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Redox Signaling-Mediated Regulation of Lipopolysaccharide-Induced Proinflammatory Cytokine Biosynthesis in Alveolar Epithelial Cells

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ABSTRACT

The regulation of cytokine gene transcription and biosynthesis involves the reduction–oxidation (redox)-sensitive nuclear factor-κB (NF-κB), whose activation is mediated by an upstream kinase that regulates the phosphorylation of inhibitory-κB (IκB). It was hypothesized that lipopolysaccharide (LPS)-induced biosynthesis of interleukin-1β, interleukin-6, and tumor necrosis factor-α *in vitro* is regulated by redox equilibrium. In alveolar epithelial cells, we investigated the role of L-buthionine-(*S,R*)-sulfoximine (BSO), an irreversible inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH biosynthesis, 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (BCNU), which inhibits glutathione oxidized disulfide reductase, pyrrolidine dithiocarbamate (PDTC), an antioxidant/prooxidant thiuram, and *N*-acetyl-L-cysteine (NAC), an antioxidant and GSH precursor, in regulating LPS-induced cytokine biosynthesis and IκB-α/NF-κB signaling. BSO blockaded the phosphorylation of IκB-α, reduced its degradation, and inhibited NF-κB activation, besides augmenting LPS-mediated biosynthesis of cytokines. BCNU up-regulated LPS-induced release of cytokines, an effect associated with partial phosphorylation/degradation of IκB-α and inhibition of the DNA binding activity. PDTC, which partially affected LPS-induced IκB-α phosphorylation/degradation, otherwise blockading NF-κB activation, reduced LPS-dependent up-regulation of cytokine release. Pretreatment with BSO did not abolish the NAC-dependent reduction of LPS-induced cytokine release, despite the fact that NAC marginally amplified IκB-α phosphorylation/degradation and suppressed NF-κB activation. These results indicate that cytokines are redox-sensitive mediators and that the IκB-α/NF-κB pathway is redox-sensitive and differentially implicated in mediating redox-dependent regulation of LPS-induced release of proinflammatory cytokines. Antioxid. Redox Signal. 4, 179–193.

INTRODUCTION

THE TRIPEPTIDE L-γ-glutamyl-L-cysteinyl-glycine, or glutathione (GSH), a ubiquitous thiol, plays an important role in maintaining intracellular reduction–oxidation (redox) equilibrium (19, 24, 28). The cysteinyl moiety provides the reactive thiol as a functional element responsible for the diverse properties of GSH, including an antioxidant potential and the regulation of cellular sulfhydryl status, redox equilibrium, and activation of redox-sensitive transcription factors, essentially involved in regulating the expression of proinflammatory genes encoding cytokines (13, 17, 24).

Under physiological conditions, the intracellular redox status of thiols is predominantly reductive. It has been reported, for instance, that GSH is elevated in lung epithelial lining

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fluid (10) and has been implicated in maintaining the integrity of the airspace epithelium *in vitro* and *in vivo* (26). On the contrary, GSH depletion has been linked to the pathophysiology of idiopathic pulmonary fibrosis (10), adult respiratory distress syndrome (9), bronchopulmonary dysplasia (37), and cystic fibrosis (35), thus highlighting its central role in maintaining the functional integrity of a physiologically competent epithelium.

The immunomodulatory potential assigned to glutathione stems from established observations (10, 13, 20, 44). An interleukin (IL)-1-induced response in mesangial cells, for example, was mediated through modulating redox equilibrium *in vitro* (36). In addition, reactive oxygen species (ROS)-dependent signaling regulating the transcription of IL-4 (25), IL-6, IL-8 (15), and tumor necrosis factor (TNF)-α (15, 29) occurred through a thiol-dependent mechanism. Interestingly, antioxidants (5, 21, 34, 46) and GSH precursors (20, 25, 32) have been shown to downregulate cytokine transcription and biosynthesis. Of note, *N*-acetyl-L-cysteine (NAC), an antioxidant and endogenous GSH precursor (7, 19, 24), has been reported to ameliorate cytokine production (20, 45) and ROS-mediated lung injury (7). In contrast, irreversible inhibition of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in GSH biosynthesis, by the action of L-buthionine-(*S,R*)-sulfoximine (BSO) (16, 17, 19, 28), has the potential to enhance cytokine release by inducing the intracellular accumulation of ROS (15, 20). We reasoned that a differential manipulation of glutathione homeostasis and shuttling may antagonistically and differentially affect a proinflammatory signal, thus bearing potential consequences for designing a novel strategy and approach in the treatment of respiratory distresses, where cytokines are recognized as major participants in their pathophysiology (30, 37).

Although the transcription factor nuclear factor-κB (NF-κB) was originally recognized as a transcriptional activity closely involved in regulating gene expression in B-cell lymphocytes (42), subsequent investigations demonstrated that it is one member of a ubiquitously expressed family of *Rel*-related transcription factors that serve as critical regulators of many genes, including those of proinflammatory cytokines (6, 12, 22, 38, 39). The translocation and activation of NF-κB in response to various stimuli are sequentially organized at the molecular level. In its inactive state, the heterodimeric NF-κB, which is composed mainly of two subunits, p50 (NF-κB_1) and p65 (RelA), the major transactivating member of the *Rel* family, is present in the cytoplasm associated with its inhibitory protein, IκB (6, 8, 22, 27, 38). Upon stimulation, such as with cytokines and lipopolysaccharide (LPS), IκB-α undergoes phosphorylation on serine/threonine residues (6, 12), ubiquitination, and subsequent proteolytic degradation, thereby unmasking the nuclear localization signal on p65 and allowing nuclear translocation of the complex. Signals emanating from membrane receptors, such as those for IL-1 and TNF-α, activate members of the MEKK-related family, including NF-κB-inducing kinase and $MEKK_1$, both of which are involved in the activation of IκB kinases, IKK_1 and IKK_2, components of the IKK signalsome. IKK_1 and IKK_2 were identified as components of the high-molecular weight complex containing a number of proteins involved in NF-κB regulation (6, 12). These kinases phosphorylate members of the IκB family, including IκB-α, at specific serines within their amino termini, thereby leading to site-specific ubiquitination and degradation by the 26S proteasome. This sequential propagation of signaling, therefore, ultimately results in the release of NF-κB subunits from IκB-α inhibitor, allowing translocation and promotion of gene transcription.

This study investigated *in vitro* the immunomodulatory role that glutathione plays, and the underlying implicated molecular mechanism is therefore explored. Specifically, glutathione depletion and repletion differentially manipulated proinflammatory cytokines, an effect antagonistically reversed by restoring redox equilibrium. In addition, this antagonistic effect of redox status further regulates the IκB-α/NF-κB downstream pathway, where we showed that it is differentially involved in mediating the role of glutathione in regulating a proinflammatory signal within the alveolar epithelium.

