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In situ assessment of oxidant and nitrogenic stress in bleomycin pulmonary fibrosis

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Abstract Reactive oxygen species (ROS) and nitric oxide (NO) have a role in the development of pulmonary fibrosis after bleomycin administration. The ROS production induces an antioxidant response, involving superoxide dismutases (SODs), catalase, and glutathione peroxidases. We compared in situ oxidative burden and antioxidant enzyme activity in bleomycin-injured rat lungs and normal controls. ROS expression and catalase, glucose-6-phosphate-dehydrogenase (G6PHD), and NOS/NADPH-diaphorase activity were investigated by using histochemical reactions. Nitric oxide synthase (e-NOS and i-NOS) and SOD (MnSOD, Cu/ZnSOD, ECSOD) expression was investigated immunohistochemically. After treatment ROS production was enhanced in both phagocytes and in type II alveolar epithelial cells. Mn, Cu/Zn, and ECSOD were overexpressed in parenchymal cells, whereas interstitium expressed ECSOD. Catalase and G6PHD activity was moderately increased in parenchymal and inflammatory cells. NOS/NADPH-d activity and i-NOS expression increased in alveolar and bronchiolar epithelia and in inflammatory cells. It can be suggested that the concomitant activation of antioxidant enzymes is not adequate to scavenge the oxidant burden induced by bleomycin lung damage. Inflammatory cells and also epithelial cells are responsible of ROS and NO production. This oxidative and nitrosative stress may be a substantial trigger in TGF-β1 overexpression by activated type II pneumocytes, leading to fibrotic lesions.

Keywords Pulmonary fibrosis · Oxidative stress · Bleomycin · Histochemistry · Superoxide dismutase · Catalase · Nitric oxide synthase · Glucose-6-phosphate-dehydrogenase

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Introduction

Bleomycins are a family of compounds, produced by *Streptomyces verticillus*, with potent tumor killing properties but also dose-dependent pulmonary toxicity. The antineoplastic activity of bleomycin is based on intracellular iron binding: the bleomycin–iron complex reduces molecular oxygen to superoxide (O_2^-) and hydroxyl radicals ($OH^\bullet$) which affects DNA integrity causing strand cleavage, lipid peroxidation, and carbohydrate oxidation (Martin and Kachel 1987; Trush 1982; Yamazaki et al. 1998; Giri et al. 1983). Experimental models have shown that in the lung, after bleomycin administration, activated phagocytes release large amounts of reactive oxygen species (ROS). The most important ROS generated by inflammatory cells are superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$), nitric oxide ($NO^\bullet$), and hydrogen peroxide (H_2O_2) (Lakari 2002; Halliwell and Gutteridge 1989). The production of ROS induces an antioxidant response, involving enzymes with ROS scavenging activity: superoxide dismutases (SODs), catalase, and glutathione peroxidases (GSP). SODs are the only enzymatic system decomposing superoxide radicals to H_2O_2, which is transformed by catalase and GSP to O_2 and H_2O. In the reaction catalyzed by glutathione peroxidases, reduced glutathione (GSH) is used as a cosubstrate to metabolize H_2O_2, resulting to H_2O, O_2, and oxidized glutathione (GSSG). GSSG can be reduced back to GSH by the enzyme GSH reductase, a reaction requiring NADPH regenerated by glucose-6-phosphate dehydrogenase

(G6PDH) (Halliwell and Gutteridge 1989). The bleomycin-mediated lung injury involves the recruitment of macrophages, neutrophils, and lymphocytes in the damaged airways (Ward and Hunninghake 1998; Manoury et al. 2005). This may lead to the development of pulmonary fibrosis, characterized by enhanced production and deposition of collagen and other matrix components, resulting in a histological appearance that is similar to idiopathic pulmonary fibrosis. The oxidative pathway activation in bleomycin-induced lung damage is not only due to inflammatory cells but also to the presence of excessive amount of ROS intracellularly generated by bleomycin (Quinlan et al. 1994).

A further molecule implicated in the oxidative stress is nitric oxide (NO), a messenger molecule with complex biological activities, which interacts with ROS producing highly reactive nitrogen intermediates. In lungs NO is produced by several cell types, such as inflammatory cells, alveolar and bronchiolar epithelia, vascular endothelia, alveolar macrophages, neutrophils, and mast cells. NO levels may be modulated by O_2^- produced by neutrophils (Jones et al. 1998). NO synthesis is catalyzed by nitric oxide synthase (NOS). Three isoforms of NOS have been described, neuronal (n-NOS), inducible (i-NOS), and endothelial (e-NOS).

