	2.76 ± 0.22	0.37 ± 0.37	0 ± 0	2.66 ± 0.23	0.77 ± 0.12	1.79 ± 1.22
TNSA (5)	2.63 ± 0.24	3.99 ± 1.39	0.22 ± 0.22	2.55 ± 0.24	0.39 ± 0.10	0.18 ± 0.18
BL (6)	8.63 ± 1.41[b]	90.5 ± 40.80[c]	5.05 ± 1.86[b]	6.75 ± 1.17[b]	6.19 ± 3.18[b]	30.2 ± 25.30
TNBL (6)	5.76 ± 1.12	11.2 ± 3.29	3.29 ± 1.70	5.24 ± 0.96[b]	3.18 ± 0.94	5.24 ± 4.93

[a]Hamsters were fed taurine (2.5%) and niacin (2.5%) in the pulverized diet and given bleomycin IT (2.5, 2.0, and 1.5 U/5 ml/kg bw) in three consecutive doses at weekly intervals. Twenty days after the last IT instillation, bronchoalveolar lavage was carried out and total and differential cell counts analyzed as described in Materials and Methods. Legend for Figure 1 explains abbreviations and experimental details. The number of animals in each group is shown in parentheses. Values are expressed as mean ± SE. [b]Significantly higher ($p < 0.05$) than corresponding SA and TNSA groups. [c]Significantly higher ($p < 0.05$) than all other corresponding groups.

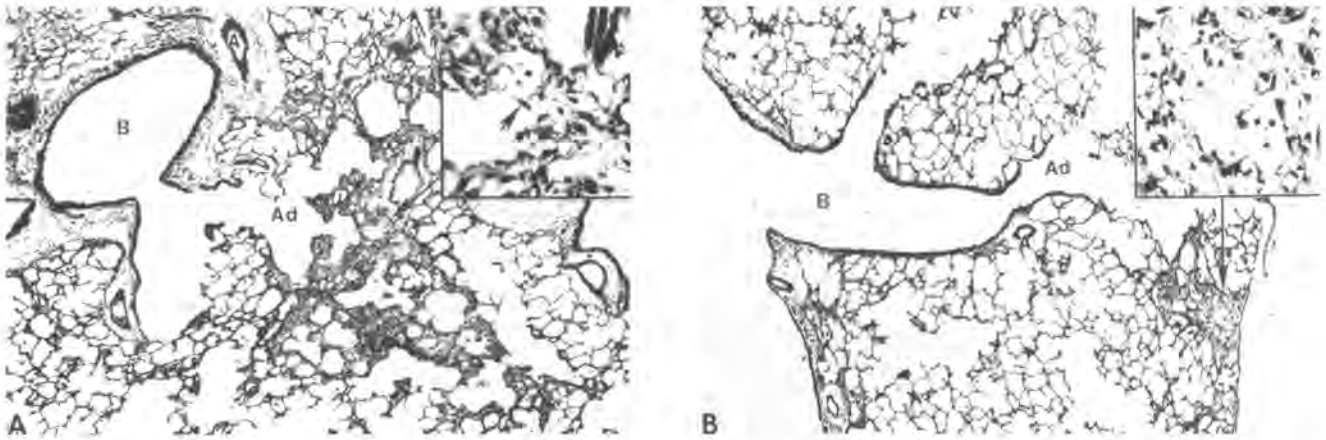

Figure 4. (*A*) A representative photomicrograph of pulmonary proximal acini from a hamster treated with bleomycin intratracheally (IT) and without taurine and niacin treatment (BL). Note the multifocal thickening of interalveolar septa by mononuclear phagocytes and fibroblasts (arrow). An arrow in the inset marks a fibroblast with adjacent fibrin in an alveolar space. (*B*) A photomicrograph of pulmonary proximal acini from a hamster treated with bleomycin IT and taurine (2.5%) and niacin (2.5%) in the diet (TNBL). Note the focus of thickened interalveolar septa that was only occasionlly observed (arrow). The subpleurally located lesion showed primarily mononuclear phagocytes (inset). B, distal bronchus; Ad, alveolar duct; A, arteriole. Paraffin sections, hematoxylin and eosin × 77, inset × 390.

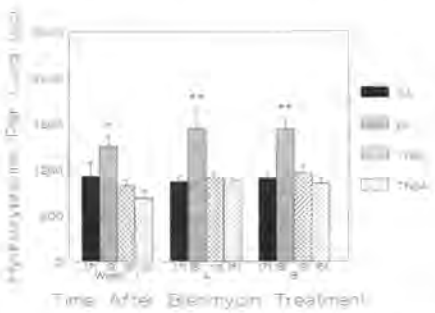

Figure 5. Effects of combined treatment with taurine and niacin on BL-induced increases in the hydroxyproline content of hamster lungs. The animals were killed at 1, 4, and 8 weeks after the last instillation and their lungs processed for the hydroxyproline assay as described in Materials and Methods. Legend for Figure 1 explains abbreviations and experimental details. The number of animals in each group is shown in parentheses below each bar and treatment groups by different bars. Each value represents mean ± SE. *Week 1: BL is significantly higher ($p \leq 0.05$) than SA, TNBL, and TNSA groups. **Week 4: BL is significantly higher ($p \leq 0.01$) than SA, TNBL, and TNSA groups. **Week 8: BL is significantly higher ($p \leq 0.01$) than SA, TNBL, and TNSA groups.

