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Effect of *N*-acetyl-L-cysteine on peroxynitrite and superoxide anion production of lung alveolar macrophages in systemic sclerosis

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Abstract

Lung macrophages may play a relevant role in oxidative processes producing both superoxide anion (O_2^-) and NO. In this view, an antioxidant therapy can be useful in the treatment of systemic sclerosis (SSc) patients. *N*-Acetylcysteine (NAC) is able to expand natural antioxidant defenses by increasing intracellular gluthatione concentration and it has been proposed as an antioxidant therapy in respiratory distress syndromes. The aim of our study was to determine whether lung macrophages obtained from SSc patient bronchoalveolar lavage (BAL) express the inducible form of nitric oxide synthase (iNOS) and whether NAC can reduce the peroxynitrite ($ONOO^-$) and O_2^- production of these cells. Alveolar macrophages were isolated from BAL of 32 patients and used for the immunocytochemical determination of iNOS, and the production of $ONOO^-$ and O_2^- was measured by fluorimetric or spectrophotometric methods, respectively. Lung macrophages obtained from SSc patients expressed a higher level of iNOS compared to healthy subject cells. NAC preincubation (5×10^{-5} M, 24 h) significantly reduced (−21%) the $ONOO^-$ production in formyl Met-Leu-Phe (fMLP)-activated cells and slightly reduced it under resting conditions, whereas NAC preincubation was unable to modify the release of O_2^- both in basal condition and in fMLP-stimulated cells. We conclude that since SSc lung macrophages express high levels of iNOS and produce a significant quantity of $ONOO^-$, NAC administration reduces $ONOO^-$ production and can be an useful treatment to alleviate SSc symptoms.

Keywords: Systemic sclerosis; Inducible nitric oxide synthase; *N*-acetylcysteine; Peroxynitrite; Superoxide anion; Alveolar macrophages

Nitric oxide (NO)[1] is physiologically produced by constitutive nitric oxide synthase isoforms (cNOS) and in several pathophysiological conditions by an inducible nitric oxide synthase isoform (iNOS). In systemic sclerosis (SSc) patients, NO is increased in exhaled air [1,2] while it is decreased in those patients with pulmonary hypertension [2,3]. Moreover, serum NO levels are enhanced [4], iNOS is highly expressed in mononuclear cells infiltrating the skin [4] and peripheral blood mononuclear cells produce increased NO levels when stimulated with IL-1 ß [5]. NO can be synthesized by different kinds of cells. Among these cells, macrophages play a pivotal role in the inflammatory infiltrate and, in patients with tubercolosis, iNOS is expressed in alveolar macrophages [6]. Inflammatory cells produce reactive oxygen species (ROS) that, reacting with NO, form tissue-damaging NO-derived inflammatory oxidants. ROS

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[1] *Abbreviations used:* NO, nitric oxide; NOS, nitric oxide synthase; SSc, systemic sclerosis; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; BAL, bronchoalveolar lavage; FCS, fetal calf serum; LPS, lipopolysaccharides; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; fMLP, formyl Met-Leu-Phe; SOD, superoxide dismutase.