MATERIALS AND METHODS

Chemicals and reagents

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

Primary cell cultures of alveolar epithelia

Fetal alveolar type II epithelial cells were isolated from the lungs of fetuses of pregnant Sprague–Dawley rats, essentially as reported elsewhere (17–19). In brief, fetal rats were removed by caesarian section at day 19 of gestation (term = 22 days); the lungs were excised, teased free from heart and upper airway tissue, finely minced, and then washed free of erythrocytes using sterile, chilled Mg^{2+}- and Ca^{2+}-free Hanks' balanced salt solution (0.5 ml/fetus). The cleaned lung tissue was resuspended in 1 ml/fetus Hanks' balanced salt solution containing trypsin (0.1 mg/ml), collagenase (0.06 mg/ml), and DNase I (0.012%, wt/vol) and agitated at 37°C for 20 min. The solution was then centrifuged at 100 *g* for 2 min to remove undispersed tissue, the supernatant was decanted into a sterile tube, and an equal volume of Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) fetal calf serum (FCS) was added to the supernatant. After the supernatant was passed through a 120-μm-pore sterile mesh, the filtrate was centrifuged at 420 *g* for 5 min, the pellet was resuspended in 20 ml of DMEM/FCS, and the cells were placed into a T-150 culture flask for 1 h at 37°C to enable fibroblasts and nonepithelial cells to adhere. Unattached cells were washed three times by centrifugation at 420 *g* for 5 min each, then seeded onto 24-mm-diameter Transwell-clear permeable supports (Costar, U.K.; 0.4-μpore size) at a density of 5×10^6 cells per filter, and allowed to adhere overnight at 152 torr (≈21% O_2/5% CO_2) at 37°C. DMEM/FCS was exchanged for 4 ml of preequilibrated serum-free PC-1 media (BioWhittaker, Walkerville, MD, U.S.A.) 24 h later, and cells were maintained as such until the experiment. The adenylate energy charge, an index of cell viability and competence, remained ≥0.7, and transepithelial monolayer resistance was monitored constantly at 250–350 Ω cm^2 or more (17–19).

Assessment of proinflammatory cytokines by enzyme-linked immunosorbent assay (ELISA)

The bioactivity of extracellularly released cytokines was measured by a two-site, solid-phase, developed sandwich ELISA (20–22). Immunoaffinity-purified polyclonal rabbit anti-rat IL-1β, IL-6, and TNF-α (2 μg/ml) primary antibodies were used to coat high-binding microtitre plates (MaxiSorp, Nunc). Recombinant rat and biotinylated immunoaffinity-purified sheep anti-rat cytokine (R&D Systems) were used as standard and recognition antibodies, respectively. The color was developed incorporating streptavidin-polyhorseradish peroxidase coupled reaction with the chromagen 3,3′,5,5′-tetramethylbenzidine dihydrochloride, and the optical density was measured at 450 nm against a filter background at 595 nm. Inter- and intraassay coefficients of variations were <10%, and the minimum detectable sensitivity for each cytokine is ;≤2 pg/ml. Results interpolated from the linear regression of the standard curve are expressed as picograms per milliliter.

LPS treatment, western analysis and electrophoretic mobility shift assay (EMSA)

For the dose–response curves, cells were pretreated with glutathione-modulating agents (see below) for 24 h before exposure for a further 24 h to LPS (1 μg/ml) from *Escherichia coli*, serotype 026:B6. Following treatments, subcellular extracts (cytosolic and nuclear) were prepared essentially as previously described (17–19). In brief, filters were washed twice in 5 ml of ice-cold, preequilibrated phosphate-buffered saline (pH 7.2–7.4), and cells ($1–2 \times 10^7$) were collected and centrifuged at 420 *g* for 5 min at 4°C. Nuclei were released by resuspending the pellet in 250 μl of buffer A containing the following (in m*M*): 10 Tris-HCl (pH 7.8), 10 KCl, 2.5

NaH_2PO_4, 1.5 $MgCl_2$, 1 Na_3VO_4, 0.5 dithiothreitol, 0.4 4-(2-aminoethyl)benzenesulfonyl fluoride-HCl, and 2 μg/ml each of leupeptin, pepstatin A, and aprotinin. The suspension was left in ice for 10 min followed by a 45-s homogenization at a moderate speed. Nuclei were collected by centrifuging the slurry at 4,500 *g* for 5 min at 4°C and resuspending in 100 μl of buffer B [buffer A adjusted to (in m*M*): 20 Tris-HCl (pH 7.8), 420 KCl, 20% (vol/vol) 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 were determined by the Bradford method using bovine serum albumin as a standard. Cytosolic and nuclear proteins (20–25 μg) were resolved over sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5%) gels at room temperature, and blotted onto nitrocellulose membrane, and nonspecific binding sites were subsequently blocked. Mouse monoclonal IgG_1 anti-IκB-α (H-4), IgG_{2b} anti-(phosphorylated) *p*IκB-α (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, U.K.) antibodies were used for primary detection. Anti-rabbit Ig-biotinylated antibody (Amersham Life Science, UK) was used for secondary detection followed by the addition of streptavidin–horseradish peroxidase conjugate and visualized on film by chemiluminescence. β-Actin standard was used as an internal reference for semiquantitative loading in parallel lanes for each variable. Western blots were scanned by NIH MagiScanII and subsequently quantitated by UN-Scan-IT automated digitizing system (version 5.1; 32-bit), and the ratio of the density of the band to that of β-actin was subsequently performed. Nuclear extracts were analyzed for the NF-κB DNA binding activity by EMSA, and specific quantitation of the corresponding DNA gel-shift bands was performed with phosphorimaging (17–19).

Redox homeostasis and thiol regulation of LPS-induced cytokine release

Redox regulation of proinflammatory cytokines is not well characterized in the alveolar epithelium. Glutathione is postulated as a negative modulator of cytokine biosynthesis, but whether this effect represents an antioxidant property has yet to be ascertained.

The role of irreversible inhibition of γ-GCS. To determine the effect of redox disequilibrium (depletion) on LPS-induced cytokine release, cells were pretreated (24 h) with BSO (0, 1, 10, 100 μ*M*), a specific and irreversible inhibitor of γ-GCS, the rate-limiting enzyme in the biosynthesis of GSH (16, 17, 28), and then exposed to LPS (1 μg/ml) for 24 h. Subcellular extracts were subsequently prepared, and cell-free supernatants were collected and assayed for cytokine release by ELISA.

The role of irreversible inhibition of glutathione oxidized disulfide reductase (GSSG-RD). Cells were pretreated with 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU; 0, 1, 10, 100 μ*M*), a specific inhibitor of GSSG-RD (23), then exposed to LPS, and assayed for cytokines.

The role of pyrrolidine dithiocarbamate (PDTC) and NAC. Cells were pretreated with PDTC (0, 1, 10, 100 μ*M*), an antioxidant/prooxidant that elevates GSSG (19, 39), or NAC (0, 1, 10, 50 m*M*), an antioxidant and a cysteine precursor (7, 20). Following pretreatments, cells were challenged with LPS (1 μg/ml) for 24 h and supernatants collected for cytokine assessment. In separate experiments, cells were pretreated with BSO (100 μ*M*) for 24 h, prior to simultaneous incubation with NAC (50 m*M*) and LPS (1 μg/ml), followed by analysis of cytokines.