The aim of the present study was to compare oxidative and antioxidant enzyme activity in situ in bleomycin-injured lungs. For this purpose, we investigated ROS expression and enzyme activity of catalase, glucose-6-phosphate-dehydrogenase, and NOS/NADPH-diaphorase with histochemical reactions. NOS (e-NOS and i-NOS) and SOD (MnSOD, Cu/ZnSOD and ECSOD, extracellular SOD) expression was investigated by immunohistochemistry.

Material and methods

Animals

Adult male Sprague–Dawley rats weighting 150 g were purchased from Charles River Italy S.p.a. (Milan, Italy). For the duration of the experiment, the animals were maintained in specific pathogen-free conditions, were fed with commercial rat food and water ad libitum with a 12-h dark/light cycle.

Induction of bleomycin-induced pulmonary fibrosis

After general anesthesia administered with intramuscular injection of tiletamine chlorohydrate (3.33 mg/100 g body weight) and zolazepam chlorohydrate (3.33 mg/100 g body weight), 15 rats received bleomycin sulfate (Nippon Kayaku Co. Ltd, Chiyoda Ku, Tokyo, Japan) 1.7 U in 0.4 ml of sterile solution (1.13 U/100 g body weight). Six control rats received only 0.4 ml of saline solution. Bleomycin and sterile solutions were prepared immediately before administration and were administered as a single dose with a 25-gauge needle directly into the trachea, exposed via a small cervical skin incision and separation of the strap muscles. Five treated and two control animals were killed at day 3, 7, and 14 after treatment by carotid exsanguination under deep anesthesia. The experimental protocol was approved by the Ethical Committee of the University of Pavia.

Lung tissue preparation

After lavage the lungs were removed en bloc, quickly weighted and placed in cold (4°C) buffer (30 mM histidine, 250 mM sucrose, 2 mM EDTA, pH 7.2). Subsequently they were cut, and a part of each lobe (4–5 mm^3) was frozen in liquid nitrogen and stored at −80°C until use for histochemical analysis. The remaining parenchyma was fixed with neutral buffered 10% formalin, embedded in paraffin according to routine laboratory protocols, and sectioned at a thickness of 4 μm for light microscopy and immunohistochemistry. Sections were stained with hematoxylin and eosin and Movat pentachrome; unstained sections were used for immunohistochemistry. Hematoxylin and eosin and Movat stained slides were examined to quantitatively assess the type and grade of tissue damage, inflammatory cell infiltration, and tissue repair and fibrosis. Semiquantitative scoring was performed in accordance with the method described by Madtes et al. (1999).

Enzyme histochemistry and ROS demonstration

Frozen lung sections, 7 μm thick, were cut on a cryostat at a cabinet temperature of −25°C. Sections were collected on silanized glass slides and stored in the cryostat cabinet until submitted to specific reactions. All media were freshly prepared just before incubation. The slides were mounted in glycerol jelly and observed and photographed immediately with a light microscope.

Catalase activity (EC 1.11.1.6) was assessed according to Van Noorden and Frederiks (1992). Cryostat sections were incubated for 30 min at 37°C in a medium containing 2% polyvinyl alcohol (PVA) in 0.1 M glycine–NaOH buffer (pH 10.5), with 5 mM DAB and 18 mM H_2O_2.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was assessed according to Van Noorden and Frederiks (1992). Sections were incubated for 5 min at 37°C in a medium containing 15% PVA in 0.1 M phosphate buffer (pH 7.4), with 10 mM glucose-6-phosphate, 0.8 mM NADP+, 0.32 mM 1-methoxyphenazine methosulfate, 5 mM $MgCl_2$, 5 mM sodium azide, and 5 mM TNBT.

To demonstrate the activity of NOS/NADPH-d (EC 1.6.99.3), cryostat sections were prefixed in 4% paraformaldehyde, buffered with 0.1 M phosphate (pH 7.4) for 15 min at 4°C, washed in phosphate buffer for 10 min, and then incubated in a medium according to

Nakos and Gossrau (1994), with our modifications (Fenoglio et al. 1997). The standard medium consisted of PVA 15% in 100 mM phosphate buffer, pH 7.4, 5 mM levamisole, 2 mM βNADPH (Sigma), 3 mM NBT (Sigma), 0.2% Triton X, and 0.2% paraformaldehyde. Incubation was performed for 50 min at 37°C in the dark. The specificity of each enzymatic reaction was tested by different control incubation in which cryostat sections were incubated in media without substrate. For NOS/NADPH-d fixed sections were alternatively incubated in NADH containing medium instead of NADPH. No reaction products were detected in any of the control sections.

