Antioxidative and Clinical Effects of High-dose *N*-Acetylcysteine in Fibrosing Alveolitis

Adjunctive Therapy to Maintenance Immunosuppression

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In fibrosing alveolitis (FA), activated phagocytes cause excessive oxidative stress in the lower respiratory tract. Additionally, levels of glutathione, a major antioxidant of the human lung, are markedly reduced. Since *N*-acetylcysteine (NAC) is a known precursor for glutathione synthesis, we investigated the effect of NAC on redox balance and lung function in FA. Eighteen patients with an established diagnosis of FA were treated with 600 mg NAC three times daily for 12 wk in addition to their latest immunosuppressive therapy. Before and after NAC therapy, pulmonary function tests (PFTs) and bronchoalveolar lavage (BAL) were performed. BAL fluid was analyzed with regard to cell differential, glutathione status, and methionine sulfoxide content of BAL proteins (Met(O)), as an indicator of oxidative stress at the alveolar surface. There was an increase of total glutathione (GSH_t = GSH + 2 × GSSG: 3.43 ± 0.30 μM versus 4.20 ± 0.66 μM, $p < 0.05$) and of reduced glutathione (GSH: 2.58 ± 0.24 μM versus 3.42 ± 0.54 μM, $p < 0.005$) in native BAL fluid and in the epithelial lining fluid (GSH_t: 267.3 ± 26.0 μM versus 367.1 ± 36.0 μM, $p < 0.005$; GSH: 204.5 ± 20.7 μM versus 302.9 ± 32.2 μM, $p < 0.005$). The increase of GSH was accompanied by a decrease of Met(O) (6.83 ± 0.71% versus 4.60 ± 0.40%, $p < 0.005$). PFTs significantly improved during NAC treatment. We conclude that high-dose NAC significantly improved the antioxidant screen of the lungs by elevating GSH levels. Moreover, the decrease of Met(O) levels indicated an antioxidant effect at the alveolar surface. These biochemical changes were accompanied by an improvement of PFTs in patients under maintenance immunosuppression. NAC supplementation should, therefore, be considered as an adjunct therapy for FA. Behr J, Maier K, Degenkolb B, Krombach F, Vogelmeier C. Antioxidative and clinical effects of high-dose *N*-acetylcysteine in fibrosing alveolitis: adjunctive therapy to maintenance immunosuppression.

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There is evidence that an imbalance between oxidants and antioxidants is involved in the pathogenesis of fibrosing alveolitis (FA) (1–6). Activated inflammatory cells accumulate in the lower respiratory tract and release increased amounts of reactive oxygen species (1–5). These may contribute to parenchymal injury and interstitial fibrosis by causing injury and cell death, modifying and disturbing the structure and function of cellular and noncellular components (7–12). A regulating factor in this oxidative pathomechanism is glutathione in its reduced form (GSH), which acts as an antioxidant (13, 14). GSH has been shown to be deficient at the alveolar epithelial surface of patients with idiopathic pulmonary fibrosis (IPF) (4, 15).

These findings indicating a pathogenetically relevant redox imbalance prompted the idea to improve the antioxidant screen of the lungs in order to avoid oxidative damage. As has been shown in paracetamol intoxication, *N*-acetylcysteine (NAC) is capable of stimulating glutathione synthesis (16, 17). NAC has also been tried in short-term pilot studies in an attempt to enhance the antioxidant screen of the lungs of patients with FA (18, 19). The results of these studies suggested that oral and intravenous application of NAC will increase pulmonary glutathione levels in the extracellular compartment of patients with pulmonary fibrosis. The discussion of these results, however, is controversial (6). It has yet to be shown that the elevation of glutathione levels observed have a measurable effect on oxidative damage. Furthermore, data indicating that the clinical course of the disease is influenced by this therapeutic intervention are lacking.

In this study, we have attempted to show antioxidative biochemical effects in the lungs and changes of lung function in patients with FA using high-dose oral NAC as a precursor for glutathione synthesis.

