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N-acetylcysteine inhibits TNF-α, sTNFR, and TGF-β 1 release by alveolar macrophages in idiopathic pulmonary fibrosis in vitro

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Abstract. *Background:* N-acetylcysteine (NAC) can act as an antioxidant. NAC slows the rate of decline of lung function in idiopathic pulmonary fibrosis (IPF) patients concurrently treated with prednisone and azathioprine. *Objective*: In this study we investigated the effect of NAC on the production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12 (p70), IL-18, transforming growth factor (TGF)-β1, and the soluble TNF receptors (sTNFR1 and sTNFR2) by alveolar macrophages (AM) in IPF patients. *Design:* AMs were harvested by bronchoalveolar lavage (BAL) from 16 IPF patients and were cultured for 24 h with RPMI medium alone, or with lipopolysaccharide (LPS) (100 ng/ml), in the presence or absence of NAC at various concentrations. *Results:* NAC suppressed the production of TNF-α, its soluble receptors, and TGF-β1 by AMs in a dose-dependent manner. At the highest concentration of NAC (10 mM), the spontaneous or LPS-stimulated production of TNF-α, sTNFR1, sTNFR2, and TGF-β1 were significantly reduced. The LPS-stimulated IL-1β production was also suppressed by 10 mM NAC. *Conclusions:* NAC has the potential to down-regulate the production of TNF-α and their soluble receptors, as well as TGF-β1 and LPS–stimulated IL-1β, by AM in IPF in vitro. NAC may have anti-inflammatory and anti-fibrotic effects. *(Sarcoidosis Vasc Diffuse Lung Dis 2009; 26: 147-154)*

Key words: alveolar macrophage, cytokine, idiopathic pulmonary fibrosis, N-acetylcysteine

Introduction

N-acetylcysteine (NAC) has been used for more than 30 years primarily as a mucolytic agent. In addition to its mucolytic action, NAC has been studied and utilized as a precursor in the synthesis of reduced glutathione (GSH) and as a free radical scavenger that protects cells from oxidant damage (1). Recently, studies have also demonstrated the capacity of NAC to exert its anti-inflammatory effects (2).

Idiopathic pulmonary fibrosis (IPF), with the histologic appearance of usual interstitial pneumonia (UIP), is characterized by progressive fibroblast proliferation, destruction of the alveolar architecture, and a relentless decline in pulmonary function (3). The precise pathogenetic mechanisms of IPF remain unclear. Several studies suggest that an oxidant-antioxidant imbalance in the lower respiratory tract plays a

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critical role in the disease process (4-6). Recent data suggest that epithelial injury and activation rather than alveolitis represent the key factor in the pathogenesis of IPF. The importance of alveolar epithelial cell and myofibroblast cross-talk in the pathogenesis of the disease has been confirmed in animal models of lung fibrosis. However the majority of data obtained in animals also suggest that the aberrant healing response of pulmonary fibrosis is initiated and regulated by molecules produced during the inflammatory response. These data suggest that cytokines could play an important role in the pathogenesis of IPF.

The multicentered European Idiopathic Pulmonary Fibrosis International study group evaluated the effects of oral NAC (1800 mg/d) in IPF patients. The study showed that therapy with NAC at a dose of 600 mg three times daily, added to prednisone and azathioprine, preserved lung function better than placebo (7). A pilot study of long-term aerosolized NAC administration in IPF suggested that NAC may delay disease progression as evidenced by exercise desaturation and high-resolution computer tomographic scanning (8).

There are no data regarding the effects of NAC on the production of cytokines by alveolar macrophages (AM) in IPF. In this study we aimed to investigate potential effects of NAC on the production of tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-6, IL-8, IL-10, IL-12 (p70), IL-18, transforming growth factor (TGF)-β1, and the soluble TNF receptors (sTNFR1 and sTNFR2) by AMs recovered by BAL in patients with IPF.

