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Original Research Communication

Alveolar Oxidative Stress is Associated with Elevated Levels of Nonenzymatic Low-Molecular-Weight Antioxidants in Patients with Different Forms of Chronic Fibrosing Interstitial Lung Diseases

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Abstract

Increasing evidence indicates that disequilibrium of the alveolar oxidant–antioxidant balance may play a role in the pathogenesis of chronic fibrosing lung diseases. Excessive production of oxidants and a differential regulation of antioxidant enzymes have been described under these conditions. We characterized for the first time numerous nonenzymatic low-molecular-weight antioxidants in bronchoalveolar lavage fluids from patients with different forms of lung fibrosis initiated either by injury to the alveolar epithelium (idiopathic pulmonary fibrosis, IPF) or by inflammation (chronic sarcoidosis/hypersensitivity pneumonitis). Footprints of oxidative stress accompanied by an increase in the majority of antioxidants assessed were observed in all patient groups: elevated levels of uric acid, ascorbic acid, retinol, and α-tocopherol were noted, whereas glutathione levels were unchanged. The expression of Nrf2, an important redox-sensitive transcriptional regulator of antioxidants, was increased in IPF lungs. Our findings were corroborated in the bleomycin model of lung fibrosis where—aside from uric acid—nonenzymatic antioxidants were elevated during the fibrotic phase. In conclusion, alveolar levels of nonenzymatic antioxidants are elevated in fibrosing lung diseases, but are incapable of restoring oxidative balance. This increase may be part of an adaptive response to oxidative stress. However, a leakage from the blood may also contribute to our findings. *Antioxid. Redox Signal.* 11, 227–240.

Introduction

IDIOPATHIC PULMONARY FIBROSIS (IPF) represents a specific form of chronic fibrosing idiopathic interstitial pneumonia and is typically characterized by a progressive and usually fatal course, with a medium survival of 2–4 years. At present, there is no effective drug therapy for IPF (1). The etiology and the exact pathogenic mechanisms at play are still unknown. However, increasing evidence indicates that alveolar oxidative stress may play a role in the pathogenesis of IPF and may contribute to the fibrotic process by (a) inducing alveolar epithelial injury and apoptosis (9, 60); (b) promoting an imbalance in protease/antiprotease systems, thereby interfering with the regulation of extracellular matrix degradation (for example, by activating matrix metalloproteinases and inactivating their inhibitors, or by mediating growth factor-induced expression of the plasminogen activator inhibitor-1; 43); (c) increasing the release and activation of inflammatory cytokines and growth factors such as transforming growth factor (TGF)-β (25, 33); or (d) by causing impairment of the pulmonary surfactant system. Biomarkers of oxidative stress, such as exhaled 8-isoprostane and ethane, are elevated in the lungs of IPF patients, are positively correlated with a poor clinical outcome, and nega-

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tively correlated with the arterial partial pressure of oxygen (PaO_2) and with the diffusion capacity of the lung for carbon monoxide (DL_{CO}) (26, 39, 40, 41, 45, 50).

Oxidative stress is also present in the lungs of patients with other forms of chronic fibrosing interstitial lung diseases, such as hypersensitivity pneumonitis (HP) and sarcoidosis (7, 26, 41, 46). For example, 8-isoprostane levels are increased in the expired breath condensate of patients with pulmonary sarcoidosis, and are positively correlated with serum angiotensin-converting enzyme levels, a marker that may reflect disease activity (46).

Generally, alveolar oxidative stress is caused by disequilibrium of the alveolar oxidant–antioxidant balance due to an increased production of oxidants and/or depletion of antioxidants (48). Antioxidants are the primary defense against oxidant burden and include enzymatic antioxidants and nonenzymatic, low-molecular-weight antioxidant scavengers (48). In IPF, in addition to an excessive production of oxidants, low expression levels of enzymatic antioxidants, including extracellular superoxide dismutase, manganese superoxide dismutase, catalase, and hemoxygenase 1, have been observed in the fibrotic areas and are thought to contribute to increased oxidative stress under these conditions as well. Of note, in the granulomas of sarcoidosis and HP patients, expression of antioxidant enzymes was found to be increased (15, 29, 34–36).

Much less is known about a potential role of nonenzymatic antioxidants in chronic fibrosing lung diseases. Glutathione has received the most attention up until now: a deficiency in glutathione has been demonstrated in the epithelial lining fluid of IPF patients and in patients with acute episodes of farmer's lung, which is the most common form of HP (2, 3, 6, 8, 37, 38). However, no data are currently available regarding the concentrations of other nonenzymatic low-molecular-weight antioxidants in chronic fibrosing interstitial lung diseases, which is, at least partially, due to the overall low levels of these molecules in the bronchoalveolar lavage (BAL) fluids, thus necessitating the use of highly sensitive analytical procedures.

In the present study, we hypothesized that nonenzymatic low-molecular-weight antioxidants may be differentially expressed in chronic fibrosing lung diseases and may in this way contribute to the disequilibrium of the alveolar oxidant–antioxidant balance as well. We also speculated that—similarly to the differences observed in enzymatic antioxidants—differences in the expression of nonenzymatic antioxidants may exist between different forms of these disorders, initiated either by injury to the alveolar epithelium (idiopathic pulmonary fibrosis, IPF) or by inflammation in the lung parenchyma (chronic sarcoidosis, chronic HP). Thus, we quantified for the first time numerous nonenzymatic low-molecular-weight antioxidants [reduced glutathione (GSH), uric acid, ascorbic acid (vitamin C), retinol (vitamin A), and α-tocopherol (vitamin E)] in BAL fluids from patients with lung fibrosis due to chronic sarcoidosis, chronic HP, or IPF. Additionally, we determined BAL fluid plasmalogen levels. Plasmalogens, the alk-1-enyl-acyl subclass of phosphatidylethanolamine and phosphatidylcholine, may act as potent antioxidants due to their vinyl ether group and are closely connected to some nonenzymatic low-molecular-weight antioxidants, particularly to the vitamins A and E, with which they are secreted by the alveolar type II cells as an integral component of the pulmonary surfactant system (52). In order to corroborate our findings, BAL fluid levels of nonenzymatic antioxidants were also assessed in the bleomycin model of lung fibrosis, in which, similarly to the patients, increased alveolar oxidative stress is thought to contribute to the fibrotic process (20).

Methods

Patient groups

The study was conducted in conformity with the principles of the Declaration of Helsinki. All investigations were approved by the local ethics committee, and written informed consent was obtained from all patients. Consecutive patients who presented themselves at the outpatient clinic for lung fibrosis of the Department of Internal Medicine, Justus Liebig University Giessen, Germany, and who fulfilled the diagnostic criteria as detailed below were enrolled. All patients were recruited between 2005 and 2007. All patients underwent bronchoscopy at the initial presentation for the purpose of ascertaining the diagnosis. In total, 16 IPF patients, 12 patients with chronic sarcoidosis, and 7 patients with chronic HP were included in the study. Diagnosis of IPF was based on the American Thoracic Society (ATS)/European Respiratory Society (ERS) consensus criteria including proof of bibasilar reticular abnormalities with minimal ground glass opacities on high-resolution computed tomography (HRCT) scans and exclusion of other known causes of interstitial lung diseases (1). In 8 patients, diagnosis was confirmed by open lung biopsy, demonstrating a usual interstitial pneumonitis pattern in each case. Diagnostic criteria of sarcoidosis are based on the joint statement of the ATS/ERS and the World Association of Sarcoidosis and other Granulomatous Disorders (WASOG; 57). All sarcoidosis patients included in the study were at least in the early fibrotic stage of the disease with a permanent impairment of pulmonary function tests and at least initial signs of lung fibrosis on HRCT scans (irregular reticular opacities, including irregular septal thickening, and/or posterior displacement of the main and upper lobe bronchi). In 8 out of 12 sarcoidosis patients, fibrous masses associated with traction bronchiectasis were even observed. Honeycombing was seen in 3 patients. However, all sarcoidosis patients also had evidence of disease activity indicated by increased serum angiotensin-converting enzyme levels (8 out of 12 patients), BAL fluid lymphocytosis, and increased CD4/CD8 ratio (all patients), and/or ground-glass opacity on HRCT scans (10 out of 12 patients). Diagnosis of HP was settled on formalized diagnostic criteria proposed by the American Academy of Allergy, Asthma, and Immunology, including evidence of exposure to appropriate antigen by history, investigation of the environment, serum precipitin test, and/or BAL fluid antibody; BAL fluid lymphocytosis; and positive "natural challenge", or by controlled inhalational challenge (44, 54). All HP patients were in the chronic stage of the disease with permanent impairment of lung function and presence of fibrosis on HRCT scans (irregular reticular opacities (all patients), traction bronchiectasis (5 out of 7 patients), and/or honeycombing (3 out of 7 patients)). Areas of ground-glass opacity and small nodular opacities indicative of active disease were observed in 6 and 5 out of 7 patients, respectively. Patients on steroids or on other immunosuppressive drugs such

