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L-Buthionine-(S,R)-sulfoximine, an irreversible inhibitor of gamma-glutamylcysteine synthetase, augments LPS-mediated pro-inflammatory cytokine biosynthesis: evidence for the implication of an IkappaB-alpha/NF-kappaB insensitive pathway

Abstract

The pro-inflammatory cytokines, including tumor necrosis factor (TNF)-alpha and interleukin (IL)-6, contribute to the exacerbation of pathophysiological conditions in the lung. The regulation of cytokines involves the reduction-oxidation (redox)-sensitive nuclear factor-kappaB (NF-kappaB), the activation of which is mediated through an upstream kinase that regulates the phosphorylation and subsequent degradation of inhibitory-kappaB (IkappaB)-alpha, the major cytosolic inhibitor of NF-kappaB. It was hypothesized that lipopolysaccharide (LPS)-induced biosynthesis of TNF-alpha and IL-6 in vitro is tightly regulated by redox equilibrium. Furthermore, the likely involvement of the IkappaB-alpha/NF-kappaB signalling transduction pathway in mediating redox-dependent regulation of LPS-induced cytokine biosynthesis was revealed. Using alveolar epithelial cells, the role of L-buthionine-(S,R)-sulfoximine (BSO), a specific and irreversible inhibitor of gamma-glutamylcysteine synthetase (gamma-GCS), the rate-limiting enzyme in glutathione (GSH - an antioxidant thiol) biosynthesis, in regulating LPS-mediated TNF-alpha and IL-6 production and the IkappaB-alpha/NF-kappaB signalling pathway was investigated. Pre-treatment with BSO, prior to exposure to LPS augmented, in a dose-dependent manner, LPS-induced TNF-alpha and IL-6 biosynthesis, an effect associated with the induction of intracellular accumulation of reactive oxygen species (ROS). Interestingly, BSO blocked the phosphorylation of IkappaB-alpha, reduced its degradation, thereby allowing its cytosolic accumulation, and subsequently inhibited the activation of NF-kappaB. These results indicate that there are ROS and redox-mediated effects regulating pro-inflammatory cytokines, and that the IkappaB-alpha/NF-kappaB pathway is redox-sensitive and differentially involved in mediating redox-dependent regulation of cytokine signaling.

Full text

INTRODUCTION

The alveolar epithelium is recognized as a dynamic barrier that plays an important role in regulating the inflammatory and metabolic responses to oxidative stress and the accompanying inflammatory signal [1-6], sepsis, endotoxemia, and other critical conditions in the lung [7-10]. The respiratory epithelium, in particular, is a primary target of inflammatory/infectious

conditions at the epithelial-blood interface and is itself capable of amplifying an inflammatory signal by recruiting inflammatory cells and by producing inflammatory mediators [2-5]. Many of the side effects of lipopolysaccharide-endotoxin (LPS), derived from the cell wall of gram-negative bacteria, are secondary to the overproduction of pro-inflammatory mediators. Inflammatory as well as autoimmune disease is often associated with deregulated expression and biosynthesis of inflammatory cytokines, which influence a plethora of cellular functions [11-13]. Therefore, the down-regulation of an inflammatory signal, and the amplification of a counter anti-inflammatory signal, is a major focus of the rational approach to the treatment of inflammatory diseases, such as chronic inflammation, sepsis and rheumatoid arthritis [14, 15]. For instance, recent novel studies by Haskó *et al.* [16] and Zidek [17] reported a potential role for extracellular purines, including adenosine and ATP, and inosine, a degradation product of these purines, as potent, endogenous, immunomodulatory molecules that inhibit inflammatory cytokine biosynthesis and protect against endotoxin-induced shock. It has also been reported, that selective inhibition of phosphodiesterases, a family of enzymes involved in the degradation of cAMP/cGMP [18, 19], steroids, such as glucocorticoids [20], pyrimidylpiperazine derivatives [21, 22], adrenoreceptor agonism/antagonism [17, 23], and ERK and p38/RK MAPK selective inhibitors [24, 25], differentially regulate the transcription and biosynthesis of inflammatory cytokines and other mediators.

The tripeptide L-gamma-glutamyl-L-cysteinyl-glycine, or glutathione (GSH), a ubiquitous thiol, plays a major role in maintaining intracellular reduction-oxidation (redox) equilibrium in the lung [2, 3, 27-29]. The cysteinyl moiety of GSH provides the reactive thiol as a functional element responsible for the diverse properties of glutathione, including, *i*) an antioxidant potential mediated by the peroxidase-coupled reaction, *ii*) regulation of cellular sulfhydryl status and redox equilibrium, *iii*) governing pathways in neuro-immune-endocrine interactions as an immunopharmacological thiol, and *iv*) regulation of the expression/activation of redox-sensitive transcription factors induced by stress-evoked responses. The pivotal role of the redox cycle in maintaining the integrity of the biological system in the face of oxidative stress and other challenges is, therefore, of particular clinical relevance. The immunopharmacological potential assigned to glutathione [2-5, 29], furthermore, stems from established observations. Interleukin (IL)-1-induced responses in mesangial cells, for instance, occur through modulating the redox

equilibrium [30]. In addition, the reactive oxygen species (ROS) signaling regulating the transcription of IL-4 [31], IL-6, IL-8 [32] and tumor necrosis factor (TNF)-alpha [32, 33] is mediated through a thiol-dependent mechanism. Interestingly, antioxidants [4, 34-36] and glutathione precursors [5, 31, 32] have been shown to down-regulate cytokine synthesis, activation and downstream processes. On the other hand, depletion of GSH is associated with the augmentation of an inflammatory signal and exacerbation of numerous pathophysiological conditions [5, 31-33].

This study has attempted, therefore, to investigate *in vitro* redox regulation of pro-inflammatory cytokine signalling. Specifically, the irreversible inhibition of gamma-glutamylcysteine synthetase (gamma-GCS), the rate-limiting enzyme in GSH biosynthesis, thereby imposing intracellular depletion, and augmented LPS-mediated TNF-alpha and IL-6 biosynthesis, an effect associated with the intracellular accumulation of ROS. Furthermore, exploring the molecular signalling mechanism likely to be implicated, revealed a quite independent, inhibitory-kappaB (IkappaB)-alpha/nuclear factor-kappaB (NF-kappaB) redox-sensitive pathway mediating the role of GSH in regulating a pro-inflammatory signal within the alveolar epithelium.

MATERIAL AND METHODS

Materials

Unless otherwise indicated, chemicals of the highest analytical grade were purchased from Sigma-Aldrich Co. (UK). All experimental procedures involving the use of live animals were approved under the Animals Legislation (Scientific Procedures) Act, 1986 (UK).