To detect ROS, cryostat sections were incubated in a medium prepared according to Kerver et al. (1997) containing 12.5 mM DAB, 5 mM $MnCl_2$, and 40 mM $CoCl_2$ dissolved in 10% PVA, in 100 mM Tris–maleate buffer (pH 8.0) at 37°C. After 30-min incubation the sections were rinsed in hot distilled water to stop the reaction and to remove the viscous incubation medium.

Two independent observers semiquantitatively evaluated the relative degree of staining. Different values of positivity were scored as weak (+), moderate (++), intense (+++), and very intense (++++). Although partially limited by the suboptimal preservation of histological detail in frozen and reacted tissue, the observers recorded the levels of staining in each cellular compartment. Bronchiolar epithelia and endothelia were recognized from the presence of the typical muscular layers and cellular shapes; we interpreted all stained cells located in the peribronchiolar/perivascular interstitium and lying free in alveolar spaces as inflammatory cells; type II and hyperplastic alveolar epithelia were recognized from their cuboidal shape and the position lining alveolar walls.

Immunohistochemistry

Immunohistochemical analysis of NOS and SOD expression was performed with antibodies and antisera against e-NOS, i-NOS (NeoMarkers, Fremont, CA), MnSOD, Cu/ZnSOD, and ECSOD (Stressgen, D.B.A. Italia S.r.l) as detailed in Table 1, on 4 μm thick paraffin sections after appropriate pretreatment. Tissue sections were incubated overnight at 4°C with primary antibodies, diluted as indicated (Dako antibody diluent, DakoCytomation, Glostrup, Denmark) (Table 1). The reactions were revealed with the avidin–biotin–peroxidase complex (Dako LSAB+ System), using diaminobenzidine tetrahydrochloride as chromogen substrate (Dako). Each reaction set included a negative control obtained with substitution of the primary antibody with dilution buffer, and positive controls as suggested by the manufacturer. Immunostained slides were examined to identify the cell types expressing antigen and to semiquantitatively score the amount of protein present in the lung.

Chemicals

The chemicals used for histochemical reactions were purchased from Sigma Chemical Co. except for sodium azide, purchased from E. Merck (Darmstadt, FRG) and magnesium chloride, manganese chloride, and cobalt chloride, purchased from Carlo Erba (Milan, Italy).

Results

Histological evaluation

Rat lung damage following bleomycin instillation did not differ from what has been described in previous studies (Borzone et al. 2001). Three days after administration, lungs began to show increased alveolar and septal cellularity, with alveolar bronchiolization, hyperplasia of type 2 pneumocytes, and focal endoalveolar hyaline membranes. The damage was more extensive at day 7, with higher inflammation scores and minimal increase of fibrosis. Starting from day 14, deposition of interstitial collagen appeared, with retraction of surrounding parenchyma, and the number of macrophages both in alveolar spaces and interstitium peaked; fibrosis scores were higher than those of inflammation. Control lungs did not show significant inflammation or fibrosis at any time point.

Catalase activity

Catalase activity was not detected in bronchiolar epithelia and endothelia at any time point. It was present in type II alveolar epithelia in control lung and was only slightly enhanced after treatment. Macrophages infiltrating the lung after bleomycin infusion showed marked catalase activity (data not shown). The results are summarized in Table 2.

Table 1 Antibodies used for NOS and SOD immunohistochemical assay

Name	Concentration	Source	Clone	Pretreatment
e-NOS	1:800	NeoMarkers (Fremont, CA)	Ab-1	MWO 750 W 5′×2 cycles
i-NOS	1:700	NeoMarkers	Ab-1	MWO 750 W 5′×2 cycles
MnSOD	1:950	Stressgen (Victoria, Canada)	SOD-110	MWO 750 W 5′×2 cycles
Cu/ZnSOD	1:2,000	Stressgen	SOD-101	None
ECSOD	1:300	Stressgen	SOD-105	MWO 750 W 5′×2 cycles

Glucose-6-phosphate-dehydrogenase activity

In control lungs, G6PDH activity was observed in bronchiolar epithelia and in some type II alveolar epithelia as well as in endothelia (Fig. 1a). After bleomycin infusion, G6PDH activity was enhanced in all cell compartments, peaking at day 7, with a slight reduction at day 14 (Fig. 1b, c). G6PDH activity was also present in alveolar macrophages, although at a lower level than other antioxidant enzymes. The results are summarized in Table 3.