Methods

Study population

Sixteen consecutive patients with IPF were investigated (11 male and 5 female; mean age ± SEM, 66.9 ± 3.2). All patients involved in this study were newly diagnosed IPF patients, no patient was taking NAC at the time of bronchoscopy. All patients were current nonsmokers (11 never-smokers and 5 ex-smokers). The patients were diagnosed according to the ATS/ERS criteria including the high-resolution computer tomography characteristics of IPF (9). 5 of the patients had a surgical biopsy showing a UIP pattern on histopathology. The lung function showed restrictive impairment with FVC, %pred: 62±4; TLC, %pred: 56±4; FEV_1/FVC: 82±3; TLco, %pred: 42±3. No patient had left ventricular cardiac failure or a history of chronic pulmonary infections. No patient was receiving treatment with corticosteroids and /or immunosuppressants at the time of BAL. Written informed consent was obtained according to institutional guidelines. The study was approved by the local Internal Review Board.

Bronchoalveolar lavage procedure

BAL was performed according to established guidelines. Sterile isotonic saline was instilled into the right middle or left lingular lobe via a fiberoptic bronchoscope in 10 x 20 ml aliquots to a total volume of 200 ml with immediate aspiration by gentle suction after each aliquot. A volume of greater than 50% was retrieved. The recovered BAL fluid was filtered through two layers of sterile gauze and subsequently centrifuged at 500 x *g* for 10 min at 4°C. The cells were counted in a haemocytometer. Cell viability was assessed by Trypan blue exclusion.

Isolation and culture of alveolar macrophages

AM cultures were performed as previously described (10). After the BAL cells were washed three times with phosphate buffered saline (PBS), the cells were resuspended to a final concentration of 1 x 10^6 cells/ml in RPMI 1640 meduim supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 200 U/ml penicillin and 200 μg/ml streptomycin (Seromed, Biochrom KG, Berlin, Germany). The cell suspension was added at 1 x 10^6 cells/well to a 24-well plastic tissue culture plate (Falkon, Becton Dickinoson, New Jersey, USA) and incubated at 37°C in a 5% CO_2 humidified atmosphere for 1 hour to permit the adherence of AM. The nonadherent cells were removed by 3 washes with culture medium. The purity of adherent AM was identified to be greater than 95% by morphology and nonspecific esterase staining. The purified AM were incubated for an additional 24 hours with 1 ml 10% RPMI medium alone; with 1 ml 10% RPMI medium and 100 ng/ml LPS (Sigma-Aldrich,St Louis, MO, USA); with 1 ml 10% RPMI in the absence and presence of 100 ng/ml LPS together with NAC (MP Biomedicals, LLC., Solon, USA) at concentrations of 1

mM, 5 mM, and 10 mM, respectively. The culture supernatants of AM were harvested and centrifuged, then stored in fractions at - 80˚C until analysis.

ELISA assays for cytokine measurements

The concentrations of cytokines in the cultured supernatants were quantified using commercially available human enzyme-linked immunosorbent assay (ELISA) kits. The concentrations of TNF-α, IL-1β, IL-6, IL-8, IL-10, and IL-12p70 were quantified using ELISA kits (Pierce Biotechnology, Inc., Rockford, USA) with sensitivities of < 1-3pg/ml; sTNFR1 and sTNFR2 were measured by ELISA kits (HyCult biotechnology, Uden, Holland) with sensitivities of < 25pg/ml; IL-18 was measured by an ELISA (Medical & Biological laboratories CO., LTD., Nagoya, Japan) with sensitivities of < 12.5 pg/ml; TGF-β1 was measured by an ELISA (Assay Designs, Michigan, USA) with a sensitivity of 3.3 pg/ml. The ELISAs were performed according to the manufacturer's instructions. All the ELISA kits are specific for the measurement of natural and recombinant human-specific cytokines without cross-reactivity with other cytokines. The analysis was done in duplicate. The concentrations of above-measured cytokine were expressed as picogram per milliliter per 10^6 AMs in order to correct for the proportion of AMs.

