Nuclear Factor-κB Blockade Attenuates but Does Not Abrogate Lipopolysaccharide-Dependent Tumor Necrosis Factor-α Biosynthesis in Alveolar Epithelial Cells

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We have investigated the role that the nuclear factor (NF)–κB plays in regulating the biosynthesis of tumor necrosis factor (TNF)–α, an inflammatory cytokine. Irreversible inhibition of the proteasome complex by carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132; 1–50 μM) had no inhibitory effect on LPS-mediated TNF–α biosynthesis. Furthermore, selective inhibition of NF–κB by the action of caffeic acid phenylethyl ester (CAPE; 1–100 μM) and sulfasalazine (SSA; 0.1–10 mM), a potent and irreversible inhibitor of NF–κB, partially attenuated, but did not abolish, LPS-dependent TNF–α secretion. Incorporation of a selectively permeant inhibitor of NF–κB, SN-50 (1–20 μM), a peptide which contains the nuclear localization sequence (NLS) for the p50 NF–κB subunit, and the amino-terminal sequence of Kaposi fibroblast growth factor to promote cell permeability, attenuated in a dose-dependent manner LPS-mediated release of TNF–α. It is concluded that the NF–κB pathway is partially implicated and that its blockade attenuates, but does not abrogate, LPS-dependent TNF–α biosynthesis in alveolar epithelial cells.

Key Words: inflammation; lipopolysaccharide; NF–κB; proteasome; TNF–α.

Tumor necrosis factor (TNF)–α is a potent inflammatory cytokine, which exerts its pleiotropic activities through ligand-induced cross-linking of specific receptors, virtually present in almost all cell types (1). During inflammation, TNF–α released in the inflammatory environment transiently activates neutrophils and macrophages, thereby causing them to release superoxide anion (O$_2^-$) as a consequence of the activation of the plasma membrane NADPH oxidase (2). This oxidative burst in leukocytes features a rapid, but transient, release of reactive oxygen species (ROS) and is a crucial part of the defense mechanism against invading microbial pathogens and tumor cell metastasis. Although TNF–α is primarily produced by macrophages, growing evidence suggested that other cell types, such as endothelial and epithelial cells, release TNF–α and other inflammatory mediators, thereby amplifying and boosting the inflammatory reaction by activating and recruiting inflammatory cells (3, 4).

Although the transcription factor NF–κB has been originally recognized in regulating gene expression in B-cell lymphocytes (5), subsequent studies 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). 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 mainly composed of two subunits, p50 (NF–κB1) and p65 (ReLA), is present in the cytoplasm associated with its inhibitory protein, IκB (7). Upon stimulation, such as with cytokines and lipopolysaccharide (LPS), IκB–α, the major cytosolic inhibitor of NF–κB, undergoes phosphorylation on serine/threonine residues, ubiquitination, and subsequent proteolytic degradation, thus unmasking the nuclear localization signal on p65 subunit, and thereby allowing nuclear translocation of the complex. This sequential propagation of signalling ultimately results in the release of NF–κB subunits from IκB–α inhibitor, promoting translocation and initiation of gene transcription (6, 7).

The present investigation has attempted, therefore, to unravel the role that NF–κB plays in regulating LPS-mediated TNF–α biosynthesis. Our results could
be highlighted as follows: (i) Irreversible inhibition of the proteasome complex by carbobenzoxy-\(\text{L-}\)-leucyl-\(\text{L-}\)-leucyl-\(\text{L-}\)-leucinal (MG-132), reported to inhibit NF-\(\kappa\)B activation (8, 9), did not abrogate LPS-induced production of TNF-\(\alpha\); (ii) Selective inhibition of NF-\(\kappa\)B by the action of caffeic acid phenethyl ester (CAPE), a potent and specific inhibitor (10), reduced LPS-mediated TNF-\(\alpha\) secretion at doses \(\geq 10 \mu\text{M}\); (iii) Similarly, pretreatment with sulfasalazine (SSA), a potent and irreversible inhibitor of NF-\(\kappa\)B (11, 12), reduced LPS-dependent TNF-\(\alpha\) biosynthesis only at the highest dose use \(10 \text{mM}\); and (iv) Incorporation of a selectively permeant inhibitor of NF-\(\kappa\)B, SN-50, a peptide which contains the nuclear localization sequence (NLS) for the p50 NF-\(\kappa\)B subunit and the amino-terminal sequence of Kaposi fibroblast growth factor to promote cell permeability (13), attenuated LPS-mediated release of TNF-\(\alpha\). It is concluded that NF-\(\kappa\)B blockade attenuates, but does not abrogate, LPS-dependent TNF-\(\alpha\) biosynthesis in alveolar epithelial cells.