as azathioprine and cyclophosphamide were excluded from the study. Table 1 documents the demographic characteristics, results of pulmonary function tests, as well as BAL fluid recovery, cell differential, lactate dehydrogenase (LDH) levels, and protein and phospholipid concentrations of the patient cohorts.

Twenty healthy volunteers without any history of cardiac or lung disease, with normal pulmonary function tests, and with no clinical signs of a pulmonary affection such as cough or dyspnea (medical staff and students at the Medical School of the Justus Liebig University, Giessen, Germany) served as controls ("healthy"). Since the control group of healthy volunteers is significantly younger and has an increased representation of females compared with the patient groups, we included a second age- and sex-matched control group consisting of 7 patients who underwent bronchoscopy either for evaluation of a pulmonary nodule ($n = 5$), or for dyspnea which was subsequently attributed to vocal cord dysfunction ($n = 2$; "controls"). Lung fibrosis was excluded by HRCT scans, and normal pulmonary function tests were noted in each patient of this control group. Additionally, for the analysis of Nrf2 expression, shock-frozen lung tissue was obtained from 15 IPF patients who underwent lung transplantation at the Department of Cardiothoracic Surgery of the Medical University of Vienna, Austria (Director: Prof. W. Klepetko). IPF diagnosis was based on both clinical criteria as well as proof of a usual interstitial pneumonitis pattern in histopathological specimens from the explanted lungs. Nonutilized donor lungs or parts of donor lungs that were not implanted due to lack of compatibility (for instance, oversized grafts) served as control ($n = 10$).

Animal studies

Animal experimentation was performed according to the Helsinki convention for the use and care of animals, and was approved by the local Committee on Animal Investigations.

Bleomycin (Almirall Prodesfarma, Barcelona, Spain) was administered as an aerosol to healthy White New Zealand rabbits (5–7 months of age), as recently described (17, 53). Briefly, rabbits of either sex were anesthetized with ketamine and xylazine, intratracheally intubated, and mechanically ventilated in a volume-controlled mode. Using an ultrasonic nebulization device (Portasonic I, Devilbis, Langen, Germany), 1.8 U bleomycin/kg b.w. were administered via the inhalational route. The rabbits were then allowed to recover from anesthesia and received food and water *ad libidum*. Animals were sacrificed at day 4 ($n = 7$), day 8 ($n = 7$), day 16 ($n = 6$), day 24 ($n = 6$), day 32 ($n = 7$), or day 64 ($n = 6$) after bleomycin challenge. As detailed previously, this model is characterized by an acute inflammatory period (until day 16), followed by lung fibrosis that is first evident at day 16 post challenge, and that is most pronounced between days 24 and 32 after bleomycin application (53). Age and sex-matched controls received 0.9 % sodium chloride ($n = 9$). Lung compliance was measured as recently described (17, 53). Briefly, following local anesthesia, a tracheotomy was performed and mechanical ventilation was started in a volume-controlled mode with tidal volumes of 6.5 ml/kg b.w. (30X/min). Upon ventilation of the animals at a FiO_2 of 1.0 for 5 min and placement of an esophageal pressure transducer, three pressure volume curves were obtained for each animal by inflating the lung up to 25 cm H_2O with subsequent deflation. Ventilation pressure, and intrathoracic pressure and volume were recorded continuously and were averaged. The slope of the linear part of the deflation limb, indicating the static lung compliance after correction for the intrathoracic pressure, was used for calculation of compliance referenced to the animal weight.

Bronchoalveolar lavage procedure

The BAL fluids were obtained from patients and controls by flexible fiberoptic bronchoscopy in a standardized manner according to the guidelines for measurement of acellular components and recommendations for standardization of BAL of the ERS Task Force Report (19). Briefly, one segment of the right middle lobe was lavaged with a total volume of 140 mL sterile saline in seven equal aliquots with a fluid recovery of ~70% (healthy volunteers: 70.7 ± 2.2%; controls: 69.0 ± 4.0%; IPF: 68.6 ± 3.3%; sarcoidosis: 69.6 ± 3.5%; HP: 71.1 ± 4.2%). The BAL fluids were obtained from bleomycin-challenged or saline-treated rabbits, as previously described (17, 53). Briefly, at the indicated time-points, a tracheotomy was performed and a ventilation tube was placed into the trachea. The lungs were lavaged with a total volume of 150 mL sterile saline in three equal aliquots. All BAL fluid fractions were pooled, filtered through sterile gauze, and centrifuged at 300 *g* (10 min, 4°C) to remove cells and membranous debris. Staining and counting of cells within the BAL fluid cell pellet were performed by routine methods (May–Gruenwald–Giemsa staining). Aliquots of rabbit BAL fluids were analyzed for total soluble collagen by reaction of Sirius Red with collagen, utilizing a commercially available test kit (Sircol Collagen Assay, Biocolor, Belfast, Ireland). BAL fluid total protein content was measured with a commercially available assay using bicinchoninic acid (BCA assay, Pierce, Rockford, Il). The activity of free LDH in BAL fluids was measured by a commercially available colorimetric assay using the ADVIA 1800 system (Bayer, Leverkusen, Germany).

Bronchoalveolar lavage fluid lipid analysis

Lipids were extracted with chloroform/methanol, according to Bligh and Dyer (4), and the phospholipid content was determined by spectrophotometric measurement of phosphorus (51). Plasmalogens were determined using gas chromatography, as previously described (14). The ratio ([weight of dimethylacetals (DMA)/weight of fatty acid methyl esters (FAME)]X2) of the detected DMA to FAME indicated the relative amount of plasmalogens within the phospholipid fraction.

Oxidant/antioxidant analysis

For antioxidant analyses, BAL fluid samples were stabilized with metaphosphoric acid, and for analyses of oxidative stress markers, BAL fluid samples were supplemented with butylated hydroxytoluene. For each parameter assessed, the intra-assay and inter-assay reproducibility (coefficient of variation; CV) were determined. For this, BAL fluids were spiked with increasing concentrations (at least three different concentrations) of the respective substance, and samples were analyzed in quintuplicate for 3 consecutive

days. Since different concentrations were assessed, a CV range is reported for each parameter.

