Protective effect of orally administered carnosine on bleomycin-induced lung injury.

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Abstract

Carnosine is an endogenously synthesized dipeptide composed by β-alanine and L-histidine. It acts as a free radical scavenger and possesses antioxidant properties. Carnosine reduces pro-inflammatory and pro-fibrotic cytokines such as TGF-β, IL-1 and TNF-α in different experimental settings. In the present study, we investigated the efficacy of carnosine on the animal model of bleomycin-induced lung injury. Mice were subjected to intra-tracheal administration of bleomycin and were assigned to receive carnosine daily by an oral bolus of 150 mg/Kg. One week after fibrosis induction, BAL cell counts and TGF-β levels, lung histology and immunohistochemical analysis for myeloperoxidase, TGF-β, inducible nitric oxide synthase (iNOS), nitrotyrosine, and poly-ADP-ribose polymerase (PARP) were performed. Finally, apoptosis was quantified by TUNEL assay. Following bleomycin administration, carnosine treated mice exhibited a reduced degree of lung damage and inflammation when compared to WT mice as shown by the reduction of: (i) loss of body weight, (ii) mortality rate, (iii) lung infiltration by neutrophils (myeloperoxidase activity, BAL total and differential cell counts), (iv) lung edema, (v) histological evidence of lung injury and collagen deposition, (vi) lung myeloperoxidase, TGF-β, iNOS, nitrotyrosine and PARP immunostaining, (vii) BAL TGF-β levels and (viii) apoptosis. Our results indicate that orally administered carnosine is able to prevent bleomycin induced lung injury likely through its direct antioxidant properties. Carnosine is already available for human use. It might prove useful as an add-on therapy for the treatment of fibrotic disorders of the lung where oxidative stress plays a role such as idiopathic pulmonary fibrosis, a disease that still represents a major challenge to medical treatment.
Introduction

Idiopathic pulmonary fibrosis (IPF) is the most common among interstitial pneumonias of unknown origin and one of the most aggressive interstitial lung diseases. It is characterized by a chronic and progressive course leading to respiratory failure with a median survival under 3 years (1; 43). Pathologic findings in this disease include temporally and spatially non homogeneous areas of inflammation, fibrosis and honeycombing. Microscopically the hyperplasia of type II pneumocytes and active fibroblasts proliferation leads to excessive matrix deposition resulting in the irreversible distortion of the lung architecture (56), which in turn is responsible for impaired gas exchanges and respiratory failure.

A number of exogenously administered agents including commonly used drugs are known to induce a iatrogenic form of pulmonary fibrosis (57). Bleomycin is an efficacious antitumour agent currently used in humans. Nevertheless, repeated administration of this drug may lead to lung inflammation and fibrosis as a side effect. Because this phenomenon is easily reproduced in different mammals, intratracheal administration of bleomycin has become the most widely used experimental model of lung fibrosis although with certain limitations. This model is characterized by an early neutrophilic response, increased collagen deposition and fibroblast proliferation (13). Bleomycin alters the balance between oxidants and antioxidant defence systems in the lung. In this particular organ the selective absence of bleomycin hydrolase activity gives an high susceptibility to bleomycin-induced oxidative stress (20). Contemporarily, hydroxyl radicals, superoxide anion radical, hydrogen peroxide, and peroxynitrite are increased by bleomycin administration (51). Reactive oxygen species (ROS) overproduction ultimately results in tissue injury, with activation of several intracellular signalling pathways leading to the production of pro-inflammatory cytokines (33). DNA is a target for ROS activity as well. Radical oxygen species by determining DNA damage in turn activates poly-(ADP-ribose) polymerase (PARP). This largely expressed nuclear protein
contributes to the maintenance of genomic stability and to the repair of oxidative DNA damage (5). Although PARP activity promotes cell survival, PARP activation depletes NAD+, decreases ATP levels, thus leading to cell death after extensive DNA strand breaks (14). Therefore, ROS produced in response to oxidative stress are able to contribute by multiple pathways to the pathogenesis of bleomycin induced lung injury.