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are considered toxic for many tissues. Among ROS, NO-derived oxidant species such as peroxynitrite [7] and hypochlorous derivatives [8] possess strong damaging properties responsible for epithelial shedding, airway hypereactivity, nerve sensitization, plasma exudation, and tyrosine nitrosylation [9]. In SSc, a ROS involvement in the disease pathogenesis has been suggested [10,11]. Indeed, in SSc the increased serum levels of antibodies against oxidized low-density lipoproteins reveal a generalized oxidative stress [12] and peripheral blood monocytes obtained from SSc produce a large amount of O_2^- anion [13] and NO [5,14]. In vitro, $ONOO^-$ production induced by combined hyperoxia and NO is cytotoxic on human alveolar epithelial and lung microvascular endothelial cells [15] and the expression of both nitrotyrosine and iNOS as a marker of $ONOO^-$ production is increased in lung macrophages, neutrophils, and alveolar epithelial cells of patients with idiopathic pulmonary fibrosis compared to healthy subjects [16]. Therefore, the toxicity of NO-derived oxidant species might be responsible for the inflammatory lung damage observed in SSc patients. Lung macrophages may produce both O_2^- anion and NO that can react together to form $ONOO^-$. In this view, an antioxidant therapy can be useful in the treatment of SSc patients [17]. *N*-Acetylcysteine (NAC) is able to expand natural antioxidant defense by increasing intracellular reduced gluthatione (GSH) concentration [18,19] and it has been proposed as an antioxidant therapy in respiratory distress syndromes [20]. Effectiveness of NAC in reducing the exacerbation of severe chronic obstructive pulmonary disease has been documented [21] and, more recently, in SSc, NAC intravenous administration has been shown to reduce significantly the number of Raynaud's phenomenon attacks [22]. In an early report, the effect of NAC (administered for 5 days) on the O_2^- anion production of rat alveolar macrophages and human peripheral neutrophiles was evaluated [23]. However, although the *in vivo* NAC treatment increases the GSH level both in human plasma and in rat bronchoalveolar lavage (BAL), the NAC effectiveness is poor in scavenging O_2^- anion [23]. According to recent data, NAC can prevent peroxynitrite-mediated oxidation in a model of ischemia–reperfusion in the rat [24] and directly reduces $ONOO^-$ formation in rat macrophages obtained from the pleural cavity in a model of carrageenan-induced pleurisy [25]. Moreover, in an acute model of renal failure produced by inferior vena cava occlusion the protective effect of NAC is dependent on the scavenging of $ONOO^-$ and is observed only when NO is physiologically produced, since the N^G-nitro-L-arginine methyl ester NOS inhibitor abolishes it [26]. Therefore, the aim of our study was to determine the iNOS expression and the effectiveness of NAC treatment in the reduction of the $ONOO^-$ and O_2^- anion production in lung alveolar macrophages obtained from BAL of SSc patients.

Methods

Patients

Lung alveolar macrophages were obtained from 27 female and 5 male patients (mean age 50.5 ± 0.6 years) affected by SSc (12 patients with cutaneous diffuse SSc and 20 with cutaneous limited SSc) with an impairment of the lung diffusion of CO (Dlco) at pulmonary function tests, an increased velocity of 99mTc DTPA pulmonary clearance rate at the alveolar membrane [27], and ground glass without reticular pattern at chest high-resolution computed tomography [28] demonstrating a lung involvement. These patients were chosen since they were considered to be in an early phase of the disease and pulmonary fibrosis was not evident. Cardiac echocolordoppler excluded the presence of pulmonary hypertension. All patients underwent BAL as a procedure for determining the presence of an active process of alveolar inflammation. According to the results of BAL cellularity, patients analyzed for iNOS expression were divided into BAL positive (>8% lymphocytes) or BAL negative (<8% lymphocytes) alveolitis. Two healthy subjects undergoing BAL for a suspected lung cancer that was demonstrated to be negative afterward were used as controls. Due to the paucity of macrophage cell recovery in the BAL, experiments investigating iNOS expression and $ONOO^-$ and O_2^- anion production were performed on different subjects. Immunocytochemical determination of iNOS was performed on 11 patients (5 with cutaneous diffuse, 6 with cutaneous limited SSc), $ONOO^-$ anion measurements were performed on 10 patients (four with cutaneous diffuse SSc and 6 with cutaneous limited SSc), and O_2^- anion measurements were performed on 11 patients (three with cutaneous diffuse SSc and eight with cutaneous limited SSc). However, in these three groups of patients, the main clinical characteristics and the age and sex distribution were homogeneous.

Alveolar macrophage isolation and preparation

After premedication with atropine and anesthetics, a bronchoscope was introduced into airways and 200 ml of sterile saline solution was injected into the lobe affected by ground glass. From the recovered fluid, macrophages were prepared by centrifugation at 700*g* for 10 min. The pellet was resuspended in RPMI 1640 medium containing antibiotics (penicillin 100 U/ml and streptomycin 0.1 mg/ml) and 10% fetal calf serum (FCS). The cell suspension was plated in Labtek chambers at a concentration of 1×10^4–10^5 cells/well for iNOS expression and cytocharacterization. In parallel experiments for iNOS expression, cells of one healthy subject and from guinea pig BAL [29] were treated with 10 μg/ml lipopolysaccharides (LPS), a known up-

regulator of iNOS expression in macrophages. After 2 h, unattached cells and debris were removed by washing, media were changed, and macrophages were used 24 h after plating as described. The macrophage cytocharacterization performed by staining the endogenous peroxidase with 3,3′-diaminobenzidine tetrahydrochloride–H_2O_2 indicated the presence of adherent macrophages ranging from 78% to 89%.