NOS/NADPH-diaphorase activity

Moderate NOS activity was present in control bronchiolar and type II alveolar epithelia, and, to a lesser extent, in endothelia (Fig. 2a). In all compartments, the activity was enhanced after treatment and high levels of reaction products were maintained up to 14 days (Fig. 2b, c). In untreated rats, alveolar macrophages showed low degrees of NOS activity, which was markedly enhanced after bleomycin treatment (Fig. 2c). The results are summarized in Table 4.

e-NOS and i-NOS expression

e-NOS was constitutively expressed in bronchiolar epithelia and endothelia; slight and focal positivity was also observed in type II alveolar epithelia. After bleomycin infusion, expression was moderately enhanced in accordance with the increased number of type II reactive alveolar cells, peaking at 7 days, with a subsequent reduction at day 14. No significant change was observed in e-NOS immunostaining in bronchiolar cells; endothelial cell expression was slightly enhanced after treatment and returned to normal at day 14. Alveolar macrophages constitutively expressed e-NOS; their number markedly increased after bleomycin treatment (data not shown).

In control rats mild i-NOS immunoreactivity was only observed in bronchiolar epithelia (Fig. 2d). After bleomycin administration, at 7 and 14 days, i-NOS expression was moderately enhanced in bronchiolar epithelia, and hyperplastic alveolar epithelia showed de novo low immunoreactivity (Fig. 2e, f). In all samples alveolar macrophages displayed low degree of i-NOS expression; however, bleomycin treated rats showed an increased number of immunoreactive macrophages. The results are summarized in Table 5.

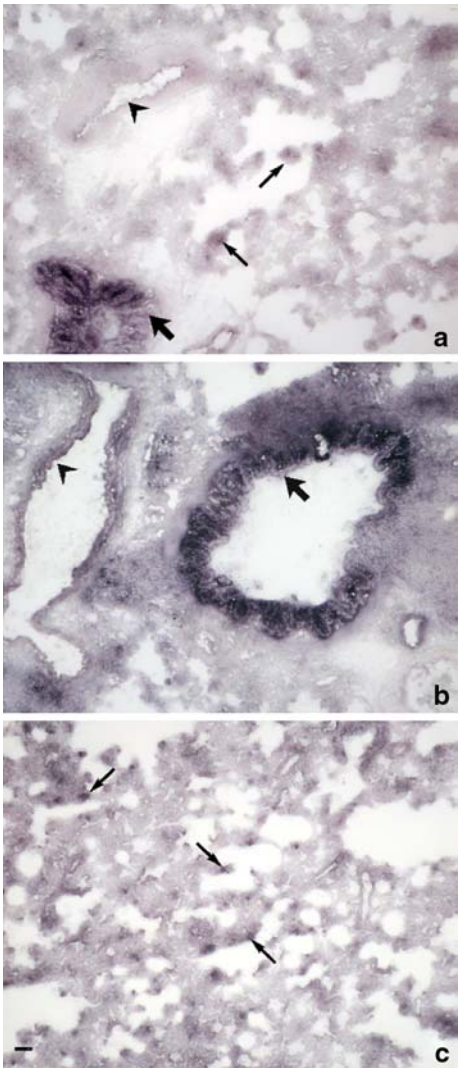

Fig. 1 G6PDH activity in control (**a**) and treated lung (**b**, **c**), respectively, 7 and 14 days after bleomycin infusion. **a** In control lung weak G6PDH activity is present in type II alveolar (*thin arrows*) and bronchiolar cells (*thick arrow*) and in endothelia (*arrowhead*). **b**, **c** Treated lung shows moderate to strong G6PDH activity in numerous type II pneumocytes (*thin arrows*) and bronchiolar cells (*thick arrow*) and in endothelia (*arrowhead*). *Scale bar* = 50 μm

Table 2 Average catalase activity

Rat group	Alveolar epithelia[a]	Bronchiolar epithelia	Endothelia	Macrophages[a]
Controls	+	–	–	+
3 days	+ +	–	–	+ +
7 days	+ +	–	–	+ + +
14 days	+ +	–	–	+ + +

[a]Increased expression is mainly due to the increased number of cells

Table 3 Average G6PDH activity

Rat group	Alveolar epithelia	Bronchiolar epithelia	Endothelia	Macrophages
Controls	–/+	+ +	+ +	+
3 days	–/+	+ + +	+ +	+
7 days	+	+ + + +	+ + +	+ +
14 days	+ +	+ + +	+ +	+ +