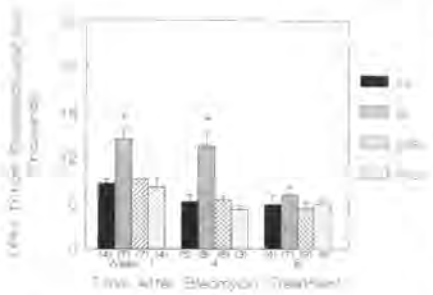

Figure 6. Effects of combined treatment with taurine and niacin on BL-induced increases in the lung lysyl oxidase activity of hamster. Legend for Figure 1 explains experimental details and abbreviations for treatment groups. Lysyl oxidase activity was measured as described in Materials and Methods. The number of animals in each group is shown in parentheses below each bar and treatment groups by different bars. Each value represents mean ± SE. *Week 1: BL is significantly higher ($p \leq 0.01$) than SA, TNBL, and TNSA groups. *Week 4: BL is significantly higher ($p \leq 0.01$) than SA, TNBL, and TNSA groups. Week 8: there are no statistically significant differences among the four groups.

showed little to no alveolitis and interstitial fibrosis with infrequent focal lesions of interstitial thickening with aggregations of mononuclear phagocytes (Figure 4*B*). Thickened septa, although infrequently observed, had numerous capillaries and some inflammatory cells. Necrotic epithelial cells were seldom observed adjacent to thick septa in TNBL hamsters. The combined treatment with taurine and niacin effectively prevented the BL-induced increases in fibrotic lesion to less than 2.3% of the pulmonary parenchyma that were not significantly different from those in the SA and TNSA groups (Table 1).

Lung Hydroxyproline and Lysyloxidase Activity

Hydroxyproline content per lung at 1, 4, and 8 weeks after the last dose of BL or saline instillation is shown in Figure 5. Bleomycin treatment in the control diet animals significantly increased the total lung hydroxyproline content to 138, 156, and 160% of the saline instilled control diet group at 1, 4, and 8 weeks, respectively. The increase in hydroxyproline content seen in the BL group was prevented by dietary administration of taurine and niacin at all three time points. Taurine–niacin treatment did not significantly decrease lung collagen content in the saline-instilled group relative to the saline-instilled group on the control diet at any of the three time points.

The lung lysyloxidase activity in different groups of hamsters is summarized in Figure 6. The activity in the BL group was significantly increased to 158 and 191% of the SA group at 1 and 4 weeks, respectively. However, taurine–niacin fed in the diet completely blocked bleomycin-induced increases in the lung lysyloxidase activity of the hamsters in the TNBL groups. Although lysyloxidase activity in the BL group was elevated at 8 weeks after the last bleomycin instillation, it was not significantly different from any other groups at that time point. The taurine–niacin in diet per se had no effect on the lysyloxidase activity in the TNSA groups.

Type I collagenase activity at 1, 4, and 8 weeks after the last bleomycin instillation is shown in Figure 7. The collagenase activity in the BL group was significantly increased to 165 and 180% of the SA group at 1 and 4 weeks after the last dose of bleomycin, respectively. The taurine–niacin fed in the diet abolished bleomycin-induced increases in the collagenase activity in the TNBL group both at 1 and 4 weeks. In fact, the activity in this group tended to be lower than the SA group at all three points. There was no significant difference in collagenase activity between the SA and BL groups at 8 weeks and the activity in the TNBL group was significantly higher than that of the TNSA group.

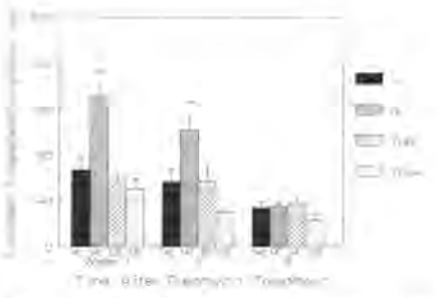

Figure 7. Effects of combined treatment with taurine and niacin on BL-induced increases in the lung collagenase activity of hamsters. Legend for Figure 1 explains experimental details and abbreviations for treatment groups. Collagenase activity was measured as described in Materials and Methods. The number of animals in each group is shown in parentheses below each bar and treatment groups by different bars. Each value represents mean ± SE. *Week 1: BL is significantly higher ($p \leq 0.01$) than SA, TNBL, and TNSA groups. **Week 4: BL is significantly higher ($p \leq 0.03$) than SA, TNBL, and TNSA groups. Week 8: TNBL is significantly higher ($p \leq 0.04$) than TNSA group.

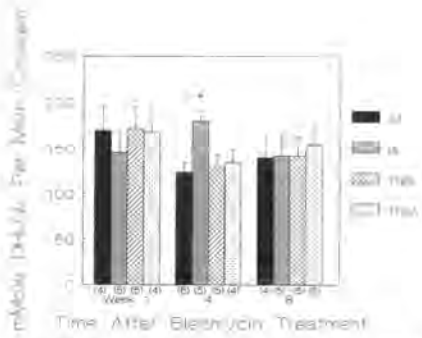

Figure 8. Effects of combined treatment with taurine and niacin on BL-induced increases in lung dihydroxylysinonoroleucine (DHLNL mmole/mole of collagen) content in hamsters. See legend to Figure 1 for experimental details and explanation of abbreviations for treatment groups. Lung DHLNL was measured as described in Materials and Methods. The number of animals in each group is shown in parentheses below each bar and treatment groups by different bars. Each value represents mean ± SE. Week 1: there are no statistically significant differences among the four groups ($p > 0.05$). *Week 4: BL is significantly higher ($p \leq 0.02$) than SA, TNBL, and TNSA groups. Week 8: there are no statistically significant differences among the four groups ($p > 0.05$).

