0026-895X/06/6906-1791–1800$20.00
MOLECULAR PHARMACOLOGY
Vol. 69, No. 6
19521/3114610
Mol Pharmacol 69:1791–1800, 2006
Printed in U.S.A.

IκB Kinase-2-Independent and -Dependent Inflammation in Airway Disease Models: Relevance of IKK-2 Inhibition to the Clinic

Mark A. Birrell, Sissie Wong, Elizabeth L. Hardaker, Matthew C. Catley, Kerryn McCluskie, Michael Collins, Saleem Haj-Yahia, and Maria G. Belvisi

Respiratory Pharmacology Group, Airway Diseases Department, Faculty of Medicine, National Heart and Lung Institute, Imperial College, London, United Kingdom (M.A.B., S.W., E.L.H., M.C.C., K.M., M.C., M.G.B.); and Royal Brompton & Harefield Hospital, London, United Kingdom (S.H.-Y.)

Received October 3, 2005; accepted March 2, 2006

ABSTRACT

Nuclear factor κB (NF-κB) is a transcription factor believed to be central in the expression of numerous inflammatory genes and the pathogenesis of many respiratory diseases. We have previously demonstrated increased NF-κB pathway activation in a steroid-sensitive animal model of lipopolysaccharide (LPS)-driven airway inflammation. It is noteworthy that this phenomenon was not observed in a steroid-insensitive model of elastase-induced inflammation in the rat. The aim of this study was to gather further evidence to suggest that these similar profiles of neutrophilic inflammation can be NF-κB-dependent or -independent by determining the impact of an IκB kinase-2 (IKK-2) inhibitor, 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1). In the LPS model, TPCA-1 blocked the increase in NF-κB DNA binding, a marker of NF-κB pathway activation. This inhibition was associated with a reduction in inflammatory mediator release [tumor necrosis factor α (TNFα)/interleukin-1β (IL-1β)/matrix metalloproteinase-9 (MMP-9)] and lung inflammatory cell burden (neutrophilia/eosinophilia). These data were paralleled with a steroid and in human cell based assays. In the elastase-driven inflammation model, in which our group has previously failed to measure an increase in NF-κB DNA binding, neither TPCA-1 nor the steroid, affected mediator release (IL-1β/MMP-9) or cellular burden (neutrophilia/lymphomononuclear cells). This is the first study to examine the effect of an IKK-2 inhibitor in well validated models that mimic aspects of the inflammatory lesion evident in diseases such as COPD. In conclusion, we have demonstrated that animal models with similar profiles of airway inflammation can be IKK-2 inhibitor/steroid-sensitive or -insensitive. If both profiles of inflammation exist in the clinic, then this finding is extremely exciting and may lead to greater understanding of disease pathology and the discovery of novel anti-inflammatory targets.

The nuclear factor-κ B (NF-κB) transcription factor plays a key role in the normal "physiological" induction of pro-inflammatory gene expression, leading to the synthesis of cytokines, adhesion molecules, chemokines, growth factors, and enzymes (Baldwin, 2001). These NF-κB regulated mediators (e.g., TNFα, IL-8, IL-6, IL-1β, MIP-1α, and GROα) have been suggested to play a central role in a variety of acute and chronic inflammatory diseases (Barnes, 2001). Therefore, it has been suggested that blocking the NF-κB pathway represents a possible disease-modifying therapy (Barnes and Adcock, 1997).

NF-κB is activated in response to a number of stimuli, including physical and chemical stress, lipopolysaccharide (LPS), double-stranded RNA, T- and B-cell mitogens, and pro-inflammatory cytokines (Rothwarf and Karin, 1999; Beyaert et al., 2000; Karin and Lin, 2002; Li and Verma, 2002). NF-κB induced gene expression is controlled by a complex series of proteins and enzymes. In resting cells, the majority of NF-κB is bound to an IκB inhibitory protein that holds the complex in the cytoplasm. Upon appropriate stimulation of the cell, the IκB protein is phosphorylated and ubiquinated, which leads to subsequent proteasome-mediated degradation. With the IκB removed, the transcription

The authors were funded by Imperial College, Imperial College Trust, or the Royal Brompton and Harefield Hospital.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.019521.

