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Thiol regulation of the production of TNF-α, IL-6 and IL-8 by human alveolar macrophages

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Thiol regulation of the production of TNF-α, IL-6 and IL-8 by human alveolar macrophages. P. Gosset, B. Wallaert, A.B. Tonnel, C. Fourneau. ©ERS Journals Ltd 1999.
ABSTRACT: Reactive oxygen intermediates exert signalling functions and modulate gene transcription, particularly for pro-inflammatory cytokines. Since exogenous as well as endogenous thiols could be potent inhibitors of the production of cytokines, the effects of *N*-acetylcysteine (NAC), glutathione (GSH) and modulated GSH synthesis on the production of tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8 by human alveolar macrophages (AMs) was evaluated, as well as the potential role of intracellular GSH depletion on the effect of exogenous thiols.

AMs were stimulated with lipopolysaccharide (LPS) and cytokine production was measured by evaluating messenger ribonucleic acid (mRNA) expression and protein secretion.

Depletion of intracellular GSH by treatment with buthionine sulphoximine (BSO) reached 45.2% after 3 h and was nearly complete at 24 h. Whereas a 24-h preincubation of AMs with BSO significantly increased LPS-induced secretion of TNF-α and IL-8, a 3-h preincubation only enhanced LPS-stimulated production of IL-8 ($p<0.05$). Treatment with NAC and GSH did not significantly increase intracellular content of GSH even after a 48-h incubation. Addition of GSH and NAC significantly reduced the secretion of TNF-α (mean±SEM 21.2±5 and 44.7±4.4% inhibition, respectively) as well as LPS-induced IL-6 and IL-8 ($p<0.05$). Similarly, NAC inhibited the production of TNF-α, IL-6 and IL-8 in GSH-depleted AMs obtained by BSO pretreatment.

In conclusion, *N*-acetylcysteine and glutathione inhibit the production of tumour necrosis factor-α, interleukin-8 and interleukin-6 by alveolar macrophages by a mechanism independent of glutathione metabolism. However, total depletion of glutathione within alveolar macrophages significantly increases tumour necrosis factor-α and interleukin-8 synthesis whereas it does not modulate interleukin-6 secretion.
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In the respiratory tract, the alveolar macrophage (AM) is one of the main cells implicated in the host defence, a role assumed in part by its ability to secrete cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6. These cytokines control the inflammatory reaction as well as the induction of the immune response [1]. In addition, TNF-α plays a key role in host defence mechanisms directed against bacteria [2]. AMs also produce large amounts of IL-8, one of the main α-chemokines. IL-8 has been identified for its neutrophil chemotactic activity, but it also induces the migration of lymphocytes, basophils and activated eosinophils (reviewed in [3]). Altered production of these cytokines may be involved in various human diseases [2].

Host defense mechanisms also involve the generation of reactive oxygen intermediates (ROIs) during various electron-transfer reactions. At high concentrations, ROIs can damage cells by peroxidizing lipids and disrupting deoxyribonucleic acid (DNA) and proteins [4]. Indeed, they are implicated in the extensive tissue injury observed in different diseases [5, 6]. At moderate levels, ROIs may exert signalling functions and regulate the transcription of genes, particularly those involved in inflammatory processes [7, 8]. ROIs may act as a transduction signal in cells activated by an exogenous stimuli such as lipopolysaccharide (LPS), or directly induce cytokine synthesis *in vitro* [9]. In contrast, the pretreatment of mice with free radical scavengers such as *N*-acetylcysteine (NAC) or glutathione (GSH) inhibits LPS-induced production of TNF-α in serum and bronchoalveolar lavage (BAL) fluid [10, 11]. Other antioxidants also decrease the production of TNF-α, IL-1β and IL-6 by blood monocytes [12]. Although IL-8 synthesis by monocytes is also modulated by oxygen metabolites, its LPS-induced production is dependent on the generation of hydroxyl radicals, whereas TNF-α and IL-1β secretion is not [13]. NAC and GSH have been used in the treatment of human lung disorders to prevent lung damage [14, 15] and to facilitate the eviction of micro-organisms. However, the effect of GSH and NAC on the human lung inflammatory reaction has not been defined, although studies in animal models have suggested that they could inhibit this reaction [16, 17]. Treatment by NAC enhances the intracellular concentration of GSH in AMs exposed to oxidant stress [17]; however, the capacity of intracellular GSH to modulate the production of cytokines by macrophages

has not been demonstrated. In rat Kupffer cells, inhibition of GSH synthesis does not prevent the inhibition of TNF-α release by NAC [18]. For these reasons, this study measured the effect of variation in intracellular GSH and of exogenous thiols on the production of TNF-α, IL-6 and IL-8 by human AMs. The study analysed: 1) the effect of the depletion of GSH intracellular content using the inhibitor of GSH synthesis, buthionine sulphoximine (BSO); and 2) the role of GSH depletion in the modulation of cytokine production by exogenous antioxidants such as NAC and GSH. Cytokine messenger ribonucleic acid (mRNA) expression as well as cytokine synthesis by AMs were evaluated under these different conditions.