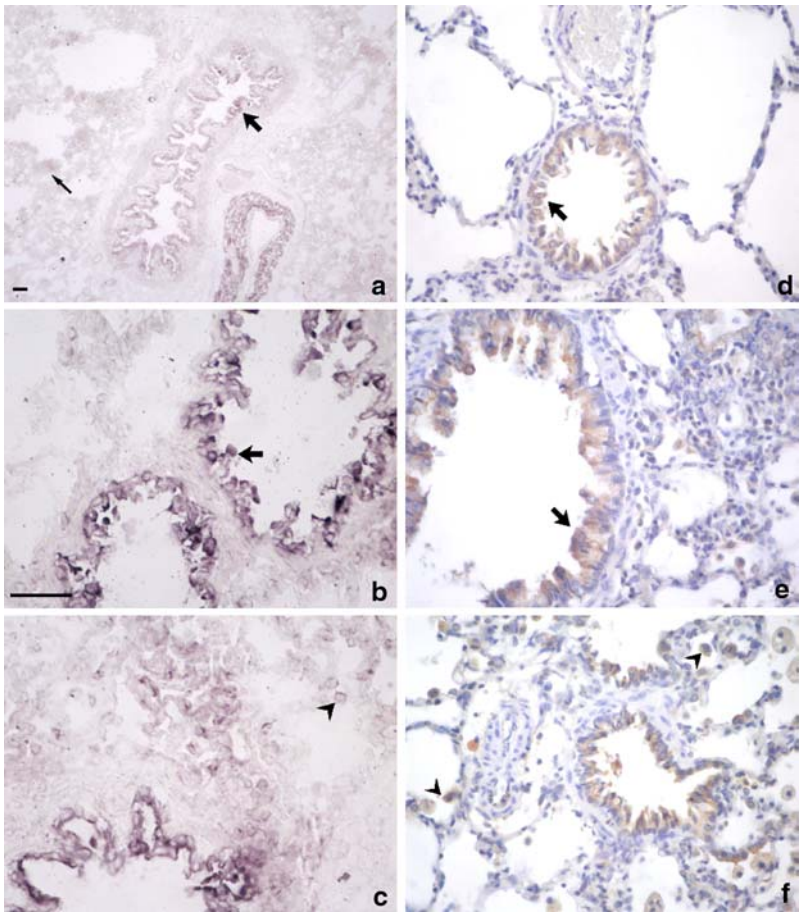

Fig. 2 NOS/NADPH-d activity (**a**–**c**). **a** NOS/NADPH-d activity in control lung. Type II pneumocytes (*thin arrow*) and bronchiolar cells (*thick arrow*) show light to moderate NOS/NADPH-d activity. **b**, **c** NOS/NADPH-d activity in treated lung at 7 and 14 days after bleomycin administration, respectively. Intense NOS/NADPH-d staining is present in bronchiolar cells (**b**, *thick arrow*) and type II pneumocytes; some alveolar macrophages (**c**, *arrowhead*) show NOS/NADPH-d activity. I-NOS immunohistochemistry (**d**–**f**). **d** In control lung bronchiolar epithelial cells are weakly immunopositive (*thick arrow*). **e**, **f** I-NOS expression in treated lung at 7 and 14 days after bleomycin administration, respectively. Moderate immunostaining in hyperplastic type II pneumocytes, bronchiolar cells (**e**, *thick arrow*), and in alveolar macrophages (**f**, *arrowheads*). *Scale bar* = 50 μm (size bar in **b** is the same from **b** to **f**)

MnSOD, Cu/ZnSOD, and ECSOD expression

In control rat lungs, MnSOD immunoreactivity was found in type II pneumocytes, in small airways epithelia, and in alveolar macrophages (Fig. 3a, b). After bleomycin infusion, the degree of expression was increased in all cell types, peaking at day 7, with subsequent reduction in epithelial cells (Fig. 3c, d). De novo expression was observed in endothelia 1 week after treatment.

Cu/ZnSOD expression was only present in bronchiolar epithelia of control lung, and was enhanced after bleomycin administration. Low degrees of de novo expression were observed 1 week after bleomycin infusion in type II pneumocytes and alveolar macrophages, but not in endothelia.

ECSOD immunoreactivity was present in the extracellular interstitium, mainly perivascular and peribronchiolar, in control lungs. The degree of expression was increased, extending to alveolar septa and fibrotic areas, after bleomycin treatment. In control samples, ECSOD was also expressed in bronchiolar epithelia and, to a lesser extent, in endothelia and alveolar macrophages.