Carnosine is an endogenously synthesized dipeptide composed of β-alanine and L-histidine, which is present abundantly in muscle and nervous tissue in many species (52). It acts as a physiological buffer, a metal ion chelator, a free radical scavenger and finally as an antioxidant (17; 35). Besides the known anti-aging properties of this dipeptide, it has been demonstrated that carnosine plays a role in inflammation. In fact, carnosine inhibited hydrogen peroxide induced IL-8 release in vitro (58). Interleukin 6 and TNF-α where reduced by the oral administration of carnosine in an animal model of diabetes (44), and finally carnosine proved to decrease the secretion of TGF-β and of various extracellular matrix components induced by high doses of glucose in vitro (34).

Given the antioxidant and anti-inflammatory properties of carnosine, we sought to investigate carnosine efficacy on lung injury caused by bleomycin administration. To this end we evaluated the following endpoints: (a) loss of body weight, (b) mortality rate, (c) infiltration of the lung with polymorphonuclear neutrophils (myeloperoxidase activity), (d) edema formation, (e) histological evidence of lung injury, (f) bronchoalveolar lavage inflammatory cells counts, (g) TGF-β expression, iNOS activity, nitrotyrosine formation and activation of the nuclear enzyme PARP and (e) lung cells apoptosis (TUNEL assay).
Methods

Animals

Male CD mice (25–35 g; Harlan Nossan; Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Experimental groups

Mice were randomly allocated into the following groups:

(i) WT + BLEO group. Mice were subjected to bleomycin-induced lung injury (N = 15), (ii) WT + saline group. Sham-operated group in which identical surgical procedures to the BLEO group was performed, except that the saline was administered instead of bleomycin, (N = 15).

(iii) Carnosine group. Same as the WT + BLEO group but mice were administered daily with an oral bolus of carnosine in PBS to a final dose of 150 mg/Kg (Sigma spa., Milan, Italy) starting 30 minutes after bleomycin intratracheal administration. (iv) Vehicle group. Same as group iii, except that only PBS was administered as control vehicle instead of carnosine (N = 15).

Induction of lung injury by bleomycin

Mice received a single intratracheal instillation of saline (0.9%) or saline containing bleomycin sulphate (1 mg/kg body weight) in a volume of 50 µl and were killed after 7 days by pentobarbitone overdose.

Measurement of fluid content in lung
The wet lung weight was measured after careful excision of extraneous tissues. The lung was exposed for 48 h at 180°C and the dry weight was measured. Water content was calculated by subtracting dry weight from wet weight.

Histological examination

Lung biopsies were taken 7 days after injection of bleomycin. Lung biopsies were fixed for 1 week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). After embedding in paraffin, the sections were prepared and stained by trichrome stain. All sections were studied using light microscopy (Dialux 22 Leitz). The severity of fibrosis was semi quantitatively assessed according to the method proposed by Ashcroft and co-workers (2). Briefly, the grade of lung fibrosis was scored on a scale from 0 to 8 by examining randomly chosen sections, with fields per sample at a magnification of $\times 100$. Criteria for grading lung fibrosis were as follows: grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; grade 3, moderate thickening of walls without obvious damage to lung architecture; grade 5, increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses; grade 7, severe distortion of structure and large fibrous areas; grade 8, total fibrous obliteration of the fields.

Immunohistochemical localization of iNOS, nitrotyrosine, PARP and TGF-β

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or ROS, was determined by immunohistochemistry as previously described (15). At the end of the experiment, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 µm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% $\text{H}_2\text{O}_2$ (v/v) in 60% (v/v) methanol for 30 min. The sections were
permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with anti-iNOS rat polyclonal antibody (1:500 in PBS, v/v), anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v) or with anti-poly (ADP-ribose) goat polyclonal antibody (1:500 in PBS, v/v), and finally anti-TGF-β rabbit polyclonal antibody (1:500 in PBS, v/v). Sections were washed with PBS, and incubated with appropriate secondary antibodies. Specific labelling was detected with biotin-conjugated IgG and avidin–biotin peroxidase complex (DBA). In order to confirm that the immunoreaction for the nitrotyrosine was specific some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mm) to verify the binding specificity. To verify the binding specificity for PAR some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections indicating that the immunoreaction was positive in all the experiments carried out.

Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay.

Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay was conducted by using a TUNEL detection kit according to the manufacturer's instructions (Apotag, HRP kit; DBA, Milan, Italy). Briefly, sections were incubated with 15 µg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H₂O₂ for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid
atmosphere at 37 °C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with antihorseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine.

**Myeloperoxidase activity assay**

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (49). At the specified time following injection of bleomycin, lung tissues were obtained and weighted, each piece homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 × g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM hydrogen peroxide. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 37°C and was expressed in milliunits per g of wet tissue.

**Bronchoalveolar Lavage (BAL)**

Seven days after bleomycin or saline solution instillation, mice were euthanized and the trachea was immediately cannulated with an I.V. polyethylene catheter (Neo Delta Ven 2, delta Med, Viadana, Italy) equipped with a 24-gauge needle on a 1 mL syringe. Lungs were lavaged once with 0.5 ml D-PBS (GIBCO, Paisley, U.K.). In >95% of the mice, the recovery volume was over 0.4 ml. The BAL fluid was spun at 800 rpm, the supernatant was removed and the pelleted cells were collected. Total BAL cells were enumerated by counting on a hemocytometer in the presence of trypan blue. Cytospins were prepared from resuspended BAL cells.
Cytospins of BAL cells were made by centrifuging 50,000 cells onto microscope slides using a Shandon Cytospin 3 (Shandon, Astmoore, U.K.). Slides were allowed to air dry and were then stained with Diff-Quick Stain Set (Diff-Quick; Baxter Scientific, Miami, FL). A total of 400 cells were counted from randomly chosen high power microscope fields for each sample. BAL supernatants were collected and analyzed in duplicate by ELISA for biologically active TGF-β1 quantification (TGFβ1 Emax ImmunoAssay System, Promega Italia s.r.l., Milan, Italy), following manufacturer instructions.

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, U.K.). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

Statistical evaluation

All values in the figures and text are expressed as mean ± standard error of the mean (SEM) of N observations. For the in vivo studies N represents the total number of animals studied, dead animals were replaced in further experiments to reach the specified number of observations. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A P-value of less than 0.05 was considered significant. Statistical analysis for survival data was calculated by Fisher's exact probability test. For such analyses, p < 0.05 was considered significant.
Results

Histological examination of lung from bleomycin treated animals revealed significant tissue damage (Fig. 1a). Compared to lung sections taken from saline-treated animals, these were characterized by extensive inflammatory infiltration of neutrophils, lymphocyte and plasma cells extending through the lung epithelium, fibrosis and granulomas were seen in perivascular region. The administration of the dipeptide carnosine in mice significantly prevented lung inflammation induced by bleomycin administration. This was confirmed by the histological grading of lung fibrosis according to Ashcroft criteria executed on Masson’s trichrome stained slides, showing a significant reduction of the fibrosis score in carnosine treated animals (Fig. 1b).

Bleomycin in WT mice elicited an inflammatory response characterized by the accumulation of water in lung as an indicator of tissue oedema, and neutrophils infiltration in lung tissues as demonstrated by the myeloperoxidase assay (Figs. 2a and 2b). Carnosine significantly reduced the fluid content and the neutrophil infiltration in bleomycin treated animals.