MATERIALS AND METHODS

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

Primary cultures of alveolar epithelia. Fetal alveolar type II (fATII) epithelial cells were isolated from lungs of fetuses, essentially as reported elsewhere (14, 25). Briefly, fetal rats were removed from pregnant Sprague-Dawley rats by caesarian section at day 19 of gestation (term = 22 days), the lungs excised, teased free from heart and upper airway tissue, and were finely minced then washed free of erythrocytes using sterile, chilled Mg\(^{2+}\) and Ca\(^{2+}\)-free Hank’s balanced salt solution (HBSS; 0.5 ml/fetus). The deaned lung tissue was resuspended in 1 ml foetus \(^{-1}\) HBSS containing trypsin (0.1 mg/ml), collagenase (0.06 mg/ml), and DNase I (0.012% w/v), and was agitated at 37°C for 20 min. The solution was then centrifuged at 100g for 2 min to remove undispersed tissue, the supernatant was saved to a fresh sterile tube and an equal volume of Dulbecco’s modified Eagle medium (DMEM) with 10% (v/v) fetal calf serum (FCS) was added to the supernatant. After passing the supernatant through a 120 \(\mu\text{m}\) pore sterile mesh, the filtrate was centrifuged at 420g for 5 min, the pellet resuspended in 20 ml DMEM/FCS and the cells were placed into a T-150 culture flask for 1 h at 37°C to enable fibroblasts and non-epithelial cells to adhere. Unattached cells were washed three times by centrifugation at 420g for 5 min each and then seeded onto 24 mm diameter Transwell-clear permeable supports (Costar; 0.4 \(\mu\text{m}\) pore size) at a density of 5 \(\times\) 10\(^5\) cells per filter and were allowed to adhere overnight at 152 Torr (\(-21\%\text{O}_2/5\%\text{CO}_2\)). DMEM/ FCS was exchanged for 4 ml of serum free PC-1 media (Blowhit-taker, MD) preequilibrated to PO\(_2\) = 152 Torr and 37°C 24 h later and cells were maintained at this PO\(_2\) until the experiment. In each case, and under conditions of independent pretreatments, the ade- nylate energy charge, an index of cell viability and competence, remained \(\geq 0.7\) and transepithelial monolayer resistance was moni- tored constantly at 250–350 \(\Omega\) cm\(^2\) or more (14, 15).

Drug pretreatment and measurement of pro-inflammatory cytokine TNF-\(\alpha\) by ELISA. Cytokines have been demonstrated to induce NF-\(\kappa\)B, whose activation has been implicated in mediating biological responses, including the expression of genes encoding cytokines (6, 7). Cells were pretreated (2 h) with various inhibitors and monolay-

ers washed twice in preequilibrated PC-1 medium and subsequently challenged with LPS (1 \(\mu\text{g/ml}\)) for 24 h (LPS was derived from Escherichia coli, serotype 026:B6). In order to determine whether LPS-induced release of TNF-\(\alpha\) is regulated, at least in part, by NF-\(\kappa\)B, we used carbobenzoxy-\(\text{L-}\)leucyl-\(\text{L-}\)leucyl-\(\text{L-}\)leucinal (MG-132; Calbiochem, UK), a potent, reversible proteasome inhibitor (K = 4 \(\mu\text{M}\)), reported to inhibit NF-\(\kappa\)B activation (8, 9). Cells were pre-treated with MG-132 (0, 1, 10, and 50 \(\mu\text{M}\)) for 2 h before exposure to LPS for further 24 h. Sulfasalazine (SSA; 0, 0.1, 1, and 10 mM; Sigma-Alrdis, UK) (11, 12) and caffeic acid phenethyl ester (CEPE; 0, 1, 10, and 100 \(\mu\text{M}\); Calbiochem, UK) (10), which are potent specific inhibitors of NF-\(\kappa\)B, were also incorporated. Cytokine release was assayed (24 h) following challenge.

Additionally, cells were pretreated for 2 h with SN-50 (0, 1, 10, and 20 \(\mu\text{M}\); Calbiochem, UK), a specific permeating inhibitor of NF-\(\kappa\)B nuclear translocation, followed by exposure to LPS (1 \(\mu\text{g/ml}\)) for 24 h. This peptide contains the nuclear localization sequence (NLS) for the p50 NF-\(\kappa\)B subunit and the amino-terminal sequence of Kaposi fibroblast growth factor to promote cell permeability (13). An inactive mutant control for SN-50 peptide (SN-50M; 20 \(\mu\text{M}\)) corresponding to the same peptide sequence with substitutions of Lys\(^{\text{363}}\) for Asn and Arg\(^{\text{364}}\) for Gly in the NLS region has been used to confirm the specificity of SN-50.