ABBREVIATIONS: NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; IL, interleukin; MIP, macrophage inflammatory protein; GRO, growth-related gene product; LPS, lipopolysaccharide; IκB, inhibitory protein for NF-κB; IKK, IκB kinase; TPCA-1, 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide; COPD, chronic obstructive pulmonary disease; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; BAL, bronchoalveolar lavage; MMP, matrix metalloproteinase; PPE, porcine pancreatic elastase.

factor can translocate to the nucleus and bind consensus sequences on DNA, which can then lead to gene transcription. The critical phosphorylation of the IκB protein, in the classic pathway, is performed by the IκB kinase (IKK) complex, which consists of at least three subunits, two catalytic subunits (IKK-1 and -2; also known as IKKα and IKKβ), and a regulatory subunit IKKγ (NEMO) (Scheidereit, 1998; Karin, 1999; Courtois et al., 2001). Of the two catalytic subunits IKK-2 is 20-fold more active than IKK-1 in the phosphorylation of IκB (Mercurio et al., 1997) and it has been shown that IKK-2, not IKK-1, is important in NF-κB activation in vivo (Hu et al., 1999; Li et al., 1999a,b,c; Takeda et al., 1999). In addition, IKK-2 has been shown to directly phosphorylate NF-κB, which in turn may enhance its transactivating potency (Sakurai et al., 2003). For this reason, there has recently been a search for a small molecular weight inhibitor of IKK-2, for the potential treatment of inflammatory diseases.

Our group has used an IKK-2 inhibitor, 2-[(aminocarbonyl)-amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1), that has a pIC_{50} of 7.7 ± 0.2 on the isolated kinase and has 22-fold selectivity over IKK-1 and >550-fold selectivity over other kinases and enzymes (Podolin et al., 2005). In cultured primary human airway smooth muscle cells (HASM), we have demonstrated that TPCA-1 inhibits IL-1β stimulated IκB phosphorylation, NF-κB DNA binding, an NF-κB reporter assay, and cytokine release. It is noteworthy that a steroid had no effect on NF-κB DNA binding and only a partial inhibitory effect on the reporter assay, suggesting that steroids and IKK-2 inhibitors do not share a common mechanism of action. Further evidence of this was observed in the same cell-based assay system in which we have shown complete inhibition of cytokines such as granulocyte colony-stimulating factor with an IKK-2 inhibitor with minimal or no inhibition evoked by a steroid (Birrell et al., 2005a). In addition, we have shown in a preclinical, steroid-sensitive, rodent model of asthma that TPCA-1 inhibits antigen-induced NF-κB pathway activation by measuring a reduction in the level of NF-κB binding to DNA. Treatment with TPCA-1 in this model resulted in an inhibition of antigen induced mediator release, at the gene and protein level, and a reduction in airway eosinophilia (Birrell et al., 2005a).

We have recently demonstrated an increase in NF-κB pathway activation in a steroid-sensitive rat model of LPS-induced airway inflammation, a model characterized by increases in neutrophilia and mediator release (e.g., TNFα, IL-1β, and MMP-9) (Birrell et al., 2005b). In addition, we have also shown that an elastase-driven rat model, with a similar profile of airway inflammation, seems to be NF-κB-independent and resistant to steroid treatment (Birrell et al., 2005c). The data generated in these two models suggest that similar profiles of airway inflammation can be NF-κB-dependent and -independent, depending on the stimulus employed. This suggestion is extremely interesting and may help in understanding the pathophysiology of steroid-resistant diseases and highlight potential novel anti-inflammatory targets.

The aim of this study was to generate additional data to aid in understanding the molecular mechanisms involved in the steroid resistance observed in the elastase model and the steroid sensitivity observed in the LPS model, as described above. We have demonstrated previously that an IKK-2 inhibitor seems to have a more comprehensive anti-inflammatory profile that differs from that of a steroid (Birrell et al., 2005a). Therefore, we believed it would be extremely interesting to profile this class of anti-inflammatory in the two models by focusing on the key cells and mediators that are thought to play a role in the pathogenesis of diseases such as COPD.