Accordingly with the extensive infiltration of inflammatory cells, bleomycin instillation in WT mice produced a significant increase of inflammatory cells compared to sham WT mice (10.2 ± 0.2 vs. 1.69 ± 0.3 cells × 10^5/mL ± SE, p < 0.001) (Fig. 3a). Carnosine treated WT mice that underwent bleomycin intratracheal instillation showed a reduced number of inflammatory cells in BAL when compared to bleomycin WT mice group (5.15 ± 0.6 vs. 10.2 ± 0.2 cells × 10^5/mL ± SE, p < 0.05). Differential cell counts showed a similar profile as compared to all of the sham groups. In bleomycin treated WT mice it was evident that there was an increase of macrophages (5.77 ± 0.6 vs. 2.06 ± 0.3 cells × 10^5/mL ± SE, p < 0.001), lymphocytes (1.49 ± 0.4 vs. 0.18 ± 0.1 cells × 10^5/mL ± SE, p < 0.01) and neutrophils (3.66 ± 1.1 vs. 0.10 ± 0.04 cells × 10^5/mL ± SE, p < 0.01) if compared to sham wild type mice respectively.
Carnosine treated mice that underwent bleomycin tracheal instillation showed a decreased content of BAL inflammatory cells when evaluated by differential cell count on cytospins preparations (Fig. 3b). In these mice macrophages (4.09 ± 0.6 vs. 5.77 ± 0.6 cells × 10^5/mL ± SE, p < 0.05), lymphocytes (0.52 ± 0.1 vs. 1.49 ± 0.3 cells × 10^5/mL ± SE, p < 0.05) and neutrophils (0.77 ± 0.3 vs. 3.66 ± 1.1 cells × 10^5/mL ± SE, p < 0.05) were significantly reduced compared to bleomycin treated WT group. Eosinophils did not show any statistically significant difference among all groups.

Immunohistochemical analysis of lung sections obtained from bleomycin-treated mice revealed a positive staining for iNOS in macrophages and neutrophils present in the alveolar space and in septal walls (fig. 4a). Carnosine treatment abolished immunostaining for iNOS in the lung of animal treated with bleomycin. We then assessed the nitration of protein by nitrotyrosine immunohistochemical staining on tyrosine residues. Lung sections obtained from bleomycin-treated mice revealed a positive staining for nitrotyrosine mostly in the inflammatory cell infiltrate present in the interstitium and also in the alveolar pneumocyte layer (Fig. 4b). In contrast, no staining for nitrotyrosine was found in the lungs of bleomycin-treated mice that underwent carnosine treatment. Moreover, mice treated with bleomycin, exhibited a substantial increase in lung PARP staining (Fig. 4c), mainly present in inflammatory cells of the interstitium and in the alveolar pneumocyte layer at the nuclear level. Carnosine administration abolished the increased staining for PARP in lung section of bleomycin treated mice. Finally, we studied total TGF-β1 in lung sections by immunohistchemistry and active TGF-β1 in BAL supernatants by ELISA. Bleomycin induced a remarkable increase of TGF-β1 staining in the alveolar epithelium and in the inflammatory infiltrate. In contrast, carnosine treated mice did not exhibit such an increase (Fig. 5a). Similarly, biologically active TGF-β1 levels of the bleomycin aperated animals were significantly reduced in the BAL of carnosine group (652±38 vs. 158±68 pg/mL±SE, p<0.01, fig. 6). There was no iNOS, nitrotyrosine, PARP or TGF-β1
increase in the lungs of sham-operated animals (Figs. 4,5). In WT mice, the severe lung injury caused by bleomycin administration was associated with a significant increase of apoptosis as demonstrated by the marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 5b). In contrast, no apoptotic cells or fragments were observed in the lung obtained from carnosine treated mice. Similarly, no apoptotic cells were observed in lung from sham animals.

In WT mice, the severe lung injury caused by bleomycin administration was associated with a significant loss in body weight and increased mortality (Figs. 5a and 5b). Carnosine administration in bleomycin treated mice significantly attenuated the loss in body weight and abolished bleomycin induced mortality at 7 days.
Discussion.

Lung fibrosis is the common endpoint of an heterogeneous group of pathological entities termed interstitial lung diseases (ILD) which are characterized by chronic inflammation and progressive fibrosis of the pulmonary interstitium (28). Although the fibrotic process has greatly attracted research efforts, there is nowadays strong evidence that the cellular redox state and the oxidant/antioxidant balance plays an important role in the pathogenesis of idiopathic pulmonary fibrosis. Indeed, oxidative stress induces apoptosis of structural cells and upregulate the synthesis of proinflammatory cytokines (45). In BAL fluid and in plasma from patients with IPF an imbalance between oxidant and antioxidants has been proven (53). Myeloperoxidase, which is a marker of neutrophil activity, is strongly increased in the BAL of these patients (29). Similarly, iNOS, which is the main source of NO during inflammation, and nitrotyrosine, a byproduct of peroxynitrite activity, are upregulated in IPF lungs (41; 55).