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, followed by *post hoc* Tukey's test, and the *a priori* level of significance at 95% confidence level was considered at $p \leq 0.05$.

RESULTS

The effect of BSO on LPS-induced proinflammatory cytokine biosynthesis

We have previously shown that BSO upregulated intracellular accumulation of ROS and subsequently induced the downstream pathway implicated in cytokine biosynthesis (20, 21). In this study, BSO expectedly augmented, in a dose-dependent manner, LPS-induced release of IL-1β (Fig. 1A), IL-6 (Fig. 1B), and TNF-α (Fig. 1C). LPS increased IL-1β secretion by 26-fold above control baseline level, and BSO increased the level of IL-1β approximately 1.3-, 2.6-, and 2.9-fold relative to LPS alone at 1, 10, and 100 μ*M*, respectively (Fig. 1A). LPS increased IL-6 secretion by 21-fold above 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 μ*M*, respectively (Fig. 1B). LPS increased TNF-α secretion by 10-fold above control baseline level, and BSO increased the level, of TNF-α approximately 1.2- (nonsignificant), 2-, and 1.7-fold relative to LPS alone at 1, 10, and 100 μ*M*, respectively (Fig. 1C).

The effect of BCNU on LPS-induced proinflammatory cytokine biosynthesis

Although BCNU suppressed ROS-dependent cytokine biosynthesis (20, 21), it augmented LPS-induced cytokine release, as shown in Fig. 2. BCNU potentiated, in a dose-dependent manner, LPS-induced release of IL-1β (Fig. 2A), IL-6 (Fig. 2B), and TNF-α (Fig. 2C). BCNU increased the level of IL-1β approximately 1.1- (nonsignificant), 1.4-, and 1.4-fold relative to LPS alone at 1, 10, and 100 μ*M*, respectively (Fig. 2A). BCNU increased the level of IL-6 approximately 1.5-, 1.9-, and 2.5-fold relative to LPS alone at 1, 10, and 100 μ*M*, respectively (Fig. 2B). BCNU increased the level of TNF-α approximately 1.3-, 1.4-, and 1.5-fold relative to LPS alone at 1, 10, and 100 μ*M*, respectively (Fig. 2C).

The effect of PDTC on LPS-induced proinflammatory cytokine biosynthesis

We have previously shown that PDTC suppressed ROS-induced cytokine biosynthesis in a GSSG-dependent mechanism (19–21). In this study, PDTC suppressed, in a dose-dependent manner, LPS-induced release of IL-

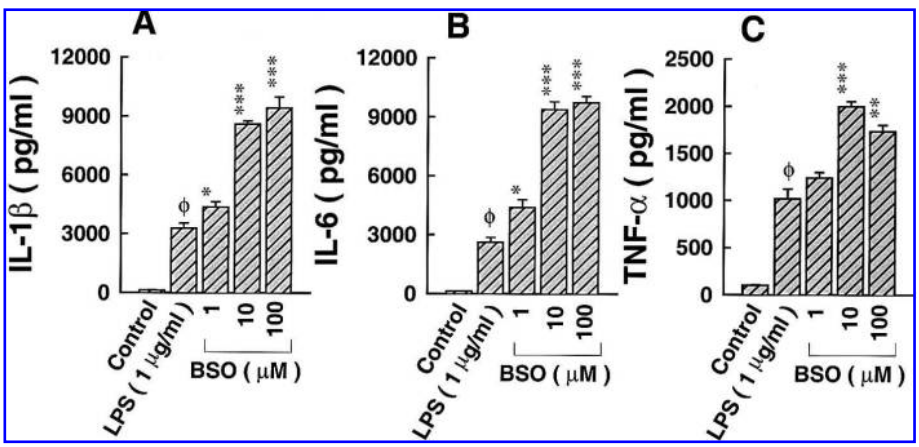

FIG. 1. The role of BSO in LPS-mediated proinflammatory cytokine biosynthesis. (**A**) LPS induced the release of IL-1β, an effect potentiated, in a dose-dependent manner, by BSO pretreatment. (**B**) The LPS-mediated increase in IL-6 biosynthesis is amplified, in a dose-dependent manner, with BSO. (**C**) The potentiating effect of BSO on LPS-induced release of TNF-α is evident at doses ≥ 10 μ*M*. $^{\phi}p < 0.05$, as compared with control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, as compared with LPS alone. $n = 4$, which represents the number of independent experiments, each run in duplicates.

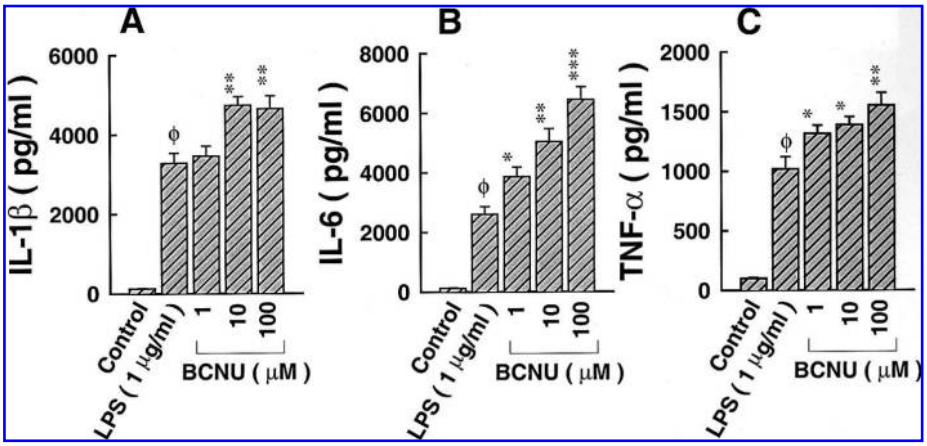

FIG. 2. The role of BCNU in LPS-mediated proinflammatory cytokine biosynthesis. (**A**) LPS induced the release of IL-1β, an effect potentiated by BCNU at doses ≥ 10 μ*M*. (**B**) The LPS-mediated increase in IL-6 biosynthesis is amplified, in a dose-dependent manner, with BCNU. (**C**) The potentiating effect of BCNU on LPS-induced release of TNF-α is dose-dependent. ϕ $p < 0.05$, as compared with control; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as compared with LPS alone. $n = 4$, which represents the number of independent experiments, each run in duplicates.

1β (Fig. 3A), IL-6 (Fig. 3B), and TNF-α (Fig. 3C). PDTC reduced the level of IL-1β approximately 1.1- (nonsignificant), 1.7-, and 2.3-fold relative to LPS alone at 1, 10, and 100 μ*M*, respectively (Fig. 3A). PDTC reduced the level of IL-6 approximately 0.85- (nonsignificant), 1.5-, and 1.7-fold relative to LPS alone at 1, 10, and 100 μ*M*, respectively (Fig. 3B). PDTC reduced the level of TNF-α approximately 0.9- (nonsignificant), 0.9- (nonsignificant), and 1.5-fold relative to LPS alone at 1, 10, and 100 μ*M*, respectively (Fig. 3C).