METHODS

Subjects

We prospectively studied a total of 20 patients (10 women, 10 men; age: 52 ± 2 yr) with FA. All patients were nonsmokers or ex-smokers

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(n = 3) who had stopped smoking at least 2 yr before entering the study.

Ten patients met the diagnostic criteria of IPF as outlined by Crystal and colleagues (20), including histologic confirmation. In 10 patients, FA in conjunction with collagen vascular disease was diagnosed following generally accepted criteria (21–23) (i.e., systemic sclerosis in seven cases and scleroderma–polymyositis overlap in three cases). All patients had significant lung function impairments (Table 1) and radiologic evidence of interstitial lung disease when enrolled in the study.

Immunosuppressive Treatment

All patients had undergone a trial period of immunosuppressive therapy for a minimum of 6 mo before entering the study. At the time of enrollment, seven patients had been off immunosuppressive drugs for at least 3 mo because of a lack of clinical benefit. The remaining 13 patients were on immunosuppressive treatment at a low maintenance dose, which had been constant for 3 mo before entering the study and which was not changed throughout the study period. All of these 13 patients received prednisolone (mean daily dose: 6.7 ± 1.8 mg). In addition, one patient received cyclophosphamide (100 mg/d) and two other patients were treated with azathioprine (50 and 150 mg/d, respectively).

Study Protocol

All patients had a set of pulmonary function tests (PFTs) within 2 to 9 mo (4 ± 1 mo) before enrollment. Another complete set of PFTs and a bronchoalveolar lavage (BAL) study were obtained immediately before starting high-dose oral NAC treatment. During the study period of 12 wk, all patients were taking 600 mg NAC three times daily. After 12 wk, another set of PFTs was obtained and a BAL study performed.

For clinical evaluation, all patients were examined by a physician (J.B. or B.D.) at the beginning of the study, after 6 wk, and after 12 wk. Symptoms and side effects were documented. Physical findings were also documented in a semiquantitative fashion.

Primary endpoints of the study were biochemical effects of high-dose NAC treatment on parameters indicative of redox balance in the BAL fluid. Secondary endpoints were changes of lung function parameters.

The study protocol was approved by the local ethical committee, and informed consent was obtained from all patients.

Biologic Samples

Serum and BAL fluid were obtained by standard techniques. Aliquots of the BAL fluid were taken for glutathione assays (*see below*), for total cell counts (Coulter counter), and for cytocentrifuge preparations for differential counts. The cells were pelleted and the supernatant was used to assess the amount of epithelial lining fluid (ELF) and the methionine sulfoxide content of proteins obtained.

Glutathione Concentration and Form

Gluthathione concentrations in serum and BAL fluid were measured using the standard techniques with only minor modifications (13, 15, 24–26). All determinations were made in triplicate, and the average value was calculated. For measurements of total glutathione (GSH_t = GSH + 2 × GSSG), immediately after BAL 100 μl of the BAL fluid supernatant (3,000 × *g* for 10 min) was mixed with 1.1 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM ethylenediamine-tetraacetic acid (EDTA), 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 63.5 μM 5.5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 4 U/ml glutathione reductase (all chemicals from Sigma Chemica Co., St. Louis, MO or Serva, Heidelberg, Germany). The rate of reduction of DTNB was recorded spectrophotometrically at a wavelengt of 412 nm. The GSH_t concentration of the BAL fluid sample was calculated using an internal standard of 0.84 μM GSH (15). GSH_t in serum was quantified accordingly.