iNOS expression

Macrophages were washed and fixed by short immersion (5 min) in cold acetone. The expression of iNOS was detected by incubating cells overnight at room temperature with the rabbit iNOS primary polyclonal antibody used at a 1:100 dilution in phosphate-buffered (PBS) saline containing 0.3% Triton X-100, 2% normal goat serum, and 0.5% bovine serum albumine (BSA). On the following day, specimens were washed and incubated for 1 h at room temperature with fluorescein-conjugated (FITC-conjugated) secondary antibody diluted 1:40 in PBS–0.5% BSA and 2% normal goat serum. Analysis was performed by imaging analysis system.

Fluorescence intensity (excitation 480 nm, emission 520 nm) was quantified in the single cell using an analogical/digital converter recognizing 256 gray levels (Magiscan, Applied Imaging) and Tardis software and was expressed as arbitrary units. Negative control for the immunostaining was obtained either by omission of the primary antibody or by use of bNOS as the primary antibody. The fluorescence of negative controls was subtracted by immunostained specimens as background fluorescence level. However, the negative control fluorescence was negligible as compared to that of the specimens (less than 1/10th). Thirty to thirty-five cells showing the typical round aspect of macrophages from subjects were analyzed and the fluorescence intensity for each subject was calculated as the mean of analysed cells. LPS-treated macrophages showed a significant increase in fluorescence ($p < 0.001$) of 1.6- and 1.5-fold compared to unstimulated human and guinea pig cells, respectively. In several experiments, 10 μM dexamethasone was added soon after centrifugation and maintained during the 24-h incubation. No significant differences in iNOS immunostaining were detected in cells incubated either in the absence or in the presence of dexamethasone, ruling out the possibility that iNOS expression may be induced during the 24-h incubation time.

Peroxynitrite anion assay

Macrophages were plated in six multiwell plates (10^6 cells/well) and washed after 2 h. Adherent macrophages were allowed to stay in the absence (control) or presence of 5×10^{-5} M NAC for 24 h. $ONOO^-$ production was evaluated by fluorimetric measure of oxidized dihydrorhodamine-123 [30] after a 60-min incubation of 10^6 macrophages under resting conditions and after stimulation with 10^{-8} M fMLP in the presence or absence of 5×10^{-5} M NAC. A blank specimen was obtained by carrying out a contemporaneous determination in the presence of either superoxide dismutase (SOD) (to quench O_2^- anion) or 1400 W, an inhibitor of iNOS [31]. No significant differences between fluorimetric measures obtained in the presence of 1400 W and SOD were observed. Therefore, this value was subtracted as an aspecific reaction of dihydrorhodamine-123.

Nitrite assay

To test whether NAC can interfere with NO production, nitrite concentration was measured using a standard Griess micromethodology as described [32]. Guinea pig lung macrophages were used for this test as described [29]. Cells were plated in six multiwell plates (10^6 cells/well) in phenol red-free RPMI 1640 medium with a composition similar to that above in the presence or absence of 5×10^{-5} M NAC, and nitrite production was measured after 24-h incubation on cell supernatant. In parallel experiments, cells were treated with 10 μg/ml LPS in the presence or absence of 5×10^{-5} M NAC for 24 h and washed, and nitrite production was assessed under resting conditions and after stimulation with 10^{-8} M fMLP in the presence or absence of 5×10^{-5} M NAC.

Superoxide anion assay

Macrophages were plated in six multiwell plates (10^6 cells/well) as described (see peroxynitrite anion assay). O_2^- production was evaluated by SOD-inhibitable reduction of ferricytochrome *c* [28] after a 60-min incubation of 10^6 macrophages under resting conditions and after stimulation with 10^{-8} M fMLP in the presence or absence of 5×10^{-5} M NAC in phosphate-buffered saline. To evaluate fMLP-induced O_2^- production, values obtained under resting conditions under different treatments were subtracted.

Statistical methods

Values are presented as means ± SE of analyzed subjects. Statistical comparisons between data groups were performed using Student's *t* test (paired and independent). A $p < 0.05$ was considered statistically significant.

