Eur Respir J 2002; 19: 906–911
DOI: 10.1183/09031936.02.00204902
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European Respiratory Journal
ISSN 0903-1936

Intracellular glutathione and bronchoalveolar cells in fibrosing alveolitis: effects of *N*-acetylcysteine

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Intracellular glutathione and bronchoalveolar cells in fibrosing alveolitis: effects of N*-acetylcysteine. J. Behr, B. Degenkolb, F. Krombach, C. Vogelmeier. ©ERS Journals Ltd 2002.*

ABSTRACT: Extracellular glutathione deficiency and exaggerated oxidative stress may contribute to the pathogenesis of fibrosing alveolitis (FA). High-dose *N*-acetylcysteine (NAC) supplementation partially reverses extracellular glutathione depletion and oxidative damage, but effects on intracellular glutathione are unknown.

Intracellular total glutathione (GSH_t) and activation of bronchoalveolar lavage cells (BAC) obtained from 18 FA patients (9 males, aged 52±2 yrs), before and after 12 weeks of oral NAC (600 mg *t.i.d.*), were assessed. Eight healthy nonsmokers (2 males, aged 36±6 yrs) served as a control group.

Intracellular GSH_t was decreased in FA (1.57±0.20 nmol·1×10^6 BAC^{-1} *versus* 2.78±0.43 nmol·10^6 BAC^{-1}). After NAC treatment, the intracellular GSH_t content increased (1.57±0.20 *versus* 1.87±0.19 nmol·1×10^6 BAC^{-1}). The spontaneous oxidative activity of BAC decreased after NAC treatment (2.7±0.8 *versus* 1.0±0.2 nmol·1×10^6 $BAC^{-1}·h^{-1}$). Interleukin-8 concentration (82.1±31.5 *versus* 80.0±22.6 pg·mL bronchoalveolar fluid (BALF), nonsignificant (NS)) and myeloperoxidase activity (1.93±0.64 *versus* 1.55±0.47 mU·mL^{-1} BALF, NS) did not change significantly, but were found to be inversely correlated to intracellular GSH_t.

In conclusion, high-dose *N*-acetylcysteine supplementation increases intracellular glutathione levels slightly. This increase is associated with a mild reduction of oxidative activity but not with a reduction of bronchoalveolar cell activation in these patients.

Eur Respir J 2002; 19: 906–911.

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Keywords: Fibrosing alveolitis, glutathione, interleukin-8, myeloperoxidase, oxidative activity

Received: January 15 2001
Accepted after revision November 2 2001

J. Behr was supported by Wilhelm Sander-Stiftung

During the last 10 yrs considerable evidence has accumulated linking increased oxidative stress and reduced antioxidative capacity to the pathogenesis of fibrosing alveolits (FA) [1–8]. Activated inflammatory cells enter the lower respiratory tract and release increased amounts of reactive oxygen species (ROS) [1, 2, 5, 7], leading to oxidative injury of the lung parenchyma [1, 3, 7, 8, 9]. Glutathione in its reduced form (GSH) prohibits oxidant injury in healthy lungs but has been shown to be lacking in pulmonary fibrosis, at least in the extracellular compartment [4, 7, 9–11], thus aggravating the pulmonary redox imbalance. In several studies it has been demonstrated that *N*-acetylcysteine (NAC) administered orally or intravenously is capable of stimulating glutathione synthesis by providing cysteine residues for the γ-glutamyl cycle located in the cytoplasm, thereby partially restoring glutathione levels in the epithelial lining fluid of fibrotic lungs [9–11]. This was accompanied by a decrease in oxidized methionine residues in extracellular proteins and by functional improvements in the patients in one study [9]. However, glutathione is also an important intracellular antioxidant, as, by influencing the intracellular redox balance, it is linked to the activation of transcription factors, such as nuclear factor (NF)-κB or activation protein (AP)-1, and may play a crucial regulatory role in inflammatory and reparative processes relevant in lung fibrosis [12–14].

Therefore, intracellular glutathione levels and parameters of bronchoalveolar inflammatory cell activation (oxidative activtivity, interleukin (IL)-8 concentrations, and myeloperoxidase (MPO) activity) in patients with FA before and after a 12-week course of high-dose NAC treatment were studied.