Oxidized glutathione (i.e., glutathione disulfide = GSSG) was determined using the method described by Adams and associates (25). After centrifugation (3,000 × *g* for 5 min), BAL fluid supernatant was mixed with an equal volume of 10 mM *N*-ethylmaleimide in 0.1 M potassium phosphate buffer, pH 6.5, containing 17.5 mM EDTA. A total of 250 μl of the mixture was passed through a SEP-PAK C_{18} cartridge (Waters Associates, Milford, MA) that had been prewashed with 3 ml methanol followed by 3 ml of aqua bidest. GSSG was eluted from the column with 1 ml of 0.1 M potassium phosphate buffer, pH 7.5, 5 mM EDTA. A total of 750 μl of the eluate was added to 250 μl of 0.1 M potassium phosphate buffer, pH 7.5, with 5 mM EDTA, 800 μM DTNB, 2 U/ml glutathione reductase, and 1 mM NADPH. The rate of reduction of DTNB was recorded spectrophotometrically at 412 nm. Standards of GSSG (Boehringer, Mannheim, Germany) of known concentrations (0.25 to 4 μM) were processed exactly as the BAL fluid samples and were used to generate standard curves. The concentration of reduced glutathione (GSH) was calculated using the following equation: GSH = GSH_t − 2 × GSSG.

Estimation of Respiratory ELF

The volume of ELF was estimated by the urea dilution method (27). Concentrations of urea in BAL fluid and serum were measured with the Urea Nitrogen 65-UV Kit (Sigma), and the dilution factor obtained was used to calculate glutathione concentrations in ELF.

Methionine Sulfoxide Content of BAL Fluid Proteins

The relative content of oxidized methionine residues (= methionine sulfoxide = Met(O)) of BAL fluid proteins was determined as previously described (3, 28, 29). Briefly, 2 ml aliquots of BALF were dialyzed against H_2O bidest, lyophilized, and then dissolved in 75% (vol/vol) formic acid. Nonoxidized methionine (Met) residues were converted to homoserine and homoserine lactone by treatment with 0.2 M cyanogen bromide (CNBr). Under these conditions, oxidized methionine (Met(O)) is stable. The CNBr-treated proteins were hydrolyzed with 6 N HCl in the presence of 5 mM dithioerythritol at 110° C for 48 h, which results in Met(O) being quantitatively reduced to Met. After precolumn derivatization with ortho-phthalaldehyde (Pierce, Rockford, IL), the samples were subjected to amino acid analysis by reversed-phase high-performance liquid chromatography (Pharmacia, Freiburg, Germany) to determine residual Met (equivalent for Met(O)) and homoserine. Results are presented as Met(O) in percentage of total methionine (= Met + Met(O)). This test was performed independently at Dr. Maier's laboratory, and all samples were blinded before analysis.

Pulmonary Function Tests

Vital capacity (VC) was determined using a spirometer (Jaeger, Würzburg, Germany). The CO-transfer factor (T_{LCO}) and residual volume (RV) were measured by a single-breath method using a gas mixture containing 0.2% CO and 8% helium. Total lung capacity (TLC) was calculated from VC and RV values. Blood gas analysis (double values) was performed with arterialized capillary blood from the earlobe at rest and during steady-state bicycle exercise at a level of 40, 60, 80, or 100 W, depending on the age and clinical status of the patient. Lung volumes were referenced to normal values as published by the European Community for Steel and Coal (30). Individual normal values for Pa_{O_2} were calculated according to Ulmer and coworkers (31). T_{LCO} was expressed as a percentage of our laboratory reference values.

As described earlier (32), the changes in three different lung function parameters (VC, T_{LCO}, exercise Pa_{O_2}) were added to each other (i.e., ΔVC + T_{LCO} + Δexercise Pa_{O_2}) to obtain a global parameter re-

TABLE 1

LUNG FUNCTION BEFORE AND AFTER 12 wk OF NAC TREATMENT

	Pre-study Observation	Before NAC Treatment	After NAC Treatment
VC, % pred	84.7 ± 4.1	80.5 ± 4.4*	82.6 ± 4.3
TLC, % pred	79.8 ± 4.4	78.3 ± 4.1	78.2 ± 4.4
T_{LCO}, % pred	61.7 ± 5.4	56.5 ± 4.0†	61.4 ± 4.6*
Resting Pa_{O_2}, mm Hg	76.1 ± 3.5	73.2 ± 3.3	74.1 ± 3.0
Exercise Pa_{O_2}, mm Hg	60.1 ± 2.4	57.1 ± 2.3†	59.8 ± 2.9