Following exposure to LPS, cell-free supernatants were assayed for pro-inflammatory (R&D Systems, UK) cytokine biosynthesis by two-site, solid phase, sandwich enzyme-linked immunosorbent assay (ELISA), essentially as recounted previously (16–19). Briefly, rabbit TNF-\(\alpha\) immunoadfinity purified sheep anti-rat cytokine antibodies were employed for pro-inflammatory (R&D systems, UK) cytokine biosynthesis by solid phase, sandwich enzyme-linked immunosorbent assay (ELISA), essentially as recounted previously (16–19).

RESULTS

The Effect of Proteasome Inhibition on LPS-Mediated TNF-\(\alpha\) Biosynthesis

As shown in Fig. 1, exposure of epithelial cells to medium alone for 24 h had no effect on the secretion of TNF-\(\alpha\). Similarly, pretreatment of monolayers with MG-132 (50 \(\mu\text{M}\)), a specific inhibitor of the proteasome complex, reportedly known to blockade the NF-\(\kappa\)B pathway (8, 9), followed by exposure to medium alone for 24 h had no effect on TNF-\(\alpha\) production either (Fig. 1). However, exposure to LPS (1 \(\mu\text{g/ml}\)) for 24 h induced TNF-\(\alpha\) biosynthesis \(\approx 3–4\)-fold relative to medium alone measured at the same time point. Pretreatment with MG-132 prior to challenge with LPS did not affect or reduce LPS-dependent release of TNF-\(\alpha\) (Fig. 1).
The Effect of CAPE on LPS-Mediated TNF-α Biosynthesis

As shown in Fig. 2, exposure of epithelial cells to medium alone for 24 h had no effect on the secretion of TNF-α. Similarly, pretreatment of monolayers with CAPE (100 μM), a specific inhibitor of NF-κB (10), followed by exposure to medium alone for 24 h had no effect on TNF-α production either (Fig. 2). Exposure to LPS (1 μg/ml) for 24 h induced TNF-α biosynthesis 3-4-fold relative to medium alone measured at the same time point. Pretreatment with CAPE prior to challenge with LPS-reduced LPS-dependent release of TNF-α at the highest dose used in this study (10 mM) (Fig. 2).

The Effect of SSA on LPS-Mediated TNF-α Biosynthesis

As shown in Fig. 3, exposure of epithelial cells to medium alone for 24 h had no effect on the secretion of TNF-α. Similarly, pretreatment of monolayers with SSA (10 mM), a potent and specific inhibitor of NF-κB (11, 12), followed by exposure to medium alone for 24 h had no effect on TNF-α production either (Fig. 3). Exposure to LPS (1 μg/ml) for 24 h induced TNF-α biosynthesis 3-4-fold relative to medium alone measured at the same time point. Pretreatment with SSA prior to challenge with LPS-reduced LPS-dependent release of TNF-α at the highest dose used in this study (10 mM) (Fig. 3).

The Effect of SN-50 and Its Mutant, SN-50M, on LPS-Mediated TNF-α Biosynthesis

As shown in Fig. 4, exposure of epithelial cells to medium alone for 24 h had no effect on the secretion of TNF-α. Similarly, pretreatment of monolayers with SN-50 (20 μM), a potent and specific permeating inhibitor of NF-κB (13), followed by exposure to medium alone for 24 h had no effect on TNF-α production either (Fig. 4). Exposure to LPS (1 μg/ml) for 24 h induced TNF-α biosynthesis 3-4-fold relative to medium alone measured at the same time point. Pretreatment

FIG. 2. The effect of CAPE on LPS-mediated TNF-α biosynthesis. Incubation of epithelial cells with medium alone (24 h) had no effect on TNF-α production, compared to preincubation with CAPE (2 h), an inhibitor of NF-κB, followed by exposure to medium alone (24 h). Exposure to LPS (1 μg/ml; 24 h) induced TNF-α secretion into the supernatant, an effect that was not affected or reversed by CAPE, at all doses tested. *P < 0.05, as compared with control; NS, nonspecific, as compared with LPS alone. n = 3, which represents the number of independent experiments run in duplicate.