Materials and Methods

Effect of TPCA-1 on LPS-Induced Cytokine Release from Human Monocytes. The human monocytic cell line THP-1, was purchased from the European Collection of Cell Cultures (ECACC, Health Protection Agency, Salisbury, Wiltshire, UK). The cells were then cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Paisley, UK) with GlutaMAX I (Invitrogen) supplemented with 10% FCS and 1% antibiotic and antimycotic solution (penicillin/streptomycin/amphotericin B; Sigma-Aldrich Co., Poole, UK) at 37°C in a humidified atmosphere [95% air, 5% (v/v) CO_2]. They were cultured into 75-cm^2 flasks, and the media was replaced every 48 to 72 h. For testing of the compounds, cells (4×10^5) were added to each well of a 24-well plate. Vehicle [0.1% (v/v) DMSO was maintained in all treatments], TPCA-1 (30 nM–10 μM), or the intra-assay positive control compound dexamethasone (1 μM) was added to wells 60 min before stimulation with vehicle or LPS (0.1 μg/ml; *Escherichia coli* serotype 0111:B4; Sigma). Twenty-four hours after stimulation, the culture fluid was collected and stored at −80°C until needed for cytokine assessment. Each experiment was performed in triplicate and repeated on three separate occasions. Cytokine levels in the supernatants were assessed using specific enzyme-linked immunosorbent assay (DuoSet; R&D Systems Europe, Ltd., Abingdon, Oxfordshire, UK) according to the manufacturer's instructions.

In parallel studies performed in six-well plates as above, the effect of TPCA-1 on cytokine gene expression was assessed using a method detailed in Birrell et al. (2005a). In brief, the cell pellet was collected 2 h after stimulation, and mRNA was extracted and converted into cDNA. Levels of gene expression were assessed using TaqMan real-time PCR (PRISM 7000 Sequence Detection System; Applied Biosystems, Warrington, UK). Results were analyzed using sequence detection software (Applied Biosystems), and the relative amount of target gene transcript was normalized to the amount of 18S internal control transcript in the same cDNA sample. The difference between 18S and target values is known as Δct. Because of the exponential nature of PCR, the delta ct values were converted to a linear form by $2^{-\Delta ct}$ (i.e., $2^{-(\text{target ct} - \text{18S ct})}$). This calculation was used to enable relative quantitation analysis between samples.

Effect of TPCA-1 on LPS Induced Cytokine Release from Human Lung Tissue Macrophages. Human lung tissue macrophages were obtained from nondiseased, lung transplant donor tissue that was not suitable for transplant as outlined below. Ethical approval for the study was obtained along with consent from the relatives. Lung tissue was cut into small pieces (approximately 3 × 3 cm) and lavaged with phosphate-buffered saline (without calcium and magnesium) (Sigma-Aldrich Co.). The pooled cell suspensions were passed through a 70-μm cell sieve and centrifuged at 250*g* for 10 min at 4°C in a chilled centrifuge (Mistral 3000i; MSE, London, UK). The supernatant was discarded, and the cell pellets were resuspended in phosphate-buffered saline (without calcium and magnesium) and layered onto six discontinuous Percoll gradients [60%/35%/25% (v/v/v)]. These gradients were then centrifuged at 1200*g* for 25 min at 20°C, with the brake set at 0. After centrifugation, the macrophage-enriched fractions were obtained from the 35% and the 60% Percoll interface and washed twice with Hanks balanced salt solution. The cells were then resuspended with RPMI 1640 medium with GlutaMAX I supplemented with 10% FCS (Invitrogen) and 1% antibiotic and antimycotic solution. Trypan blue exclusion was performed to assess cell viability and/or cell purity of the macrophage enriched fraction was determined with Kimura stain. The cell sus-

pension was diluted in RPMI 1640 with GlutaMAX I, supplemented with 10% FCS and 1% antibiotic and antimycotic solution, and 500 μl of 400,000 cells per well were added to 24-well plates (Costar, UK). These plates were then incubated for 60 min at 37°C in a humidified atmosphere [95% air, 5% (v/v) CO_2]. After 60 min, the nonadherent cells were removed and fresh medium was added. The adherent purified macrophages were incubated overnight, for treatment the following day. After discarding the supernatant, the macrophages were treated in the similar way as the THP-1 cells, as detailed above.