In detail, the following assays were applied:

Glutathione. Total glutathione (GSX) and oxidized glutathione (GSSG) were determined by the enzymatic recycling assay originally described by Tietze (58), adapted for microtiter plates. The GSSG was measured after masking GSH with *N*-ethylmaleimide (NEM) and separation of GSH-NEM using solid-phase extraction. The concentration of GSH was calculated according to the equation GSH = GSX − 2 × GSSG. The detection limit for this assay was 0.005 μM. The intra-assay and inter-assay reproducibility (CV) ranged from 1.7% to 4.8%, and from 2.5% to 6.0%, respectively ($n = 5$ and $n = 3$, respectively).

Ascorbic and uric acids. Ascorbic acid (vitamin C) and uric acid were determined by high pressure liquid chromatography (HPLC), according to the method of Iriyama *et al*. (22). Detection was accomplished using a HP 1049A electrochemical detector (Agilent, Waldbronn, Germany), equipped with a Ag/AgCl working electrode and set at 800 mV. The detection limits for ascorbic and uric acid were 0.029 and 0.03 μM, respectively. The intra-assay and inter-assay reproducibility (CV) for ascorbic acid ranged from to 1.8% to 4.5% and from 2.3% to 6.3%, respectively ($n = 5$ and $n = 3$, respectively). The intra-assay and inter-assay reproducibility (CV) for uric acid ranged from 2.5% to 4.7% and from 2.6% to 6.8%, respectively ($n = 5$ and $n = 3$, respectively).

Retinol and α-tocopherol. The hydrophobic vitamins retinol (vitamin A) and α-tocopherol (vitamin E) were simultaneously analyzed by HPLC and diode array detection, according to the method of Catignani and Bieri (10). Retinol and α-tocopherol were simultaneously detected at 324 and 292 nm, respectively. The purity of the constituents eluting within the peak of interest was verified using the corresponding ultraviolet spectrum. Analytes were quantified by means of standard curves for each vitamin, after correction for internal standard (retinol acetate) variability. The detection limits for retinol and α-tocopherol were 0.87 and 5.8 n*M*, respectively. The intra-assay and inter-assay reproducibility (CV) for retinol ranged from 2.0% to 4.2% and from 1.9% to 5.0%, respectively ($n = 5$ and $n = 3$, respectively). The intra-assay and inter-assay reproducibility (CV) for α-tocopherol ranged from 1.4% to 3.9% and from 2.0% to 4.6%, respectively ($n = 5$ and $n = 3$, respectively).

F2-Isoprostanes. The F2-isoprostanes were quantified by means of an EIA (Cayman Chemical, Ann Arbor, MI). Briefly, samples (500 μL BAL fluid) were purified and concentrated by solid-phase extraction (3 mL Chromabond® C18 ec cartridges; Macherey Nagel, Dueren, Germany). The F2-isoprostanes were eluted and subjected to EIA, according to the manufacturer's instructions. The detection limit for this assay was 8.5 pM. The intra-assay and inter-assay reproducibility (CV) ranged from 2.8% to 5.4%, and from to 3.0% to 6.1%, respectively ($n = 5$ and $n = 3$, respectively).

Thiobarbituric reactive substances (TBARS). Levels of thiobarbituric reactive substances (TBARS) were determined by HPLC, according to the method of Draper and Hadley (13). The detection limit for this assay was 6.9 n*M*. The intra-assay and inter-assay reproducibility (CV) ranged from 2.9% to 4.9% and from 3.4% to 6.7%, respectively ($n = 5$ and $n = 3$, respectively).

Preparation of lung homogenate and Western blot analysis for the detection of Nrf2

Peripheral lung tissue samples were pulverized in liquid nitrogen by using mortar and pestle and then incubated on ice in lysis buffer [50 m*M* tris-HCl [pH 7.5], 150 m*M* NaCl, 1% (wt/vol) triton X-100, 0.5% (wt/vol) Na-deoxycholate, 5 m*M* EDTA, and 2 m*M* phenylmethylsulphonyl fluoride (PMSF)] for 1 h. Cell debris was removed from crude extracts by centrifugation at 10,000 *g* for 10 min. Equal amounts of protein (50 μg/lane) of the lysates were separated on a 10% SDS polyacrylamide gel under reducing conditions, followed by electrotransfer to a polyvinylidene fluoride (PVDF) membrane. After blocking, the membranes were probed with an anti-human Nrf2 antibody (1:1,000, rabbit polyclonal) or with an anti-human β-actin antibody (1:3,000, mouse monoclonal; both from Abcam, Cambridge, UK). Afterwards, blots were washed and incubated with respective horseradish peroxidase-conjugated secondary antibodies (DakoCytomation, Hamburg, Germany; anti-mouse-IgG, anti-rabbit IgG, 1:2,000 diluted in blocking buffer). Final detection of proteins was performed using the ECL Plus chemiluminescent detection system (Amersham Biosciences, Freiburg, Germany), and band intensity of exposed film was analyzed by densitometric scanning and quantified using AlphaEase®FC Imaging System (San Leandro, CA).

Immunohistochemistry for Nrf2

Immunohistochemistry for the detection of Nrf2 in formalin-fixed paraffin-embedded lung tissue sections (3 μm) was performed with a commercially available system based on the streptavidin–biotin method, according to the manufacturer's instructions (ZytoChem-Plus AP Kit, Broad Spectrum; Zytomed Systems, Berlin, Germany). Briefly, lung sections were incubated with SuperBlock-solution for 5 min, followed by overnight incubation at 4°C with an anti-human Nrf2 antibody (1:50, rabbit polyclonal, Abcam). For safe identification of alveolar epithelial type II cells, immunohistochemical staining was performed on serial sections using an antibody directed against human pro-surfactant protein C (1:750, rabbit polyclonal, Chemicon International, Inc., Temecula, CA). A polyvalent secondary biotinylated antibody was applied (15 min), followed by incubation with alkaline phosphatase-conjugated streptavidin (15 min). Sections were then developed with Fast Red substrate solution. The sections were counterstained with hemalaun and mounted in Glycergel (DakoCytomation). Controls were performed by substituting the primary with the respective isotype control antibodies (normal rabbit IgG, from Acris Antibodies, Hiddenhausen, Germany). Lung tissue sections were scanned with a Mirax Desk Digital Slide scanning device (Zeiss, Goettingen, Germany) and analyzed at 100X, 200X, and 400X original magnification. All images illustrated are representative of at least six other areas per section, seen on at least three independent sections per patient and control, respectively. Fifteen IPF patients and 10 controls were examined.

Statistics

Data are presented as mean and standard error of the mean (SEM). Differences between the patient and control groups were tested by analysis of variance (ANOVA), differences between bleomycin-treated animals and control rabbits were tested with Student's *t* test. Relationships between variables were analyzed by means of Pearson correlations. A Bonferroni adjustment for multiple testing was performed. The level of statistical significance was set at 5%.