* $p < 0.05$ versus preceding test.
† $p < 0.01$ versus preceding test.

flecting lung function (i.e., the lung function index [LFC index]). For the NAC treatment period, changes in VC and TL_{CO} were calculated by subtracting the test value before starting NAC from that after 12 wk of NAC treatment (measured values expressed as a percentage of the predicted value). Changes in exercise Pa_{O_2} were calculated by subtracting the measured exercise Pa_{O_2} value before starting NAC from the corresponding value after 12 wk of NAC treatment (values expressed as mm Hg). Analogous calculations were performed for the pre-study period of 4 ± 1 mo of follow-up, and the results were compared with those of the NAC study period.

Statistics

Data are expressed as the arithmetic mean value ± SEM. For statistical analysis, the Wilcoxon U-test for dependent samples was employed. For differences, p values < 0.05 were considered significant with the two-tailed test. The statistical computations were performed with the aid of SPSS/PC+ software.

RESULTS

Side Effects of High-dose Oral NAC Treatment

Of the 20 patients who entered the study, two dropped out: one male patient (62 yr) because of persistent diarrhea, which ceased promptly after NAC treatment was stopped, and one female patient (61 yr) because of abdominal surgery unrelated to this study. Thus, the drug-related drop-out rate was 1 of 20 (5%).

The remaining 18 patients completed the study. Three (17%) experienced a single episode of diarrhea during the treatment period, which ceased in all cases after a treatment pause of 2 to 3 d. Upon continuation of high-dose NAC treatment, there was no recurrence of diarrhea. Four patients (22%) complained of temporary mild nausea, which ceased without medication. Coughing was intensified in three patients (17%) and improved in one (6%). Increased amounts of sputum were reported by three patients (17%).

The following data were calculated from the results of 18 patients who completed the study.

BAL Total and Differential Cell Counts

The BAL differential cell counts did not show significant changes after high-dose NAC treatment in terms of relative as well as absolute cell numbers (Table 2).

Oxidant-Antioxidant Balance

Plasma levels of GSH_t increased slightly during NAC treatment (3.1 ± 0.3 μM versus 3.4 ± 0.3 μM, $p > 0.1$). In contrast, there was a significant increase in the GSH_t concentration in native BAL fluid and in ELF (Table 3). The GSSG concentration remained unaltered in native BAL fluid and ELF, respectively (Table 3). Consequently, there was a significant increase of reduced glutathione (GSH) in native BAL fluid and ELF (Table 3; Figure 1).

The methionine sulfoxide content of BAL fluid-derived proteins was 6.83 ± 0.71% of total methionine before and decreased significantly to 4.60 ± 0.40% after high-dose NAC treatment ($p < 0.005$) (Figure 1).

TABLE 2

BAL TOTAL AND DIFFERENTIAL CELL COUNTS

	Percentage of BAL Cells		BAL Cells (10^4/ml)	
	Before NAC	After NAC	Before NAC	After NAC
Macrophages	71.5 ± 4.6	71.1 ± 4.3	12.9 ± 1.2	14.1 ± 1.3
Neutrophils	12.3 ± 2.3	12.1 ± 1.6	2.3 ± 0.5	2.8 ± 0.5
Eosinophils	3.8 ± 1.2	4.2 ± 1.3	0.8 ± 0.3	0.8 ± 0.2
Mast cells	0.3 ± 0.2	0.5 ± 0.2	0.06 ± 0.03	0.11 ± 0.04
Lymphocytes	12.1 ± 3.9	12.2 ± 3.9	2.3 ± 0.9	3.4 ± 1.5

TABLE 3

GLUTATHIONE CONCENTRATION AND FORM

	Concentration in Native BAL Fluid (μM)		Concentration in ELF (μM)	
	Before NAC	After NAC	Before NAC	After NAC
GSH_t	3.43 ± 0.30	4.20 ± 0.66*	267.3 ± 26.0	367.1 ± 36.0
GSSG	0.43 ± 0.06	0.40 ± 0.08	31.6 ± 4.7	33.5 ± 4.5
GSH	2.58 ± 0.24	3.42 ± 0.54†	204.5 ± 20.7	302.9 ± 32.2

Definition of abbreviations: GSH_t = total glutathione (i.e., GSH + 2 × GSSG); GSSG = glutathione disulfide (i.e., oxidized form of glutathione); GSH = reduced form of glutathione.