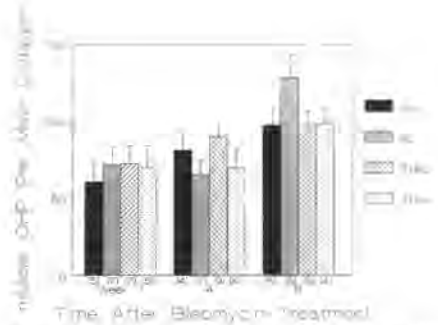

Figure 9. Effects of combined treatment with taurine and niacin on BL-induced increases in the lung hydroxypyridinium (OHP mmole/mole of collagen) content in hamsters. Legend for Figure 1 explains experimental details and abbreviations for treatment groups. OHP was measured as described in Materials and Methods. The number of animals in each group is shown in parentheses below each bar and treatment groups by different bars. Each value represents mean ± SE. Week 1: there are no statistically significant differences among the four groups ($p > 0.05$). Week 4: there are no statistically significant differences among the four groups ($p > 0.05$). *Week 8: BL is significantly higher ($p \leq 0.05$) than SA, TNBL, and TNSA groups.

Lung Collagen Cross-link Content

DHLNL content per mole of lung collagen at 1, 4, and 8 weeks after the last bleomycin or saline instillation is shown in Figure 8. DHLNL in the BL group was significantly increased to 146% of the SA group at the four week time point. There were no significant differences among the four groups at the 1- and 8-week time points. Taurine–niacin treatment prevented the bleomycin-induced increase in DHLNL content per mole of collagen at 4 weeks.

OHP content per mole of lung collagen at 1, 4, and 8 weeks after the last BL or saline instillation is shown in Figure 9. At the 1- and 4-week time points, there were no statistically significant differences among the four groups. However, the OHP content in the BL group was 131% of the values of the SA, TNBL, and TNSA groups ($p \leq 0.05$) at 8 weeks.

Discussion

In the present study, we have demonstrated that dietary supplementation with taurine and niacin abolished the BL-induced lung collagen accumulation in hamsters. One of the underlying mechanisms for the pathogenesis of BL-induced lung injury includes the generation of reactive oxygen species (ROS)(*40*). It is generally believed that under aerobic conditions an intracellular BL-Fe^{2+} complex undergoes a cyclic oxidation-reduction in the presence of a reducing equivalent (*41*). This complex functions as a minienzyme, catalytically reducing molecular oxygen leading to generation of various types of ROS. The mechanism by which the dietary supplementation with taurine and niacin offered protection against BL-induced lung fibrosis is not clearly understood. However, the results of this study suggest that the beneficial effect of the combined treatment with taurine and niacin may be attributed to their ability to suppress the BL-induced increases in the lung collagen reactivity and offer protection against the deleterious effects of ROS generated by BL.

The following lines of evidence suggest that the dietary supplementation with taurine and niacin inhibits the lung collagen reactivity in the BL-hamster model of lung fibrosis. First, dietary supplementation with taurine and niacin inhibited the BL-induced increases in the lung prolyl hydroxylase activity and a decrease in the activity of this enzyme paralleled a marked reduction in the lung collagen accumulation. It has been demonstrated by several investigators that the accumulation of collagen in the BL-rodent model of lung fibrosis usually follows the increase in the lung prolyl hydroxylase activity (*24,42*). The prolyl hydroxylase catalyzed hydroxylation of proline is a critical posttranslational event in the processing of mature collagen (*43*). A reduction in BL-induced increases in prolyl hydroxylase activity would allow the deposition of a more pliable and soluble form of collagen that is more susceptible to degradation by collagenases than the mature collagen fibers.

Second, dietary supplementation with taurine and niacin inhibited the BL-induced increases in the lung lysyl oxidase activity. Lysyloxidase oxidatively deaminates peptidyl lysine and hydroxylysine residues in the collagen molecule (*29,44*). The deamination is not only an obligatory but it possibly represents a rate-limiting step in collagen cross-linking. The lysyloxidase activity is shown to be increased in BL-induced lung fibrosis (*45*) and treatment with β-aminopropionitrile was found to block both the BL-induced increases in the lung lysyloxidase activity and collagen accumulation (*46,47*). Our findings are very similar since dietary intake of taurine–niacin blocked both bleomycin-induced increases in the lung lysyloxidase activity at 1 and 4 weeks and the collagen accumulation at 1, 4, and 8 weeks after the last dose of bleomycin. It appears that the increased lung lysyl oxidase activity in BL-instilled animals, as found in this as well as in other studies, may be essential for the accumulation of collagen in the lung and that inhibition of this enzyme by dietary intake of taurine–niacin, as reported in this study, may constitute another possible mechanism for their antifibrotic effect.