The effect of NAC on LPS-induced proinflammatory cytokine biosynthesis

We have previously shown that NAC suppressed ROS-induced cytokine biosynthesis in a GSH-dependent mechanism (20, 21) Here, NAC suppressed, in a dose-dependent manner, LPS-induced release of IL-1β (Fig. 4A), IL-6 (Fig. 4B), and TNF-α (Fig. 4C). NAC reduced the level of IL-1β approximately 1.4-, 2.6-, and 6.1-fold relative to LPS alone at 1, 10, and 50 m*M*, respectively (Fig. 4A). NAC

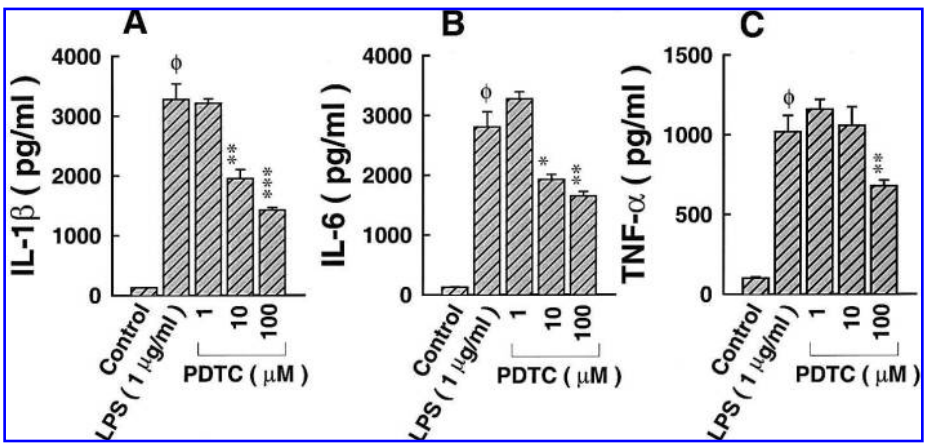

FIG. 3. The role of PDTC in LPS-mediated proinflammatory cytokine biosynthesis. (**A**) LPS induced the release of IL-1β, an effect blocked by PDTC at doses ≥ 10 μ*M*. (**B**) The LPS-mediated increase in IL-6 biosynthesis is inhibited with PDTC at doses ≥ 10 μ*M*. (**C**) The inhibitory effect of PDTC on LPS-induced release of TNF-α is prominent at 100 μ*M*. ϕ $p < 0.05$, as compared with control; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as compared with LPS alone. $n = 4$, which represents the number of independent experiments, each run in duplicates.

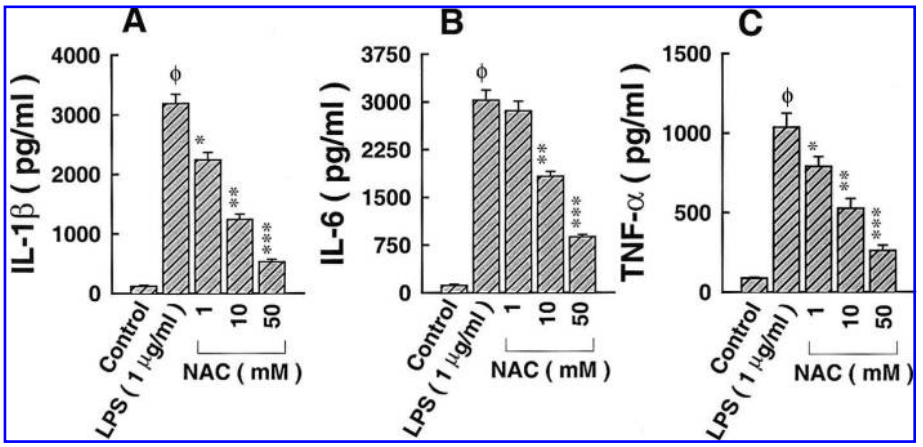

FIG. 4. The role of NAC in LPS-mediated proinflammatory cytokine biosynthesis. (**A**) LPS induced the release of IL-1β, an effect blocked by NAC in a dose-dependent manner. (**B**) The LPS-mediated increase in IL-6 biosynthesis is inhibited with NAC at doses ≥ 10 m*M*. (**C**) The inhibitory effect of NAC on LPS-induced release of TNF-α is dose-dependent. $^{\phi}$ $p < 0.05$, as compared with control; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as compared with LPS alone. $n = 4$, which represents the number of independent experiments, each run in duplicates.

reduced the level of IL-6 approximately 1.1-(nonsignificant), 1.7-, and 3.4-fold relative to LPS alone at 1, 10, and 50 m*M*, respectively (Fig. 4B). NAC reduced the level of TNF-α approximately 1.3-, 2-, and 4-fold relative to LPS alone at 1, 10, and 50 mM, respectively (Fig. 4C).

The role of glutathione-modulating agents in IκB-α/NF-κB signaling

To determine an integral role for GSH precursors and antioxidants in regulating LPS-mediated cytokine release and whether the IκB-α/NF-κB pathway is likely to be involved, we tested the effect of glutathione-depleting and -repleting agents on IκB-α phosphorylation and degradation, and NF-κB subunit nuclear localization and activation. Under baseline conditions, we found that IκB-α protein is constitutively expressed and that exposure to LPS induced its cytosolic degradation in a time-dependent (4–96 h) and dose-dependent (0–10 μg/ml) manner (22). As shown in Fig. 5A, BSO blockaded, in a dose-dependent manner, LPS-induced degradation of IκB-α in the cytosolic compartment, as compared with the negative (LPS; 0 μg/ml) and positive (LPS; 1 μg/ml) controls. This effect was paralleled by inhibiting IκB-α phosphorylation, thus allowing its cytosolic accumulation. On the other hand, although BCNU suppressed LPS-mediated degradation of IκB-α at 1 and 10 μ*M*, thus restoring its abundance within this range, the degradation of IκB-α was comparable to the positive control at the highest dose (100 μ*M*) (Fig. 5B). The effect of BCNU occurred in parallel to a marginal, dose-dependent, up-regulation of IκB-α phosphorylation, as shown in Fig. 5B. PDTC, an antioxidant/prooxidant thiuram, mildly affected LPS-mediated degradation of IκB-α, in parallel to a marginal dose-independent up-regulation of its phosphorylation state (Fig. 6A). The effect of PDTC was similar to that observed with NAC, an antioxidant and a precursor of GSH (Fig. 6B). In parallel to differentially regulating LPS-induced IκB-α phosphorylation and degradation, thiol-modulating agents retarded the induced nuclear localization of specific NF-κB subunits (Fig. 7). LPS up-regulated the nuclear accumulation of NF-κB_1 (p50), RelA (p65), the major transactivating member of the *Rel* family, RelB (p68), and c-Rel (p75), but not NF-κB_2 (p52) (Fig. 7). BSO, BCNU, PDTC, and NAC similarly, and in a dose-independent manner, abolished LPS-induced NF-κB nuclear localization, as shown in Fig. 7. In addition to blockading NF-κB nuclear localization, BSO, BCNU, PDTC, and NAC reduced, in a dose-independent manner, the DNA binding activity to specific κB moieties (Fig. 8A). Histogram analysis