Statistical analysis

All data are expressed as mean ± SEM. The data were analyzed using one way ANOVA. A value of $p < 0.05$ was accepted as statistically significant.

Results

Effects of NAC on the spontaneous cytokine production from AMs in patients with IPF

As shown in Table 1 and Figure 1a-3a, NAC induced a dose dependent suppression of TNF-α, sTNFR1, sTNFR2 and TGF-β1. At the highest NAC concentration (10 mM) the production was significantly reduced in comparison with the spontaneous baseline production ($p<0.05$; 0.01; 0.001; 0.01, respectively). The spontaneous production of the other cytokines was not significantly inhibited by NAC at all tested concentrations.

Effects of NAC on the LPS-stimulated cytokine production from AMs in patients with IPF

As shown in Table 2 and Figure 1b-3b, the LPS-stimulated production of all cytokines was significantly higher than the spontaneous cytokines production. NAC suppressed the release of LPS-stimulated TNF-α, sTNFR1, sTNFR2, IL-1β, and TGF-β1 in a dose dependent manner. The difference was significant at the highest concentration of NAC ($p<0.01$; $p<0.05$; $p<0.05$; $p<0.05$; $p<0.05$, respectively). No effect was found on the production of other cytokines.

Discussion

This study showed that NAC induces a dose dependent suppression of spontaneous and LPS-stimulated release of TNF-α and its soluble recep-

Table 1. Spontaneous production of cytokines by BAL macrophages in IPF

	Spontaneous (pg/ml/10^6)	NAC (1mM) (pg/ml/10^6)	NAC (5mM) (pg/ml/10^6)	NAC (10mM) (pg/ml/10^6)
TNF-α	255.5±84.6	269.1±96.7	159.8±57.1	115.0±40.5*
sTNFR1	54.4±4.6	43.3±8.8 2	28.0±5.9*	17.1±5.9**
sTNFR2	190.9±20.1	155.6±24.2	98.4±16.3**	67.9±12.5***
IL-1β	81.4±70.7	65.9±44.2	96.3±74.2	46.0±37.6
IL-6	1,283.2±637.9	1,357.4±668.8	1,114.8±532.3	1,180.2±652.4
IL-8	25,548.4±11,288.1	24,300.2±10,184.6	18,843.8±7,911.1	11,630.2±3,188.1
IL-10	22.6±6.9	31.2±14.4	21.2±9.1 2	22.3±10.3
IL-12p70	3.2±0.5	3.2±0.3	2.7±0.2	2.7±0.3
IL-18	23.8±4.6	18.9±3.4	17.1±3.0	23.6±3.1
TGF-β1	7,749.0±1,564.2	6,693.4±1,114.1	6,000.9±996.9	5,131.4±719.0**

Data are shown as mean ± SEM. NAC: N-acetylcysteine; TNF-α: tumor necrosis factor-alpha; TNFR: TNF receptor; IL: interleukin; TGF-β: transforming growth factor-beta. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ (compared to spontaneous)

Table 2. LPS-stimulate production of cytokines by BAL macrophages in IPF

	LPS (pg/ml/10^6)	LPS+NAC (1mM) (pg/ml/10^6)	LPS+NAC (5mM) (pg/ml/10^6)	LPS+NAC (10mM) (pg/ml/10^6)
TNF-α	13,060.1±2,630.3	8,719.9±1,095.1	8,481.3±1,844.4	5,866.2±866.4**
sTNFR1	101.4±15.2	93.7±18.0	69.3±21.9	39.7±18.6*
sTNFR2	1,085.2±205.7	1,018.4±280.3	735.3±234.2	510.3±201.1*
IL-1β	273.3±131.1	242.8±102.4	255.4±126.3	165.4±94.5*
IL-6	33,890.0±6,736.2	32,540.3±6,629.1	38,430.1±8,300.2	31,190.4±8,127.2
IL-8	274,400.2±21,150.1	339,043.1±26,114.2	339,694.8±27,941.1	274,423.2±21,154.1
IL-10	813.7±200.4	848.1±210.5	801.7±147.6	522.1±111.4
IL-12p70	3.3±0.6	2.9±0.3	3.1±0.3	3.1±0.3
IL-18	208.7±93.9	192.1±96.5	141.9±72.9	210.3±104.2
TGF-β1	10,570.1±2,670.4	10,760.2±4,239.8	11,839.3±4,324.4	5,144.3±1,005.7*