Materials and methods

Patients and protocol

The patients studied and the study protocol used were identical to those described in detail in a previous study [9]. In brief, a total of 20 patients (10 males, aged 52±2 yrs) were enrolled for a prospective study of NAC 600 mg *t.i.d.* Two patients dropped out, one because of diarrhoea and the other because of a surgical procedure not related to the study drug. Of the remaining 18 patients (9 males) who completed the study, nine suffered from idiopathic pulmonary fibrosis (histologically confirmed) and nine had collagen vascular disease with lung involvement. Thirteen of 18 patients were under maintenance immunosuppression with prednisolone (mean daily

dose 6.7±1.8 mg), three of which received additional azathioprine (n=2) or cyclophosphamide (n=1). The remaining five patients were off any immunosuppressive treatment because of lack of effect. All patients were nonsmokers.

The study protocol included a bronchoalveolar lavage (BAL) study before and after 12 weeks of NAC therapy. Written informed consent was obtained from all patients and the protocol was approved by the local ethical committee.

Controls

A standard BAL was performed in eight healthy volunteers (2 males, age 36±6 yrs) without a history of atopy or bronchopulmonary disease and with normal pulmonary function tests. All controls had never smoked. BAL cell differential was within the laboratory normal range. Written informed consent was obtained from all controls and the protocol was approved by the local ethical committee.

Biological samples

BAL fluid (BALF) was obtained by standard techniques. Aliquots of freshly obtained bronchoalveolar cells (BAC) were taken for glutathione assays (see later), total cell counts (Coulter counter), and cytocentrifuge preparations for differential cell counts. The cells were pelleted and the supernatants were used to assess the MPO activity and IL-8 concentration.

Intracellular glutathione levels

Total glutathione (GSH_t=GSH+2×oxidized glutathione (GSSG)) concentrations were measured using standard techniques, as reported previously [7]. Immediately after the BAL procedure, cells were gently centrifuged (300×*g* for 10 min), the supernatant was then removed and the cells were resuspended in phosphate-buffered saline. An aliquot of 1×10^6 BAC was lysed by incubation in 1 mL of distilled water for 5 min. After centrifugation (3,000×*g* for 5 min), 100 μL of the lysate was mixed with 1.1 mL of 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM ethylenediamine tetraacetic acid, 0.2 mM nicotinamide adenine dinucleotide phosphate, 63.5 μM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Serva, Heidelberg, Germany), and 4 $U\cdot mL^{-1}$ glutathione reductase. The rate of reduction of DTNB was recorded spectrophotometrically at a wavelength of 412 nm. All measurements were made in triplicate and the average value was calculated. The GSH_t concentration of the BAC lysate sample was calculated using an internal standard of 0.84 μM GSH [7]. The GSH_t content of BAC was expressed as $nmol\cdot1\times10^6$ BAC^{-1}.

Oxidative activity

For measurement of the oxidative activity, *ex vivo* cultures of BAC were used as previously described [7]. BAC obtained by BAL were cultured in standard medium (Roswell Park Memorial Institute (RPMI) 1640, Sigma Chemical Co., St Louis, MO, USA) using 6-well, flat-bottomed, plastic-culture dishes (Flacon, NJ, USA). After incubation for 30 min at 37°C with 5% carbon dioxide, nonadherent cells were removed, generating BAC monolayer cultures consisting mainly of alveolar macrophages (>95%) [7]. For measurement of oxidative activity, BAC cultures were incubated with Hank′s balanced salt solution (Sigma) containing 50 μM of GSH ±160 nM phrobol myristate acetate (PMA). Blanks without BAC were processed in exactly the same way. After 1 h of incubation the supernatant was removed and catalase (Sigma) was added to a final concentration of 80 $U\cdot mL^{-1}$ to stop hydrogen peroxide (H_2O_2)-dependent reactions [15]. The samples were mixed with DTNB (final concentration 250 μM) and after 5 min of incubation the absorption was measured at 412 nm (Lambda 19 Spektrophotometer; Perkin Elmer, Ueberlingen, Germany). The concentration (nmol) of GSH, which was oxidized by 1×10^6 $BAC^{-1}\cdot h^{-1}$ spontaneously or with PMA stimulation, was calculated from the decrease of the GSH concentration in the presence of BAC (±PMA) as compared to the blanks. All measurements were performed in duplicate. The results were expressed as $nmol\cdot1\times10^6$ $BAC^{-1}\cdot h^{-1}$.