FIG. 3. The role of the proteasome complex and its inhibition in LPS-mediated TNF-α biosynthesis. Incubation of epithelial cells with medium alone (24 h) had no effect on TNF-α production, compared to preincubation with MG-132 (2 h), a proteasome inhibitor, followed by exposure to medium alone (24 h). Exposure to LPS (1 μg/ml; 24 h) induced TNF-α secretion into the supernatant, an effect that was not affected or reversed by MG-132, at all doses tested. *P < 0.05, as compared with control; NS, nonspecific, as compared with LPS alone. n = 3, which represents the number of independent experiments run in duplicate.

FIG. 4. The role of the proteasome complex and its inhibition in LPS-mediated TNF-α biosynthesis. Incubation of epithelial cells with medium alone (24 h) had no effect on TNF-α production, compared to preincubation with MG-132 (2 h), a proteasome inhibitor, followed by exposure to medium alone (24 h). Exposure to LPS (1 μg/ml; 24 h) induced TNF-α secretion into the supernatant, an effect that was not affected or reversed by MG-132, at all doses tested. *P < 0.05, as compared with control; NS, nonspecific, as compared with LPS alone. n = 3, which represents the number of independent experiments run in duplicate.
with SN-50 prior to challenge with LPS-reduced, in a dose-dependent manner, LPS-dependent release of TNF-α (Fig. 4). Mutation of the SN-50 peptide (SN-50M; 20 μM) reversed the inhibitory effect of this peptide on LPS-mediated TNF-α biosynthesis (Fig. 4). The 50% minimum effective inhibitory concentration (IC₅₀) of selective NF-κB inhibitors on LPS-mediated TNF-α biosynthesis is given in Table 1.

DISCUSSION

There is increasing evidence implicating the alveolar epithelium, in particular, as a dynamic barrier that plays an important role in regulating the inflammatory and metabolic responses to oxidative stress and the accompanying inflammatory signal, sepsis, endotoxemia, and other critical illnesses in the lung (4, 12, 14–22). The respiratory epithelium is a primary target of an inflammatory/infectious condition at the epithelial-blood interface and is itself capable of amplifying an inflammatory signal by recruiting inflammatory cells and by producing inflammatory mediators (14–19). Many of the side effects of LPS, derived from the cell wall of gram negative bacteria, are secondary to the overproduction of pro-inflammatory mediators, including cytokines such as TNF-α. Inflammatory as well as autoimmune disease is often associated with deregulated expression and biosynthesis of pro-inflammatory cytokines, which influence a plethora of cellular functions. Therefore, the down-regulation of

**FIG. 3.** The effect of SSA on LPS-mediated TNF-α biosynthesis. Incubation of epithelial cells with medium alone (24 h) had no effect on TNF-α production, compared to preincubation with CAPE (2 h), an inhibitor of NF-κB, followed by exposure to medium alone (24 h). Exposure to LPS (1 μg/ml; 24 h) induced TNF-α secretion into the supernatant, an effect that was reversed by SSA only at a dose of 10 mM. *P < 0.05, as compared with control; NS, nonspecific, as compared with LPS alone; **P < 0.01, as compared with LPS alone. n = 3, which represents the number of independent experiments run in duplicate.

**FIG. 4.** The effect of SN-50 and its mutant, SN-50M, on LPS-mediated TNF-α biosynthesis. Incubation of epithelial cells with medium alone (24 h) had no effect on TNF-α production, compared to preincubation with SN-50 (2 h), a permeating inhibitor of NF-κB, followed by exposure to medium alone (24 h). Exposure to LPS (1 μg/ml; 24 h) induced TNF-α secretion into the supernatant, an effect that was reversed by SN-50 in a dose-dependent manner. Mutation of the wild-type peptide (SN-50M; 20 μM) reversed the inhibitory effect of SN-50 on LPS-dependent TNF-α secretion. *P < 0.05, as compared with control; NS, nonspecific, as compared with LPS alone. n = 3, which represents the number of independent experiments run in duplicate.