Primary cell cultures of alveolar epithelia

Fetal alveolar type II (fATII) epithelial cells were isolated from the lungs of fetuses of pregnant *Sprague-Dawley* rats, essentially as reported in detail elsewhere [2, 3]. The adenylate energy charge (EC), an index of cell viability and competence, remained > = 0.7, and transepithelial monolayer resistance (R_f) was monitored constantly at 250-350 omégacm^2 or more [2, 3]. In addition, the total protein content of cells pre-treated with 1, 10 and 100 mu M BSO for 24 hours did not vary significantly from control cultures [2, 3]. Alveolar pre-treatment with

BSO has also been shown to intervene specifically at the level of the cell cycle events (such as with p53), and at the level of factors that are key components of the signalling pathways governing apoptosis (such as with Bax and Bcl-2 proto-oncogenes), suggesting specificity rather than necrotic toxicity (data not shown).

Measurement of intracellular reactive oxygen species (ROS)

The biochemical pathway inducing the generation of $O_2^{-\cdot}$ by beta-NADPH oxidation allows H_2O_2 formation by superoxide dismutase (SOD). In order to measure the accumulation of H_2O_2, cell supernatants were collected and centrifuged at 2 000 g for 5 min at 4° C, and then treated with 1 M NaOH. The change in absorbency at 600 nm was monitored against phenol red solution (PRS), containing (in mM): 140 NaCl, 10 K_3PO_4, 5.5 glucose, 0.28 phenol red, and 20 U/ml horseradish peroxidase. Standard curves using PRS and H_2O_2 were prepared (0-100 muM), and results extrapolated from the linear regression were converted to nmoles/mg protein [4, 5]. For the quantification of $O_2^{-\cdot}$ release, the supernatant was discarded and cells were either exposed to LPS (1 mug/ml; 24 hours), pre-treated with BSO (100 muM) for 24 hours, or pre-treated with various doses of BSO and exposed to LPS for 24 hours. Cells were subsequently re-incubated for 24 hours (37° C) in the presence of 80 muM ferricytochrome c (fc) suspended in HBSS. The amount of O_2^- released was determined by measuring the absorbency at 550 nm against blanks containing fc and SOD (300 U/ml). All experiments were performed in duplicate and data are presented as the amount of O_2^- released based on nmoles of reduced fc/min/mg protein [4, 5]. The Fenton reaction is a biochemical conversion of dihydrorhodamine (DHR) into fluorescent rhodamine. One particular intermediate is $^{\cdot}OH$, which reacts with DHR yielding water and a tertiary free radical, which is quite stable. This radical is able to undergo rearrangement of the pi electrons, leading to formation of fluorescent rhodamine. Cells were cultured at 10^5/well in flat-bottomed, microtitre plates at 37° C. Wells were washed with sterile, pre-equilibrated saline, then culturing was continued in Kreb's/Ringer solution containing 50 muM DHR (24 hours). Fluorescence was measured at excitation/emission wavelengths of 485/535 nm. The $^{\cdot}OH$ level measured at 24 hours under standard medium treatment was calibrated to 100%, and variables were plotted against this level as fluorescence units [4, 5].

Drug pre-treatment, western analysis and electrophoretic mobility shift assay

*Pre-treatment with L-buthionine-(*S,R*)-sulfoximine (BSO) and exposure to lipopolysaccharide (LPS).* For the dose-response curve, epithelial cells were pre-treated for 24 hours with glutathione-depleting agent, BSO (0, 1, 10, 100 muM). Cell filters were subsequently washed twice in pre-equilibrated PC-1 medium before being exposed for a further 24 hours to LPS (1 mug/ml) from *Escherichia coli*, serotype 026:B6.

Subcellular extraction and western immunoelectroblotting analysis. Following pre-treatment with BSO, sub-cellular extracts (cytosolic and nuclear) were prepared, essentially as previously described [2, 3, 37]. Briefly, filters were washed twice in 5 ml ice-cold, pre-equilibrated phosphate-buffered saline (PBS; pH7.2-7.4) and cells (1-2 **x** 10^7) were collected and centrifuged at 420 g for 5 min at 4° C. Nuclei were released by re-suspending the pellet in 250 mul buffer A containing (in mM): 10 Tris-HCl (pH7.8), 10 KCl, 2.5 NaH_2PO_4, 1.5 $MgCl_2$, 1 Na_3VO_4, 0.5 dithiothreitol (DTT), 0.4 4-2-aminoethyl-benzene sulfonyl fluoride-HCl (AEBSF), and 2 mug/ml each of leupeptin, pepstatin A and aprotinin. The suspension was left in ice for 10 min followed by a 45-second homogenization at a moderate speed. Nuclei were collected by centrifuging the slurry at 4,500 g for 5 min at 4° C, and re-suspending in 100 mul buffer B (Buffer A adjusted to (in mM): 20 Tris-HCl (pH7.8), 420 KCl, 20% (v/v) glycerol). The supernatant thus formed is termed the cytosolic extract. The nuclei were then lysed at 4° C for 30 min with gentle agitation, the debris cleared by centrifugation at 10,000 g for an additional 30 min at 4° C, and the supernatant frozen in liquid nitrogen and stored at - 70° C until used. In all cases, protein contents was determined by the Bradford method using BSA as a standard. Cytosolic and nuclear proteins (20-25 mug) were resolved over SDS-PAGE (7.5%) gels at RT, blotted onto nitrocellulose membrane, and non-specific binding sites were subsequently blocked. Mouse monoclonal IgG_1 anti-IkappaB-alpha (H-4), IgG_{2b} anti-(phosphorylated) *p*IkappaB-alpha (B-9), rabbit polyclonal IgG anti-p50 (NLS), anti-p52 (K-27), anti-p65 (RelA; A), anti-p68 (RelB; C-19), and anti-p75 (c-Rel; N) (Santa Cruz Biotechnology, UK) antibodies were used for primary detection. Anti-rabbit Ig-biotinylated antibody (Amersham Life Science, UK) was employed for secondary detection followed by the addition of streptavidin-HRP conjugate, and visualized on film by chemiluminescence. beta-actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable.

Western blots were scanned by NIH MagiScanII and subsequently quantitated with the UN-Scan-IT automated digitizing system (version 5.1; 32-bit), and the ratio of the density of the band to that of beta-actin was subsequently calculated.

*Analysis of NF-*kappa*B DNA binding activity by electrophoretic mobility shift assay (EMSA).* Custom deoxy-oligonucleotide probe sequences were purchased from Genosys, UK: NF-kappaB, 5'-AGTTGAGGGGACTTTCCCAGGC-3' (binding sequence underlined). Gel-purified double-stranded DNA was end-labeled with [gamma^{32}P]-ATP (NEN Life Sciences Products). Identical amounts of radioactive probe (1-2 **x** 10^4 counts/min) were added to binding reactions containing 1-5 mug of nuclear extracts in a final volume of 40 mul in DNA binding buffer (20 mM HEPES (pH7.9); 1 mM $MgCl_2$; 4% FicoII). Reaction mixtures were incubated for 30 min at 25° C before separating on non-denaturing, 4% polyacrylamide gels at RT, and subjected to electrophoresis with 1:10 5X Tris-borate-EDTA buffer. A non-specific competitive polydeoxyinosinic-deoxycytidylic acid [poly(dI-dC)] (Amersham Pharmacia Biotech, UK) was added to reaction mixtures after addition of labeled probe. Gels were transferred to ion-exchange chromatography paper, vacuum dried and then electronically visualized on a Packard Instant phosphorimager. Specific quantitation of the corresponding DNA gel shift bands was performed with phosphorimaging [2, 3, 37].