Results

Following the guidelines of the ERS Task Force Report (19), all measurements are reported per mL or per L of BAL fluid recovered. The most abundant antioxidant molecule in BAL fluids from both control groups was GSH, followed by uric acid and ascorbic acid. The levels of GSH were not significantly altered in any patient group (Fig. 1) In contrast, uric acid levels were significantly higher in all patient groups compared with controls: a more than twofold elevation was observed in BAL fluids from IPF and sarcoidosis patients, whereas in the case of BAL fluids from HP patients, the increase was ~3.5-fold (Fig. 1). Similarly, a statistically significant increase in ascorbic acid concentrations was observed in BAL fluids from sarcoidosis (0.96 ± 0.19 μM) and IPF patients (0.87 ± 0.14 μM *vs.* 0.54 ± 0.06 and 0.48 ± 0.04 μM, respectively, in controls), while ascorbic acid levels in HP patients were basically unaltered. Due to the lack of material, the lipophilic antioxidants α-tocopherol (vitamin E) and retinol (vitamin A) were not determined in BAL fluids from HP patients. In IPF, as well as in sarcoidosis patients, BAL fluid concentrations of both lipophilic antioxidants were strongly increased compared with controls (α-tocopherol: IPF: 53.68 ± 7.02 nM; sarcoidosis: 47.45 ± 10.51 nM; controls: 26.95 ± 3.21 and 25.43 ± 4.17 nM, respectively; retinol: IPF: 11.58 ± 2.83 nM; sarcoidosis: 9.52 ± 2.16 nM; controls: 3.97 ± 0.66 and 4.46 ± 0.61 nM, respectively; Fig. 1). In contrast, plasmalogen concentrations did not significantly differ between the patients' BAL fluids and the controls, although a slight tendency towards a decrease was noted in IPF and HP patients. No statistically significant correlation was found between the levels of antioxidants examined and pulmonary function tests (forced vital capacity) or gas exchange abnormalities (DLCo, PaO_2; data not shown). In all patient groups, oxidative stress was noted as evident by increased BAL fluid concentrations of GSSG and F2-isoprostanes as products of lipid peroxidation (Fig. 1).

In line with the guidelines of the ERS Task Force Report (19), all parameters were also calculated in relation to the total phospholipid concentration to compensate for dilution variability during the BAL procedure (Fig. 2). However, the changes in antioxidant levels and oxidative stress markers were very similar, independent of whether absolute or relative concentrations were employed in the analyses. To further explore the potential mechanisms that may underlie the observed increase in antioxidant levels, we determined the activity of free LDH and the total protein content in the patients' BAL fluids as indicators of cell damage/lysis and permeability of the capillary alveolar barrier, respectively, and we analyzed the expression of Nrf2, which is an important transcriptional regulator of antioxidants (11, 24, 61), in lung tissue from IPF patients. The LDH activity in the BAL fluids did not significantly differ between the patient groups and the controls (Table 1). Although six IPF patients as well as three and four patients with sarcoidosis and HP, respectively, showed elevated LDH activity, there was no correlation between the LDH activity and the levels of antioxidants (data not shown). The BAL fluid total protein content was slightly, albeit significantly, increased in all patient groups (Table 1), however, there was no statistically significant correlation between any of the antioxidants assessed and the BAL fluid protein content (data not shown). Nrf2 protein expression was found to be more than fivefold increased in IPF lungs compared with controls, and this increased expression was localised to alveolar epithelial type II cells (Fig. 3).

Afterwards, antioxidant levels and oxidative stress markers were determined in the BAL fluids of bleomycin-challenged rabbits. In this model, the early acute inflammatory phase of the disease is followed by a fibrotic phase starting with day 16 post bleomycin challenge and evidenced by significantly increased BAL fluid soluble collagen levels and severely impaired lung compliance (Fig. 4). The alterations in antioxidant concentrations detected in this model closely resembled those observed in patients: the BAL fluid GSH concentration was decreased in the early phase, but was unaltered in the late fibrotic phase compared with controls (Fig. 5). Increased ascorbic acid concentrations were observed over the entire observation period, however, the highest concentrations were found in the late fibrotic phase (day 24: 6.59 ± 0.59 μM; day 32: 5.71 ± 0.61 μM; day 64: 12.36 ± 4.18 *vs.* 2.34 ± 0.59 μM in controls). Similarly, the lipophilic antioxidant α-tocopherol exhibited persistent elevation during the 64-day observation period, where the highest levels were observed in the late fibrotic phase, with a more than fivefold increase at day 32 (554.71 ± 111.01 *vs.* 101.1 ± 17.01 nM in controls). The concentration of the second lipophilic antioxidant, retinol, was significantly increased during the early inflammatory and the late fibrotic phases with peak levels observed on day 16 (48.77 ± 10.09 *vs.* 6.05 ± 4.8 nM in controls; Fig. 5). In contrast to the patients, uric acid levels were unaltered in the late fibrotic phase of bleomycin-treated rabbits compared with controls, and plasmalogen levels were significantly increased (day 32: 14.98 ± 2.28 *vs.* 7.23 ± 0.55 μg/ml in controls). Oxidative stress was noted in the late fibrotic phase of bleomycin-injured rabbit lungs as evident by increased BAL fluid concentrations of GSSG and thiobarbituric reactive substances. In contrast to the findings in sarcoidosis and HP patients, F2 isoprostanes were not elevated in the animals during the fibrotic phase of the disease (Fig. 5).

Discussion

In the present study, we investigated for the first time whether nonenzymatic antioxidants are differentially expressed in chronic fibrosing lung diseases and may, in this way, contribute to the disequilibrium of the alveolar oxidant–antioxidant balance. Keeping in mind the evidence of increased oxidative stress in these conditions, we expected the alveolar levels of nonenzymatic antioxidants to be decreased rather than increased. However, elevated levels of the majority of the antioxidants analyzed were noted, and this observation was independent of whether patients with lung fibrosis due to chronic sarcoidosis, chronic HP, or IPF