Materials and methods

Patients and bronchoalveolar lavage procedure

Informed consent for BAL was obtained from healthy subjects free of lung disease as judged by the following criteria: normal examinations and chest radiographs, normal pulmonary function tests and the absence of routine use of medication. The study was approved by the ethics committee of Lille (CCPRB No. 9307). Nineteen subjects (12 males and seven females, mean±SEM age 42.4±4.5 yrs) (12 smokers, seven nonsmokers) were studied. BAL was performed by the instillation of 250 mL saline solution into the bronchoalveolar tree under fibreoptic bronchoscopic observation. Total and differential cell counts were determined. AM viability was (mean±SEM) 92.3±0.9% as assessed by trypan blue exclusion. The percentages (mean±SEM) of macrophages, lymphocytes, neutrophils and eosinophils were 89.2±2.3, 8.6±2, 1.6±0.4 and 0.6±0.16%, respectively; epithelial cells were undetectable in the BAL fluid.

Alveolar macrophage isolation and culture

AMs were isolated by adherence as previously described [19]. Briefly, the lavage fluid was filtered through sterile surgical gauze and centrifuged at 400 × *g* for 10 min at 4°C. After three washings, the pellet was resuspended at a concentration of 1.5×10^6 cells·mL^{-1} in Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% heat inactivated foetal calf serum and 2 mM L-glutamine (Gibco BRL, Eragny, France). Endotoxin contamination of the medium was determined by the limulus amoebocyte test (Boehringer Ingelheim Bioproducts, Heidelberg, Germany) and was <50 pg·mL^{-1}. This quantity was insufficient to trigger TNF-α mRNA expression and secretion as previously demonstrated [19]. Cells were allowed to adhere to plastic Petri dishes (2 mL in a 35-mm diameter well) for 2 h at 37°C. The nonadherent cells were removed by three washings with RPMI. Adherent cells contained >95% AMs and <3% lymphocytes.

Alveolar macrophage activation

AMs were treated with oxygen radical scavengers such as NAC and GSH (Sigma Co., St Louis, MI, USA) at different concentrations (0.1, 1 and 10 mM for NAC and 1 mM for GSH). In other culture wells, 0.5 mM BSO was added, a concentration previously shown to be optimal for GSH inhibition [20]; BSO had no cytotoxic effect at this concentration as defined by lactate dehydrogenase (LDH) release (data not shown). The cultures treated with BSO, GSH and NAC were either activated simultaneously or pre-incubated at 37°C (for 3 and 24 h with BSO; for 24 h with GSH and NAC) before the stimulation. Control experiments were performed using cells in medium alone. In some experiments, the cells were preincubated for 3 and 24 h, after which they were washed and fresh medium was added. The activators of cytokine production used in this study included LPS type 055B5 (100 ng·mL^{-1}; Difco, Detroit, MI, USA), opsonized zymosan (0.2 mg·mL^{-1}; Sigma Co.) and phorbol myristate acetate (PMA) (100 nM; Sigma Co.). Depending on the number of AMs, different protocols were performed with the cells from one subject (namely, the effect of NAC and GSH and/or of BSO and/or of NAC on BSO-pretreated AMs). After stimulation, AMs were incubated for 24 h, and the supernatants collected and stored at -30°C until cytokine assays performed.

Measurement of glutathione levels

At the end of the incubation, adherent AMs were lysed in 2 mL of 0.05% v/v Triton-X-100 in buffer (125 mM sodium phosphate, 6.3 mM sodium ethylenediamine tetraacetic acid (Na-EDTA), adjusted to pH 7.5). Samples of the lysates (760 μL) were acidified with 40 μL of 0.1 N HCl. The proteins were precipitated by the addition of 40 μL 50% w/v sulphosalicylic acid and removed by centrifugation (1,000 × *g*, 10 min, 4°C) . The supernatants were assayed for total glutathione (reduced and oxidized GSH (GSSG)) by an enzymatic recycling procedure [21]. GSH was sequentially oxidized by 5-5′-dithiobis-2-nitrobenzoic acid (DTNB) and reduced by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of glutathione reductase at 30°C. Formation of 2-nitro-5-thiobenzoic acid was monitored at 6 min intervals by comparing the absorbance at 412 nm with that of a standard sample prepared under the same conditions. For the glutathione disulphide assay, 10 μL triethanolamine and 10 μL 2-vinylpyridine (all from Sigma Co.) were added to 200 μL supernatant of the same cellular sample. After 1 h incubation, the assay was the same as that for total glutathione. Results were expressed in ng·mL^{-1}.

Concentrations of GSH and GSSG in untreated AMs were 468±102 and 69.2±20 ng·mL^{-1}, respectively. Activation by LPS did not significantly modulate GSH concentrations.

Cytokine assays

TNF-α concentration was evaluated sequentially by the L929 cytotoxic test and an immunoenzymetric assay. Biological activity was estimated from a standard curve as the amount required to kill 50% of actinomycin-treated L929 mouse fibroblasts after 18 h of culture [22]. Subsequently, the TNF-α level was determined by the use of an immunoenzymetric assay (Medgenix, Fleurus, Belgium) after adequate dilution. A highly significant correlation was found between the two methods ($r=0.85$, $p<0.001$). The TNF-α concentration was expressed as ng·mL^{-1} as defined by the standards provided by the manufacturer.