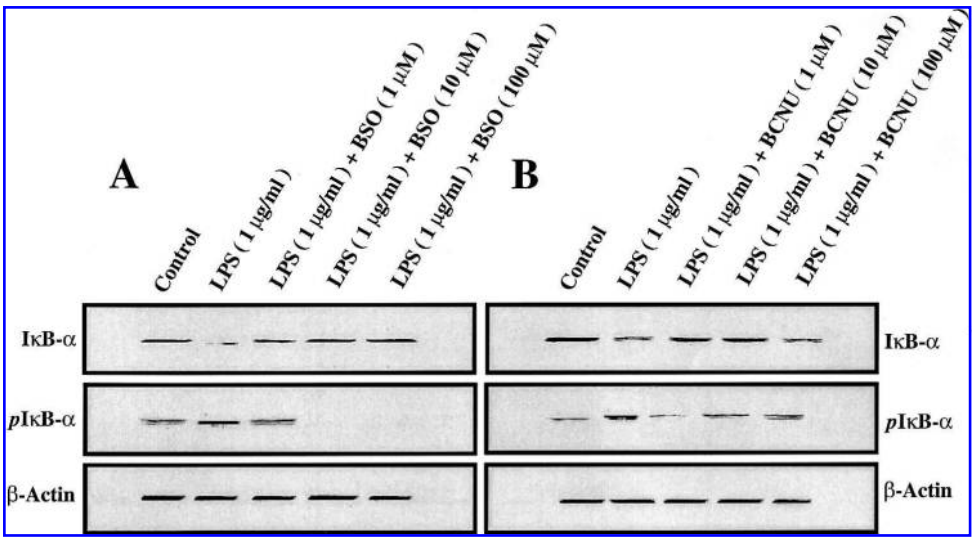

FIG. 5. Western analysis of thiol-dependent regulation of LPS-mediated IκB-α phosphorylation/degradation. (**A**) The effect of BSO on LPS-induced phosphorylation/degradation of IκB-α. BSO reversed LPS-induced IκB-α degradation, allowing its cytosolic accumulation. This effect was accompanied by a reduction in LPS-induced IκB-α phosphorylation, in a dose-dependent manner. (**B**) The effect of BCNU on LPS-induced phosphorylation/degradation of IκB-α. BCNU reversed LPS-induced IκB-α degradation, allowing its cytosolic accumulation, an effect not observed at 100 μ*M*. BCNU did not potentiate LPS-induced phosphorylation of IκB-α, except mildly at 100 μ*M*. The housekeeping β-actin protein was used as an internal reference for semiquantitative loading in parallel lanes. $n = 4$, which represents the number of independent experiments.

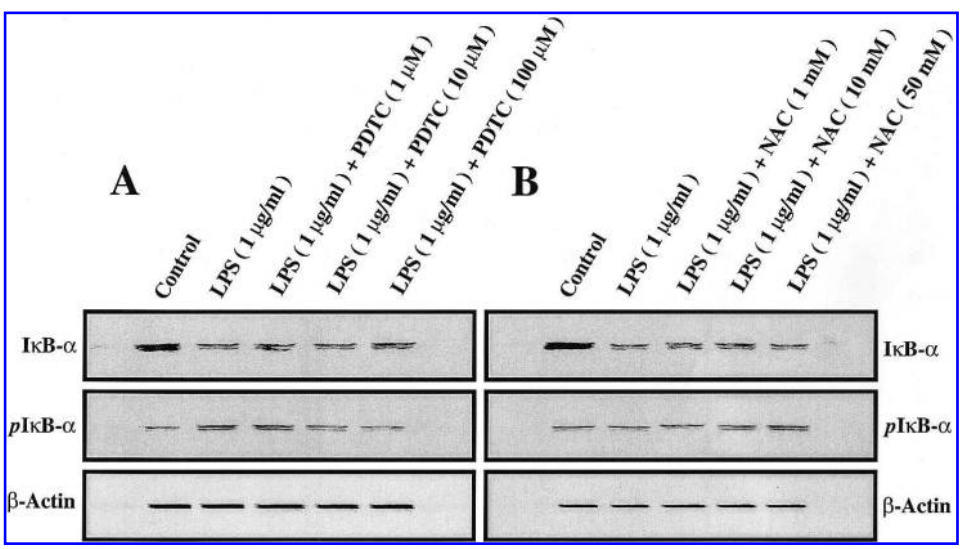

FIG. 6. Western analysis of thiol-dependent regulation of LPS-mediated IκB-α phosphorylation/degradation. (**A**) The effect of PDTC on LPS-induced phosphorylation/degradation of IκB-α. The PDTC effect on LPS-induced IκB-α degradation is similar to LPS alone, but it mildly enhanced LPS-induced phosphorylation in a dose-independent manner. (**B**) The effect of NAC on LPS-induced phosphorylation/degradation of IκB-α. NAC did not potentiate LPS-induced IκB-α degradation, an effect accompanied by a marginal, dose-dependent, increase in LPS-induced IκB-α phosphorylation. The housekeeping β-actin protein was used as an internal reference for semiquantitative loading in parallel lanes. $n = 4$, which represents the number of independent experiments.

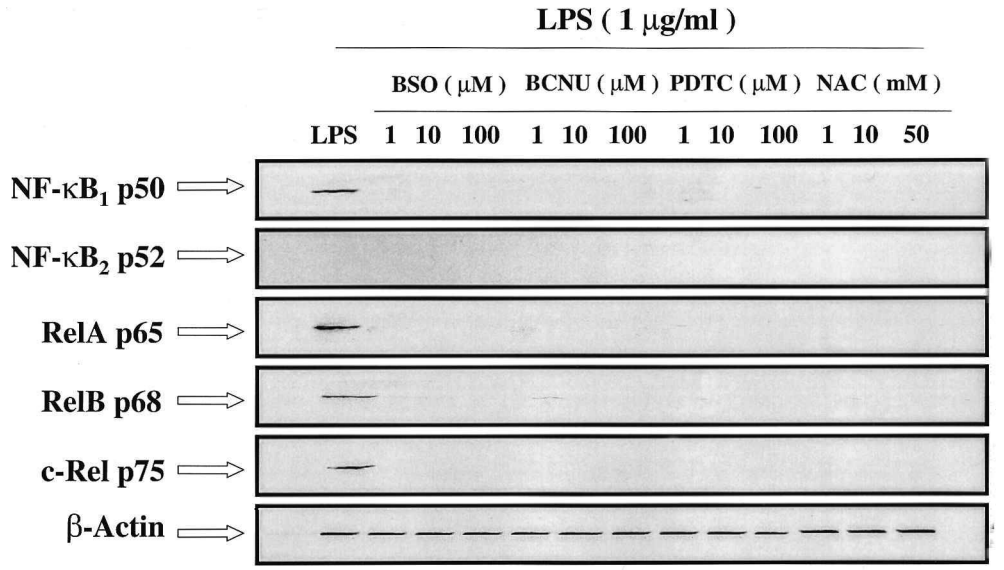

FIG. 7. Western analysis of thiol-dependent regulation of LPS-mediated NF-κB nuclear localization. LPS up-regulated the nuclear accumulation of NF-κB_1 (p50), RelA (p65), RelB (p68), and c-Rel (p75), but not NF-κB_2 (p52). The thiol-modulating agents BSO, BCNU, PDTC, and NAC blocked, in a dose-independent manner, LPS-induced NF-κB nuclear localization. The housekeeping β-actin protein was used as an internal reference for semiquantitative loading in parallel lanes. $n = 4$, which represents the number of independent experiments.