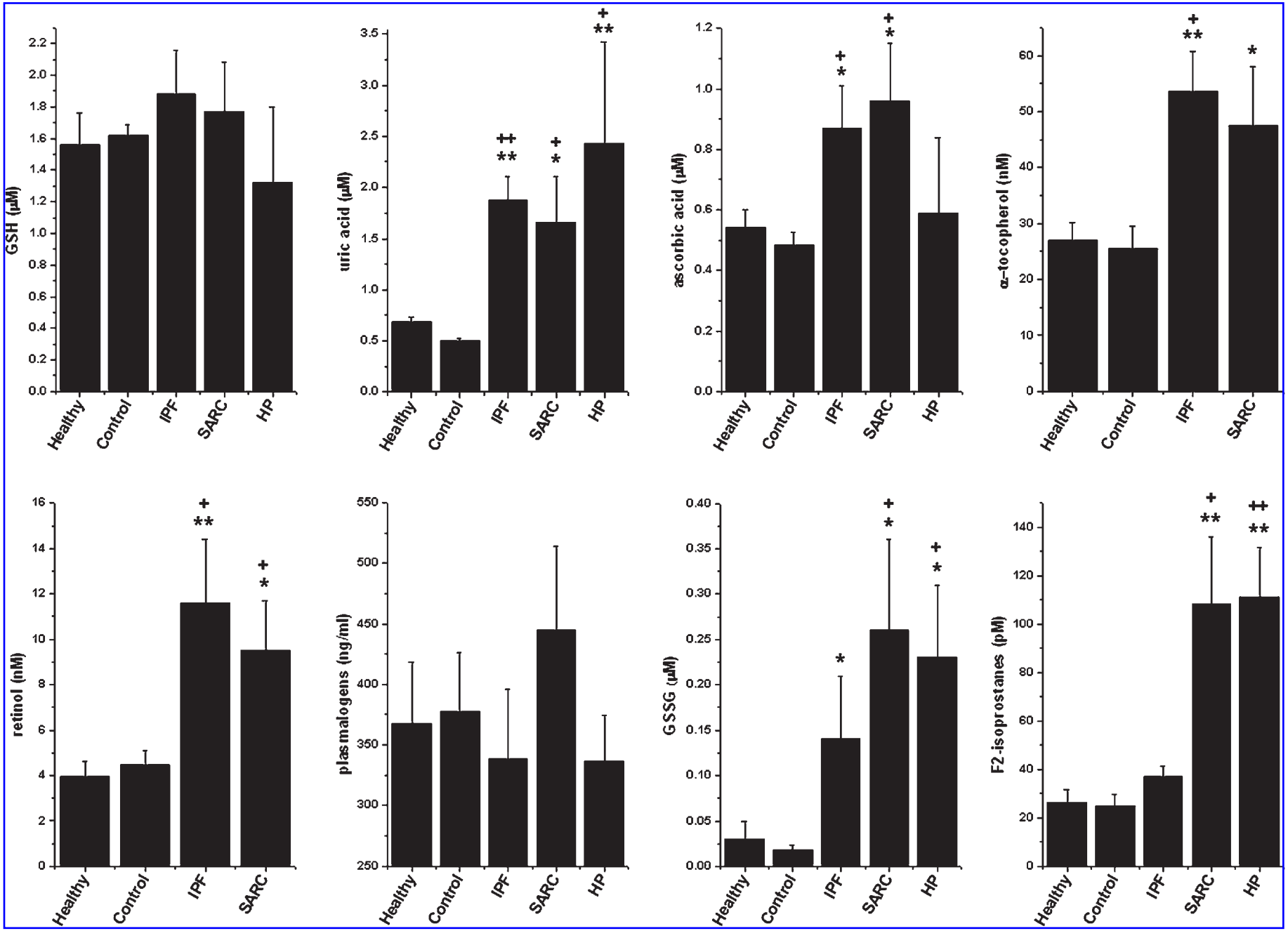

FIG. 1. Absolute concentrations of antioxidants and oxidative stress markers in BAL fluids from patients with IPF, sarcoidosis, and hypersensitivity pneumonitis. Data are presented as mean ± standard error of the mean (SEM). Significance levels are indicated (*$p < 0.05$, **$p < 0.01$, all *versus* healthy volunteers ("healthy"); $^{+}p < 0.05$, $^{++}p < 0.01$, all *versus* control). BAL, bronchoalveolar lavage; GSH, reduced glutathione; GSSG, oxidized glutathione; HP, hypersensitivity pneumonitis ($n = 7$); IPF, idiopathic pulmonary fibrosis ($n = 16$); SARC, sarcoidosis ($n = 12$).

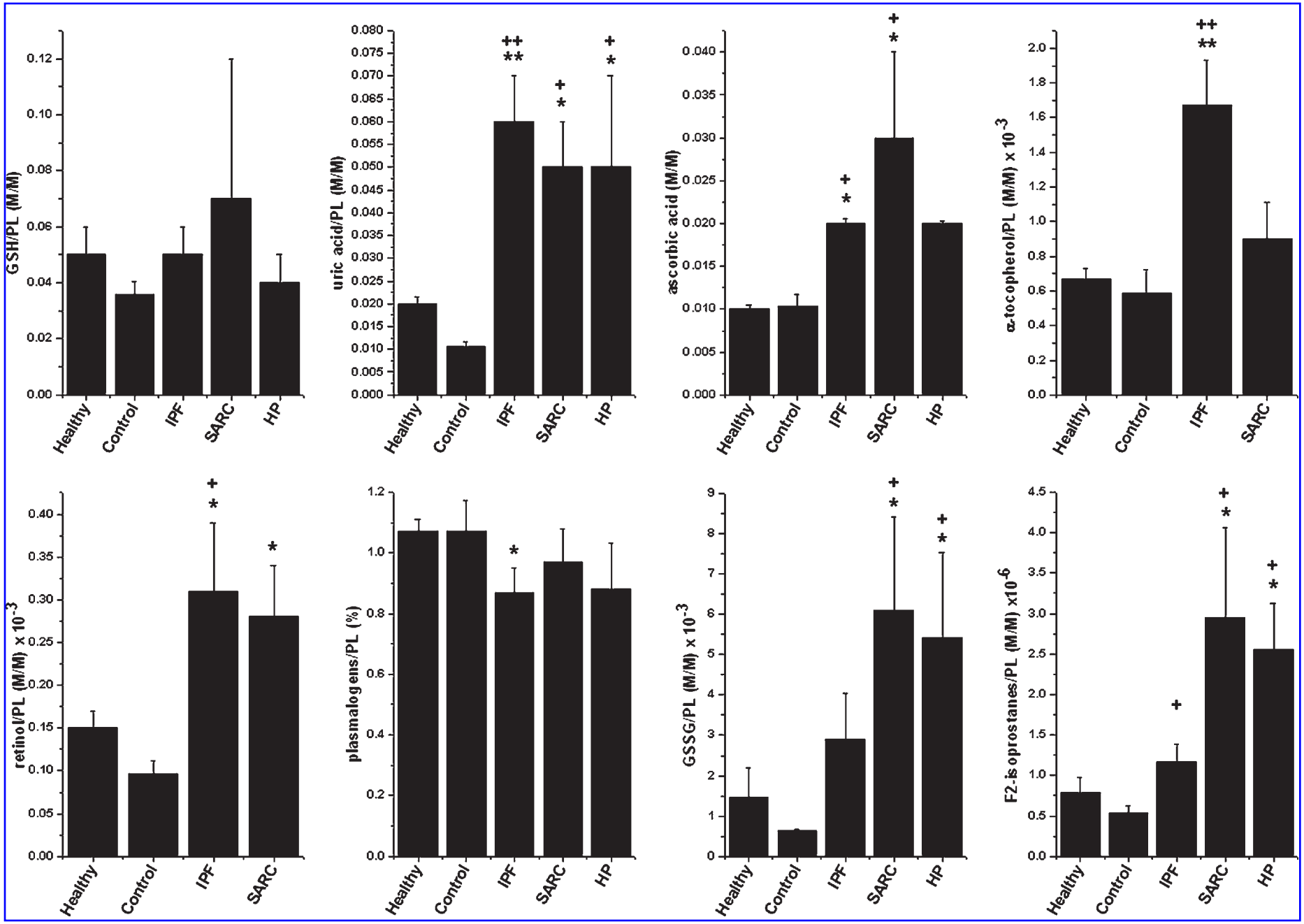

FIG. 2. Relative concentrations of antioxidants and oxidative stress markers in BAL fluids from patients with IPF, sarcoidosis, and hypersensitivity pneumonitis. Parameters are given in relation to the total phospholipid concentration. Data are presented as mean ± standard error of the mean (SEM). Significance levels are indicated (*$p < 0.05$, **$p < 0.01$, all *versus* healthy volunteers ("healthy"); $^{+}p < 0.05$, $^{++}p < 0.01$, all *versus* control). BAL, bronchoalveolar lavage; GSH, reduced glutathione; GSSG, oxidized glutathione; HP, hypersensitivity pneumonitis ($n = 7$); IPF, idiopathic pulmonary fibrosis ($n = 16$); PL, phospholipids; SARC, sarcoidosis ($n = 12$).

Table 1. Demographic Characteristics, Results of Pulmonary Function Tests, and BAL Fluid Analysis of the Patient Cohorts

	Healthy	*Controls*	*IPF*	*Sarcoidosis*	*HP*
Patients (*n*)	20	7	16	12	7
Age (years)	29.3 ± 1.9	57.4 ± 3.6	59.3 ± 2.3	40.6 ± 3.3	54.9 ± 6.3
Sex, female/male (*n*)	10/10	2/5	5/11	3/9	3/4
FVC (% predicted)	101.5 ± 2.1	98.8 ± 2.9	62.3 ± 12.2	77.5 ± 9.2	68.5 ± 7.9
DL_{Co} (% predicted)	98.9 ± 3.5	95.7 ± 3.8	38.9 ± 10.8	52.6 ± 7.9	47.3 ± 8.6
BAL fluid recovery (%)	70.7 ± 2.20	69.0 ± 4.0	68.6 ± 3.3	69.6 ± 3.5	71.1 ± 4.2
BAL fluid cell differential					
Macrophages (%)	93.0 ± 0.8	91.9 ± 1.2	73.2 ± 6.1	73.4 ± 8.1	57.4 ± 7.5
Neutrophils (%)	1.8 ± 0.6	3.3 ± 0.8	16.5 ± 4.3	4.7 ± 2.6	11.3 ± 4.1
Lymphocytes (%)	5.0 ± 0.6	4.0 ± 1.0	6.8 ± 3.2	21.3 ± 5.5	29.7 ± 8.0
Eosinophils (%)	0.2 ± 0.2	0.8 ± 0.4	3.5 ± 1.3	0.6 ± 0.4	1.6 ± 0.5
BAL fluid phospholipid content (μg/ml)	32.1 ± 3.4	35.1 ± 2.6	33.6 ± 4.3	37.3 ± 7.5	35.5 ± 3.3
BAL fluid protein (mg/ml)	0.07 ± 0.01	0.08 ± 0.00	0.19 ± 0.02*‡	0.25 ± 0.06†§	0.21 ± 0.05*‡
BAL fluid LDH levels (U/l)	6.7 ± 1.2	6.3 ± 1.4	9.8 ± 2.3	8.0 ± 1.5	11.5 ± 2.8

Data are presented as *n* or mean ± standard error of the mean (SEM).