The mechanisms as to how BL instillation increases the lung lysyloxidase activity and how dietary intake of taurine–niacin prevents this increase are not known. It is,

however, possible that taurine and niacin either somehow decrease the synthesis and/or increase the inactivation of this enzyme protein. It is also possible that taurine–niacin treatment may simply block or ameliorate the BL-induced initial tissue damage and this then prevents the inflammatory events reaching the intensity that is essential for generation of an array of mediators involved in triggering an increase in the lysyloxidase activity.

Dietary supplementation with taurine and niacin also caused a marked reduction in the BL-induced increases in the lung collagenase activity. The role of collagenase in the accumulation of collagen in the fibrotic lung is unclear. Laurent and McAnulty (*48*) and others have suggested that decreased degradation is in part responsible for the accumulation of collagen seen in the fibrotic lungs. Phan et al. (*49*) found a marked decrease in the breakdown of collagen *in vitro* in a single-dose bleomycin model of lung fibrosis in rats. Selman et al. (*50*) found that the release of hydroxyproline from lung biopsy samples from 11 patients suffering from idiopathic pulmonary fibrosis was markedly decreased *in vitro* relative to controls. Montano et al. (*51*) also found lowered hydroxyproline release from lung biopsy samples taken from patients with pulmonary fibrosis *in vitro*.

In contrast to these findings, a number of researchers have found that collagen degradation is increased in pulmonary fibrosis. Elevated Type I collagenase activity was found in BALF from 15 of 21 patients with pulmonary fibrosis (*52*). Christner et al. (*53*) found increased Type I collagenase activity in the BALF of 12 of 17 patients with idiopathic pulmonary fibrosis. The BALF in rats intratracheally instilled with bleomycin contained elevated levels of Type I collagenase (*54*).

Our own results indicate that Type I collagenase activity was elevated in the BL group at the 1- and 4-week time points and declined to the level of the SA group by 8 weeks. The rise and decline in collagenase activity following the last BL instillation roughly paralleled the increase and subsequent decline in inflammatory cells over time, as found by Zia et al. (*14*). The increased collagenase activity in BL-instilled hamster lung is probably due in part to collagenase secreted by neutrophils (*55*) and alveolar macrophages (*56*). It should be noted that alveolar macrophages isolated from single-dose, BL-instilled rats showed an increase in secreted collagenase activity relative to control animals (*54*).

Bleomycin instillation may well cause an increase in extracellular collagenolytic peptides. A role for an increased amount of collagenolytic peptides is suggested by the work of several investigators. Malone et al. (*57*) found that collagenase digests of Type I collagen can recruit peripheral mononuclear cells. Riley et al. (*47*) found that intratracheal instillation of collagen fragments could cause an influx of neutrophils. Postlethwaite et al. (*58*) found Type I, II, and III collagens, collagen-derived peptides, and tripeptides with hydroxyproline were chemotactic to dermal fibroblasts. It is possible that the role of extracellular collagenolytic peptides during the fibrotic process is to amplify and sustain the inflammatory response, as has been suggested by Crouch (*1*).

Another line of evidence that dietary supplementation with taurine and niacin suppressed the BL-induced increases in the lung collagen reactivity was a marked diminution of lung collagen cross-links in the TNBL group. We had postulated that taurine and niacin somehow could be involved in blocking the normal process of Schiff base formation between residues of hydroxyallysine and hydroxylysine, thus preventing formation of DHLNL. This mechanism has been suggested for the inhibitory effect of penicillamine on collagen cross-link formation (*59*). However, other mechanisms are now thought to play a major role in inhibiting the formation of collagen cross-links (*60,61*). If DHLNL formation were blocked, the formation of mature stable cross-link OHP would also be subsequently blocked. There is good evidence that mature cross-linked collagen does not turn over in either the BL-induced fibrotic lung or the normal lung (*62,63*). Thus, a blockage in cross-link formation by dietary supplementation with taurine and niacin could at least, in part, be responsible for minimizing the BL-induced accumulation of collagen in the lung since collagen deficient in cross-links is generally more susceptible to turnover than cross-linked collagen.

In addition to a diminution of collagen reactivity, taurine and niacin are known to possess other beneficial effects that protect the tissue against the oxidant-induced tissue damage. For instance, the membrane-stabilizing antioxidant ROS and HOCl scavenging properties of taurine seem to be somehow involved in its protective effect (*64–68*). BL-induced pulmonary toxicity is usually attributed to the generation of ROS (*40*) and their involvement in the pulmonary toxicity is reflected by the increase in lung SOD activity as a defense mechanism to protect the tissue against the deleterious effects of superoxide radicals (*24,69*). In the present study, dietary supplementation with taurine and niacin significantly inhibited the BL-induced increase of lung SOD activiry. This indicates that taurine and/or niacin may prevent the initial BL-induced production of ROS or block their injurious effects by virtue of the taurine's antioxidant property.

Several laboratories, including our own, have demonstrated that BL-induced acute inflammatory phase is always associated with an excessive accumulation of neutrophils in vascular, interstitial, and intraalveolar compartments of the lung (*70*). The activated neutrophils by virtue of the enzyme myeloperoxidase are known to oxidize halides Cl^-, Br^-, or I^- to their corresponding hypohalous acid in a reaction involving H_2O_2 (*71*). The biocidal property of HOCl in oxidizing a variety of biologically significant substances is well known. The beneficial effect of the combined treatment with taurine and niacin against BL-induced lung damage may also be attributed to a decreased influx of neutrophils in the various compartments of the lung as well as to the ability of taurine to act as a trap for HOCl. Recently, it has been demonstrated that of all the amino acids, taurine was the most effective inhibitor of HOCl-induced lysis of erythrocytes (*72*). Thus, it is possible that taurine could be effective in preventing the biomembrane damage caused by BL. This then also would explain why the BL-induced increases in pulmonary vascular permeability and acid phosphatase activity of the BALF were markedly suppressed by the combined treatment with taurine and niacin.