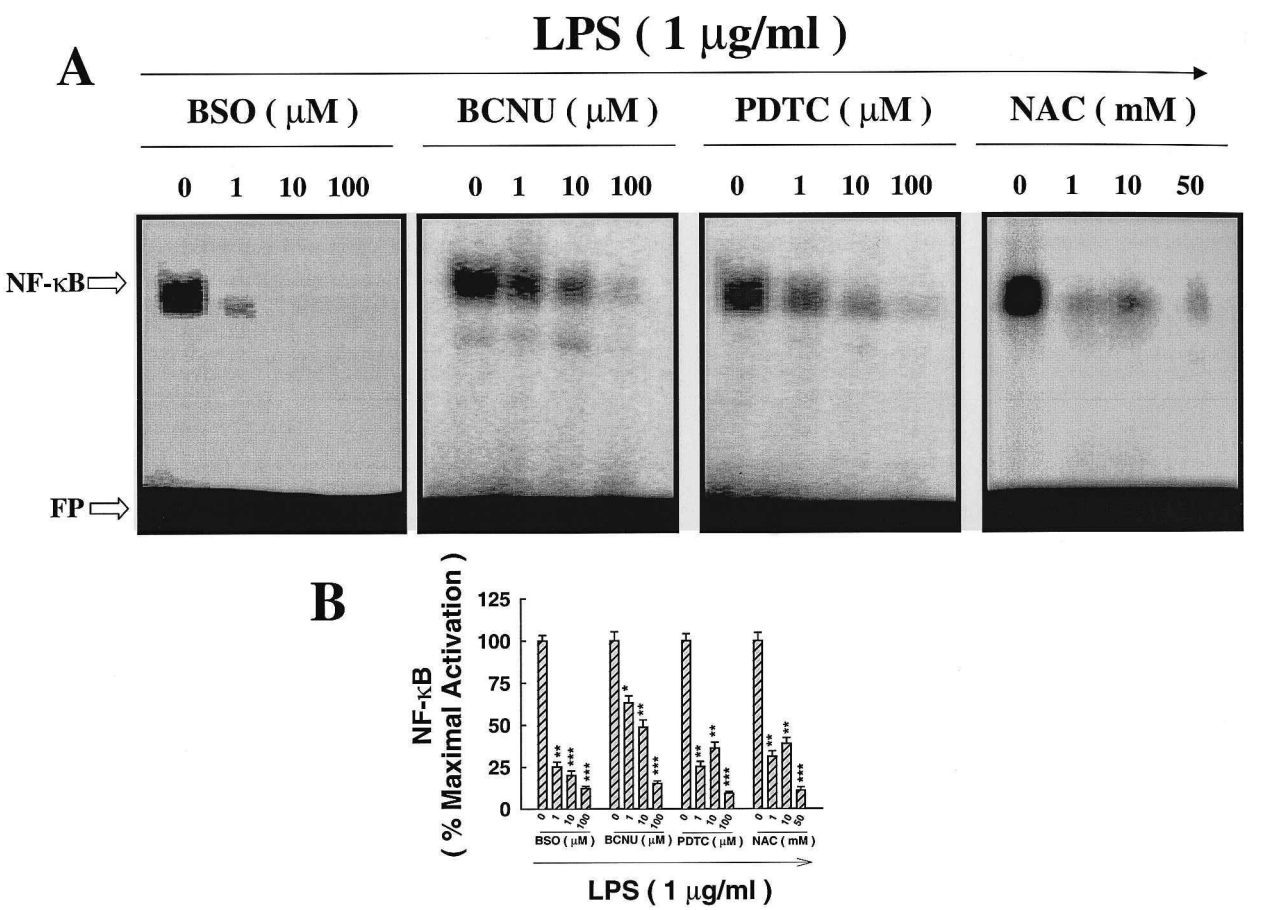

FIG. 8. An EMSA showing the inhibitory effect of thiol-modulating agents on LPS-induced NF-κB activation. (**A**) BSO, BCNU, PDTC, and NAC reduced LPS-induced NF-κB activation (24 h). (**B**) Histogram analysis of the corresponding gel-shifted bands (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as compared with control).

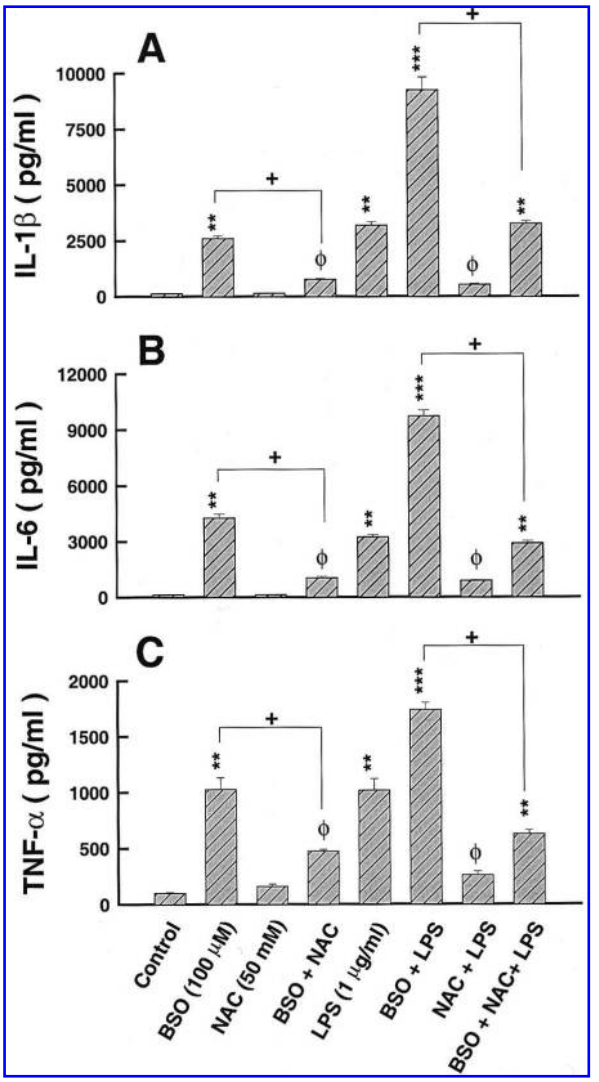

FIG. 9. The effect of BSO on NAC-induced down-regulation of LPS-induced cytokine release. NAC reduced BSO-induced up-regulation of (**A**) IL-1β, (**B**) IL-6, and (**C**) TNF-α, suggesting that its inhibitory effects are independent of GSH biosynthesis (**$p < 0.01$, ***$p < 0.001$, as compared with control; $^{+}p < 0.05$; $^{\phi}p < 0.05$, as compared with BSO).

of the corresponding shifted bands is shown in Fig. 8B.