Interleukin-8

The IL-8 concentration was determined in BALF supernatants using a commercially available enzyme-linked immunosorbent assay (ELISA; Endogen Human Interleukin-8 ELISA; Endogen, MA, USA). All determinations were performed in duplicate. The results are expressed as $pg\cdot mL\cdot BALF^{-1}$.

Myeloperoxidase activity

MPO activity was measured as described by SUZUKI *et al.* [15]. In brief, 400 μL of fresh BALF supernatant (3,000×*g* for 10 min) were mixed with 420 μL of 150 mM sodium hydrogen phosphate buffer and 80 μL of 20 mM tetramethyl benzidine (Serva). After 5 min of incubation at room temperature, the reaction was started by adding 100 μL of 3 mM H_2O_2 and absorption was recorded by spectrometer at 655 nm. All measurements were performed in duplicate. The results are expressed as $mU\cdot mL^{-1}$ BALF.

Statistics

Data are expressed as mean±SEM. For statistical analysis, t-tests for dependent or independent samples were employed, as appropriate. Correlations were calculated according to Pearson correlation coefficient. p-Values <0.05 were considered significant.

Results

Bronchoalveolar lavage differential cell counts

The results of absolute and relative cell counts are summarized in table 1. Total cell count and absolute numbers of alveolar macrophages, neutrophils, eosinophils and lymphocytes were significantly elevated in FA patients, both before and after NAC treatment, compared with healthy controls. The percentage of alveolar macrophages decreased in FA patients before and after NAC, as compared to the control group, whereas the percentages of neutrophils, eosinophils and lymphocytes increased. In contrast, absolute and relative cell counts did not differ significantly between FA patients before and after NAC treatment (table 1).

Glutathione content

The GSH_t content of BAC from patients with FA before NAC treatment was significantly lower compared to healthy nonsmokers (1.57±0.20 nmol·1×10^6 BAC^{-1} *versus* 2.78±0.43 nmol·1×10^6 BAC^{-1}, $p<0.05$). This finding of a 44% lower cellular GSH_t content in FA patients may, at least in part, be due to the different composition of total BAC from controls and FA patients, particularly the 23.4% lower percentage of alveolar macrophages in FA patients before NAC therapy. It should be noted that the control group was younger than the patient group. However, there was no relationship between age and intracellular GSH_t in the control and patient groups, and no reports were found in the literature.

After 12 weeks of NAC supplementation, there was a significant increase of the GSH_t content of BAC from FA patients (1.57±0.20 nmol·1×10^6 BAC^{-1} *versus* 1.87±0.19 nmol·1×10^6 BAC^{-1}, $p<0.05$). Although intracellular GSH_t did not normalize, the difference was no longer significant when compared with controls (p=0.082). As shown in figure 1, 13 out of 18 patients (72%) had higher intracellular GSH_t levels after NAC treatment, one (6%) remained unchanged, and four (22%) experienced a small decline of intracellular GSH_t (fig. 1). This finding cannot be explained by different BAC compositions, because there was no significant change of absolute or relative BAC numbers during the treatment period (table 1).

Table 1. – Differential cell counts in bronchoalveolar lavage fluid (BALF) in controls and in subjects with fibrosing alveolitis (FA) before and after *N*-acetylcysteine (NAC) treatment