Values are expressed as mean ± SEM.

* $p < 0.05$ versus before NAC.

† $p < 0.005$ versus before NAC.

Physical Findings and Subjective Dyspnea Scoring

Auscultatory findings were recorded semiquantitatively before and after NAC treatment and were found to be unchanged in 14 cases, whereas a slight reduction of intensity and/or spreading of crackles was noted in four patients (22%). Nine patients (50%) had the impression that their dyspnea improved during the study period, seven (39%) felt no change, and two (11%) reported an increase of dyspnea.

Lung Function

During the pre-study observation period (4 ± 1 mo), there was a slight but significant deterioration of lung function parameters, whereas a positive trend of lung function parameters was observed during the 12-wk NAC treatment period (Table 1). When the LFC index was calculated, the majority of patients (n = 13, 72%) showed a negative value during the pre-study observation period. In contrast, there was a shift to

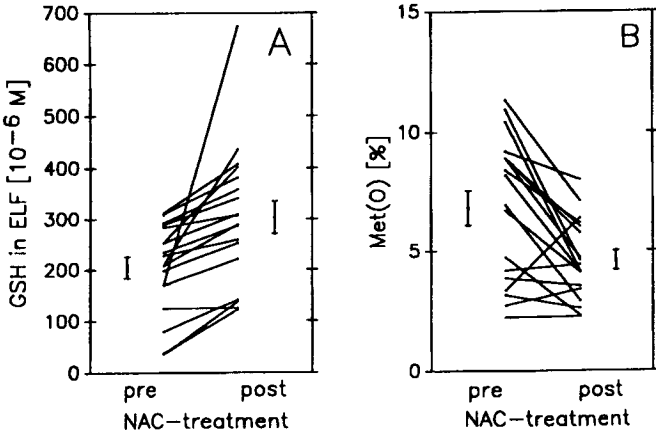

Figure 1. Influence of high-dose NAC supplementation on pulmonary redox imbalance. *Panel A* shows the changes in the concentration of the reduced form of glutathione (GSH) in the epithelial lining fluid (ELF) for each individual patient. Mean values ± SEM are indicated by *filled squares* with *error bars*; the difference was statistically significant ($p < 0.005$). *Panel B* shows the individual change of the oxidized methionine (Met(O)) content of BAL fluid-derived proteins. Mean values ± SEM are indicated by *filled squares* with *error bars*; the difference was statistically significant ($p < 0.005$).

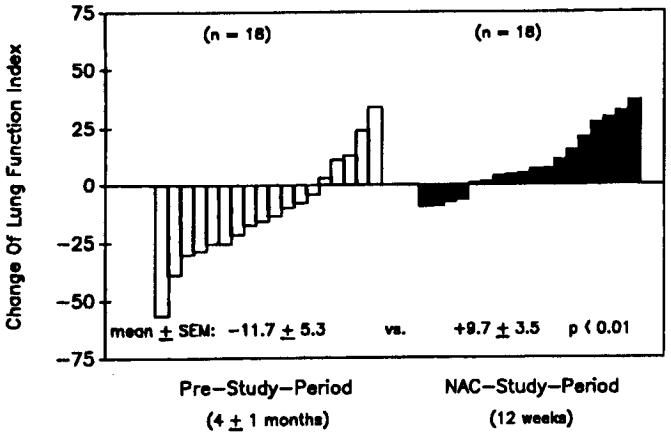

Figure 2. Follow-up of lung function without (pre-study period, *open bars*) and with (NAC study period, *filled bars*) NAC treatment in 18 patients with fibrosing alveolitis. Changes of lung function are expressed as lung function index (*see* METHODS). Each bar represents follow-up data of a single patient. A positive index signifies improvement; a negative index indicates deterioration.