BAL, bronchoalveolar lavage; DL_{Co}, diffusion capacity of the lung for carbon monoxide; FVC, forced vital capacity; HP, hypersensitivity pneumonitis; IPF, idiopathic pulmonary fibrosis.

Significance levels are indicated (*$p < 0.05$, †$p < 0.01$, all *versus* healthy volunteers ("healthy"); ‡$p < 0.05$, §$p < 0.01$, all *versus* control).

were analyzed. This was not necessarily predictable, since differences clearly exist between the diseases investigated: in sarcoidosis and HP, the fibrotic response is initiated by pronounced inflammation in the lung parenchyma, whereas IPF is essentially a disorder of alveolar epithelial injury with limited inflammation (1, 44, 54, 57). In previous studies, it was demonstrated that few enzymatic antioxidants, such as hemoxygenase 1, are predominantly expressed in interstitial lung diseases associated with significant inflammation, but not in end-stage fibrosis as in the case in IPF (15, 29, 34–36). However, as shown in the current study, increases in the levels of nonenzymatic low-molecular-weight antioxidants in the alveolus appear to be independent of the driving forces of the fibrotic process and may reflect a rather uniform response pattern to chronic lung injury associated with oxidant burden. All parameters assessed in the patients were compared with those in 20 healthy volunteers, whose lavages were obtained exclusively for the purpose of serving as controls for clinical studies. However, the healthy volunteers are significantly younger and have an increased representation of females compared with the patients, which may have influenced our results. Thus, we included a second age- and sex-matched control group consisting of seven patients who underwent bronchoscopy either for evaluation of a pulmonary nodule or for dyspnea which was subsequently attributed to vocal cord dysfunction. However, the results obtained with this second control group were very similar compared with the healthy volunteers. Additionally, we did not find a statistically significant correlation between any of the antioxidant parameters assessed and age, and no difference between male and female BAL parameters was noted (not shown). Moreover, it has to be noted that none of the patients were on steroid treatment while the BAL was obtained; steroids represent another variable that may affect the oxidant/antioxidant balance and, thus, could have influenced our results.

To corroborate our findings, antioxidant levels were also determined in bleomycin-challenged rabbits. This model has several limitations, in particular, with regard to the comparisons drawn with IPF. Amongst other reasons, the use of the bleomycin model is complicated by the fact that it is primarily driven by inflammation leading to early fibrosis, while the natural course and history of IPF is unknown, and clinically diagnosed IPF patients are usually in the late fibrotic stage with little evidence of inflammation (1, 20, 42, 53). However, the bleomycin model is the currently best-characterized and most widely used animal model of lung fibrosis and has led to the discovery of many pathological pathways, mediators, and cells that are believed to be important in IPF as well (42). In line with these considerations, our results from the bleomycin-treated rabbits are very similar compared with the patients: unaltered GSH levels, increased levels of ascorbic acid, α-tocopherol, and retinol, as well as clear demonstration of oxidative stress were noted during lung fibrosis in this model. Of note, at day 64 post bleomycin challenge, α-tocopherol levels were only slightly albeit still significantly increased, while retinol and TBARS levels were already normalized. This observation is most likely due to the partial reversibility of this model (20, 42, 53) that is also reflected by the soluble collagen levels and the lung compliance which show a tendency to normalization at this time point. The most striking differences between the patients and animals are the unaltered uric acid levels in the fibrotic phase of bleomycin-treated rabbits, whilst uric acid levels were significantly increased in all patient groups. One possible explanation for this observation is that the bleomycin model does not completely correlate with the human diseases investigated. Alternatively, species differences may contribute to the varying uric acid levels. Particularly, the fact that rabbits, in contrast to human beings, can convert uric acid to allantoin and urea may explain the relatively low uric acid levels in the animals. Unfortunately, experi-