	Healthy controls	FA before NAC	FA after NAC
Subjects n	8	18	18
Total cell count			
10^4·mL $BALF^{-1}$	7.1±1.0*	18.4±1.5	21.2±1.6
AM 10^4·mL BAL F^{-1}	6.6±0.7*	12.9±1.2	14.1±1.3
AM %	93.3±2.7*	71.5±4.6	71.1±4.3
PMN 10^4·mL $BALF^{-1}$	0.3±0.09*	2.3±0.5	2.8±0.5
PMN %	4.3±1.4*	12.3±2.3	12.1±1.6
EOS 10^4·mL $BALF^{-1}$	0.05±0.03*	0.8±0.3	0.8±0.2
EOS %	0.9±0.7*	3.8±1.2	4.2±1.3
Mast 10^4·mL $BALF^{-1}$	0	0.06±0.03	0.11±0.04
Mast %	0	0.3±0.2	0.5±0.2
lym 10^4·mL $BALF^{-1}$	0.12±0.09*	2.3±0.9	3.4±1.5
lym %	1.6±1.2*	12.1±3.9	12.2±3.9

Data are presented as mean±SEM unless otherwise stated. AM: alveolar macrophages; PMN: neurophilic granulocytes; EOS: eosinophilic granulocytes; Mast: mast cells; lym: lymphocytes. *: $p<0.05$ *versus* FA before and after NAC.

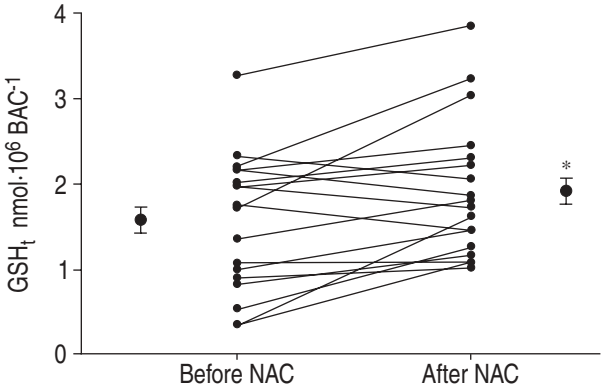

Fig. 1. – The total glutathione (GSH_t) contents of bronchoalveolar cells (BAC) of 18 patients with FA before and after *N*-acetylcysteine (NAC). Data are presented as mean±SEM. *: $p<0.05$.

The extracellular glutathione concentrations in these patients have been previously published [11]. The concentrations of GSH_t and GSH increased significantly during the 12-week course of NAC supplementation [11]. Interestingly, there was a significant positive correlation between GSH_t concentration in the epithelial lining fluid and the glutathione content of BAC ($r=0.46$; $p<0.005$).

Oxidative activity of bronchoalveolar cells

The spontaneous oxidative activity of *ex vivo* cultivated BAC declined significantly from 2.70±0.83 nmol·1×10^6 BAC^{-1}·h^{-1} to 1.02±0.21 nmol·1×10^6 BAC^{-1}·h^{-1} ($p<0.05$) after NAC treatment, whereas the PMA-stimulated oxidative activity was only slightly diminished (8.26±1.32 nmol·1×10^6 BAC^{-1}·h^{-1} *versus* 7.13±1.00 nmol·1×10^6 BAC^{-1}·h^{-1}, nonsignificant (NS)) (fig. 2). As BAC composition did not change significantly during NAC treatment (table 1) [11], these changes do not appear to be attributable to differences in BAC composition, athough in this study it was not possible to establish which cell population(s) was (were) responsible for the reduction in oxidative activity. Correlation analysis showed a slight trend toward an inverse relationship between intracellular GSH_t and spontaneous or PMA-stimulated oxidative activity of BAC ($r=-0.277$, NS, and $r=-0.237$, NS, respectively).

Interleukin-8 concentrations

As reported previously [16, 17], IL-8 concentrations in BALF were significantly increased in FA

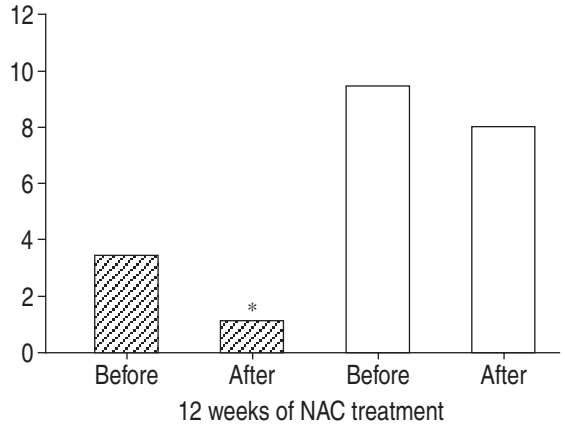

Fig. 2. – Spontaneous (▨) and phorbol myristate acetate-stimulated (□) oxidative activity of *ex vivo* cultivated bronchoalveolar cells (BAC) before and after *N*-acetylcysteine (NAC) treatment. For further details see text. *: $p<0.05$ pre- *versus* post-NAC treatment.