### TABLE 1

<table>
<thead>
<tr>
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<th>CAPE (μM)</th>
<th>SSA (mM)</th>
<th>SN-50 (μM)</th>
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<tr>
<td>MG-132</td>
<td>93.15 ± 5.42</td>
<td>≥10 mM</td>
<td>21.86 ± 2.57</td>
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Note. Data are presented as mean ± SEM. n = 3, each. IC₅₀ was determined from the negative slope of the linear regression curves.
an inflammatory signal is a major focus of the rational approach to the treatment of inflammatory diseases, such as chronic inflammation, sepsis, and rheumatoid arthritis. For instance, a novel recent study by Haskó et al. (23) 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, in addition, that selective inhibition of phosphodiesterases, a family of enzymes involved in the degradation of cAMP (24, 25), steroids, such as glucocorticoids (26), pyrimidylpiperazine derivatives (19, 27–29), and ERK and p38/RK MAPK selective inhibitors (30, 31) differentially regulate the transcription and biosynthesis of inflammatory cytokines. The promoters of genes encoding cytokines contain multiple cis-acting motifs including those that bind such transcription factors as NF-κB. Furthermore, the release of free NF-κB upon extracellular stimulation due to IκB phosphorylation and degradation by the proteasome complex, leads to DNA binding to specific κB moieties in order to initiate transcription of related genes, including immunoreceptors, cytokines, and, interestingly, its own inhibitor, IκB (6, 7, 18). Two unique features of the NF-κB/IκB complex system are deduced from its feedback regulation. The transcriptional activation of NF-κB triggers the synthesis of IκB and NF-κB-activated transcription is maintained by continuous degradation of IκB, which is sustained by an extracellular stimulus (6, 7, 18). Thus, the expression of IκB parallels both NF-κB activity and the duration of the activating extracellular stimulation, suggesting that this temporal parallelism between IκB accumulation/degradation and an effective external stimulation is a mechanism allowing dual regulation of NF-κB within the alveolar space. The selective interference with the functioning of the proteasome complex (8, 9) in regulating the translocation and activation of NF-κB and the expression of its inhibitor IκB-α suggested that the IκB-α/NF-κB pathway is partially implicated in regulating LPS-mediated biosynthesis of TNF-α. This is rather unequivocally reinforced with the observation reported in this study that selective inhibition of the NF-κB pathway attenuated, but did not abrogate, the inductive effect of LPS on TNF-α production. Therefore, the IκB-α/NF-κB pathway could be partially dissociated from the presumably downstream pathway regulating TNF-α signalling, indicating the involvement of a possible cross-talk among several pathways working independently or in coherence to integrate signalling mechanisms governing the regulation of cytokines in the alveolar epithelium.

Many extracellular stimuli, including pro-inflammatory cytokines and other inflammatory mediators (32), elicit specific cellular responses through the activation of mitogen-activated protein kinase (MAPK) signalling pathways (30, 31). MAPKs are a proline-targeted serine-threonine kinases that transduce environmental stimuli to the nucleus and they themselves are activated by upstream MAPK kinases (MAPKs) on both threonine and tyrosine residues within an "activation loop" (30, 31). Once activated, MAPKs can phosphorylate and activate other kinases or nuclear proteins, including potential transcription factors and substrates. Furthermore, inflammatory mediators, such as TNF-α, were shown to activate the MAPK pathway in several cell types (33). Since MAP kinase regulation has been recently implicated in regulating pro-inflammatory cytokine biosynthesis (34) and transcription (35), it is possible that this pathway is directly involved in regulating TNF-α biosynthesis in the alveolar epithelium. We have recent evidence from our laboratory indicating a potential role for the MAPK pathway, and in particular MAPK p38, in regulating pro-inflammatory cytokines (Haddad and Land, unpublished observations). We are currently extending these observations to investigate the immunoregulatory role that MAPK p38 plays in regulating inflammatory mediator transcription and biosynthesis and whether there is a potential cross-talk between MAPK p38 and IκB-α/ NF-κB signalling pathways.

In summary our results could highlighted as follows: (i) Irreversible inhibition of the proteasome complex by carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132), reported to inhibit NF-κB activation, did not abrogate LPS-induced production of TNF-α; (ii) Selective inhibition of NF-κB by the action of caffeic acid phenethyl ester (CAPE), a potent and specific inhibitor, reduced LPS-mediated TNF-α secretion at doses ≥10 μM; (iii) Similarly, pretreatment with sulfasalazine (SSA), a potent and irreversible inhibitor of NF-κB, reduced LPS-dependent TNF-α biosynthesis only at the highest dose use (10 mM); and (iv) Incorporation of a selectively permeant inhibitor of NF-κB, SN-50, a peptide which contains the nuclear localization sequence (NLS) for the p50 NF-κB subunit and the aminoterminal sequence of Kaposi fibroblast growth factor to promote cell permeability, attenuated LPS-mediated release of TNF-α. It is concluded that NF-κB blockade attenuates, but does not abrogate, LPS-dependent TNF-α biosynthesis in alveolar epithelial cells

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