Assessment of cytokine production by enzyme-linked immunosorbent assay (ELISA)

The production of pro-inflammatory cytokines released extracellularly was measured by a two-site, solid-phase, developed sandwich enzyme-linked immunosorbent assay (ELISA) [4, 5]. Immunoaffinity purified polyclonal rabbit anti-rat TNF-alpha (2 mug/ml) and IL-6 (2 mug/ml) primary antibodies were used to coat high-binding microtitre plates (MaxiSorp, Nunc, UK). Recombinant rat TNF-alpha and IL-6 and biotinylated immunoaffinity purified sheep anti-rat TNF-alpha and IL-6 (R&D Systems, UK) were used as standard and recognition antibodies, respectively. The colour was developed incorporating streptavidin-poly-horseradish peroxidase coupled reaction with the chromagen 3,3',5,5'-tetramethyl-benzidine dihydrochloride (TMB), and the optical density (O.D.) was measured at 450 nm against a filter background at 595 nm. Inter- and intra-assay coefficients of variations (CV) were < 10%, and the minimum detectable

sensitivity for each cytokine is ¾ 2 pg/ml. Results interpolated from the linear regression of the standard curve are expressed as pg/ml.

Statistical analysis and data handling

Data are the means and the error bars the SEM. Statistical analysis of the difference in mean separation was performed by one-way analysis of variance (ANOVA), followed by *post hoc* Tukey's test, and the *a priori* level of significance at 95% confidence was considered to be P ¾ 0.05.

RESULTS

*The dose- and time-dependent effects of LPS on TNF-*alpha *and IL-6 biosynthesis*

Exposure of epithelial cells to LPS induced, in a time- and dose-dependent manner, TNF-alpha biosynthesis, as shown in Figures 1A and 1B, respectively. LPS doses ¾ 10 ng/ml were ineffective in inducing the extracellular accumulation of TNF-alpha. The minimum effective concentration of LPS was 100 ng/ml, and induced TNF-alpha production continued to increase in a dose-dependent manner up to 10,000 ng/ml, the highest dose used in this study, as measured 24 hours-post induction (Figure 1A). In addition, LPS (10 mug/ml) induced a time-dependent release of TNF-alpha, with immediate and maximum induction at 2-3 hours, persistently significant up until 24 hours, declining thereafter (48-96 hours) (Figure 1B). The control levels of TNF-alpha secretion in the supernatants of cells exposed to medium alone, in the absence of LPS were not statistically significant (data not shown). Exposure of epithelial cells to LPS induced, in a time- and dose-dependent manner IL-6 biosynthesis, as shown in Figures 1C and 1D, respectively. LPS doses ¾ 10 ng/ml were ineffective in inducing the extracellular accumulation of IL-6. The minimum effective concentration of LPS was 100 ng/ml and induced IL-6 production continued to increase in a dose-dependent manner up to 10,000 ng/ml, as measured 24 hours-post induction (Figure 1C). In addition, LPS (10 mug/ml) induced a time-dependent release of IL-6, with induction at 2-3 hours, maximal at 24 hours, thereafter declining but still significantly elevated (48-96 hours) (Figure 1D). The control levels of IL-6 secretion in the supernatants of cells exposed to medium alone, in the absence of LPS were not statistically significant (data not shown).

The effect of L-buthionine-(S,R)-sulfoximine (BSO) on LPS-induced TNF-alpha/IL-6 biosynthesis

The degree of glutathione depletion (homeostasis) obtained in epithelial cells pre-treated with BSO for 24 hours is given in Table 1. BSO is a specific and irreversible inhibitor of gamma-glutamylcysteine synthetase (gamma-GCS), the rate-limiting enzyme in the biosynthesis of GSH [3, 26, 27]. It has been previously shown that pre-treatment (24 hours) of alveolar epithelial cells with BSO, followed by exposure to ascending deltapO_2 regimen (oxyexcitation), induced, in a dose- and time-dependent manner, the intracellular accumulation of hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical ($^{\cdot}OH$)$^-$ [4, 5]. In addition, BSO has been reported to block, in a dose-dependent manner, oxyexcitation (deltapO_2) and chemioxyexcitation (deltapO_2/ROS)-mediated nuclear translocation and activation of the redox-sensitive transcription factor NF-kappaB [4, 5]. In this study, BSO augmented, in a dose-dependent manner, LPS-induced release of TNF-alpha (Figure 2A). LPS (1 mug/ml; 24 hours) increased TNF-alpha secretion by 5-7 fold above the control baseline level and BSO substantiated the increase approximately 1.4 (non-significant), 2.8 and 2.4 fold relative to LPS alone at 1, 10 and 100 muM, respectively (Figure 2A). Although BSO at a concentration of 1 muM, tended to mildly augment LPS-induced TNF-alpha production, the levels obtained did not reach statistical confidence. Pre-treatment with BSO (24 hours), followed by exposure to medium alone for an additional 24 hours in the absence of exogenous LPS, had no effect on TNF-alpha secretion, similar to the control (Figure 2A). Similarly, BSO augmented, in a dose-dependent manner, LPS-mediated release of IL-6 (Figure 2B). LPS (1 mug/ml; 24 hours) increased IL-6 secretion 21 fold above the control baseline level, and BSO increased the level of IL-6 approximately 1.7, 3.6 and 3.7 fold relative to LPS alone at 1, 10 and 100 muM, respectively (Figure 2B). Pre-treatment with BSO (24 hours), followed by exposure to medium alone for an additional 24 hours in the absence of exogenous LPS, had no effect on IL-6 secretion, similar to the control (Figure 2B).

The effect of BSO and LPS on intracellular accumulation of reactive oxygen species (ROS)

The profile of H_2O_2 formation in response to LPS and BSO is shown in Figure 3A. The level of H_2O_2 increased after LPS treatment for 24 hours, an effect associated with the increase in H_2O_2 accumulation in cells pre-treated with BSO

for 24 hours. Cells pre-treated with BSO for 24 hours, followed by exposure to LPS for another 24 hours, showed an increase, in a dose-dependent manner, in H_2O_2 accumulation (Figure 3A). The profile of $O_2^{-\cdot}$ formation in response to LPS and BSO is shown in Figure 3B. The level of $O_2^{-\cdot}$ did not increase after LPS treatment for 24 hours, an observation similar to that found in cells pre-treated with BSO for 24 hours. Cells pre-treated with BSO for 24 hours, followed by exposure to LPS for another 24 hours, showed an increase, in a dose-dependent manner, in $O_2^{-\cdot}$ accumulation (Figure 3B). The profile of $^{\cdot}OH$ formation in response to LPS and BSO is shown in Figure 3C. The level of $^{\cdot}OH$ marginally increased after LPS treatment for 24 hours, an effect associated with the increase in $^{\cdot}OH$ accumulation in cells pre-treated with BSO for 24 hours. Cells pre-treated with BSO for 24 hours, followed by exposure to LPS for another 24 hours, showed an increase, in a dose-dependent manner, in $^{\cdot}OH$ accumulation (Figure 3C).