Selective modulation of redox-sensitive enzymes and cytokines

The effect of BSO on NAC-induced down-regulation of cytokine release is shown in Fig. 9. NAC significantly reduced BSO/LPS-induced IL-1β (Fig. 9A), IL-6 (Fig. 9B), and TNF-α (Fig. 9C) release.

DISCUSSION

This investigation has dealt with the hypothesis that modulating redox equilibrium regulates proinflammatory cytokine biosynthesis and that the IκB-α/NF-κB pathway is differentially implicated in mediating redox-dependent regulation of cytokines in the alveolar epithelium. Irreversible inhibition of glutathione biosynthesis augmented LPS-induced release of proinflammatory cytokines in a ROS-dependent manner, regardless of its ability to down-regulate IκB-α phosphorylation/degradation and subsequently intervene in NF-κB activation. Blockading redox cycle through the conversion of GSSG → 2 GSH (17–19), thereby accumulating intracellular GSSG, reversed LPS-induced IκB-α degradation, thus allowing its cystosolic accumulation, an effect not reproducibly observed at high doses. Whilst this mechanism partially potentiated LPS-induced phosphorylation of IκB-α, it up-regulated LPS-induced release of cytokines despite blockading NF-κB activity. The antioxidant/prooxidant thiuram, PDTC, mildly potentiated LPS-induced IκB-α degradation and marginally affected LPS-induced phosphorylation, retarded the nuclear localization/activation of NF-κB, however, and suppressed cytokine biosynthesis. Analysis of the mechanism of action of the antioxidant and GSH precursor, NAC, revealed that its ability to down-regulate proinflammatory cytokine release is independent of its capacity to act as a GSH precursor, but requires, at least partially, the involvement of a NF-κB-sensitive pathway. Taken together, these results indicate that cytokines are redox-sensitive and that the IκB-α/NF-κB pathway is differentially implicated in redox-dependent regulation of LPS-mediated biosynthesis of proinflammatory cytokines in the alveolar epithelium.

We hitherto report *in vitro* regulatory mechanisms of pharmacological thiols on proinflammatory cytokines. This approach bears clinical relevance to the pediatric treatment of respiratory distresses, where cytokines are crucial elements in their pathophysiology. Growing evidence implicated an

association between redox signaling and the regulation of a proinflammatory state, thereby placing more demand on the utilization of intracellular glutathione (1, 40). As such, the respiratory epithelium becomes more engaged in regulating enzymes involved in maintaining redox homeostasis (17–19). Whilst the glutathione biosynthetic machinery is overwhelmed in disease, up-regulating cytokines may exacerbate the clinical symptoms. Although cytokine participation in the pathogenesis of respiratory distress has been considerably 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 m*M*; $K_{m\ Cysteine}$ = 0.3 m*M*) (17–21, 24). This pathway is selectively blocked by BSO, a specific and irreversible inhibitor of γ-GCS (16, 19, 28). 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 (17–19). This subsequently leads to ROS up-regulation, whose inappropriate disposition, accumulation, and intracellular localization augment a proinflammatory signal that is dependent, in part, on the activation of redox-sensitive transcription factors, such as NF-κB (39, 40, 41, 43). This is consistent with the observation that the expression of γ-GCS was shown to suppress TNF-α-induced activation of NF-κB (27). We believe that the pathway mediating BSO-induced up-regulation of cytokines in the alveolar epithelium involves a secondary mediator, the most likely candidate being the hydroxyl radical (·OH) (20). This conforms to the evidence that GSH is involved in hydrogen peroxide (H_2O_2) reduction, a major source of ·OH, through the GSH-peroxidase coupled reaction (17–19, 24), suggesting that in the case of BSO preincubation, GSH depletion seems involved in its effect (20). It is possible that GSH depletion, by blocking its biosynthesis, reduces the capacity of the epithelium to dispose accumulating H_2O_2, with the resulting increase in ·OH production, consistent with other observations (17, 19, 27). It remains to be defined, however, whether GSH depletion is implicated in up-regulating cytokines in association with lung disease, because the degree of depletion necessary to evoke cytokines *in vitro* is higher than that observed in pathophysiology *in vivo* (1, 15, 20, 33, 40).

The intriguing ability of BSO to block the translocation/activation of NF-κB, yet up-regulate the presumably downstream cytokine pathway remains of particular interest. We have previously reported that BSO blocked the oxyexcitation (ΔpO_2)-dependent nuclear localization of RelA (p65), the major transactivating member of the *Rel* family, and subsequently suppressed NF-κB activation (17–19). However, in additional studies, we also showed that BSO is capable of inducing intracellular accumulation of ROS, particularly ·OH (20, 21). Taken together, these data argue for ROS as potential second messengers for cytokine biosynthesis in the alveolar epithelium; however, ROS might not be favorably universal messengers in the activation of NF-κB (27, 38, 39). Furthermore, the ability of BSO to inhibit the phosphorylation/degradation of IκB-α, the major cytosolic inhibitor of NF-κB (22), yet up-regulate cytokine biosynthesis, demonstrated that this transcription factor is partially involved in regulating redox-mediated pathways governing proinflammatory cytokines in the alveolar epithelium. Although a NF-κB consensus binding site is present in the promoter region of IL-1β, IL-6, IL-8, and TNF-α genes (2, 12, 33), we are unable to conclude from the present data alone whether these specific κB 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, we are able to dissociate between ROS-dependent and -independent pathways governing the translocation/activation of NF-κB in the alveolar epithelium and that this pathway, along with the downstream proinflammatory cytokine pathway, is tightly regulated by redox signaling. Furthermore, evidence supported a separation of oxidant-initiated and redox-regulated mecha-

nisms in the NF-κB signal transduction pathway (1, 2). Therefore, the IκB-α/NF-κB pathway could be partially dissociated from that of redox-dependent regulation of cytokines, indicating that this transcription factor is not exclusively ROS-sensitive, but differentially implicated in regulating proinflammatory cytokine signaling, suggesting the involvement of a possible cross-talk among several pathways working independently or in coherence to integrate ROS and redox signaling mechanisms governing the regulation of cytokines.