Intratracheal instillation of the antitumour agent bleomycin is the most commonly used animal model for pulmonary fibrosis. Earlier reports point out that the pathogenesis of bleomycin-induced fibrosis, at least in part, is mediated through the generation of reactive oxygen species which cause the peroxidation of membrane lipids and DNA damage (26).

Carnosine is a dipeptide (β-alanyl-L-histidine) discovered nearly 100 years ago. Since then many functions have been proposed for this compound including wound healing promoter, ion chelant agent, antioxidant and free-radical scavenger (52). Carnosine prevents cellular toxicity in vitro, with a direct anti-peroxidative activity on proteins (31), lipids (50) and DNA bases (38). The anti-oxidant and metal ion-chelator properties of carnosine have been successfully tested on animal models of stomatitis, duodenal and gastric ulcers, and on different ocular disorders (3; 36). Furthermore, carnosine has been proven to affect inflammation directly by modulating cytokine release. In an animal model of diabetes, carnosine reduced IL-6 and TNF-α (44),
while TGF-β and extracellular matrix deposition were reduced by carnosine after stimulation with high doses of glucose in vitro (34).

In the present study we show a significant reduction of tissue damage and cellular apoptosis in lungs of bleomycin-treated mice treated with carnosine. Not only the extracellular matrix deposition evaluated histologically in lung sections of treated mice showed a reduced degree of fibrosis, but also the alveolar architecture was preserved, indicating that the treatment with this antioxidant significantly prevented lung damage induced by bleomycin.

Furthermore, carnosine proved efficacious to significantly lower total and biologically active TGF-β1 levels. TGF-β1 plays a central role in fibrotic disorders in different organs, including fibrosis of the lung. In fact, it stimulates collagen and fibronectin production in fibroblasts (21), on the other hand, it can suppress the production of proteases that degrade the extracellular matrix (59). TGF-β1 has been shown to be increased in bleomycin induced lung fibrosis in the alveolar inflammatory infiltrate (32). Secretion of active TGF-β1 by alveolar macrophages is augmented after bleomycin administration in mice, whereas latent TGF-β1 secretion remains elevated for a prolonged length of time and it is probable that the extent of inflammation and fibrosis in this model depends on the quantity of active TGF-β1 available (37). Finally, the increase of TGF-β1 mRNA precedes the biosynthesis of type I and type III procollagen in lung fibrosis (32).

Lung edema and fall of body weight were virtually absent and inflammation significantly reduced in carnosine treated animals. Leukocytes recruited into the tissue can contribute to tissue destruction by the production of reactive oxygen metabolites, granule enzymes, and cytokines that further amplify the inflammatory response. These responses are integral part of the antibacterial defences. For example, neutrophil-derived myeloperoxidase uses hydrogen peroxide produced by dismutation of superoxide to produce hypochlorous acid, a compound with relevant antibacterial properties (62). On the other hand, reactive oxygen species can
mediate tissue injury. An increased susceptibility to bleomycin has been reported for mice lacking extracellular superoxide dismutase (SOD1), which indicates that superoxide anion radicals play a main role in experimental fibrosis(19). Recently, carnosine and some carnosine derivatives have been shown to scavenge superoxide anion radical radicals(39), and to chelate copper(II) leading to a complex that shows SOD1-like activity with a catalytic constant equal to that found for native SOD1(6; 7). Moreover, this natural dipeptide has been shown to protect primary astroglial cell cultures from iNOS induced oxidative stress by scavenging NO radical(12). Superoxide reacts with nitric oxide to generate highly reactive metabolites such as peroxynitrite. This compound is able to oxidize proteins, resulting in direct nitration of tyrosine residues. Protein structure and function can be subsequently altered and enzymatic activity affected. Proteins containing nitrotyrosine residues have been detected in different pathologies associated with enhanced oxidative stress and increased levels of peroxynitrite (30). Consistent with these data, carnosine reduced the expression of the inducible nitric oxide synthase (iNOS) and the nitration of tyrosine residues in lung sections. Nitric oxide (NO) mediates vaso- and broncho-dilatation, and it is synthesized from L-arginine by 2 constitutive forms of NOS which are involved in the physiological regulation of airway function(48). However, the inducible form of the nitric oxide synthase generates much larger quantities of NO than the constitutive isoforms, and it is directly involved in host defence from infections (46) and in various models of inflammation (16; 61). Exogenous NO is able to stimulate in vitro fibroblast proliferation(24), while iNOS upregulation in lung fibroblasts is associated with the early proliferative response to cytokine stimulation(54). Finally, the pharmacological inhibition and the genetic disruption of iNOS have been shown to reduce the development of inflammatory responses and fibrosis in lung of bleomycin treated animals(25).