Effect of TPCA-1 on LPS-Induced Airway Inflammation in the Rat. Male Wistar rats (175–200g) were purchased from Harlan-Olac (Bicester, UK) and kept for at least 5 days before initiating experiments. Food and water were supplied ad libitum. United Kingdom Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed. Experiments were conducted with groups of $n = 8$ animals.

Rats were orally dosed with vehicle (2% DMSO, 10% CremophorEL, and 5% ethanol in distilled water, at a dose volume of 3 ml/kg) or TPCA-1 (3, 10, 30, or 60 mg/kg) 1 h before and 2 h after an aerosol challenge of endotoxin-free saline (for 30 min) or LPS (1 mg/ml). This dosing regimen was used because it was found to give adequate compound exposure as assessed by pharmacokinetic studies and efficacy in an in-house antigen-driven model of allergic inflammation (Birrell et al., 2005a). The glucocorticoid budesonide (3 mg/kg), which is a commonly used oral steroid in humans, was used as a positive control in these in vivo experiments because it has previously been shown to inhibit LPS-induced neutrophilia in the rat (Birrell et al., 2005b). A similar dosing regimen that had been validated in our previous studies was adopted (Belvisi et al., 2001; Birrell et al., 2005b).

Quantification of Airway Inflammation. Six hours after saline or LPS challenge, animals were euthanized with sodium pentobarbitone (200 mg/kg, i.p.) and the trachea was cannulated. Cells were recovered from the airway lumen by bronchoalveolar lavage (BAL), this involves flushing the airways with 10 ml/kg of RPMI delivered through the tracheal cannula and removed after a 30-s interval. This procedure was repeated, and samples were then pooled for each animal. The inflammatory cells were extracted from the lung tissue by collagenase digest as described by Underwood et al., (1997). The remaining lung tissue was either flash-frozen in liquid nitrogen for gene expression assessment or insufflated with 10% neutral buffered formalin at a pressure of 20 mm Hg for demonstration of inflammatory status in the lung. After remaining overnight in the fixative, the lungs were cleared and processed into paraffin blocks. Paraffin sections (3 μM) were cut and stained with Mayer's hematoxylin and eosin for assessment of cellular inflammation.

Total white cell numbers in the BAL and lung tissue samples were determined on the Sysmex F820 hematology analyser according to the manufacturer's instructions and after calibration of the machine using a standard protocol and sample of standard whole blood (Linford Wood, Milton Keynes, UK). Cytospins of these samples were prepared by centrifugation of 100-μl aliquots in a cytospin (Shandon, Runcorn, UK) at 700 rpm for 5 min at low acceleration at room temperature. Slides were fixed and stained on a Hema-tek 2000 (Ames Co., Elkhart, IN) with modified Wright-Giemsa stain. Four-part differential counts on 200 cells per slide were performed according to standard morphological criteria, and the percentage of eosinophils, lymphomononuclear cells, and neutrophils was determined as described by Underwood et al. (1997).

Mediator Determination in the Lung after LPS Challenge. Levels of TNFα and IL-1β in the lung were determined by enzyme-linked immunosorbent assay using rat DuoSets according to manufacturer's instructions. The levels of MMP-9 in the lung were determined by zymography as described by Birrell et al. (2005b).

Effect of TPCA-1 on Porcine Pancreatic Elastase-Induced Airway Inflammation. Male Sprague-Dawley rats (260–300g) were purchased from Harlan-Olac (Bicester, UK) and kept for at least 5 days before initiating experiments. Food and water were supplied ad libitum. UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed. Experiments were conducted with groups of $n = 8$ animals.

Rats were orally dosed with vehicle (2% DMSO, 10% CremophorEL, and 5% ethanol in distilled water, 3 ml/kg) or TPCA-1 (3, 10, 30, or 60 mg/kg) 1 h before and 6, 22, 30, and 46 h after an intratracheal dose of endotoxin-free saline (1 ml/kg) or porcine pancreatic elastase (PPE) (120 U/kg). As above, budesonide (3 mg/kg) was included in the study and dosed using the same regimen.