Data are shown as mean ± SEM. NAC: N-acetylcysteine; LPS: lipopolysaccharide TNF-α: tumor necrosis factor-alpha; TNFR: TNF receptor; IL: interleukin; TGF-β: transforming growth factor-beta. *: p<0.05; **: p<0.01; (compared to LPS-stimulated)

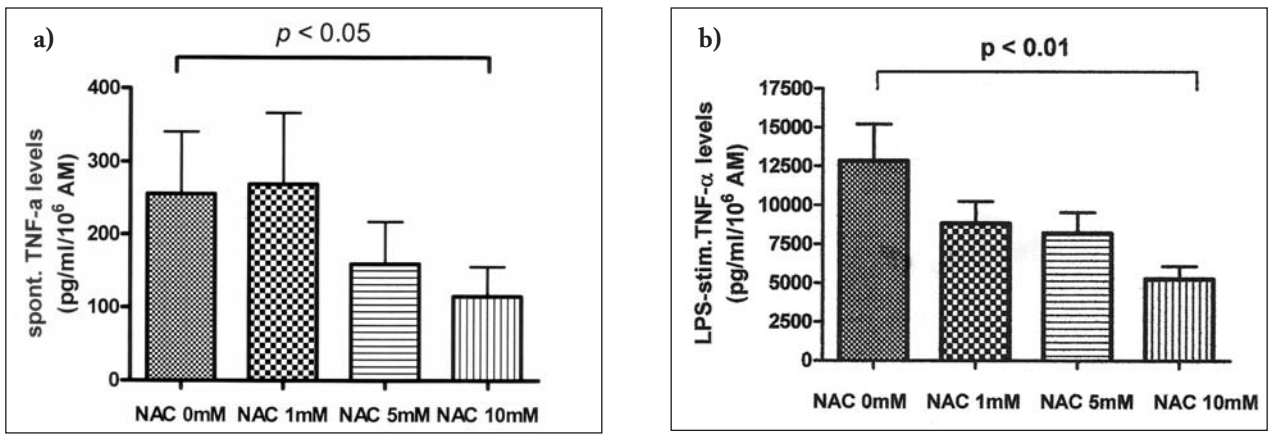

Fig. 1. a) The effect of N-acetylcysteine (NAC) on spontaneous tumor necrosis factor -alpha (TNF-α) production from alveolar macrophages (AMs) in idiopathic pulmonary fibrosis patients (IPF) (Data are presented as mean ± SEM); **b)** The effect of N-acetylcysteine (NAC) on lipopolysaccharide (LPS) -stimulated tumor necrosis factor -alpha (TNF-α production from alveolar macrophages (AMs) in idiopathic pulmonary fibrosis patients (IPF) (Data are presented as mean ± SEM)

tors from AM in IPF in vitro. To a lesser degree, the production of TGF-β1 and LPS-stimulated IL-1β was suppressed in IPF.

TNF-α is secreted by LPS stimulated macrophages and plays a significant role in many lung disorders. There is compelling evidence for an important role of TNF-α in the pathophysiology of IPF. TNF-α stimulates proliferation of fibroblasts and collagen synthesis in vitro (11). Adenoviral transfer of TNF-α complementary DNA (cDNA) to lungs of normal adult rats results in the increased TGF-β expression followed by the development of fibrosis (12). In a murine model of lung fibrosis the expression of TNF-α is strongly enhanced after the induction of fibrosis by bleomycin (13), whereas concomitant treatment with soluble TNF-α receptors inhibits fibrotic changes (14). Enhanced expression of TNF-α has been detected in the lungs of patients with IPF (15- 16). Moreover, elevated levels of TNF-α released by BAL-cells have been observed in IPF (17). However, the recent placebo-controlled trial of the TNF antagonist etanercept in the treatment of IPF failed to find a clinically significant impact on disease progression though a trend towards a decreased rate of disease progression was observed on several measures (18).