The role of BSO in regulating LPS-mediated inhibitory-kappa*B (I*kappa*B-*alpha*) signalling*

In a previous study, the role that LPS plays in regulating IkappaB-alpha signalling, recognized as the major cytosolic inhibitor of NF-kappaB, was investigated [37]. LPS induced, in a time- and dose-dependent manner, IkappaB-alpha degradation in the cytosolic compartment, with no apparent effects on its expression within the nucleus [37]. It was found that LPS (1 mug/ml) mediated degradation of IkappaB-alpha significantly beginning after 4 hours, and continuing to disappear in the cytosol in the presence of LPS up until 96 hours. This was associated with a time- and dose-dependent up-regulation of the IkappaB-alpha phosphorylation state [37]. As shown in Figure 4A, pre-treatment with medium or BSO (100 mum) alone for 24 hours, followed by exposure to medium for additional 24 hours, did not affect the constitutive protein expression of IkappaB-alpha. Exposure to LPS (1 mug/ml) for 24 hours induced IkappaB-alpha degradation in the cytosolic compartment, reaffirming our previous observation [37]. However, pre-treatment with BSO for 24 hours, followed by exposure to LPS (1 mug/ml; 24 hours) reversed, in a dose-dependent manner, the degradation effect of LPS on IkappaB-alpha, thereby restoring its cytosolic abundance and accumulation (Figure 4A). The inhibitory effect of BSO on LPS-induced IkappaB-alpha degradation was associated with the suppression of LPS-induced IkappaB-alpha phosphorylation, in a dose-dependent manner (Figure 4A). BSO at a dose of 100 muM completely abolished

LPS-induced IkappaB-alpha phosphorylation. The housekeeping protein, beta-actin, was used as an internal reference for semi-quantitative loading in parallel lanes (Figure 4A). The dose-dependent reversal by BSO of LPS-induced IkappaB-alpha degradation is illustrated by histogram analysis in Figure 4B. Similarly, the histogram analysis of the effect of BSO on IkappaB-alpha phosphorylation is shown in Figure 4C.

*The role of BSO in regulating LPS-mediated NF-*kappa*B signalling*

The effect of BSO on LPS-induced nuclear localization of selective NF-kappaB subunits. As shown in Figure 5, pre-treatment with BSO reduced LPS-mediated nuclear translocation of selective NF-kappaB subunits. Although LPS (1 mug/ml; 24) induced the expression of NF-kappaB_1 (p50), RelA (p65), RelB (p68) and c-rel (p75), it had no apparent effect on NF-kappaB_2 (p52) (Figure 5). Pre-treatment with BSO (100 muM; 24 hours) followed by exposure to medium alone in the absence of LPS had a weak effect on NF-kappaB subunits. Pre-treatment with BSO followed by exposure to LPS reduced, in a dose-dependent manner, the translocation of NF-kappaB subunits (Figure 5). BSO at the dose-response curve spanned had no effect on the housekeeping protein beta-actin, as shown in Figure 5.

*The effect of BSO on LPS-induced NF-*kappa*B activation*

The inhibitory effect of BSO on LPS-induced nuclear translocation of selective NF-kappaB subunits was associated with the interference of BSO in the binding of NF-kappaB to specific kappaB consensus sequences. Figure 6A shows a representative electrophoretic mobility shift assay (EMSA) revealing the effect of BSO on LPS-induced NF-kappaB activation. The control lane contained no nuclear extracts, and BSO (100 muM; 24 hours) pre-treatment followed by exposure to medium alone had weak effect on NF-kappaB DNA binding activity, similar to the control (Figure 6A). Whereas LPS (1 mug/ml; 24 hours) induced the NF-kappaB DNA binding activity, BSO pre-treatment abrogated this response, in a dose-dependent manner (Figure 6A). Histogram analysis of the inhibitory effect of the dose-response curve for BSO is shown in Figure 6B. The specificity of NF-kappaB DNA binding activity was previously determined, where we have shown that the NF-kappaB/DNA complex is formed mainly of p50-p65 subunits [2, 3, 37]. Furthermore, utilization of a mutant oligonucleotide (3 bp mutation of

the wild type oligonucleotide used in this study) and 100X cold competitor abolished the NF-kappaB DNA activity [2, 3, 37].

DISCUSSION

The "biomarkers" of oxidative stress, such as antioxidant inefficiency, redox disequilibrium and derivation of oxidant radicals may arise from conditions other than hyperoxia (oxidizing signals) *per se*, such as hypoxia/re-oxygenation and cytokine-dependent processes [1, 4, 5, 7-9]. In physiological conditions, the intracellular redox status of thiols is predominantly reductive [2, 3, 26]. GSH, for example, is present in high concentrations in lung epithelial lining fluid [2, 3, 38], and has been reported to maintain the integrity of the airspace epithelium *in vitro* and *in vivo* [7, 8, 26, 28, 39]. In contrast, GSH depletion has been linked with the pathophysiology of idiopathic pulmonary fibrosis [38], adult respiratory distress syndrome [40], bronchopulmonary dysplasia [41] and cystic fibrosis [42], thus highlighting its central role in maintaining the functional integrity of a physiologically competent epithelium. There is growing evidence moreover, supporting the notion that oxidative conditions modulate redox-linked pathways by altering the dynamic equilibrium of glutathione homeostasis [2, 3, 26-28]. Exogenous/endogenous agents which induce the formation of reactive oxygen species (ROS), for example, can affect redox homeostasis by up-regulating antioxidant enzymes, particularly glutathione peroxidase and enzymes involved in glutathione recycling and biosynthesis [2, 3, 26, 29, 39]. Furthermore, ROS signaling could be mediated by cytokines, peptide hormones and immunoregulators, whose participation in cellular pathways is modulated by redox status [4, 5, 30-35]. Conversely, cytokines which themselves are mediators of oxidative stress have the potential to alter redox equilibrium, thereby affecting GSH/GSSG shuttling and recycling [43-45].