IL-6 was assayed as described by VAN SNICK *et al.* [23] with some modifications. Briefly, the hybridoma cells 7TD1 (a generous gift from J. Van Snick, Institut Ludwig, Brussels, Belgium) were incubated with serial dilutions of cell supernatants in microtitre plates. After four days of incubation, the number of cells was evaluated by a colorimetric method. A 4-mg·mL^{-1} solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma Co.) was added at 10%. After a 4-h incubation, the MTT precipitate was dissolved by an acidified solution of sodium dodecyl sulphate (Sigma Co.). After homogenization, the optical density was read in a multiwell spectrophotometer (Anthos Labtec, Salzburg, Austria) at 570 nm with a reference at 650 nm. All analyses were performed in duplicate and concentrations were calculated by a probit analysis in comparison with a standard of recombinant human IL-6 (Boehringer Mannheim, Mannheim, Germany). The results were expressed in U·mL^{-1}. The specificity of the test was controlled by the inhibition of cell proliferation after addition of a neutralizing anti-IL-6 rabbit antibody (Endogen, Boston, MA, USA). In all cases, specific antibody addition to AM supernatants induced an inhibition >90%. GSH, NAC and BSO, at the concentrations used, did not interfere with this bioassay.

IL-8 concentration was measured using a sandwich immunoenzymetric assay (CLB, Amsterdam, the Netherlands). Samples were assayed after adequate dilution. The results were expressed in ng·mL^{-1}.

Expression of messenger ribonucleic acid encoding for cytokines

AMs were activated as previously described and ribonucleic acid (RNA) was collected 4 h after LPS stimulation. Total RNA was isolated using Trizol solution (Gibco BRL, Paisley, Scotland) as described by the manufacturer. The mRNA was reverse-transcribed to first strand complementary DNA (cDNA) using Moloney mouse leukaemia virus (MMLV)-reverse transcriptase (Gibco BRL) and oligo-deoxythymidine (dT) primers (Boehringer). The first strand cDNA was amplified with primer sets for reduced glyceraldehyde-phosphate dehydrogenase (GAPDH), TNF-α, IL-6 and IL-8 (Eurogentec, Seraing, Belgium) in a 25 µL reaction using *Taq* polymerase (Perkin Elmer, Foster City, CA, USA), $MgCl_2$, deoxyribonucleoside triphosphate (dNTP) and 10-fold concentrated buffer. Nucleotide sequences for the oligonucleotide 5′- and 3′-primers are shown in table 1.

A 21-cycle and a 22-cycle polymerase chain reaction (PCR) was performed for GAPDH and cytokines, respectively, in a DNA thermal cycler (Mastercycler 5330; Eppendorf, Hinz, Germany) using a denaturation step of 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The number of cycles was defined by a kinetic experiment and gave a linear dose-response curve. After PCR, a 10-µL aliquot of the PCR products was electrophoresed on a 1.5% agarose-ethidium bromide gel. The optical density of each band was measured using a video-camera coupled with a computer equipped with the Bio-Profil software package (Vilber Lourmat, Marne la Vallée, France). Results for cytokines were expressed as a ratio with the corresponding GAPDH optical density.

Statistical analysis

The number of experiments was equal to the number of subjects from which AMs were used. To evaluate the effect of NAC, GSH and BSO on cytokine production, the results were also expressed as per cent change compared with the control (% of control value). Results were expressed as mean±SEM for the % of control value or as median with interquartile ranges for the net values. Statistical analyses were performed using Wilcoxon sign rank test for paired observations.

Results

Depletion of intracellular glutathione

GSH and GSSG contents were not altered in AMs incubated in medium alone. Treatment with BSO did not increase LDH release in comparison with stimulated or unstimulated AMs. AMs were then cultured for 24 h and the supernatants were collected for measurement of cytokine levels. BSO quickly depleted the intracellular content of GSH (fig. 1). After 3 h incubation with BSO, the concentration of GSH was reduced by 45.2±10% and a 90% decrease was observed after 24 h incubation. When the incubation was continued for 48 h without BSO, AMs partially reconstituted their GSH content.

Unstimulated AMs released low levels of TNF-α, IL-6 and IL-8 (0.35±1.5 ng·mL^{-1}, 256±724 U·mL^{-1} and 94.5±76 ng·mL^{-1}, respectively) in culture supernatants; LPS activation significantly increased their secretion (116.5±241 ng·mL^{-1}, 26,015±28,895 U·mL^{-1} and 3,393±4,520 ng·mL^{-1}, respectively) ($p<0.05$). The production of cytokines by AMs from smokers compared to nonsmokers did not differ significantly. Addition of BSO at the time of LPS stimulation (time=0) did not significantly alter the secretion of cytokines (fig. 2). When AMs were preincubated for 3 h with BSO, only IL-8 synthesis was increased ($p<0.05$). When LPS activation was performed after 24 h of treatment with BSO, a significant increase of TNF-α and IL-8 secretion was detected (fig. 2, $p<0.05$), whereas IL-6 synthesis was not altered. This effect was not dependent of the presence of BSO which was not added to

Table 1. – Nucleotide sequences of the 5′ and 3′ oligonucleotide primers used in the study

Target gene	5′ primer	3′ primer
GAPDH	GTCTTCACCACCATGGAGA	CCAAAGTTGTCATGGATGACC
TNF-α	ACAAGCCTGTAGCCCATGTT	AAAGTAGACCTGCCCAGACT
IL-6	TCAATGAGGAGACTTGCCTG	GATGAGTTGTCATGTCCTGC
IL-8	TTGGCAGCCTTCCTGATT	AACTTCTCCACAACCCTCTG

Primers are written in the 5′→3′ direction. GAPDH: reduced glyceraldehyde-phosphate dehydrogenase; TNF-α: tumour necrosis factor-α; IL: interleukin.