Table 4 Average NOS/NADPH-diaforase activity

Rat group	Alveolar epithelia[a]	Bronchiolar epithelia	Endothelia	Macrophages[a]
Controls	+	+ +	−/+	−/+
3 days	+	+ +	−/+	−/+
7 days	+ +	+ + +	−/+	+ +
14 days	+ +	+ + +	+	+ + +

[a]Increased expression is mainly due to the increased number of cells

Table 5 e-NOS and i-NOS expression

Rat group	Alveolar epithelia		Bronchiolar epithelia		Endothelia		Macrophages	
	e-NOS[a]	i-NOS	e-NOS	i-NOS	e-NOS	i-NOS	e-NOS	i-NOS
Controls	−/+	−	+ +	−	+/+ +	−	−	+
3 days	−/+	−	+ +	+	+ +	−	−	+
7 days	+/+ +	+	+ +	+ +	+ +	−	+ +/+ + +	+ +
14 days	+	−/+	+ +	+ +	+/+ +	−	+ +	+ +

[a]Increased expression is mainly due to the increased number of cells

After treatment, the reactivity was slightly increased in endothelia and airways epithelia and moderately in macrophages. De novo expression was observed in type II pneumocytes (Table 6).

ROS demonstration

In control lungs, ROS production was present in type II alveolar epithelia but not in bronchiolar epithelia and endothelia (Fig. 3e, f). After bleomycin infusion, very weak reactivity was observed in bronchiolar epithelia, from day 7 to day 14. In alveolar epithelia, bleomycin treatment induced overexpression peaking at 7 days (Fig. 3g, h). In all samples alveolar macrophages constitutively expressed ROS; however, their number and ROS expression within the cytoplasm increased after bleomycin infusion (Fig. 3g, h). The results are summarized in Table 7. ROS was also present in smooth muscle of the vascular and bronchiolar walls in normal and treated lungs (Fig. 3f, h). In accordance with recent in vitro observations (Caporossi et al. 2003), in this setting ROS was interpreted as a physiological modulator of skeletal muscle functions independent of bleomycin toxicity.

Discussion

In the present study, to evaluate in situ oxidant–antioxidant balance in bleomycin-injured rat lungs, we analyzed the expression of ROS and the activity or expression of enzymes involved in ROS scavenging at different molecular levels: SOD decomposing superoxide radicals to H_2O_2, catalase that further reduces H_2O_2 to water and oxygen, and G6PDH, whose activity was