The ability of taurine to prevent an excess influx of Ca^{2+} by its membrane stabilizing effect might have also played a key role in ameliorating BL-induced lung fibrosis. This hypothesis draws support from two lines of evidence: *a*) BL-induced lung fibrosis is always associated with an intracellular overload of Ca^{2+} (*73*); and *b*) biochemical (*65,67*) and morphologic findings (*74*) provided by other investigators suggest that taurine does indeed prevent an excess influx of Ca^{2+}. In the presence of an excessive amount of intracellular Ca^{2+}, the ability of mitochondria to synthesize ATP is impaired, and Ca^{2+} accumulates at the inner surface of the cell membrane, activating the membrane-bound enzymes including proteases and phospholipases that in turn damage the cell membrane leading to cell death.

Calcium also stimulates phospholipase-A_2, a rate-limiting enzyme, in the metabolism of arachidonic acid. Metabolites of arachidonic acid appear to be involved in the BL-induced lung fibrosis (*75*). Activation of PLA_2 stimulates the peroxidation of membrane lipids (*76*) that is measured by the amount of MDAE. The generation of MDAE, in addition to being an index of lipid peroxidation, also reflects DNA damage (*77*). A significant suppression in BL-induced increases in the lung MDAE by combined treatment with taurine and niacin as reported at this conference indicates that this treatment might have inhibited BL-induced lipid peroxidation and DNA damage. This is not surprising since taurine and niacin are known to inhibit lipid peroxidation (*67,78*) and DNA damage (*79*), respectively.

The beneficial effect of niacin against paraquat-induced lung toxicity has been previously reported (*80*). It is generally believed that niacin offers protection against tissue damage caused by a variety of oxidants by virtue of its ability to increase intracellular level of NAD and to inhibit overactivation of poly(ADP-ribose) polymerase (*79,81*). An overstimulation of this chromosomal enzyme leads to intracellular depletion of NAD (*82*). This hypothesis is consistent with our earlier findings that IT instillation of BL increased the lung poly(ADP-ribose) polymerase activity and caused a marked depletion of NAD (*28*). This will explain why the combined treatment with taurine and niacin markedly suppressed the BL-induced overstimulation of poly(ADP-ribose) polymerase, and presumably allowed an adequate availability of NAD, as reported in the present study. The availability of NAD would maintain vital cell functions, including the DNA repair of injured lung epithelium. This hypothesis is further supported by our recent finding that the daily treatment with niacin in BL-treated hamsters not only caused significant increases in the lung levels of NAD and ATP, but it also minimized the lung toxicity at 10 and 14 days following a single IT instillation of bleomycin (*83*). It is believed that the proliferation of interstitial cells in the lung are normally hampered by the intact epithelial cells; under normal conditions, the lung injury is repaired (*84*). However, if the injured epithelial cells remain unrepaired, it would trigger the proliferation of interstitial cells leading to an excessive synthesis and deposition of collagen in the lung (*85,86*). Our data suggest that the combined treatment with taurine and niacin helps repair the BL-induced injury of epithelium and thus minimizes the interstitial cell proliferation and subsequent build-up of collagen.