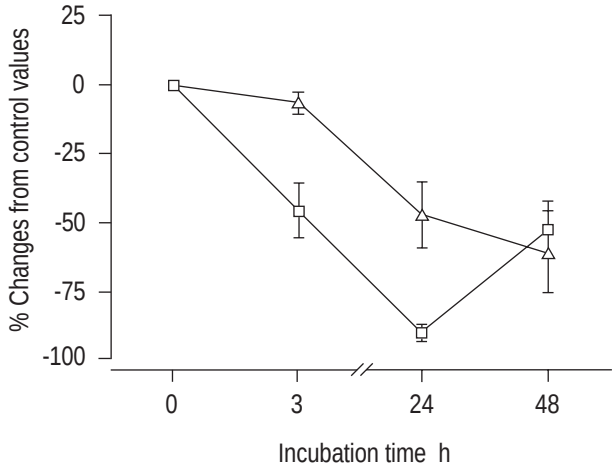

Fig. 1. – Modulation of intracellular concentration of reduced (□) and oxidized (Δ) glutathione in alveolar macrophages incubated with buthionine sulphoximine (added at time 0) for 3 and 24 h (n=9). The cells were washed at 24 h and incubated in medium alone until 48 h. Glutathione was evaluated at time 0, 3 h, 24 h and 48 h and the results expressed as the per cent change (±SEM) from control values (at time 0).

the culture medium between 24 and 48 h. Therefore, complete depletion of the GSH intracellular reserve increased the production of TNF-α and IL-8, induced by LPS activation whereas partial decrease of this content only modified IL-8 secretion.

Effect of glutathione and N*-acetylcysteine on the production of cytokines by alveolar macrophages*

NAC and GSH (1 mM) totally inhibited anion superoxide and hydrogen peroxide generation by PMA-stimulated AMs. At 0.1 mM, NAC had no significant effect on cytokine production, whereas 10 mM NAC was more potent than 1 mM (fig. 3). However, NAC at 10 mM had a

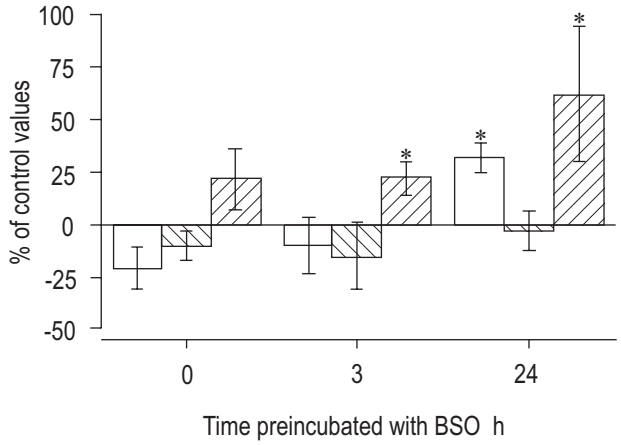

Fig. 2. – Modulation of alveolar macrophage (AM)-derived cytokine secretion after treatment with buthionine sulphoximine (BSO) (n=16). Tumour necrosis factor-α (□), interleukin (IL)-6 (▨) and IL-8 (▨) secretion was determined 24 h after stimulation of AMs with lipopolysaccharide (LPS). LPS was added at 0, 3 and 24 h after preincubation of the AM cultures with BSO. Results are expressed as the per cent change (±SEM) from control values (LPS stimulated AMS at 0, 3 and 24 h without BSO preincubation). *: p<0.05, compared with LPS-activated AMs.

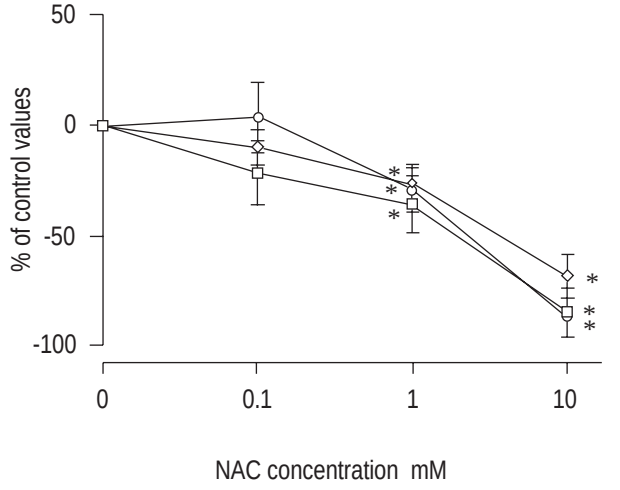

Fig. 3. – Effect of *N*-acetylcysteine (NAC) on tumour necrosis factor-α (□), interleukin (IL)-6 (◇) and IL-8 (○) production by lipolysaccharide (LPS)-stimulated alveolar macrophages (AMs) (n=10). Concentrations of cytokines were determined in LPS-activated AMs 24 h after incubation with the different concentrations of NAC. Results are expressed as the per cent change (±SEM) from controls (LPS-activated AMs). *: p< 0.05, compared with controls.

cytotoxic effect on AMs as shown by LDH release (data not shown). Thus, all the further experiments were performed with 1 mM of both antioxidants. NAC and GSH had no significant effect on intracellular GSH concentration (7.4±3.5 and 27.8±20.5% of control values, respectively), whereas only the addition of GSH significantly increased the level of GSSG (p<0.05) (-10.1±6.1 and 35.7±9.7% of control values, respectively).