Materials

fMLP, penicillin–streptomycin (antibiotic solution), SOD, NAC, dihydrorhodamine-123, secondary anti-rabbit

FITC conjugate monoclonal antibodies, and ferricytochrome *c* were purchased from Sigma–Aldrich s.r.l. (Milan, Italy); RPMI 1640 medium and FCS were obtained from Gibco BRL-Life Technologies (Milan, Italy) and 1400 W, iNOS, and bNOS from Calbiochem (La Jolla, CA). Cell culture plastic supports were purchased from Costar (Corning Costar Co., Costar Italia, Milan, Italy). All other reagents were of analytical grade.

Results

All the SSc patients showed a ground glass appearance at high-resolution computed tomography of the lung without reticular pattern. The BAL cellularity, performed in the affected lobe, identified two groups of patients, one with an increased number of lymphocytes (BAL positive) and the other with a normal number of lymphocytes (BAL negative).

In both groups, all macrophages immunostained with the primary antibody showed a detectable level of fluorescence for iNOS. No difference was found in the two groups, the group with a positive BAL being insignificantly higher than that with a negative BAL. However, as shown in Fig. 1, lung macrophages obtained from SSc patients expressed a higher level of iNOS than those from healthy subjects.

No correlations between iNOS expression and lymphocytes or other bronchoscopic and clinical parameters were found.

Since the iNOS expression was high in patient lung macrophages, we investigated the cell production of $ONOO^-$ and O_2^- anions and the effect of NAC on these levels. As shown in Figs. 2A and B the basal production

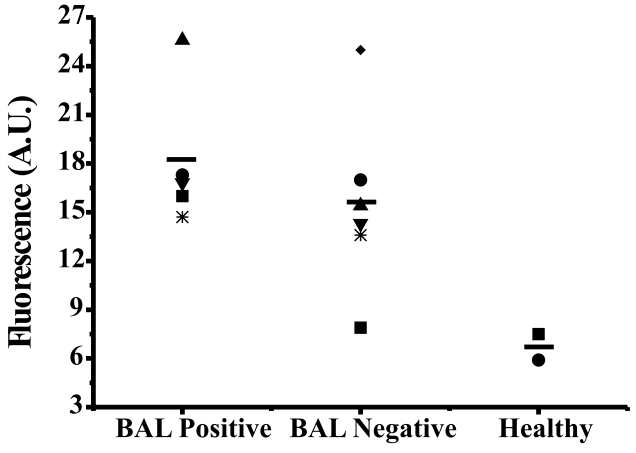

Fig. 1. iNOS expression in lung macrophages obtained by bronchoalveolar lavage from SSc patients with (BAL Positive, $n=5$) or without (BAL Negative, $n=6$) alveolitis and healthy subjects ($n=2$). Each point represents the immunostaining fluorescence mean of 30–35 cells from the same specimen quantified by imaging analysis. Bars are the mean of each groups. Vertical scale: fluorescence, arbitrary units.

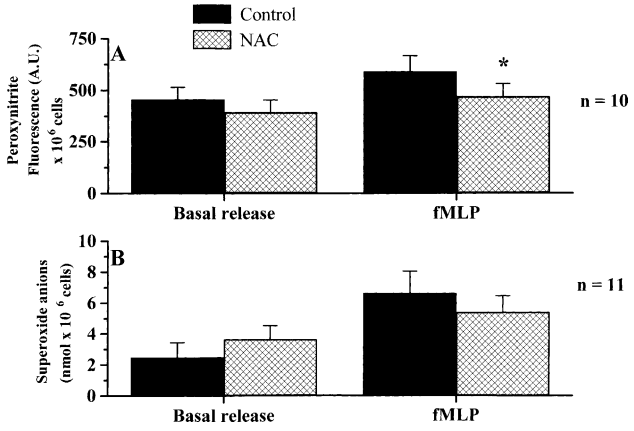

Fig. 2. Effect of NAC (5×10^{-5} M, 24 h) on the release of peroxynitrite ($ONOO^-$, A) and superoxide anion (O_2^-, B) in lung macrophages obtained by bronchoalveolar lavage from SSc patients. Values are the mean (±SE) of the indicated number of experiments; $^*p < 0.05$.