In vitro the regulatory effect of a pharmacological thiol (BSO) on pro-inflammatory cytokines has been hitherto reported. This approach bears clinical relevance to the pediatric treatment of respiratory distress, where cytokines are recognized as crucial elements in its pathophysiology [11-13]. Growing evidence implicates an association between redox signalling and the regulation of a pro-inflammatory state, thereby placing more demand on the utilization of intracellular glutathione [4, 5, 26-29]. As such, the respiratory epithelium becomes more engaged in regulating enzymes involved in maintaining redox homeostasis [4, 5, 26]. Whilst the glutathione biosynthetic machinery is

overwhelmed in disease, up-regulating cytokines may exacerbate the clinical symptoms. Although the participation of pro-inflammatory cytokines, including TNF-alpha and IL-6, in the pathogenesis of respiratory distress has been widely recognized, the mechanisms involved have not been clearly defined.

The rate-limiting substrate for GSH biosynthesis is glutamate-cysteine ($K_{m\ Glutamate}$ = 1.6-2 mM; $K_{m\ Cysteine}$ = 0.3 mM) [3, 4, 26, 27]. This pathway is selectively blocked by BSO, a specific and irreversible inhibitor of gamma-GCS [26, 27]. Consequently, the capacity of the epithelium to replenish intracellular stores of GSH is dramatically affected, thereby modulating the optimum equilibrium necessary to evoke a defense strategy in pathophysiology [26, 38, 40-42]. This subsequently leads to ROS up-regulation, whose inappropriate disposition, accumulation and intracellular localization augment a pro-inflammatory signal that is mediated, at least in part, by the activation of redox-sensitive transcription factors [2, 3, 29, 39]. This is consistent with the observation that the expression of gamma-GCS was shown to suppress TNF-alpha induced activation of NF-kappaB [46]. I believe that the pathway mediating BSO-induced up-regulation of pro-inflammatory cytokines in the alveolar epithelium involves a secondary mediator, the most likely candidate being the hydroxyl radical (·OH), an observation in accordance with other investigations [3, 4, 26]. This conforms to the evidence that GSH is involved in H_2O_2 reduction, a major source of ·OH, through the GSH-peroxidase coupled reaction [2, 26], suggesting that in the case of BSO pre-incubation, GSH depletion seems involved in its effect [3-5, 28]. It is possible that GSH depletion, by blocking its biosynthesis, reduces the capacity of the epithelium to dispose of accumulating H_2O_2, with the resulting increase in ·OH production, consistent with other observations [3, 5, 26, 29, 46]. It remains to be defined, however, whether GSH depletion is implicated in up-regulating cytokines in association with lung disease, since the degree of depletion necessary to evoke cytokines *in vitro* is higher than that observed in pathophysiology *in vivo* [38, 40-42, 47-49].

The intriguing ability of BSO to blockade the translocation/activation of NF-kappaB yet up-regulate the presumably downstream cytokine pathway remains of particular interest. It was previously reported that BSO blocked the oxyexcitation (deltapO_2)-dependent nuclear localization of RelA (p65), the major transactivating member of the *Rel* family [37], and subsequently suppressed NF-kappaB activation [2, 3]. However, in additional studies, BSO was also shown to be capable of inducing intracellular accumulation of ROS,

particularly ·OH [4, 5]. Taken together, these data argue for ROS as potential second messengers for cytokine biosynthesis in the alveolar epithelium; however, ROS might not be universal messengers in the activation of NF-kappaB [46, 50]. Furthermore, the ability of BSO to inhibit the phosphorylation/degradation of IkappaB-alpha, yet up-regulate cytokine biosynthesis, demonstrated that this transcription factor could be partially involved, but not exclusively required, in regulating redox-mediated pathways governing pro-inflammatory cytokines in the alveolar epithelium. Although a NF-kappaB consensus binding site is present in the promoter region of IL-1beta, IL-6, IL-8 and TNF-alpha genes [51, 52], it is difficult to conclude from the present data alone whether these specific kappaB moieties are indispensable for regulating cytokine expression and release apart from coupling this mechanism to intracellular redox state, and whether other transcription factors, such as AP-1 and Oct-1, are likely to be involved. However, there is apparently a dissociation between ROS-dependent and independent pathways governing the translocation/activation of NF-kappaB in the alveolar epithelium, and this pathway, along with the downstream pro-inflammatory cytokine route, is tightly regulated by redox signaling. This is supported by unequivocal evidence indicating a separation of oxidant-initiated and redox-regulated mechanisms in the NF-kappaB signal transduction pathway [50]. Therefore, the IkappaB-alpha/NF-kappaB pathway could be partially dissociated from that of redox-dependent regulation of pro-inflammatory cytokines, indicating that this transcription factor is not exclusively ROS-sensitive and differentially implicated in regulating pro-inflammatory cytokine signaling, suggesting the involvement of possible cross-talk among several pathways working independently or in coherence to integrate ROS and redox signalling mechanisms governing the regulation of cytokines. In this respect, we are extending these observations to investigate a potential role for the mitogen-activated protein kinase (MAPK) pathway, with particular emphasis on the possible immunoregulatory effect that this pathway has on IkappaB-alpha/NF-kappaB signalling and the regulation of pro-inflammatory cytokine transcription and biosynthesis [24, 25].

CONCLUSION

In summary, the results therein reported could be summarised as follows: *i*) irreversible inhibition of glutathione biosynthesis blocked the phosphorylation of IkappaB-alpha and subsequently reduced its degradation, thus allowing its cytosolic accumulation; and *ii*) BSO blocked the activity of NF-kappaB, an effect

uncoupled from its ability to augment LPS-mediated biosynthesis of pro-inflammatory cytokines in an ROS-sensitive mechanism. It is concluded that there are oxidant-initiated and redox-mediated mechanisms regulating inflammatory cytokine biosynthesis and that the IkappaB-alpha/NF-kappaB signaling transduction pathway is redox-sensitive and differentially implicated in redox-mediated regulation of cytokine signalling in the alveolar epithelium.