Induction of cellular inflammatory reactions, enhancement of oxidative stress and increased expression of various proinflammatory molecules contribute to the biological action of TNF-α. Over-ex-

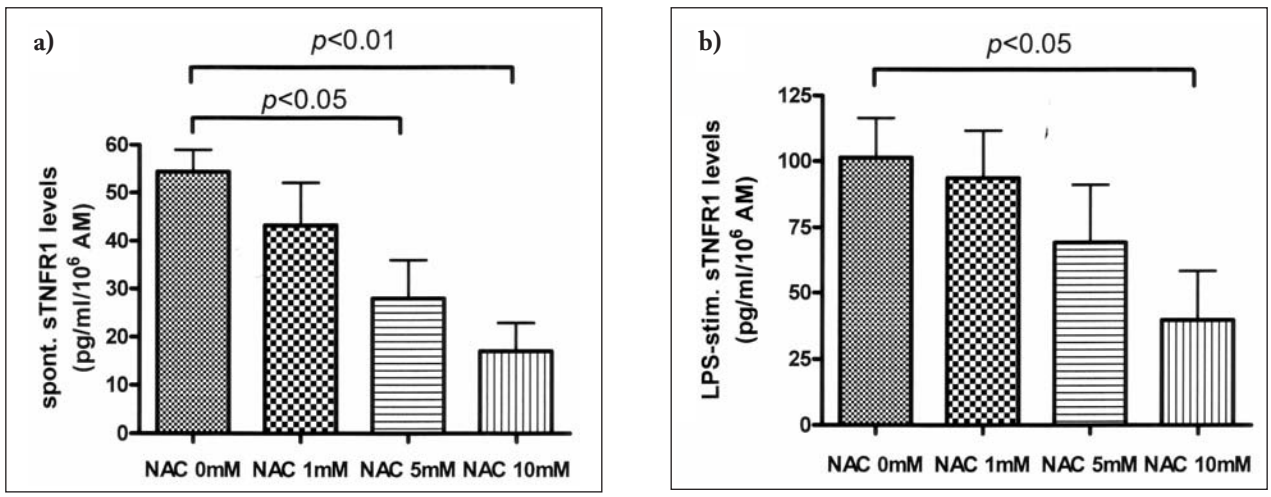

Fig. 2. a) The effect of N-acetylcysteine (NAC) on spontaneous soluble tumor necrosis factor receptor (TNFR)1 production from alveolar macrophages (AMs) in idiopathic pulmonary fibrosis patients (IPF) (Data are presented as mean ± SEM); **b)** The effect of N-acetylcysteine (NAC) on lipopolysaccharide (LPS) -stimulated soluble tumor necrosis factor receptor (TNFR) 1 production from alveolar macrophages (AMs) in idiopathic pulmonary fibrosis patients (IPF) (Data are presented as mean ± SEM)