Nitrotyrosine immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation of peroxynitrite (4). There is, however, recent evidence
that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine (18). Increased nitrotyrosine staining is considered, therefore, as an indication of "increased nitrative stress" rather than a specific marker of the generation of peroxynitrite. Nevertheless, our results confirm previous data on the activity of carnosine on tyrosine nitration in vitro (22).

We finally tested a novel pathway of inflammation relying on the nuclear PARP enzyme activation by superoxide and peroxynitrite. Poly(ADP-ribose) polymerase contributes to the maintenance of genomic stability and to the repair of oxidative DNA damage (5). However its activity can deplete NAD+, interfere with glycolysis and ATP metabolism, ultimately leading to cell death because of extensive DNA strand breaks (14).

Although the exact role of PARP in human lung fibrosis has not been investigated, it has been shown that PARP is implicated in experimental fibrosis and that PARP inhibition confers protection from inflammation and fibrosis in different animal models including the bleomycin model (60). Indeed, we have previously shown that PARP is elevated in the lung of bleomycin treated mice (25). In this study we do confirm that bleomycin administration PARP levels, while carnosine greatly reduced the expression of this enzyme. Interestingly, treated animals showed a reduced degree of lung cellular apoptosis. Therefore, it is conceivable that the protection from bleomycin injury might derive, at least in part, from the carnosine scavenging ability on superoxide and peroxynitrite, which in turn prevents PARP activation and the depletion of crucial metabolites to cellular activities.

The beneficial activity of carnosine administration following bleomycin was reflected by some favourable clinical outcomes. Most notably, the beneficial effects given by this treatment resulted in the complete abrogation of the bleomycin-induced mortality.

Taken together, our data further support the rationale for antioxidant therapy in ILD.
Similarly to other proposed therapeutic chelating molecules, carnosine and its derivatives form very stable mono- and poly-nuclear copper complexes (40; 47), suggesting a dual action, as an antioxidant and also as a chelating agent. In example, tetrathiomolybdate (TM) is a copper chelating agent (11) which has been proposed recently for the treatment of cancer(8), and against bleomycin-induced pulmonary fibrosis in mice (10). The TM metal chelating effect lowers systemic copper(II) levels thus inhibiting several pro-angiogenic cytokines in cancer(27) and modulating pro-fibrotic and pro-inflammatory cytokines such as TGF-β and TNF-α (9; 23; 42). TM efficacy in experimental lung fibrosis has been associated with a significant decrease of systemic copper level indeed. Considering these results, carnosine may be considered a potential multifunctional drug with both chelating and antioxidant activity; these properties may prove useful for the treatment and the prevention of diseases in which ROS are thought to play a major role such as the interstitial pathologies of the lung.
Grants:

This work was funded by the Italian Ministry of University and Research (MIUR): grants id. PRIN 2005, # 2005069290_003 and FIRB RBNE03PX83_001.
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Figure legend

Figure 1: Effect of carnosine on lung fibrosis.
(a) Bleomycin administration in WT mice caused extensive inflammation, inflammatory cells infiltration and fibrosis. Carnosine treated animals showed patchy areas of inflammation with minimal fibrosis. Each figure is representative of at least 3 experiments performed on different experimental days. Trichrome stain. Original magnification: × 150. (b) Histological grading of lung fibrosis. Black bar represents vehicle group, grey bar carnosine group. Data are means ± SEM from 15 mice for each group. *p < 0.01 versus sham. °p < 0.01 versus bleomycin.