wards a positive LFC index during the high-dose NAC treatment period, which resulted in a positive LFC index in 14 patients (78%); this change of the LFC index was statistically significant (-11.7 ± 5.4 versus 9.7 ± 3.5, $p < 0.01$) (Figure 2). Interestingly, those four patients who showed a further decline of their LFC index during NAC supplementation were off immunosuppressive therapy. The remaining three patients without accompanying immunosuppressive therapy were stable during NAC supplementation.

DISCUSSION

In our study, a significant increase of GSH in native BAL fluid and in ELF after a 12-wk treatment period with high-dose oral NAC in patients with FA was found. The GSH concentration thereby increased by about 50%. Meyer and colleagues reported a significant increase of total glutathione levels (i.e., GSH + 2 × GSSG) in native BAL fluid of IPF patients after 5 d of high-dose oral NAC (i.e., 3 × 600 mg/d) without a significant increase of the GSH_t concentration in ELF (18). This difference may be explained by the substantially longer treatment period in our study, which may lead to a more homogeneous elevation of glutathione concentrations within the study population.

As shown, the increase of total glutathione in our study was almost completely due to an increase of the reduced form of glutathione (GSH), which has antioxidative properties, whereas the oxidized form of glutathione (GSSG), which is not functional as an antioxidant, remained constant. This finding agrees with another short-term study showing an increase of reduced glutathione in native BAL fluid and ELF 3 h after intravenous application of 1.8 g NAC in patients with fibrotic lung disease of various etiologies (19).

Borok and coworkers (33) reported that inhalative application of GSH to IPF patients over 3 d increases total glutathione levels at the alveolar surface, with an increase of GSSG to an average of about 50% of total glutathione. This finding was interpreted to indicate that GSH had been consumed by the inflammatory process (33). In contrast, our data suggest that oral NAC leads to elevated GSH levels without increasing GSSG. Thus, the physiologic GSH/GSSG ratio is maintained by oral NAC, whereas deposition of GSH directly at the epithelial surface by inhalation leads to accumulation of GSSG The NAC approach may thus be more efficient as a long-term antioxidant treatment.

None of the previous studies dealing with augmentation of pulmonary GSH levels by NAC evaluated the consequences of an increase in GSH levels on oxidative damage within the lungs. Since we previously demonstrated that increased levels of oxidized methionine in BAL-derived proteins reflect the imbalance between oxidants and antioxidants at the alveolar epithelial surface (3, 15, 29) and are linked to lung function impairment (3), we measured this parameter before and after high-dose NAC treatment. The results clearly demonstrate that in parallel to the rise in GSH levels a significant decrease of the methionine sulfoxide content of BAL fluid proteins ensues. This finding indicates that GSH generated by NAC therapy actually exerts an antioxidative effect at the alveolar epithelial surface.

With respect to pulmonary function, we could show that compared to a pre-study period without NAC supplementation there was a significant improvement of lung function during the NAC study period. These findings were accompanied by a subjective improvement of dyspnea reported by 50% of the patients who completed the study. Serious side effects did not occur. The overall acceptance of the NAC treatment was good, and those nine patients who felt subjective improvement continued NAC therapy beyond the study period.

Interestingly, the positive effect of NAC treatment on clinical course and lung function was restricted to patients who received immunosuppressive maintenance therapy, whereas those four patients who experienced further deterioration were off standard anti-inflammatory therapy. Therefore, high-dose NAC therapy may be considered an adjunct treatment for patients with FA. We believe that based on our results a placebo-controlled clinical trial to further evaluate the effect of oral high-dose NAC therapy should now be undertaken.

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