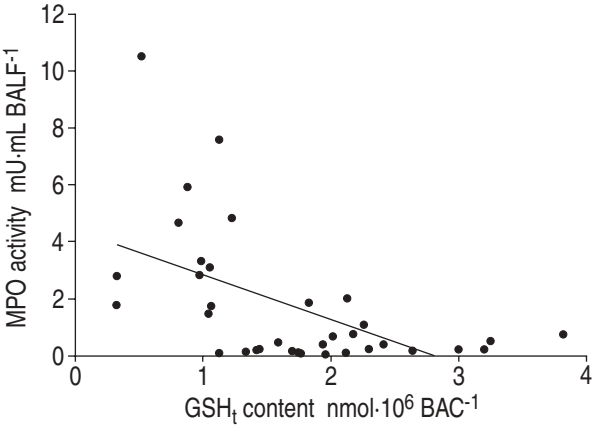

Fig. 4. – Inverse correlation between intracellular total glutathione (GSH_t) content of bronchoalveolar cells (BAC) and myeloperoxidase (MPO) activity in bronchoalveolar lavage fluid (BALF) in 18 patients with FA before and after *N*-acetylcysteine treatment.

patients when compared to healthy controls (82.1±31.5 pg·mL·BALF^{-1} *versus* 18.1±2.0 pg mL·BALF^{-1}, $p<0.01$). The IL-8 concentrations in native BALF were not significantly altered after NAC treatment (82.1±31.5 pg·mL·BALF^{-1} *versus* 80.0±22.6 mL^{-1} BALF, NS). However, a significant inverse correlation existed between the GSH_t content of BAC and IL-8 levels in BALF, as shown in figure 3 ($n=36$, $r=0.45$, $p<0.05$).

Myeloperoxidase activity

MPO is a marker enzyme of neutrophilic inflammation and is responsible for enhanced oxidative stress mediated by hypohalides. MPO activity in native BALF was slightly reduced after NAC supplementation (1.93±0.64 mU·mL·BALF^{-1} *versus* 1.55±0.47 mU·mL·BALF^{-1}, NS). However, a significant inverse correlation was observed between GSH_t content of BAC and MPO activity in native BALF ($n=36$, $r=0.54$, $p<0.01$) (fig. 4).

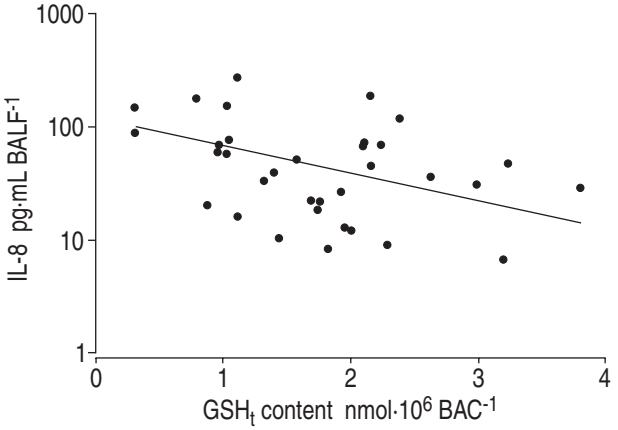

Fig. 3. – Inverse correlation between intracellular total glutathione (GSH_t) content of bronchoalveolar cells (BAC) and interleukin (IL)-8 concentration in bronchoalveolar lavage fluid (BALF) in 18 patients with FA before and after *N*-acetylcysteine treatment.