of $ONOO^-$ and O_2^- anions was significantly increased after stimulation with 10^{-8} M fMLP, a dose that, according to preliminary data obtained in alveolar lung human macrophages and our previous works on guinea pig lung macrophages [29], was maximal. The $ONOO^-$ and O_2^- anion levels in both groups of patients did not correlate with lymphocytosis or other bronchoscopic and clinical parameters. NAC preincubation (5×10^{-5} M) significantly reduced the $ONOO^-$ production in fMLP-activated cells and slightly reduced it under resting conditions (Fig. 2A). An NAC inhibitory effect was observed only when NAC preincubation lasted overnight, while 1-h preincubation was ineffective. On the other hand, 5×10^{-5} M NAC preincubation was unable to modify the release of O_2^- anion, both under resting conditions and in fMLP-stimulated cells, although in fMLP-stimulated cells NAC slightly decreased it (Fig. 2B).

To test whether NAC can directly interfere with NO production, we measured nitrite production of guinea pig macrophages. The preincubation with 5×10^{-5} M NAC reduced nitrite production in the 24-h cell supernatant from $4.5 \pm 0.56\,\mu M$ (control) to $1.2 \pm 0.22\,\mu M$ (NAC, $p < 0.001$, paired Student's *t* test, $n=4$). Moreover, NAC reduced nitrite production from $4.9 \pm 0.73\,\mu M$ (control) to $3.6 \pm 0.49\,\mu M$ (NAC, $p < 0.05$, paired Student's *t* test, $n=4$) in fMLP-stimulated LPS-pretreated guinea pig lung macrophages, whereas NAC was unable to reduce nitrite production in LPS-pretreated unstimulated cells ($4.1 \pm 0.58\,\mu M$ control vs $3.3 \pm 0.39\,\mu M$ NAC, $n=4$).

Discussion

Our data clearly demonstrate that SSc lung macrophages express high levels of iNOS and produce a

significant quantity of $ONOO^-$ and O_2^- anions. However, since no correlation among lung inflammatory parameters and iNOS expression has been found, the higher iNOS expression in SSc might be considered an early but aspecific pathological sign suggesting that previous lung inflammation (ground glass without increased cellularity) or current lung inflammation (ground glass with increased cellularity), priming macrophages for iNOS expression, may be an aspecific event secondary to the process of systemic inflammation. The up-regulation of iNOS in macrophages might contribute to the increased levels of exhaled NO [1,2]. Our data also demonstrate that alveolar macrophages are not functionally impaired since fMLP can increase both $ONOO^-$ and O_2^- anion production. The NO production together with the concomitant release of O_2^- anion and hypochlorous acid may determine high local concentrations of reactive NO-derived, very potent inflammatory oxidants. This process can greatly contribute to the inflammatory process and enhance tissue damage leading to alveolar injury.

The data obtained with NAC preincubation are in agreement with the work previously performed by Sambo et al. [13] on SSc circulating monocytes. In our work,NAC preincubation reduces $ONOO^-$ anion release without affecting O_2^- anion production after an in vitro overnight treatment of SSc macrophages. These data are in agreement with early reports showing similar NAC effects on different cell types [23–25] and in vivo [26].

According to the current literature, NAC can exert its beneficial action on inflammation and inflammatory-linked tissue damage through several mechanisms. In our in vitro experiments it seems likely that NAC can reduce $ONOO^-$ formation by reducing nitrite production, since in experiments performed on guinea pig lung macrophages, NAC can directly reduce nitrite formation. This effect may be dependent on a direct reaction of NAC thiol group and NO, thus producing a nitrothiol compound. The formation of these nitrothiol compounds can preserve and accumulate NO in a biologically active form. A similar mechanism could explain the NAC protective effect described by Conesa and coauthors in their acute renal failure in vivo model [26] in which the presence of NO is essential for attempting NAC beneficial effect. On the other hand, NAC is unable to modify the O_2^- production, ruling out a scavenging effect on this ROS. Moreover, no significant differences between iNOS expression levels have been detected during the incubation time in the control condition, ruling out a possible NAC effect on iNOS induction.

Although we have tested the in vitro NAC effect, it is probable also that in vivo NAC therapeutical administration may reduce $ONOO^-$ formation by alveolar macrophages, not only in SSc patients, but also in other pulmonary pathologies.

Therefore, NAC by its reducing effect on $ONOO^-$ might become a useful antioxidant therapy not only by systemic infusion [22] but also by aerosol delivery in pulmonary airways, synergizing other therapies in the treatment of SSc patients.

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