Although the involvement of GSSG in pathways determining the induction of cytokines in the alveolar epithelium is not well characterized, its role in regulating redox equilibrium is established (8, 19, 28, 39). Replenishing GSH is not only γ-GCS rate-limited, but also determined by the degree of NADPH-dependent GSSG recycling (17–19, 24). Thus, favoring oxidation equilibrium by elevating GSSG has been reported to activate signaling pathways that down-regulate the activation of transcription factors (1, 2, 40). This is consistent with the observation that BCNU, which we have shown leads to GSSG accumulation, retarded NF-κB translocation and activation (17, 19). However, GSSG augmented LPS-induced release of cytokines although it suppressed the activity of NF-κB. On the mechanism of action of BCNU, we have revealed an involvement of an IκB-α-sensitive pathway. BCNU-mediated partial reversal of IκB-α degradation seems to be independent of its phosphorylation, highlighting whether this mechanism is solely involved in NF-κB regulation (1, 2, 12, 22, 40). In preference to this conclusion, we and others have observed that NF-κB translocation/activation is compartmentalized and subsequently controlled by stimuli pressing their differential effects, depending on whether they are acting within the cytoplasm or nucleus (17–19, 22, 33, 38–42). For instance, raising nuclear levels of GSSG has been reported to blockade the binding of NF-κB to specific DNA moieties (8, 19). In addition, direct phosphorylation of RelA (p65) by the mitogen-activated protein kinase/reactive kinase (p38/RK) was found to be a prerequisite for initiating a transcriptional activity of NF-κB, regardless of the status of the up-stream IκB-α pathway (11). Our results are in agreement with the notion that the ability of GSSG to create an inactive NF-κB/DNA complex is dissociated from redox-dependent regulation of proinflammatory cytokines, an event that is likely to be insensitive to the up-stream IκB-α pathway (3, 6).

Dithiocarbamates, including PDTC, induce differential effects on redox equilibrium according to (a) their ability to decrease single-electron radical species (a reduction property), and (b) their capacity to oxidize GSH and related thiol compounds, thereby modulating glutathione recycling potential (an oxidation property) (8, 19, 39). As with other dithiocarbamates, PDTC thus possesses the capacity to exert both anti- and prooxidant effects, the former being mediated through dithiocarboxy scavenging of H_2O_2, superoxide anion ($O_2^{-\cdot}$), and ·OH, and the latter being mediated by its oxidation by reactive oxygen and nitrogen species, generating dithiocarbamate thiyl radicals and thiuram disulfides that directly oxidize GSH to GSSG, a potent regulator of several transcription factors and signal transducing pathways (19, 33, 39, 41). Dithiocarbamates inhibit the phosphorylation-dependent release of NF-κB from its cytosolic inhibitory subunit, IκB (8), suggesting that the mechanism of ROS-mediated activation of this transcription factor involves a redox-sensitive kinase (6, 12). However, GSSG also promotes the formation of a NF-κB/disulfide complex, directly inhibiting DNA binding (8, 19, 39). Although PDTC has been shown to elevate GSSG at the expense of GSH (8, 19), the suppression of LPS-induced cytokine release is rather secondary to its antioxidant potential in scavenging ROS, rather than a direct effect through NF-κB inhibition, although the latter mechanism could not be excluded. This is supported by the rather unequivocal evidence that BCNU-dependent intracellular accumulation of GSSG augmented LPS-induced cytokine biosynthesis despite blockading the NF-κB pathway, arguing therefore in favor of ROS as potential messengers in regulating cytokines. We have evidence supporting the notion that intracellular localization of ROS is redox-dependent and

likely to regulate the expression/synthesis of proinflammatory genes (20–22). Therefore, this sequential trajectory of ROS signaling down through the cytokine pathway allowed us to discriminate between the antioxidant and prooxidant effects of PDTC, a mechanism that is not secondary, at least in part, to NF-κB inhibition. It remains to be determined, however, whether PDTC is involved in regulating NF-κB-dependent proinflammatory gene expression and transactivity in the alveolar epithelium.

The ability of NAC to provide cysteine for GSH biosynthesis (4, 7, 14, 19), along with that of BSO to block *de novo* synthesis (13, 16, 17, 28), provided the criteria to establish whether NAC inhibitory effects on LPS-induced cytokine release are rate-limited by glutathione biosynthesis. Interestingly, BSO did not affect NAC-mediated down-regulation of cytokine secretion, suggesting that its inhibitory effect is independent of its role as a GSH precursor. As the cysteine provided by NAC eventually cannot feed into the biosynthetic pathway because of irreversible inhibition of γ-GCS (16), it is very likely that NAC is acting either on components of the cytokine signaling transduction pathway or through an alternative metabolic machinery. In this respect, the antioxidant, scavenging action against ROS-induced cytokine release is a possible mechanism (4, 20, 21), thereby providing evidence for dissociating between the antioxidant properties of NAC and its ability to act as a glutathione precursor.

A recent study reported by Parmentier and colleagues (31) suggested that NAC-dependent regulation of IL-1β is tightly regulated by NF-κB. Interestingly, these authors showed an elevation in IL-1β following coincubation with LPS and NAC, which also up-regulated the activation of the p50–p65 complex (31). Other investigators, however, have reported antagonistic effects of NAC on proinflammatory cytokine biosynthesis and NF-κB regulation (5, 7, 14, 45). We cannot exclude the possibility, however, that NAC-mediated inhibitory effects on cytokines are linked with the IκB-α/NF-κB pathway; rather our data favored an antioxidant potential consistent with the notion that ROS are key messengers in the regulation of the cytokine pathway. Whether this effect of NAC is secondary to inhibition of NF-κB is possible; however, the fact that inhibition of this transcription factor by BSO, for instance, which augmented ROS accumulation (20, 21), but otherwise had no inhibitory effect on cytokine release, favors an antioxidant mechanism involved in the regulatory potential of glutathione-modulating agents.

In summary, the results are highlighted as follows: (a) irreversible inhibition of GSH biosynthesis blockaded IκB-α phosphorylation and reduced its degradation, allowing its cytosolic accumulation; (b) BSO suppressed the activity of NF-κB, uncoupled from its ability to augment cytokines; (c) blockading redox cycle up-regulated LPS-induced release of cytokines, an effect associated with partial phosphorylation/degradation of IκB-α and inhibition of the DNA-binding activity; (d) dithiocarbamates marginally affected LPS-induced IκB-α phosphorylation/degradation, otherwise suppressed NF-κB activation, and reduced cytokine release; and (e) blockading GSH biosynthesis did not abolish NAC-dependent reduction of LPS-mediated cytokine biosynthesis, an effect accompanied by inhibition of NF-κB activation. It is concluded that there are oxidant-initiated and redox-mediated mechanisms regulating proinflammatory cytokines and the IκB-α/NF-κB signaling transduction pathway.

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ABBREVIATIONS

BCNU, 1,3-*bis*-(2-chloroethyl)-1-nitrosourea; BSO, L-buthionine(*S*,*R*)-sulfoximine; DMEM, Dulbecco's modified Eagle medium; ELISA,

enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; γ-GCS, γ-glutamylcysteine synthetase; GSH, L-γ-glutamyl-L-cysteinyl-glycine; GSSG, glutathione oxidized disulfide; H_2O_2, hydrogen peroxide; IκB, inhibitory protein; IKK, inhibitory κB kinase; IL, interleukin; LPS, lipopolysaccharide; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor-κB; ·OH, hydroxyl radical; PDTC, pyrrolidine dithiocarbamate; redox, reduction–oxidation; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

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