Treatment with GSH and NAC (at time=0) significantly decreased TNF-α secretion by unstimulated (-30.5±10.5 and -34.7±7.2% of control values, respectively) and LPS-activated AMs (-21.2±5 and -44.7±4.4% of control values, respectively) (fig. 4). GSH and NAC did not significantly modify IL-6 and IL-8 secretion by unstimulated AMs

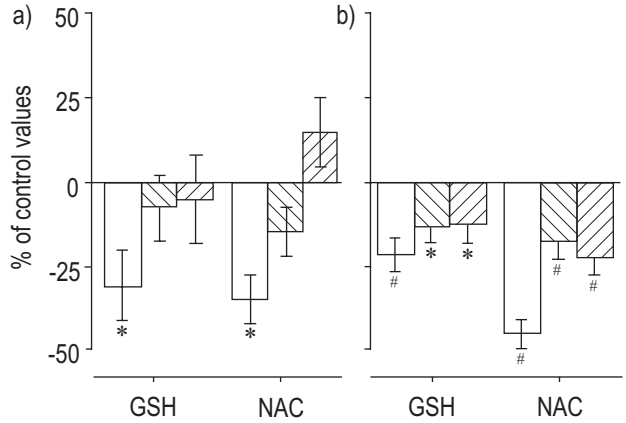

Fig. 4. – Effect of glutathione (GSH) and *N*-acetylcysteine (NAC) on cytokine secretion by a) unstimulated alveolar macrophages (AMs) and b) lipopolysaccharide (LPS)-stimulated AMs. Concentrations of tumour necrosis factor-α (□), interleukin (IL)-6 (▨) and IL-8 (▨) were determined in AM supernatants after 24 h incubation with GSH or NAC. Results are the per cent change (±SEM) from the corresponding controls (unstimulated and stimulated AMs without incubation with GSH or NAC). *: p<0.05 compared with unstimulated and LPS-activated AMs; #: p<0.01 compared with LPS-activated AMs.

(fig. 4), but significantly inhibited the production of IL-6 (-12.8±4.9 and -17.1±5.5% of control values, respectively) and IL-8 (-11.9±6.1 and -21.9±5.9% of control values, respectively) by LPS-stimulated AMs ($p<0.05$). In contrast, oxidized GSH (GSSG) did not modify the production of the three cytokines (data not shown).

Quantification of GSH and GSSG content in AMs showed that incubation for 48 h with GSH and NAC did not alter the intracellular concentrations in GSH (13±10 and 8.1±6.4% of control values, respectively) and GSSG (1.8±5.2 and -15.1±12.9% of control values, respectively). Evaluation of cytokine production by AMs preincubated with GSH and NAC for 24 h showed that there was no modulation of TNF-α, IL-6 and IL-8 secretion measured after 48 h (for NAC, -0.4±15, 3.6±13 and -2.2±12% of control values, respectively). Readdition of GSH or NAC between 24 and 48 h restored their inhibitory effect on cytokine synthesis suggesting that their presence was necessary for their action (data not shown).

Activation of alveolar macrophages by different stimuli: effect of N*-acetylcysteine and glutathione*

Opsonized zymosan and PMA increased cytokine secretion by AMs compared with cells in medium alone ($p<0.05$), however the levels of cytokines were significantly lower compared with LPS-stimulated AMs ($p<0.05$). Whereas GSH did not significantly modify the cytokine secretion induced by PMA and zymosan, addition of NAC decreased the production of TNF-α, IL-6 and IL-8 with both stimuli (fig. 5) ($p<0.05$, except for the effect of NAC on IL-6 production for which the difference was not significant). The level of the NAC inhibitory effect on zymosan and PMA-induced cytokine production ranged 17–34%.

Effect of N*-acetylcysteine on cytokine production by glutathione-depleted alveolar macrophages*

AMs pretreated with BSO for 24 h spontaneously repleted, between 24 and 48 h, their stock of GSH to ~50% of the control value (fig. 1). Addition of NAC between 24 and 48 h to GSH-depleted AMs did not significantly increase the GSH concentration compared to those cells incubated in medium alone (data not shown).

As described above, 24-h preincubation with BSO induced an increase of TNF-α and IL-8 secretion by LPS-stimulated AMs. NAC inhibited the LPS-induced production of TNF-α, IL-6 and IL-8 at a similar level in AMs pretreated with BSO (-31.2, -34.6 and -53.2% of control values, respectively) or not (-33.7, -36.4 and -50.4% of control values, respectively) (fig. 6). These data demonstrated that NAC also inhibited cytokine secretion by GSH-depleted AMs.