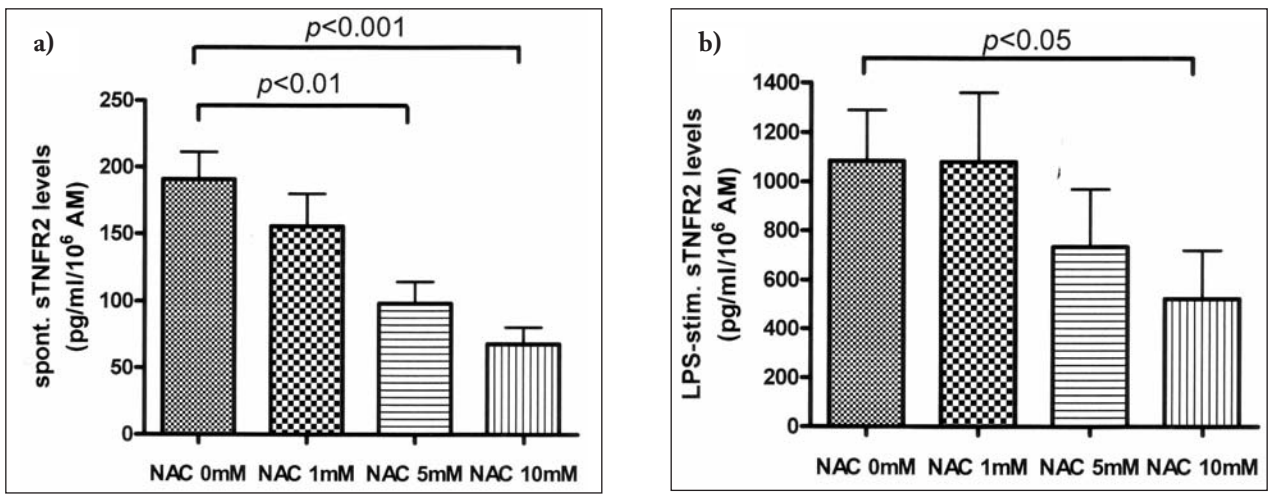

Fig. 3. a) The effect of N-acetylcysteine (NAC) on spontaneous soluble tumor necrosis factor receptor (TNFR) 2 production from alveolar macrophages (AMs) in idiopathic pulmonary fibrosis patients (IPF) (Data are presented as mean ± SEM); **b)** The effect of N-acetylcysteine (NAC) on lipopolysaccharide (LPS) -stimulated soluble tumor necrosis factor receptor (TNFR) 2 production from alveolar macrophages (AMs) in idiopathic pulmonary fibrosis patients (IPF) (Data are presented as mean ± SEM)

pression of TNF-α in transgenic mice induce differential changes in redox status and glutathione-regulating enzymes by depleting the total cellular GSH levels (19). GSH plays a central physiological role in maintaining the body homeostasis and protecting cells against oxidants, toxic substances, and DNA-damaging agents. Unfortunately, the large GSH molecular is not transported efficiently into cells. Furthermore, L-cysteine, the rate-limiting amino acid in the intracellular synthesis of GSH, is toxic to humans. NAC is readily deacetylated in cells to yield L-cysteine thereby promoting intracellular GSH synthesis. GSH is essential for the activation of T-lymphocytes and polymorphonuclear leukocytes as well as for cytokine production, and therefore for mounting successful immune responses (20).

NFκB is involved in the inducibible transcription of several immunologically important genes including TNF-α (21). The activity of NFκB can be potentially enhanced by changing redox states. The inflammatory environment with its oxidative conditions may be the optimal site to induce the nuclear translocation of NFκB, whereas the subsequent migration of the leukocyte to a less oxidative environment may provide more favorable conditions for NFκB DNA binding. Antioxidants such as NAC have been shown to be potent inhibitors of NFκB activation. This may be the mechanism how NAC can inhibit the production of TNF-α.

Reduced production of TNF-α by NAC at similar concentrations as used in our experiments has also been reported by other researchers. BAL derived AM from human lung transplant recipients produce less TNF-α when cocultured with NAC, both in terms of protein and mRNA levels (22). NAC has been shown to prevent silica-induced production of TNF-α and IL-1β in vitro (23). In vivo, NAC prevented LPS-induced increased production of TNF-α, IL-1β, or MCP in rats (24). NAC inhibited the production of TNF-α, IL-6 and IL-8 in GSH-depleted human AM in vitro (25).