Figure 2: Effect of carnosine on tissue edema and myeloperoxidase activity in the lung.
Bleomycin in WT mice elicited an inflammatory response characterized by the accumulation of water in lung (a) and increased myeloperoxidase activity (b). Carnosine significantly reduced the edema formation and myeloperoxidase activity.
Black bar represents vehicle group, grey bar carnosine group. Data are means ± SEM from 15 mice for each group. *p < 0.01 versus sham. °p < 0.01 versus bleomycin.

Figure 3: Effect of carnosine on bleomycin-induced total and differential cellularity in bronchoalveolar lavage (BAL).
Total BAL cellularity for sham and bleomycin treated mice with and without carnosine administration (a). In b differential cells counts for macrophages, lymphocytes, neutrophils and eosinophils per milliliter of BAL fluid are shown. White bar represents sham group, black bar bleomycin group, grey bar carnosine group. Data, expressed as means ± SEM, are representative of 15 mice for each group. + p < 0.001 vs. sham, ++ p < 0.05 vs. bleomycin treated WT, ° p < 0.01 vs. sham, *p < 0.05 vs. bleomycin treated WT.
Figure 4: Effect of carnosine on lung iNOS, nitrotyrosine and PAR immunostaining.
(a) After bleomycin injection in WT mice, a positive staining for iNOS in macrophages and neutrophils was present in the alveolar space and in septal walls. Carnosine treatment abolished immunostaining for iNOS in the lung of animal treated with bleomycin.
(b) Bleomycin administration increased nitrotyrosine staining mainly in inflammatory infiltrate present in the interstitium but also in the alveolar pneumocyte layer. In contrast, no staining for nitrotyrosine was found in the lungs of bleomycin-treated mice that underwent to carnosine treatment.
(c) Mice treated with bleomycin, exhibited a substantial increase in lung PAR staining in inflammatory cells of the interstitium but also in the alveolar pneumocyte layer at the nuclear level. Carnosine administration abolished the increased staining for PAR in lung section of bleomycin treated mice.
Original magnification: 150×. These microphotographs are representative of at least 3 experiments performed on different experimental days.

Figure 5: Effect of carnosine on lung TGF-β1 immunostaining and TUNEL assay.
(a) After bleomycin injection in WT mice, a positive staining for TGF-β1 in the inflammatory infiltrate and in the alveolar epithelium was present in lung sections. Carnosine treatment strongly diminished immunostaining for TGF-β1 in the lung of animal treated with bleomycin.
(b) Bleomycin-induced apoptosis was measured by terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL)-like staining. One week after bleomycin administration, lung tissue demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments. No apoptotic cells or fragments were observed in the tissues obtained from bleomycin mice treated with carnosine. Similarly, no apoptotic cells were observed in
section obtained from sham animals. Original magnification: 150×. These microphotographs are representative of at least 3 experiments performed on different experimental days.

Figure 6: Bronchoalveolar lavage active TGF-β1 levels.

Bleomycin in WT mice elicited a relevant increase TGF-β1 levels in the BAL. Carnosine significantly reduced biologically active TGF-β1 levels after one week from bleomycin administration.

Black bar represents vehicle group, grey bar carnosine group. Data are means ± SEM from 15 mice for each group. *p < 0.01 versus sham. °p < 0.01 versus bleomycin.

Figure 7: Effect of carnosine on body weight and mortality rate.

(a) Body weight was recorded immediately before bleomycin administration and daily during the experimental period. Carnosine administration significantly prevents the loss of body weight.

(b) Survival was significantly improved in carnosine treated mice in comparison to mortality rate of the bleomycin-treated WT mice. ♦ represents bleomycin group, ● carnosine treated animals. Data are means ± SEM from 15 mice for each group. *p < 0.01 versus vehicle.
Figure 1
Figure 2

(a) Wet/dry lung weight ratio

(b) Lung myeloperoxidase (U/g wet tissue)
Figure 3
Figure 4
Figure 5
Figure 7