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Figures

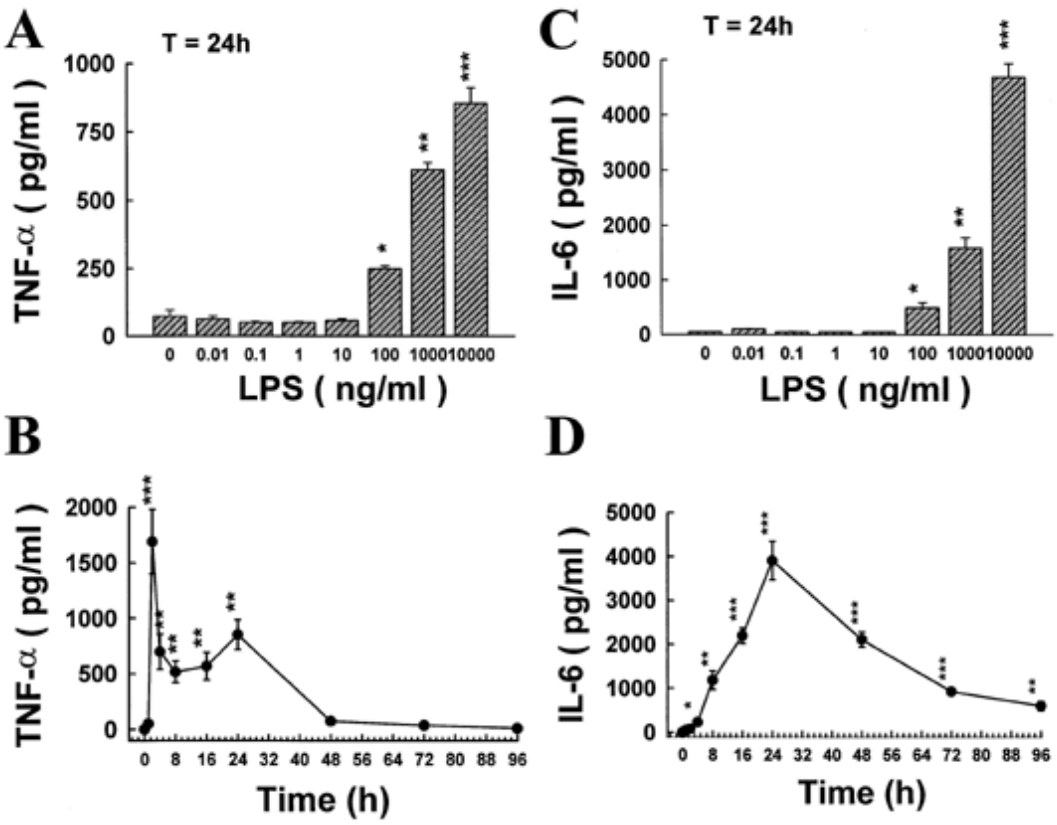


Figure 1

The dose- and time-response curves for the effect of lipopolysaccharide-endotoxin (LPS) on pro-inflammatory cytokine biosynthesis. A: the dose-response curve for LPS-mediated TNF-alpha biosynthesis at 24 hours, showing a minimum effective concentration of 100 ng/ml. LPS doses ¾ 10 ng/ml did not elicit any TNF-alpha production as determined by ELISA in culture supernatants. The highest induction obtained in comparison with lower LPS concentrations was at 10,000 ng/ml. **B:** the time-response curve (0-96 hours) with an LPS concentration of 10,000 ng/ml. TNF-alpha was significantly increased 2-3 hours post-addition of LPS, and remained significant up to 24 hours, thereafter declining (48-96 hours). **C:** The dose-response curve for LPS-mediated IL-6 biosynthesis at 24 hours, showing a minimum effective concentration of 100 ng/ml. LPS doses ¾ 10 ng/ml did not elicit any IL-6 production. **D:** the time-response curve (0-96 hours) with LPS concentration of 10,000 ng/ml. IL-6 was significantly increased 2-3 hours

post-addition of LPS, maximal at 24 hours, and remained significant up to 96 hours, despite thereafter declining. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared with control (LPS = 0 mug/ml; T = 0 hour). $n = 4$, which represents the number of independent cell cultures performed and experiments were run in duplicate for the determination of the accumulation of inflammatory cytokines in the supernatant.

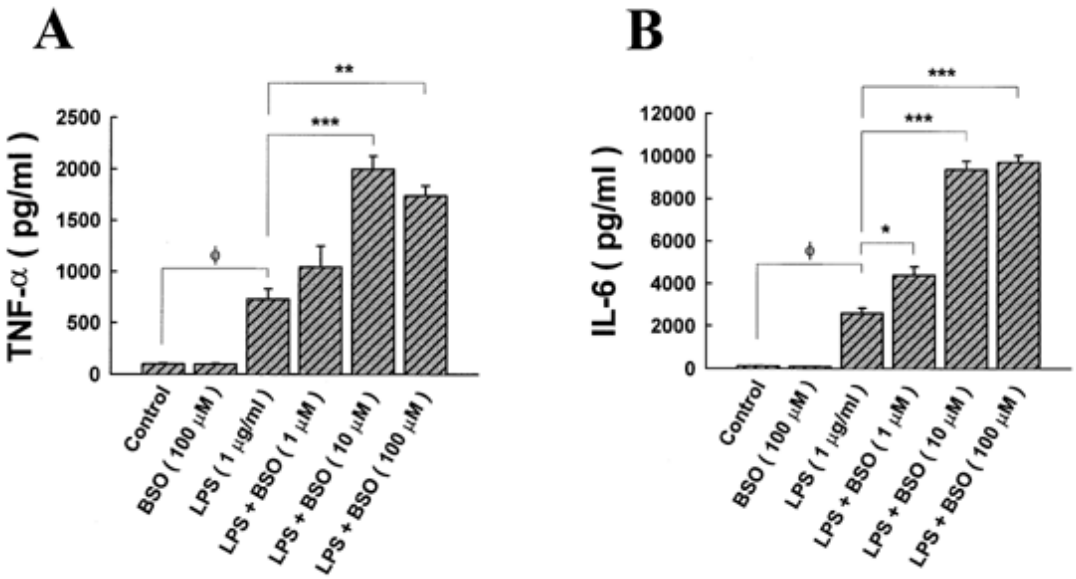


Figure 2

The role of L-buthionine-(*S,R*)-sulfoximine (BSO) in regulating LPS-mediated pro-inflammatory cytokine production. A: Pre-treatment with BSO (24 hours) prior to exposure to LPS (1 mug/ml; 24 hours) induced, in a dose-dependent manner, the release of TNF-alpha, evident at doses > = 10 muM. **B:** pre-treatment with BSO (24 hours) prior to exposure to LPS (1 mug/ml; 24 hours) induced, in a dose-dependent manner, the release of IL-6, evident at doses > = 1 muM. [phi] $P < 0.05$, as compared with control; ** $P < 0.01$, *** $P < 0.001$, as compared with LPS alone. $n = 4$, which represents the number of independent cell cultures performed and experiments were run in duplicate for the determination of the

accumulation of inflammatory cytokines in the supernatant.