TNF-α exerts its function by binding to membrane-bound receptors. The soluble TNFRs are considered to be liberated from the cell surface by proteolytic cleavage of membrane-bound TNF receptors. They act as competitors to membrane-bound TNFRs and block the actions of TNF-α. However, at low concentrations, sTNFRs can enhance TNF-α effect by stabilizing its structure. Therefore, sTNFRs can function as TNF antagonists and TNF stabilizers. Their effects vary depending on their concentrations at the site of TNF-α action and the ratios of the sTNFR to TNF-α. sTNFRs are present at low concentrations in the blood of normal healthy individuals (26), whereas elevated levels of sTNFs in human biological fluids have been found in a variety of TNF-α involved inflammatory disorders (27-29). TNF-α itself can also augment the production of the sTNFRs (30). In the current study, NAC was found to suppress the production of sTNFRs by AM to the same extent as the NAC-induced suppression of TNF-α production.

TGF-β mediates remodeling of lung tissue and fibrosis. TGF-β1 stimulates fibroblast differentiation to the myofibroblast phenotype and suppresses myofibroblast apoptosis (31). TGF-β is the most potent direct stimulator of procollagen production in vitro (32); it can also induce the transcription and synthesis of various other components of the extracellular matrix (33-34). In addition to promoting the synthesis of matrix protein, TGF-β stabilizes the newly formed extracellular matrix proteins by inhibiting their degradation. Macrophages are an important source of TGF-β in chronic lung diseases, and TGF-β is also a chemoattractant for monocytes and macrophages (35). Inhibition of TGF-β1 arrests or decreases lung fibrosis. Anti-TGF-β1 antibodies reduce fibrosis in bleomycin-induced lung fibrosis (36-37). Intratracheal administration of a soluble TGF-β type II receptor significantly reduced bleomycin-induced fibrotic lung histopathogy and hydroxyproline accumulation in hamsters (38). An increased expression of TGF-β messenger RNA and protein has been documented in the lungs of IPF patients (39). In the current study, NAC partially suppressed the TGF-β1 production from AM of IPF patients in vitro.

IL-1β is a product of activated macrophages and has potent inflammatory effects which overlap with those of TNF. Early studies reported the expression of IL-1β in alveolar macrophages from the lungs of patients with IPF (40-41). Studies on animal models have confirmed the role of IL-1β in pulmonary tissue injury and repair. In a rodent in vivo lung injury model, transient overexpression of IL-1β in lung epithelial cells caused acute inflammation and tissue destruction, followed by production of fibrogenic cytokines, such as TGF-β, and progressive interstitial fibrosis (42). As shown, IL-1β also exerts its profibrotic effects by inducing the expression in lung fibroblasts of osteopontin, a multifunctional matrix cellular protein up-regulated in IPF as well as in bleomycin-induced fibrosis (43). Elevated expression of IL-1β has been detected in cultured AM supernatants in IPF patients and in bleomycin-induced pulmonary fibrosis in rat (44-45). Inhibition of IL-1β in animal models of fibrosis results in milder disease (46), in theory suggesting the rationale of inhibiting IL-1 in IPF. A NAC-induced down-regulation of IL-1 / IL-1β release from alveolar epithelium has been reported previously (47). In the present study we found that NAC partially suppressed the production of IL-1β from AM, especially when stimulated by LPS, in IPF patients.

This study has some limitations. First, since we did not have a control group, it remains unclear whether our observations are unique for IPF derived AM. This needs to be clarified in further studies. Second, the mechanism of these anti-inflammatory in vitro effects of rather high NAC concentrations remains obscure. The concentrations of NAC used in our in vitro experiments do not correspond to the clinical situation where NAC is rapidly metabolized. We did not evaluate whether ex-vivo cultured AM are able to metabolize NAC and to synthesize increased amounts of GSH. This is considered to be the major mode of action of NAC as an antioxidant in vivo. Therefore it would be premature to explain the clinical effects of NAC in IPF (7, 8) by our in vitro experiments.

In conclusion, this study reveals evidence of NAC to down-regulate the production of TNF-α and their soluble receptors, as well as TGF-β1 and LPS -stimulated IL-1β, by AM in IPF in vitro. This indicates that NAC may have anti-inflammatory and anti-fibrotic effects.

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