REFERENCES

1. Crouch E. Pathobiology of pulmonary fibrosis. Am J Physiol 259:159–184 (1990).
2. Giri SN. Pharmacologic perspective in pulmonary fibrosis research. In: Focus on Pulmonary Pharmacology and Toxicology (Hollinger MA, ed). Boca Raton, FL:CRC Publications, 1990;19–55.
3. Chandler DB, Hyde DM, Giri SN. Morphometric estimates of infiltrative cellular changes during the development of bleomycin-induced pulmonary fibrosis in hamster. Am J Pathol 112:170–177 (1983).
4. Clark JG, Overton JE, Marino BA, Uitto J, Starcher BC. Collagen biosynthesis in bleomycin-induced pulmonary fibrosis in hamsters. J Lab Clin Med 96:943–953 (1980).
5. Reiser KM, Tryka AF, Lindenschmidt RC, Last JA, Witschi HP. Changes in collagen cross-linking in bleomycin-induced pulmonary fibrosis. J Biochem Toxicol 83:83–91 (1986).
6. Seyer JM, Hutchenson ET, Kang AH. Collagen polymorphism in idiopathic chronic pulmonary fibrosis. J Clin Invest 57:1498–1507 (1976).
7. Garnick MB. Advanced testicular cancer: treatment choice in the "land of plenty." J Clin Oncol 3:294–297 (1985).
8. Crooke ST, Bradner WT. Bleomycin, a review. J Med 7:333–427 (1976).
9. Giri SN, Wang Q. Mechanisms of bleomycin-induced lung injury. Comments Toxicol 3:145–176 (1989).
10. Snider GL, Hayes JA, Korthy AL. Chronic interstitial pulmonary fibrosis produced in hamster by endotracheal bleomycin: pathology and stereology. Am Rev Respir Dis 177:1099–1108 (1978).
11. Thrall RS, McCormack JR, McReynolds RM, Ward PA. Bleomycin-induced pulmonary fibrosis in rats. Am J Pathol 95:117–127 (1979).
12. Hyde DM, Giri SN. Polyinosinic-polycytidylic acid, an interferon inducer ameliorates bleomycin-induced lung fibrosis in mice. Exp Lung Res 16:533–546 (1990).
13. O'Neill CA, Giri SN, Wang A, Perricone MA, Hyde DM. Effects of dibutyryl-cylic adenosine monophospate on bleomycin-induced lung toxicity in hamsters. J Appl Toxicol 12:97–111 (1992).
14. Zia S, Hyde DM, Giri SN. Development of a bleomycin hamster model of subchronic lung fibrosis. Pathology 24:155–163 (1992).
15. Wang Q, Hyde DM, Giri SN. Abatement of bleomycin-induced increases in vascular permeability, inflammatory cell infiltration and fibrotic lesions in hamster lungs by combined treatment with taurine and niacin. Lab Invest 67:234–242 (1992).
16. Nakashima JM, Levin JR, Hyde DM, Giri SN. Repeated exposure to enzyme-generated oxidants causes alveolitis, epithelial hyperplasia and fibrosis in hamsters. Am J Pathol 139:1485–1499 (1991).
17. Wilcox M, Kervitsky A, Watters LC, King TE Jr. Quantification of cells recovered by brochoalvedar lavage. Comparison of cytocentrifuge preparations with the filter method. Am Rev Respir Dis 138:74–80 (1988).
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275 (1951).
19. Moss DW. Acid phosphatases. In: Methods of Enzymatic Analysis (Bergmeyer HU, Bergmeyer J, Grassl M, eds). Deerfield Beach, FL:Chemie Verlag, 1983:92–106.
20. Scherle WF. A simple method of volumetry of organs in quantitative stereology. Mikroskopie 26:57–60 (1970).
21. James J, Bosch KS, Zuyderhoudt FMJ, Houfkopper JM, Van Gool J. Histophotometric estimation of volume density of collagen as an indicator of fibrosis in rat liver. Histochemistry 85:129–133 (1986).
22. Elias H, Hyde DM. A Guide to Practical Steriology. New York:Karger, 1983;25–34.
23. Woessner JF Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 93:440–447 (1961).
24. Giri SN, Misia HP, Chandler DB, Chen Z. Increases in lung prolyl hydroxylase and superoxide dismutase activities during bleomycin-induced lung fibrosis in hamsters. Exp Mol Pathol 39:317–326 (1983).
25. Ohkawa H, Ohismi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358 (1979).
26. Misra HP, Fridovich I. The role of superoxide anion in the

autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247:3170–3175 (1972).
27. Thompson M, Walsh JN. A Handbook of Inductively Coupled Plasma Spectrometry. Glasgow:Blackie & Son Ltd, 1983.
28. Hussain MZ, Giri SN, Bhatnager RS. Poly(ADP-ribose) synthetase activity during bleomycin-induced lung fibrosis in hamsters. Exp Mol Pathol 43:162–176 (1985).
29. Pinnell SR, Martin GR. The cross-linking of collagen and elastin: enzymatic conversion of lysine in peptide linkage to aminoadipic-semialdehyde (allysine) by extract from bone. Proc Natl Acad Sci USA 61:708–716 (1968).
30. Kagan HM, Sullivan KA. Lysyl oxidase: preparation and role in elastin biosynthesis. In: Methods in Enzymology 82: Structural and Contractile Proteins; Part A (Cunningham LW, Frederiksen DW, eds). New York:Academic Press, 1982;637–659.
31. Cawston TE, Murphy G. Mammalian collagenases. In: Methods in Enzymology 80: Proteolytic Enzymes; Part C (Lorand L, ed). New York:Academic Press, 1981;711–722.
32. Fukae M, Mechanic GL. Maturation of collagenous tissue: temporal sequence of formation of peptidyl lysine-derived cross-linking aldehydes and cross-links in collagen. J Biol Chem 255:6511–6518 (1980).
33. Reiser KM, Lasat JA. Analysis of collagen composition and biosynthesis by HPLC. Liquid Chromat 1:498–501 (1983).
34. Eyre DR, Kobb TJ, Van Ness KP. Quantitation of hydroxypyridinium cross-links in collagen by high-performance liquid chromatography. Anal Biochem 137:380–388 (1984).
35. Gallop PM, Blumenfield OO, Henson E, Schneider AL. Isolation and identification of alpha-amino aldehydes in collagen. Biochemistry 7:2409–2430 (1968).
36. Reiser KM, Hennessy SM, Last JA. Analysis of age associated changes in collagen cross-linking in the skin and lung in monkeys and rats. Biochem Biophys Acta 926:339–348 (1987).
37. Fujimoto D, Moriuchi T. A non-reducible cross-link of collagen. J Biochem 83:863–867 (1978).
38. Koob TJ, Eyre DR, Van Ness KP. Defection and photolysis of hydroxypyridinium cross-links in cartilage collagen *in situ*. Trans Ortho Res Soc 8:23–25 (1983).
39. Karlinski JB, Goldstein CH. Fibrotic lung diseases: a perspective. J Lab Clin Med 96:939–942 (1980).
40. Sugiura U, Kikuchi T. Formation of superoxide and hydroxyl-radicals in iron (II)-bleomycin-oxygen system: electron spin resonance detection by spin trapping. J Antibiot (Tokyo) 31:1310–1312 (1978).
41. Caspary MK, Lanzo DA, Niziak C. Effect of deoxyribnucleic acid on the production of reduced oxygen by bleomycin and iron. Biochemistry 21:334–338 (1982).
42. Kelley J, Newman RA, Evans JN. Bleomycin-induced pulmonary fibrosis in the rat. Prevention with an inhibitor of collagen synthesis. J Lab Clin Med 96:954–964 (1980).
43. Miller RL, Undenfriend S. Hydroxylation of proline residues in collagen nascent chains. Arch Biochem Biophys 139:104–113 (1970).
44. Siegel RC. Lysyloxidase. Int Rev Connect Tis Res 8:73–118 (1979).
45. Counts DF, Evans JN, Dipetrillo TA, Sterling KM, Kelley J. Collagen lysyl oxidase activity in the lung increase during bleomycin-induced lung fibrosis. J Pharmacol Exper Ther 219:675–678 (1981).
46. Zuckerman JE, Hollinger MA, Giri SN. Evaluation of antifibrotic drugs in bleomycin-induced pulmonary fibrosis in hamsters. J Pharmacol Exp Ther 213:425–431 (1980).
47. Riley DJ, Kerr JS, Berg RA, Lanni BB, Pietra GG, Edelman NH, Prockop DJ. β-aminoproprionitrile prevents bleomycin-induced pulmonary fibrosis in the hamster. Am Rev Resp Dis 125:67–73 (1982).
48. Laurent GJ, McAnulty RJ. Protein metabolism during bleomycin-induced pulmonary fibrosis in rabbits. *In vivo* evidence for collagen accumulation because of increased synthesis and decreased degradation of the newly synthesized collagen. Am Rev Respir Dis 128:82–88 (1983).
49. Phan SH, Thrall RS, Williams C. Bleomycin-induced pulmonary fibrosis. Effect of steroid on lung collagen metabolism. Am Rev Respir Dis 124:428–434 (1981).
50. Selman M, Montano M, Ramos C, Chapela R. Concentration, biosynthesis and degradation of collagen in idiopathic pulmonary fibrosis. Thorax 41:355–359 (1986).
51. Montano M, Ramos C, Gonzalez G, Vadillo F, Pardo A, Selman M. Lung collengenase inhibitors and spontaneous and latent collagenase in idiopathic pulmonary fibrosis and hypersensitivity pneumonitis. Chest 96:1115–1119 (1989).
52. Gadek JE, Kelman JA, Fells Gg, Weinberger SE, Horwitz AL, Reynolds HY, Fulmer JD, Crystal RG. Collangenases in the low respiratory tract of patients with idiopathic pulmonary fibrosis. N Eng J Med 301:737–742 (1979).
53. Christner P, Fein A, Goldberg S, Lippmann M, Abrams W, Weinbaum G. Collagenase in the lower respiratory tract of patients with adult respiratory distress syndrome. Am Rev Respir Dis 131:690–695 (1985).
54. Marom Z, Weinberg DS, Fanburg BL. Effect of bleomycin on collagenolytic activity of the rat alveolar macrophage. Am Rev Respir Dis 121:859–867 (1980).
55. Horwitz AL, Hance AJ, Crystal RG. Granulocyte collagenase:selective digestion of type I relative to type II collagen. Proc Natl Acad Sci USA 74:897–901 (1977).
56. Welgus HG, Campbell EJ, Bar-Shavit Z, Senior RM, Teitelbaum SL. Human alveolar macrophanges produce a fibroblast-like collagenase and collagenase inhibitor. J Clin Invest 76:19–24 (1985).
57. Malone JD, Richards M, Jeffrey JJ. Recruitment of peripheral mononuclear cells by mammalian collagenase digests of type I collagen. Matrix 11:289–295 (1991).
58. Postlethwaite AE, Seyer JM, Kang AH. Chemotactic attraction of human fibroblasts to type I, II, and III collagens and collagen-derived peptides. Proc Natl Acad Sci USA 75:871–875 (1978).
59. Nimni ME, Deshmukh K, Genth N. Collagen defect induced by penicillamine. Nature (London), New Biol 240:220–222 (1972).
60. Ledson, MJ, Bucknall RC, Edwards SW. Inhibition of neutrophil oxidant secretion by D-penicillamine of H_2O_2 and HOCl. Ann Rheum Dis 51:321–325 (1992).
61. McQuaid A, Lamand M, Mason J. The interactions of penicillamine with copper *in vivo* and the effect on hepatic metallothionein levels and copper zinc distribution. The implications for Wilson's disease and arthritis therapy. J Lab Clin Med 6:744–750 (1992).
62. Last JA, Summers P, Reiser KM. Biosynthesis of collagen cross-links II. *In vivo* labeling and stability of lung collagen in rats. Biochem Biophys Acta 990:182–289 (1989).
63. Last JA, Reiser KM. Biosynthesis of collagen cross-links III. *In vivo* labeling and stability of lung collagen in rats with bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol 1:111–117 (1989).
64. Wright CE, Tallan HH, Lin YY, Gaull GE. Taurine: biological update. Annu Rev Biochem 55:427–452 (1986).
65. Huxtable RJ. From heart to hypothesis: a mechanism for the calcium modulatory actions of taurine. Adv Exp Med Biol 217:371–387 (1987).
66. Wright CE, Lin T, Lin YY, Sturman JJ, Gaull GE. Taurine scavenges oxidized chlorine in biological systems. In: Taurine: Biological Actions and Clinical Perspective (Oja SS, Antee L, Kontro D, Paasonen MK, eds). New York:Alan R Liss,1985;112–123.
67. Azuma J, Hamaguchi T, Ohta H, Takihara K, Awata N, Sawamurg A, Harada H, Tanaka Y, Kishimoto S. Calcium overload-induced myocardial damage caused by isoproterenol and by adriamycin:possible role of taurine in its prevention. Adv Exp Med Biol 217:167–179 (1987).
68. McLoughlin DM, Stapleton DD, Bloomfield FJ. Influence of taurine and a substituted taurine on the respiratory burst pathway in the inflammatory response. Biochem Soc Trans 19:73–78 (1991).
69. Giri SN, Chen Z, Younker WR, Schiedt MJ. Effects of intratracheal administration of bleomycin on GSH-shuttle enzymes,