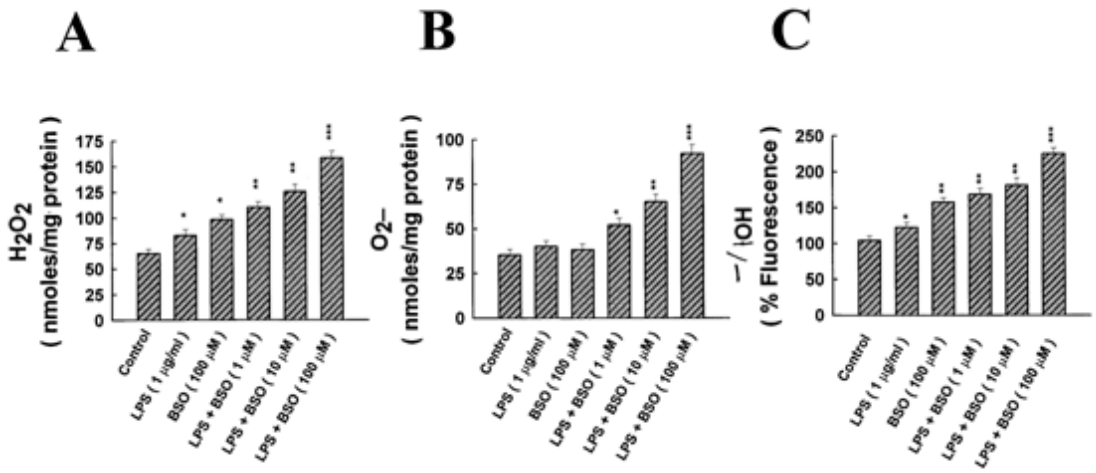


Figure 3

Analysis of intracellular ROS in response to LPS, BSO and their combination. A: the formation of H_2O_2. Exposure to LPS (24 hours) induced the production of H_2O_2, similar to BSO (24 hours) pre-treatment. BSO pre-treatment, followed by exposure to LPS, induced, in a dose-dependent manner, the accumulation of H_2O_2. **B:** the formation of $O_2^{-\cdot}$. LPS alone or BSO pre-treatment alone, followed by exposure to medium, had no effect on $O_2^{-\cdot}$. BSO pre-treatment followed by exposure to LPS induced, in a dose-dependent manner, the accumulation of $O_2^{-\cdot}$. **C:** The formation of ·OH. LPS alone marginally induced the accumulation of ·OH, similar to BSO pre-treatment alone. BSO pre-treatment followed by exposure to LPS induced, in a dose-dependent manner, the accumulation of ·OH. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared with control. Data are means ± SEM of 4 independent experiments in duplicate.

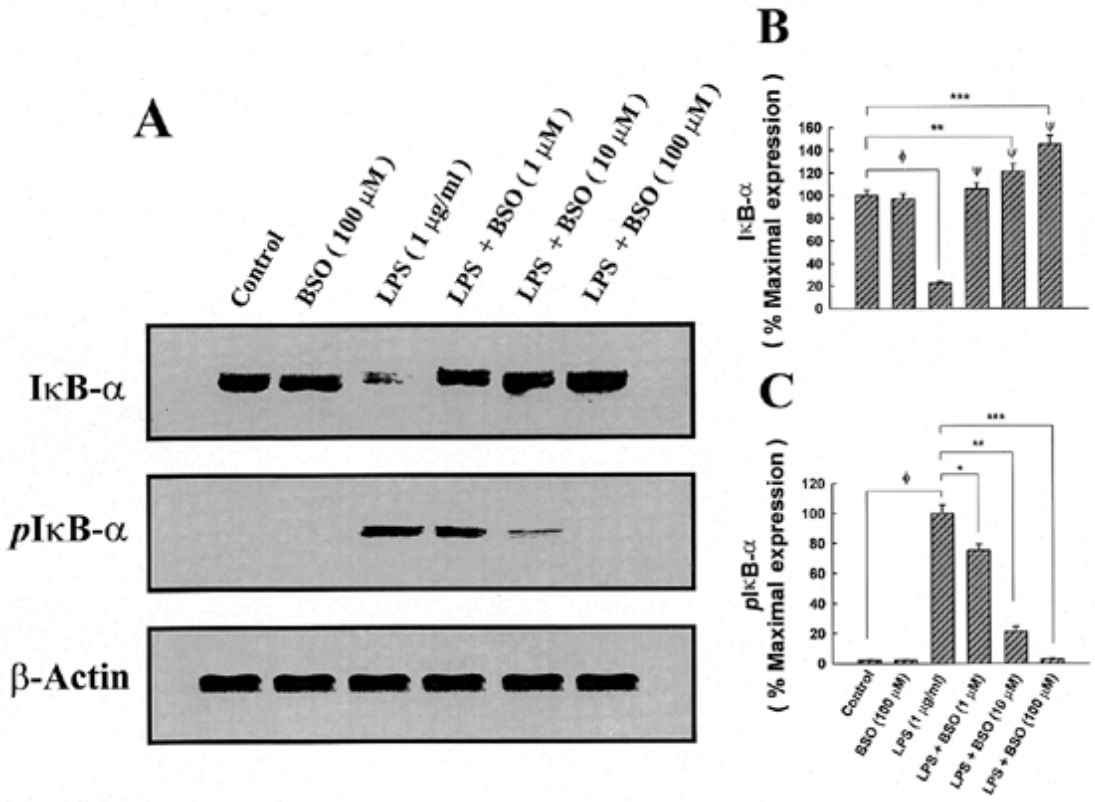


Figure 4

BSO-mediated regulation of LPS-induced NF-kappaB subunit nuclear translocation. LPS (1 mug/ml; 24 hours) induced the nuclear localization of NF-kappaB_1 (p50), RelA (p65), RelB (p68) and c-Rel (p75), with no apparent effect on NF-kappaB_2 (p52). Pre-treatment with BSO abrogated, in a dose-dependent manner, LPS-induced nuclear translocation of p50, p65, p68 and p75. The housekeeping beta-actin protein was used as an internal reference for semi-quantitative loading in parallel lanes. *n* = 3, which represents the number of independent experiments.