Effect of antioxidants on cytokine synthesis

In some experiments (n=4), mRNA expression for cytokines was evaluated by semi-quantitative PCR analysis with GAPDH mRNA as a reference. Treatment of LPS-activated AMs with NAC significantly inhibited mRNA expression for TNF-α, IL-6 and IL-8, whereas GSH only downregulated TNF-α mRNA expression (fig. 7). Levels of mRNA inhibition were comparable to those observed for cytokine secretion with NAC (-22.4, -22 and -20% of control values for TNF-α, IL-6 and IL-8, respectively). In contrast, BSO-pretreatment for 24 h did not significantly modulate cytokine mRNA expression in the presence of LPS.

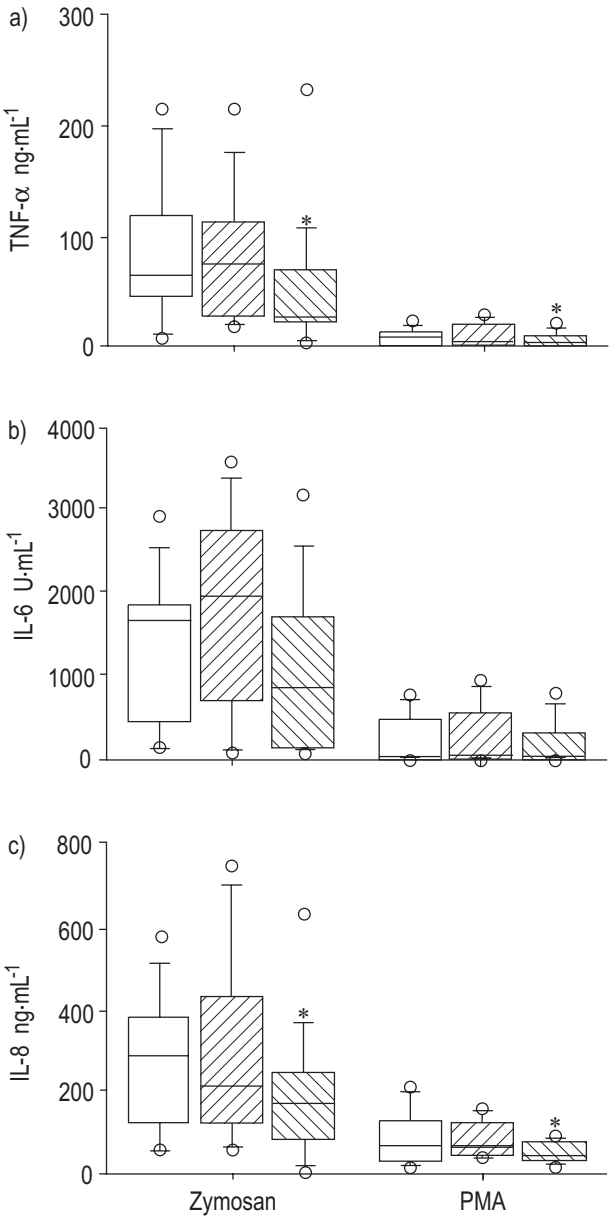

Fig. 5. – Effect of glutathione (▨) and *N*-acetylcysteine (▩) on the production of tumour necrosis factor-α (a), interleukin (IL)-6 (b) and IL-8 (c) by opsonized zymosan- and phorbol myristate acetate (PMA)-stimulated alveolar macrophages (AMs) compared to nonstimulated control (□) AMs. (n=9). Antioxidants and activators were added simultaneously; cytokine secretion was evaluated after 24 h. Box plots with horizontal lines represent the median, columns the 25th and the 75th percentiles, the error bars the 10th and the 90th percentiles. ○: outliers below 10th and above 90th percentiles; *: $p<0.05$ compared with the corresponding control.