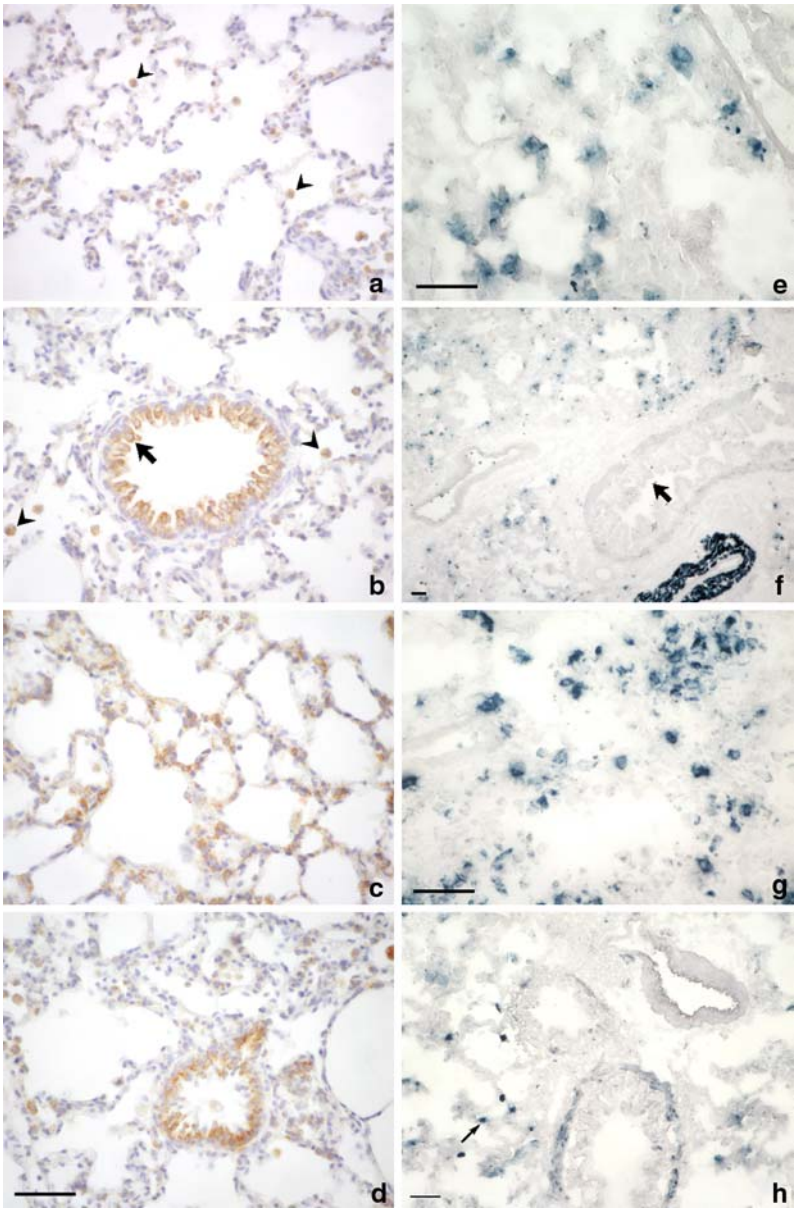

Fig. 3 MnSOD immunostaining (**a**–**d**). **a**, **b** In control lung MnSOD immunostaining is weak to moderate in type II pneumocytes, in bronchiolar cells (*thick arrow*), and alveolar macrophages (*arrowheads*). **c**, **d** MnSOD expression in treated lung at 7 and 14 days after bleomycin administration, respectively. Type II pneumocytes, alveolar macrophages, and bronchiolar epithelial cells show intense (**c**) to moderate (**d**) immunostaining. ROS expression (**e**–**h**). **e, f** In control lung type II pneumocytes are weakly stained. **f** Note negative bronchiolar cells (*arrow*). **g**, **h** ROS expression in treated lung 7 and 14 days after bleomycin infusion, respectively. Alveolar epithelial cells and inflammatory cells show intense ROS staining. A mild ROS expression is present in bronchiolar epithelial cells; type II pneumocytes are also stained (**h**, *arrow*). *Scale bar* = 50 μm (size bar in **d** is the same from **a** to **d**)

Table 6 MnSOD, Cu/ZnSOD, and ECSOD[a] expression

Rat group	Alveolar epithelia			Bronchiolar epithelia			Endothelia			Macrophages		
	Mn[b]	Cu/Zn	EC	Mn	Cu/Zn	EC	Mn	Cu/Zn	EC	Mn[b]	Cu/Zn	EC
Controls	+	–	–	++	++	+/++	–	–	–/+	++	–	–/+
3 days	+	–	–/+	++	+++	+	–	–	–/+	++	–	–/+
7 days	++/+++	–/+	++	++/+++	++	++	+	–	–/+	+++	–/+	++
14 days	++	–	++	++	++	++	+/++	–	+	+++	–/+	+/++

[a]ECSOD expression in extracellular interstitium was present in control lungs and increased after treatment
[b]Increased expression is mainly due to the increased number of cells

Table 7 Average ROS expression

Rat group	Alveolar epithelia	Bronchiolar epithelia	Endothelia	Macrophages[a]
Controls	+	–	–	+
3 days	++	–	–	++
7 days	+++	–/+	–	++++
14 days	++	–/+	–	++++

[a]Increased expression is mainly due to the increased number of cells

considered as an indirect indicator of the H_2O_2 metabolism by GSP, which could not be assessed in situ. Moreover, we investigated the activity of NOS/NADPH-d and the expression of endothelial and inducible NOS isoforms.

A global increase in oxygen metabolites and detoxifying enzymes has been previously documented in lungs exposed to bleomycin by means of biochemical assays on lung homogenates (Fantone and Phan 1988; Giri et al. 1983; Matalon et al. 1985). Partial data exist on the cellular compartments involved in ROS production and antioxidant activity in lung: the pivotal role of ROS produced by inflammatory cells in bleomycin lung toxicity was recently confirmed by a study documenting that p47 $^{phox}-/-$ (KO) mice, deficient for ROS production in phagocytes through the NADPH-oxidase pathway, fail to develop pulmonary fibrosis after bleomycin administration (Manoury et al. 2005). In our study, for the first time, in situ ROS production was observed in both phagocytes and in type II alveolar epithelial cells in control lung, whose number was increased after bleomycin. In addition, a very slight expression was observed in bronchiolar epithelia after treatment. Type 2 pneumocytes and bronchiolar epithelia are involved in detoxification as well, along with phagocytes: increased SOD and GSP activity was observed in type II pneumocytes and in bronchiolar epithelia in our and previous studies (Karam et al. 1998) after bleomycin infusion. In contrast, the constitutive expression of catalase in type II alveolar epithelia of rats did not show induction after hyperoxia or inflammatory cytokines (Engstrom et al. 1990), and only moderate increase in our bleomycin model. As to G6PDH, an essential enzyme involved in the response to toxic and oxidative stress (Pandolfi et al. 1995; Ursini et al. 1997), its activity was constitutively expressed in all lung structural cells where it appeared to be moderately enhanced after bleomycin infusion.