catalase, lipid peroxidation and collagen content in the lungs of hamsters. J Toxicol Appl Pharmacol 71:132–141 (1983).
70. Giri SN, Hyde DM, Nakashima JM. Analysis on bronchoalveolar lavage fluid from bleomycin-induced pulmonary fibrosis in hamsters. Toxicol Pathol 14:149–157 (1986).
71. Klebanoff SJ. Phagocytic cells: products of oxygen metabolism. In: Inflammation: Basic Principles and Clinical Correlates (Gallin JL, Goldstein IM, Snyderman R, ed). New York:Raven Press, 1988;391–444.
72. Nakamori K, Koyama I, Nakamura T, Yoshida T, Umeda M, Inouc K. Effectiveness of taurine in protecting biomembrane against oxidant. Chem Pharm Bull (Tokyo) 38:3116–3119 (1990).
73. Giri SN, Nakashima JM, Curry DL. Effects of intratracheal administration of bleomycin or saline in pair-fed and control-fed hamsters on daily food intake and on plasma level of glucose, cortisol and insulin and lung level of calmodulin, calcium, and collagen. Exp Mol Pathol 42:206–219 (1985).
74. Gordon RE, Heller RF, Del Valle JR. Membrane perturbations and mediation of gap junction formation in response to taurine treatment in normal and injured alveolar epithelia. Exp Lung Res 15:895–908 (1989).
75. Wang Q, Giri SN, Hyde DM. Characterization of a phospholipase A_2 in hamster lung and *in vitro* and *in vivo* effects of bleomycin on this enzyme. Prostaglandins Leukot Essent Fatty Acids 36:85–92 (1989).
76. Chien KR, Adams J, Serroni A, Martin JT, Farber JL. Accelerated phospholipid degradation and associated membrane dysfunction in irreversible ischemia liver cell injury. Biol Chem 253:4809–4819 (1978).
77. Trush MA, Mimnaugh EG, Ginsburg E, Gram TE. Studies on the interaction of bleomycin A_2 with rat lung microsomes II. Involvement of adventitious iron and reactive oxygen in bleomycin-mediated DNA chain breakage. J Pharmacol Exp Ther 221:159–165 (1982).
78. Nakashima T, Takino T, Kuriyama K. Therapeutic and prophylactic effects of taurine administration on experimental liver injury. In: Sulfur Amino Acids: Biochemical and Clinical Aspects (Kuriyama K, Huxtable R, Iwata H, eds). New York:Alan R Liss, 1983;449–459.
79. Weitberg AB. Effect of nicotinic acid supplementation *in vivo* on oxygen radical-induced genetic damage in human lymphocytes. Mutat Res 216:197–201 (1989).
80. Brown OR, Heitkamp M, Song CS. Niacin reduce paraquat toxicity in rats. Science 212:1510–1512 (1981).
81. Wang Q, Giri SN, Hyde DM, Nakashima JM, Javadi I. Niacin attenuates bleomycin-induced lung fibrosis in the hamster. J Biochem Toxicol 5:13–22 (1990).
82. Stubberfield CR, Cohen GM. NAD+ depletion and cytotoxicity in isolated NAD^+ hepatocytes. Biochem Pharmacol 37:3967–3974 (1986).
83. O'Neill CA, Giri SN. Biochemical mechanisms for the attenuation of bleomycin-induced lung fibrosis by treatment with niacin in hamsters: The role of NAD and ATP. Exp Lung Res 20:41–56 (1994).
84. Witschi HP, Haschek WM, Klein-Szanto AJP, Hakkinen PJ. Potentiation of diffuse lung damage by oxygen: determining variables. Am Rev Respir Dis 123:98–102 (1981).
85. Haschek WM, Witschi HP. Pulmonary fibrosis: a possible mechanism. Toxicol Appl Pharmacol 51:475–487 (1979).
86. Witschi HP, Godfrey G, Frome E, Lindenschmidt RC. Pulmonary toxicity of cytostatic drugs. Cell kinetics. Fundam Appl Toxicol 8:253–262 (1987).