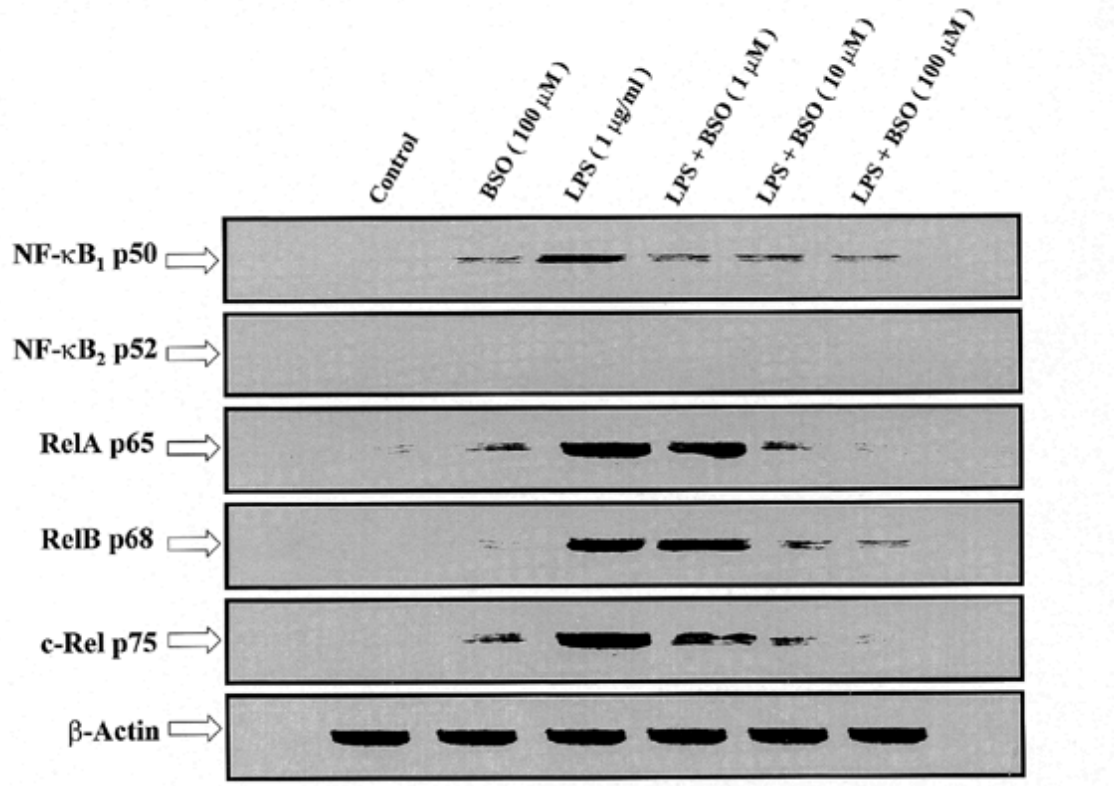


Figure 5

Western analysis of BSO-dependent regulation of LPS-mediated IkappaB-alpha phosphorylation/degradation. **A:** the effect of BSO on LPS-induced phosphorylation/degradation of IkappaB-alpha. BSO reversed LPS-mediated IkappaB-alpha degradation, allowing its cystosolic accumulation, in a dose-dependent manner. This effect was associated with the reduction of LPS-induced IkappaB-alpha phosphorylation, in a dose-dependent manner. The housekeeping beta-actin protein was used as an internal reference for semi-quantitative loading in parallel lanes. **B:** histogram analysis of the effect of BSO on LPS-induced IkappaB-alpha degradation. Control was set to 100% and other treatments were determined relative to control. **C:** histogram analysis of the effect of BSO on LPS-induced IkappaB-alpha phosphorylation. LPS treatment alone was set to 100% and all other values were determined relative to LPS. ^{phi} $P < 0.05$, as compared with control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared with control; ^{PSI} $P < 0.05$, as compared with LPS alone. $n = 3$, which represents the

number of independent experiments where data were pooled and histograms subsequently plotted.

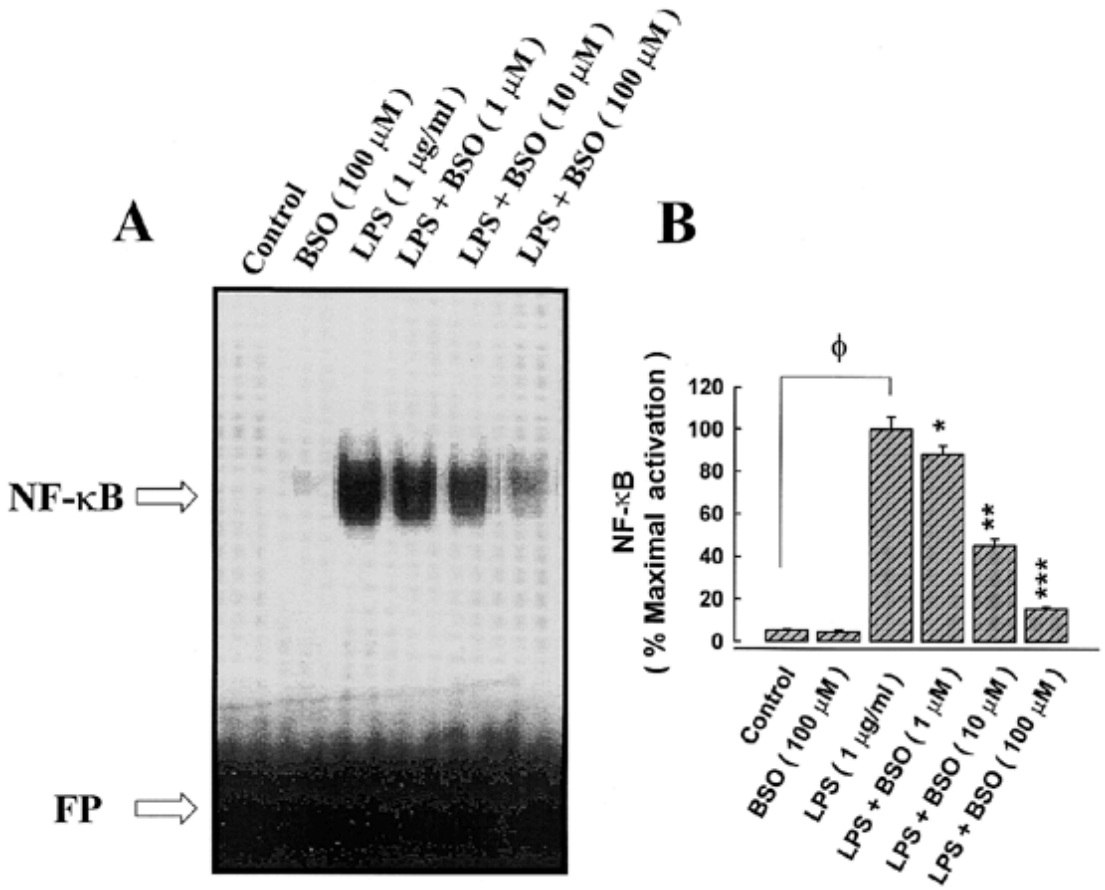


Figure 6

The effect of BSO on LPS-mediated NF-kappaB activation. A: representative EMSA showing the dose-dependent depression of NF-kappaB DNA binding activity in nuclear samples extracted from epithelial cells pre-treated with BSO. BSO reduced, in a dose-dependent manner, the DNA binding activity, but on its own had no effect on NF-kappaB activation, similar to control lane which contained no nuclear extracts. **B:** histogram analysis of the dose-response curve, where results were plotted relative to LPS.^{phi} $P < 0.05$, as compared with control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared with LPS. $n = 3$, which represents the number of independent experiments where data were pooled and histograms subsequently plotted.

Table 1
Glutathione homeostasis in epithelial cells pre-treated with BSO for 24 hours at 37° C

Total glutathione content (GSH + 2GSSG) (µmoles/mg protein)				
Degree of depletion with BSO (µM)				
0	1	10	50	100
128.5 ± 10.5	102.8 ± 11.5	46.6 ± 3.7**	21.4 ± 2.8***	08.7 ± 1.1***

Data are means ± SEM. BSO (0; n = 7), (1 µM; n = 5), (10 µM; n = 6), (50 µM; n = 6) and (100 µM; n = 7). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared to control (0), without BSO pre-treatment.

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human recombinant interleukin-1 beta, myelopoiesis, myelodepression, cancer chemotherapy.

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