Discussion

The most important finding in this study was that glutathione deficiency in FA was not restricted to the extracellular compartment as reported previously [4, 7, 9–11], but that there was also an intracellular glutathione deficiency in BAC. However, it was not possible to establish whether the lack of intracellular glutathione in BAC cells was restricted to a specific cell population or was caused by the presence of apoptotic cells. It was also unclear whether the lower GSH_t content of BAC was the cause or consequence of BAC activation in FA.

Despite the limitations of this study, the finding that intracellular GSH_t content in BAC was reduced in FA patients is of interest, as intracellular glutathione is linked to important immunological, inflammatory, and reparative cell functions and may have relevant consequences for the course of the disease [12–14, 18, 19]. Therefore, it is of particular interest that oral NAC supplementation, at a high dose of 1,800 mg·day^{-1} over a period of 12 weeks, was able to significantly increase intracellular GSH_t, although it did not normalize GSH_t levels. These findings parallel previous reports, which showed that NAC increases extracellular glutathione levels in patients with pulmonary fibrosis [9–11]. A significant positive correlation between intra- and extracellular glutathione levels was observed. This data, therefore, provides additional confirmation that NAC works as a glutathione precursor not only in the liver but also in the lungs.

Previous reports on the anti-inflammatory actions of NAC suggest it has an antioxidative effect and may play a role as a glutathione precursor [14, 18, 20–22]. However, there are conflicting reports regarding the effect of oral NAC on oxidant release from phagocytes [20, 23]. In this study, there was a significant drop in the spontaneous oxidative activity of BAC after 12 weeks of NAC treatment, whereas PMA-stimulated oxidative activity did not change significantly. This could be due to slight anti-inflammatory

actions of NAC. The findings in this study parallel the results of Borok *et al.* [24], who also observed a decrease in spontaneous superoxide anion release by alveolar macrophages after 3 days of glutathione aerosol administration (600 mg every12 h) in idiopathic pulmonary fibrosis patients. The data from this study suggests that the decrease of spontaneous oxidative activity is linked, at least indirectly, to the increase of intracellular GSH_t after NAC supplementation, although it is not clear whether it is the cause or the consequence.

It has been well documented that oxidative stress plays a key role in the regulation of IL-8 secretion [25]. The finding that IL-8 concentrations in BALF are inversely correlated to intracellular GSH_t levels supports the idea that increased oxidative stress triggers IL-8 gene expression and secretion. However, NAC induced an average increase of intracellular GSH_t of 19%, which was insufficient to induce a significant reduction in IL-8 concentrations in BALF.

MPO is a marker of neutrophil activation and was used here as an indicator of neutrophil inflammatory activity, which could be modified by altered intra- and/or extracellular glutathione levels. A decrease in the MPO concentration in serum from smokers after 8 weeks of treatment with NAC (200 mg *t.i.d.*) has been reported previously [20]. The reduction in MPO activity in BALF observed in this study was statistically ns. Nonetheless, this data suggests that MPO release is inversely linked to intracellular glutathione. Although evidence of a causal relationship between intracellual GSH_t and IL-8 or MPO secretion could not be generated, the present study′s results are in agreement with this notion.

To conclude, the data in this study has shown for the first time, that intracellular total gluthione is decreased in fibrosing alveolitis and that this decrease is partially reversed by high-dose oral *N*-acetylcysteine supplementation. Despite the limitations of the study, the observations are consistent with the hypothesis that an increase in intracellular glutathione, by virtue of its antioxidative effect, may have a slight anti-inflammatory effect on bronchoalveolar cell activation, as measured by spontaneuos oxidant release. The known pathways of oxidative activation of transcription factors and key cytokines, such as interleukin-8, provide the pathophysiological background for profound anti-inflammatory actions of anti-oxidative therapy [13, 14, 18, 19, 25]. However, significant changes in interleukin-8 and myeloperoxidase levels after *N*-acetylcysteine supplementation were not shown, although intracellular total glutathione content was inversely correlated with these pro-inflammatory mediators. Overall, the changes of inflammatory cell activation observed were small and may be clinically nonsignificant. However, prolonged treatment or the use drugs that are more effective in elevating intracellular glutathione, may provide new and more potent anti-inflammtory treatment strategies.

Acknowledgements. The authors would like to thank B. Braun and A. Neuber for expert technical assistance.

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