Although comparing protein activity and expression data can be methodologically criticized, the increased expression of SOD isoforms in lung structural cells (MnSOD, Cu/ZnSOD, and ECSOD) and in interstitium (ECSOD) further indicates a global activation of antioxidant pathways.

Increased NOS/NADPH-d activity has been documented in bleomycin-treated rat lungs (Yildirim et al. 2004; Ozyurt et al. 2004; Gurujeyalakshmi et al. 2000), associated with increased expression of i-NOS and e-NOS (Gurujeyalakshmi et al. 2000; Jang et al. 2004). We assessed for the first time, to our knowledge, in situ activity of NOS after bleomycin lung injury. We documented an increase in enzyme activity in all cell populations; activity was highest in inflammatory cells, and in bronchiolar epithelia, moderate in alveolar epithelia, and very low in endothelia. Interestingly, the enzyme histochemistry of NOS/NADPH-d paralleled the changes of i-NOS expression, the most powerful responsible of NO synthesis.

Overall, it may be argued that the antioxidant response elicited by the ROS production subsequent to toxic injury, mainly sustained by antioxidant enzymes, is not effective in balancing bleomycin-induced oxidative and nitrosative stress and in preventing both tissue damage and reparative fibrosis. Similar results were also reported by Fantone and Phan (1988). Further support to the role of oxidative damage in bleomycin damage to lung cells and tissue is the protective effect exerted by antioxidants such as *N*-acetyl-L-cysteine, thioredoxin, and ambroxol in various experimental settings (Gon et al. 2000, 2001; Hong et al. 2003). However, it must be noted that the bleomycin-induced lung damage in rats eventually evolves toward subtotal repair, which makes the model unfit for comparison with human idiopathic pulmonary fibrosis as proposed by Borzone et al. (2001).

As recently reviewed by Kinnula et al. (2005), oxidant–antioxidant imbalance, as well as nitrosative stress, may be relevant in usual interstitial pneumonia (UIP), the histopathological correlate of idiopathic pulmonary fibrosis (ATS/ERS 2002). It is extremely interesting, in this perspective, the ability of reactive oxygen and NO intermediates to directly stimulate the production of

transforming growth factor β1 (TGF-β1) in alveolar epithelia (Bellocq et al. 1999) and to activate TGF-β1 by separating it from its latency-associated peptide. TGF-β1 is a well-known fibrogenic mediator involved in bleomycin lung fibrosis (Zhang et al. 1995; Azuma et al. 2005), as well as in human lung fibrosis (Broekelmann et al. 1991; Ziesche et al. 1999). TGF-β1 is at the center of a recently proposed hypothesis on the pathogenesis of UIP. It has been shown that TGF-β1 stimulation can induce epithelial–mesenchymal transition in type II alveolar epithelial cells (Willis et al. 2005), leading to the appearance of fibroblastic foci, which are the histopathological hallmark of UIP (Chilosi et al. 2003).

The results of the present study, in accordance with previous data (Fantone and Phan 1988; Karam et al. 1998), confirm that bleomycin infusion in the rat lung induces a burst of reactive oxygen and NO intermediates both in inflammatory and in alveolar epithelial cells. From our observations, it can be suggested that the observed concomitant activation of antioxidant enzymes such as catalase, G6PDH, and SOD is not adequate to scavenge the oxidant burden. The oxidative and nitrosative stress after bleomycin lung damage may be a substantial trigger in TGF-β1 overexpression by activated type II pneumocytes (Khalil et al. 1991, 1996), leading to fibrotic lesions. In UIP, a similar pattern of redox imbalance has been observed, with increased amounts of oxidants released by BAL inflammatory cells (Cantin et al. 1987; Strausz et al. 1990; Behr et al. 1995), reduction of glutathione concentration and of global antioxidant capacity in the BAL fluid (Cantin et al. 1989; Rahman et al. 1999), evidence of oxidative damage (Kuwano et al. 2003), and NOS overexpression (Lakari et al. 2002) in epithelial cells. On the other hand, increased catalase and GSP expression was observed in UIP epithelial, fibroblast, and inflammatory cells (Lakari et al. 2000). The observed similarity in oxidant–antioxidant balance and cell distribution pattern in experimental and human pulmonary fibrosis suggests that oxidative disequilibrium and subsequent oxidative stress, mediated by inflammatory but also by epithelial cells may have a leading role in the development of TGF-β1 mediated lung fibrosis (Kinnula et al. 2005). The recently reported inhibition of epithelial–mesenchymal transition (Barth et al. 2005) in bleomycin-treated cell lines and animals, however, suggests that the final pathway of fibrogenesis of bleomycin lung injury is divergent from the mechanism of the human disease.

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