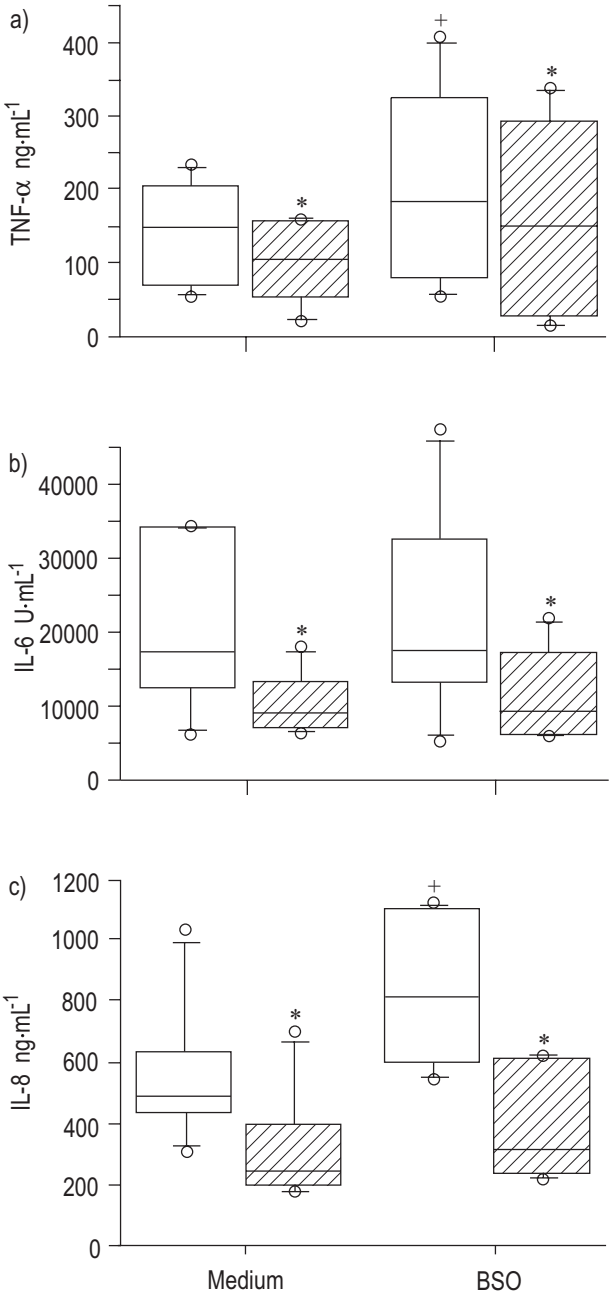

Fig. 6. – Modulation of: a) tumour necrosis factor (TNF)-α; b) interleukin (IL)-6; and c) IL-8 production by *N*-acetylcysteine (NAC) in alveolar macrophages (AMs) preincubated for 24 h in medium alone (Medium) and in butathione sulphoxamine (BSO) (n=6). After 24 h, AMs were washed and activated with lipopolysaccharide (LPS) with (▨) or without (□) NAC (1 mM). Cytokines were evaluated in supernatants collected at 48 h. Box plots with horizontal lines representing the median, columns the 25th and the 75th percentiles, and the error bars the 10th and the 90th percentiles. ○: outliers below 10th and above 90th percentiles. $^{+}$: $p<0.05$ compared with AM cultivated in medium alone during the first 24 h; *: $p<0.05$ compared with the corresponding control without NAC.

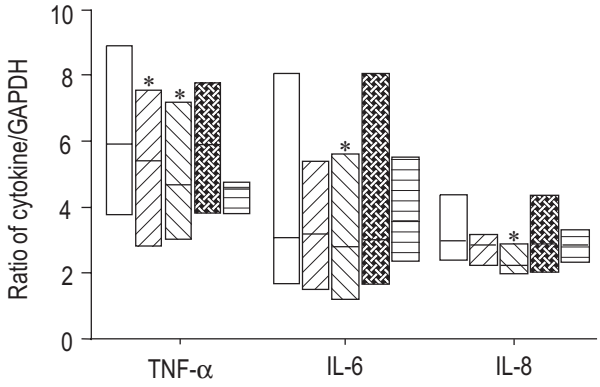

Fig. 7. – Messenger ribonucleic acid (mRNA) expression of tumour necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-8 in alveolar macrophages (n=4) stimulated by lipopolysaccharide (LPS; □), LPS and glutathione (▨) or LPS and *N*-acetylcysteine 1 mM (▧) and in AMs preincubated for 24 h with medium alone (▩) or with butathionine sulphoxamine 0.5 mM (▤) before LPS activation. mRNA expression was evaluated by reverse transcriptase-polymerase chain reaction with reduced glyceraldehide-phosphate dehydrogenase (GAPDH) mRNA as a reference. Box plots with horizontal lines representing the median and columns the 25th and the 75th percentiles. *: $p<0.05$ compared with LPS-activated AMs.

Discussion

In this study, the effect of antioxidants and inhibitors of GSH synthesis on cytokine production by AMs has been evaluated. Results showed that NAC and GSH significantly inhibited LPS-induced production of TNF-α and IL-6, the effect of NAC being dose dependent. Between 30 and 50% of TNF-α production was inhibited by NAC treatment and the effect on IL-6 and IL-8 secretion seemed slightly lower. In addition, GSH and NAC significantly decreased the mRNA expression for TNF-α, IL-6 and IL-8. The LPS concentration used in this study was suboptimal in order to simultaneously evaluate the positive and negative effect on cytokine production.

Previous reports have demonstrated that in a mouse model, oral administration of NAC decreased TNF-α synthesis induced by LPS, whereas BSO administration enhanced cytokine production [10]. The authors suggested that the effect was associated with the modulation of intracellular GSH. Moreover, they reported that NAC treatment of mice did not modulate IL-1 and IL-6 secretion. The current data showed that both antioxidants did not significantly increase reduced-GSH levels in AMs, whereas previous reports demonstrated an increase in GSH synthesis after treatment with NAC [17]. It is important to stress that, in this study, AMs were incubated in RPMI 1640 with 5% FCS containing 3 μM GSH and L-cystine, and that GSH concentration in AMs cultured for 48 h did not significantly decrease in these culture conditions. Alternatively, it has been shown that an increase in plasma GSH after *in vivo* treatment with NAC was clearly detected only in patients deficient in GSH and not in subjects with normal concentrations of GSH [24]. Nevertheless, it has been shown that cytokine secretion by AMs and by GSH-depleted AMs was significantly inhibited by the addition of GSH and NAC without modulation of the intracellular GSH level. The doses of thiols used in this study are relatively low compared to *in vitro* experiments reported in the literature (10–30 mM); however, at 1 mM, NAC and GSH had a strong inhibitory effect on ROI production [25, 26] and a cytotoxic effect on AMs at 10 mM was observed.