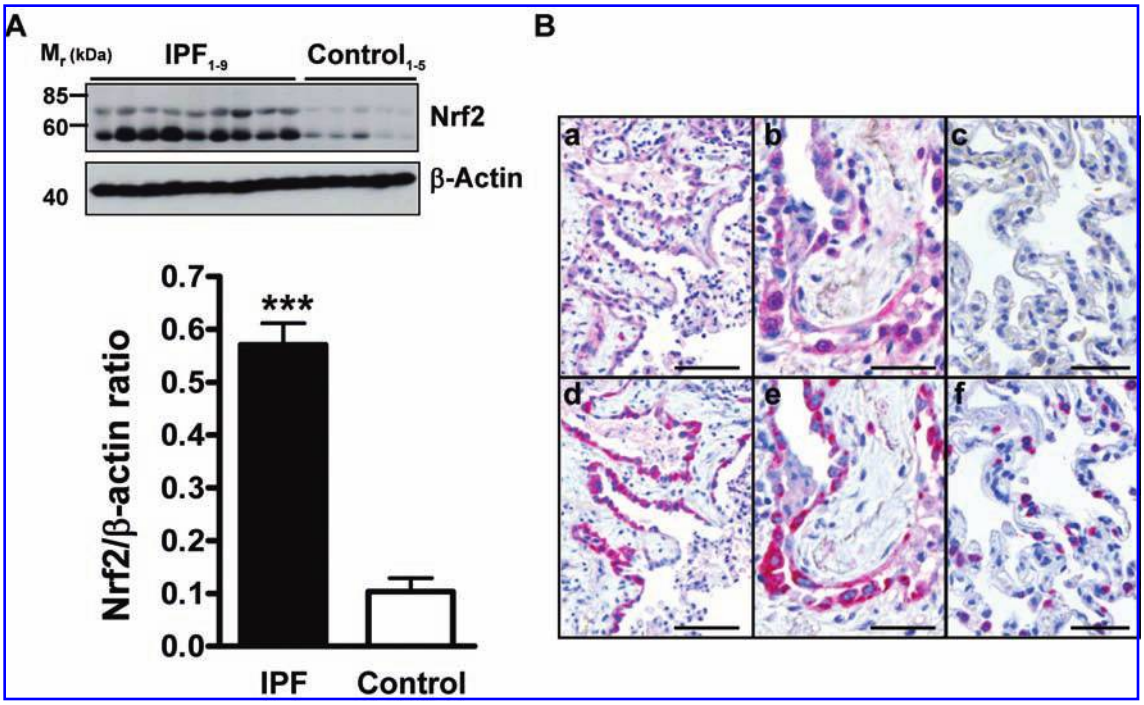

FIG. 3. Nrf2 protein expression in IPF lungs compared with controls. (**A**) Nrf2 protein expression in lung tissue from IPF patients and controls (human donor lungs) was analyzed by Western blot analysis using specific antibodies against Nrf2 and β-actin as loading control. Representative patients (9/15) and controls (5/10) are shown. Nrf2 protein appears as a 57 kDa band and a higher molecular weight band of ~70 kDa that is due to posttranslational modification (*top*). The staining intensity of the bands from all patients and controls was densitometrically quantified and normalized to β-actin (*bottom*; mean ± SEM). The significance level is indicated by $^{***}p < 0.001$ for IPF *versus* control. (**B**) Immunohistochemistry for the detection of Nrf2 in lung tissue from IPF patients (*a*, *b*) and controls (*c*). For the identification of alveolar epithelial type II cells, serial sections from IPF patients (*d*, *e*) and controls (*f*) were stained for pro-surfactant protein C. In IPF, expression of Nrf2 was localized to (hyperplastic) alveolar epithelial type II cells (*a*). Nrf2 positive alveolar epithelial type II cells were often found in close proximity to fibroblast foci (*b*). In controls, no notable staining for Nrf2 was observed (*c*). One representative IPF patient out of 15 and one control out of 10 are shown. All images illustrated are representative of at least six other areas per section, seen on at least three independent sections per patient and control, respectively. Bar = 50 μm (*b, c, e, f*) and 100 μm (*a, d*), respectively.

mental models other than the bleomycin model which may be more representative for human lung fibrosis, in particular for IPF, are currently not available, and other species commonly used for animal experiments, such as mice or rats, can, like rabbits, further convert uric acid to allantoin and urea as well. Thus, the question whether the difference in uric acid levels between the patients and the bleomycin-challenged rabbits is due to the bleomycin model used or due to differing species cannot be answered definitively at the present time. Species differences and/or the use of the bleomycin model may also underlie the observed differences in F2-isoprostane levels that were not elevated during lung fibrosis in the bleomycin model, and in plasmalogen levels that tended—in contrast to all other antioxidants investigated—towards decreased expression in IPF and HP. Possible explanations for this decrease include increased degradation in the alveolus and/or altered synthesis or release by alveolar type II cells. Interestingly, other antioxidants that are also secreted by alveolar type II cells such as vitamins A and E are elevated in the BAL fluids of IPF and HP patients, possibly indicating a differential regulation on the cellular level.

Different mechanisms may underlie the observed increase in alveolar levels of nonenzymatic antioxidants in fibrosing lung diseases. First, increased damage or lysis of cells in the patients may have contributed to our findings. However, BAL fluid LDH levels were only slightly but not significantly increased in the patients compared with controls, which makes this possibility unlikely. Second, leakage from the blood due to a disturbed integrity of the capillary alveolar barrier may lead to elevated alveolar antioxidant levels. In fact, BAL fluid total protein content as an index of permeability was increased in all patient groups compared with controls. However, this increase was relatively small compared to patients with acute inflammatory lung diseases such as pneumonia (18), and we did not find a statistically significant correlation between BAL fluid antioxidants and the protein content. Nevertheless, increased endothelial and epithelial permeability may contribute to our findings. Third, the elevation of nonenzymatic antioxidants in chronic fibrosing lung diseases may represent an adaptive attempt to compensate for the increased oxidant burden. Although not directly proven, our observation that the expression of the redox-sensitive transcription factor Nrf2, which is a key cellular sensor of oxidative stress and an important transcriptional regulator of antioxidants (11, 24, 61), is strongly increased in IPF lungs, points to the possibility that an

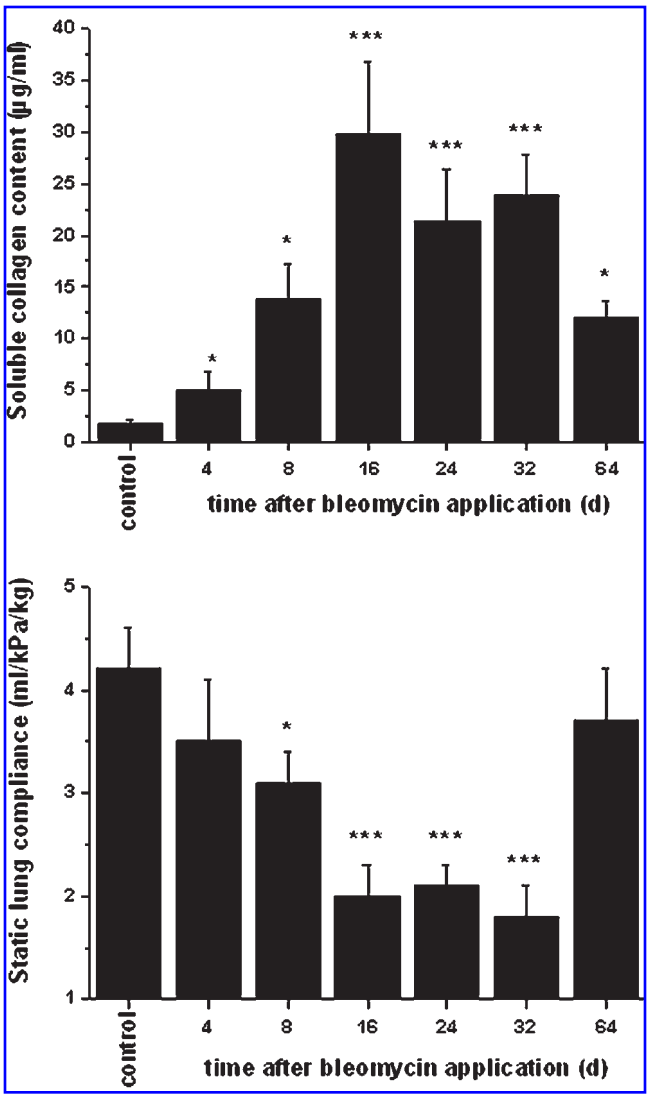

FIG. 4. BAL fluid soluble collagen content and static lung compliance in bleomycin-challenged rabbits. Data are presented as mean ± standard error of the mean (SEM). Significance levels are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, all *versus* sham-treated controls).

upregulation of the antioxidant defense system occurs in these conditions and that our findings may report part of a specific and adaptive response to oxidative stress. Of particular interest, the increased expression of Nrf2 in IPF-lungs is localized to alveolar epithelial cells whose chronic injury is thought to be a crucial pathogenetic event in IPF. An increased expression of Nrf2 has also been noted in bleomycin-injured mice lungs, and a protective role of Nrf2-antioxidant response element (ARE)-pathways in lung fibrosis is underscored by the recent observation that Nrf2-/- mice are more susceptible to bleomycin-induced lung fibrosis as compared to wild-type mice (11). In support of these considerations, different lung cells including alveolar and bronchial epithelial cells were previously shown to respond rapidly and sensitively to oxidant stress, leading to increased antioxidant expression (47, 56). Additionally, animal studies have demonstrated induction and an increase in the levels of different antioxidants (for example, uric acid, glutathione, and antioxidant enzymes) in lung tissue, bronchial epithelial cells, BAL fluid cells, and BAL fluid in response to sublethal oxidative stress, and this may result in tolerance to and protection from subsequent oxidant challenge (5, 16, 21, 23, 31, 32, 49, 59). Future studies are required to exactly clarify the relative importance of leakage from blood and specific response to oxidant stress for the observed increase in BAL fluid antioxidant levels.