GSH metabolism in AMs can also modulate cytokine production induced by LPS, since depletion of intracellular

GSH by treatment with BSO for 24 h increased the production of TNF-α, and IL-8. In this case, IL-6 secretion was not altered showing that intracellular GSH is not involved in IL-6 synthesis. Partial depletion of GSH, at a level of ~50% (3 h preincubation) only increased IL-8 secretion, whereas simultaneous addition of LPS and BSO had no effect. These data, particularly the lack of effect at time zero, suggest that the increased cytokine secretion was associated with the effect of BSO on GSH metabolism. In the case of BSO preincubation, GSH depletion seems involved in its effect. It remains to be defined if such a GSH-depletion might be implicated in cytokine production associated with lung diseases, since the percentage of decrease necessary to obtain TNF-α modulation is high compared to that observed in lung diseases. It seems possible that at the cellular level, a low percentage of AMs are transiently depleted in GSH and that the upregulation of cytokine synthesis can take place in these cells.

The mechanisms implicated in the effect of either NAC and GSH or BSO on cytokine production are probably different. In contrast to BSO, GSH and NAC seem to modulate mainly the mRNA expression of cytokines. It has been demonstrated that thiol antioxidants are potent scavengers for radical hydroxyl, hypochlorous acid and carbon centered radicals but not for anion superoxide [26, 27]. The involvement of ROIs in TNF-α production has been demonstrated with mouse peritoneal macrophages and blood monocytes [9, 28], whereas the presence of H_2O_2 enhanced TNF-α production, iron chelators and free radical scavengers inhibited the cytokine release. EUGUI *et al.* [12] showed that some antioxidants such as tetrahydropapaveroline (THP) and butylated hydroxyanisole (BHA) inhibit production of pro-inflammatory cytokines by blood monocytes, whereas other antioxidants (*e.g.* α-tocopherol and butylated hydroxytoluene) are ineffective. DEFORGE *et al.* [13] provided data showing that an hydroxyl radical scavenger (dimethylsulphoxide) selectively inhibited IL-8 production, whereas addition of H_2O_2 induced it. Moreover, antioxidants and thioredoxin which have a strong reducing activity for thiols, inhibited the translocation of nuclear factor (NF)-κB and activator protein (AP)-1 towards the nucleus in blood monocytes activated by LPS [29]; both of these NFs control the trancription of genes encoding for pro-inflammatory cytokines [30, 31]. Endogenous GSH participates in the elimination of hydrogen peroxides and lipid hydroperoxides and it alters enzyme activity through the oxido-reduction of thiol groups or chelation of divalent cations [32]. In rat Kupffer cells, NEUSCHWANDER-TETRI *et al.* [18] suggested that GSH formed endogenously or provided (in addition to NAC) can alter the intracellular thiol cell-redox equilibrium leading to the suppression of NF-κB activation. It is proposed that the activity of thiols is related to their reducing activity since GSSG had no effect on cytokine synthesis. The activity of thiols on NF activation probably involved the modulation of protein tyrosine kinase and protein tyrosine phosphatase involved in signal transduction [33]. In this study, some differences were noted between the effect of exogenous and endogenous thiols. In contrast with NAC and the addition of GSH, depletion of endogenous GSH did not alter IL-6 production as well as TNF-α and IL-8 mRNA expression. Although the secretion of the three cytokines was significantly inhibited by NAC and GSH, TNF-α production was more sensitive than IL-6 and IL-8 to the inhibition by these antioxidants; a sensitivity probably linked to the transcription factor(s) or to the signal transduction involved in the respective gene transcription.

The inhibitory effect was also related to the activator used for cytokine production. Whereas NAC had a similar activity with the three activators, GSH did not inhibit the cytokine secretion induced by zymosan and PMA. It has been shown in other experimental models [29, 34] that thiols can inhibit the activation of different transcription factors (NF-κB, AP-1 and/or NF-IL-6) depending on the activator used. Since, in this study, NAC had a higher inhibitory activity than GSH, it may also be assumed that the effect of GSH is not sufficient to neutralize the zymosan-induced cytokine secretion.

The observation that antioxidants, particularly NAC, inhibit AM-derived cytokine production induced by activators able to induce inflammation raises some questions about the role of such a mechanism in lung physiology. TNF-α and IL-6 have a dual role. Firstly, they participate in the development of defence mechanisms by the regulation of the immune response and the modulation of effector cell functions. Secondly, TNF-α plays a key role in inflammation, and upregulation of its secretion is involved in several lung diseases [2]. IL-8 mainly par-ticipates in leukocyte recruitment, particularly neutrophils [3]. As mentioned for TNF-α and IL-6, an increased IL-8 secretion was demonstrated in lung pathology associated with lung neutrophilia. This study reports that GSH and NAC have a moderate inhibitory effect on cytokine production (~30%). These data suggest that treatment with NAC, which is the more effective of the two free radical scavengers, could limit lung injury without a significant impairment of lung defense mechanisms even in a situation with impaired GSH metabolism.

In conclusion, these results support the continued interest of antioxidants treatment of lung diseases involving an exaggerated cytokine production by alveolar macrophages.

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