To date, very few studies have investigated nonenzymatic antioxidants in chronic fibrosing lung diseases, mostly focusing on glutathione. In line with our observations, alveolar glutathione levels were recently found to be unchanged in sarcoidosis (38). Similarly, in HP unaltered glutathione levels were noted in the current study. In a previous study, BAL fluid glutathione levels were decreased in patients with farmer's lung (2). However, the patient groups are not directly comparable, since this previous study examined patients with acute episodes of the disease with normal baseline lung function, whereas in the current study patients with chronic HP and a significant impairment of pulmonary function tests were investigated. In the current study, the levels of GSH were also unaltered in IPF BAL fluids. This is in contrast to some previous studies in which a glutathione deficiency was noted in IPF. Most likely this apparent discrepancy is due to the fact that in the majority of the previous studies glutathione levels were quantified in the epithelial lining fluid using the urea method (3, 8, 37, 38). The urea method is controversial and prone to pitfalls, mainly due to rapid diffusion of urea, in particular under conditions of a leaky barrier. Thus, in the current study, all parameters were calculated per mL or L of BAL fluid recovered. Furthermore, BAL fluid introduction volume as well as percentage recoveries are quoted to demonstrate that quantitative differences are not merely a reflection of these variables. Additionally, the BAL fluid phospholipid concentration was used as a denominator to compensate for dilution variability during the BAL procedure. However, GSH concentrations calculated relative to phospholipids were also not significantly different between the patients and controls. Moreover, the changes in all other antioxidants investigated were very similar, independent of whether absolute or relative concentrations were employed in the analyses.

Despite increased concentrations of most of the antioxidants assessed, oxidative stress was noted in all patient groups and in bleomycin-injured lungs, which is in line with previous studies (7, 20, 26, 39, 40, 41, 45, 46, 50). This observation indicates that the elevation of particular low-molecular-weight antioxidants is apparently incapable of entirely counterbalancing the increased oxidant burden. The relative or absolute deficiency of alveolar antioxidants in chronic fibrosing lung diseases suggests that the application of antioxidants may be a promising therapeutic approach in these disorders. Both nonenzymatic antioxidant scavengers and enzymatic antioxidants attenuate lung fibrosis in animal models (27, 28, 30, 55). In humans, a beneficial effect on the progression of IPF has been observed by the addition of *N*-acetylcysteine to standard therapy with prednisone and azathioprine (12). Future trials will demonstrate whether other antioxidants will prove effective in the clinical treatment of patients with chronic fibrosing lung diseases as well, and may help to clarify the relative importance of single antioxidants in protecting the lung from oxidative burden.

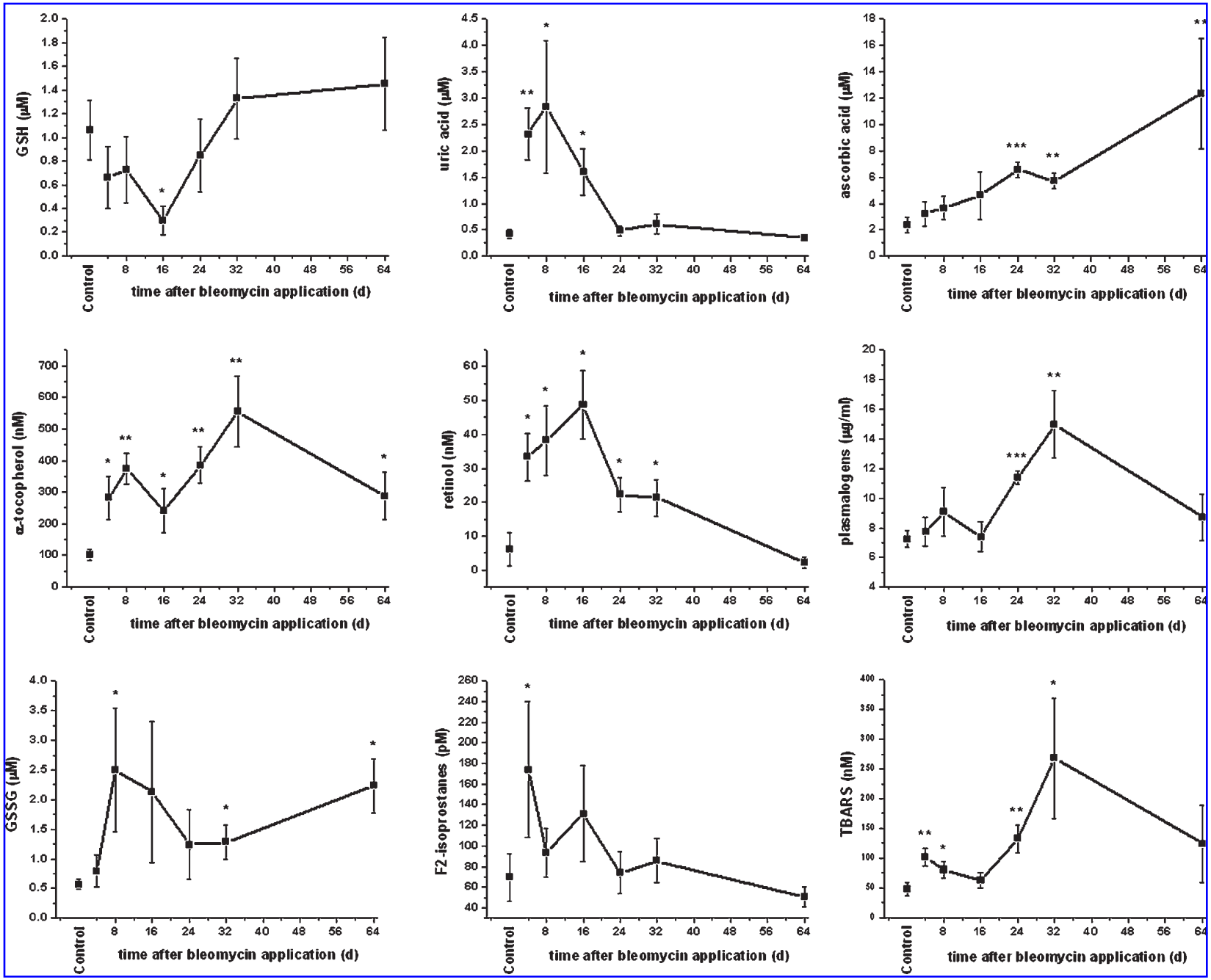

FIG. 5. Time course of antioxidant and oxidative stress marker concentrations in BAL fluids from bleomycin-injured rabbits. Data are presented as mean ± standard error of the mean (SEM). Significance levels are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, all *versus* sham-treated controls). BAL, bronchoalveolar lavage; GSH, reduced glutathione; GSSG, oxidized glutathione; TBARS, thiobarbituric reactive substances.

In conclusion, levels of nonenzymatic low-molecular-weight antioxidants are elevated in the alveolar compartment of patients with different forms of chronic fibrosing lung diseases, but are incapable of restoring oxidative balance. This increase may be part of an adaptive response to oxidative stress. The regulation and relative importance of single antioxidants in these conditions remain to be elucidated.

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Abbreviations

ARE, antioxidant response elements; ATS, American Thoracic Society; BAL, bronchoalveolar lavage; $DL_{Co,}$ diffusion capacity of the lung for carbon monoxide; DMA, dimethylacetals; ELISA, enzyme linked immunosorbent assay; ERS, European Respiratory Society; FAME, fatty acid methyl esters; GSH, reduced glutathione; GSSG, oxidized glutathione; GSX, total glutathione; HP, hypersensitivity pneumonitis; HPLC, high pressure liquid chromatography; HRCT, high-resolution computed tomography; IPF, idiopathic pulmonary fibrosis; NAC, N-acetyl cysteine; NEM, *N*-ethylmaleimide; PaO_2, arterial partial pressure of oxygen; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene fluoride; SEM, standard error of the mean; TBARS, thiobarbituric reactive substances; TGF, transforming growth factor; WASOG, World Association of Sarcoidosis and Other Granulomatous Disorders.

Disclosure Statement

No competing financial interests exist.

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