Forty-eight hours after PPE administration, the lungs were assessed for cellular infiltration and inflammatory mediators as above. Assessment of airway tissue inflammation was performed in formalin-insufflated (pressure of 20 mm Hg) fixed lung tissue by an experienced histologist. Levels of overall inflammatory cell burden and macrophages were assessed using a doubling of severity scoring system consisting of: no score, focal inflammation, 1, 2, 3 and 4. These scores were then converted into arbitrary values (0, 10, 20, 40, 80, and 160).

Levels of inflammatory mediator gene expression (TNFα, IL-1β, inducible nitric-oxide synthase, and MMP-9) in the frozen lung tissue from the time course experiment performed in Birrell et al. (2005c) were determined using TaqMan real-time PCR using the methods outlined above.

Assessment of NF-κB Pathway Activation. To assess the effect of the compound on NF-κB DNA binding in the LPS-driven in vivo model, EMSA assays were carried out. Details of the assay used are outlined in Birrell et al. (2005c); in brief, nuclear extracts were prepared from the lung tissue using an NXTRACT kit (Sigma-Aldrich Ltd), and assessed for protein levels using a Bradford assay. EMSA oligonucleotide probes were labeled using 5′ end labeling with T4 polynucleotide kinase. The labeling reactions, containing 1× kinase buffer (Promega, Southampton, UK) supplemented with 3.5 pmol of double-stranded probe NF-κB consensus oligonucleotide (Promega), 20 μCi of [γ-^{32}P]ATP, and 10 units of T4 polynucleotide kinase, were incubated for 1 h at 37°C. Labeled oligonucleotide was separated from residual [γ-^{32}P]ATP by G25 Sephadex spin column.

EMSA reactions containing equal concentrations of nuclear extracts in 18 μl of buffer D and 5 μl of 5× EMSA binding buffer (Promega) was incubated for 20 min on ice before the addition of 2 μl of labeled probe. Specificity was determined by the addition of a 100-fold excess of unlabeled competitor consensus oligonucleotide. After an additional 1 h on ice, the reactions were stopped by the addition of 3 μl of EMSA loading buffer [50% (v/v) glycerol, 0.05% (w/v) bromphenol blue], loaded onto a 6% polyacrylamide gel and electrophoresed until the dye front runs to the end of the gel, typically approximately 2 h. The experiments were carried out on ice to prevent protein degradation. The gels were then vacuum dried and exposed to BioMax MS-1 film (Sigma) at −80°C until defined bands were visible.

Statistical Analysis. Values are expressed as mean ± S.E.M. of n independent observations. Statistical comparisons were made using a Mann-Whitney test for two groups of nonparametric data; one-way analysis of variance followed by Dunnett's post test for multiple comparisons of parametric data or Kruskal-Wallis one-way analysis of variance followed by Dunn's post test for multiple comparisons of nonparametric data.

Results

Effect of TPCA-1 on LPS-Induced Cytokine Release From Cultured Human Cells. Stimulation with LPS of cultured THP-1 cells caused an increase in a range of inflammatory cytokines: TNFα, IL-8, IL-6, IL-1β, and MIP-1α (Fig. 1). Treatment with TPCA-1 caused a concentration related decrease in all cytokines measured, similar in magnitude to the intra-assay steroid positive control (Fig. 1). The potency of the compound in this assay (IC_{50} of approximately 100–

300 nM) seemed to be comparable with that reported by Podolin et al. (2005). Assessment of the gene expression of the same cytokines suggests that the IKK-2 inhibitor acted at the transcriptional level but post-transcriptional effects (e.g., mRNA stability) cannot be ruled out (Fig. 2). In primary human lung tissue macrophages, LPS stimulation increased the production of a similar range of inflammatory cytokines: TNFα, IL-8, IL-6, MIP-1α, and GROα (Fig. 3). Similar to the result with the THP-1 cells, treatment with TPCA-1 caused a concentration-related decrease in all cytokines measured in macrophages; this was similar in magnitude to the intra-assay positive control, a steroid (Fig. 3).

Effect of the IKK-2 Inhibitor on Airway Inflammation in Two Rodent Models. To demonstrate that inhibi-

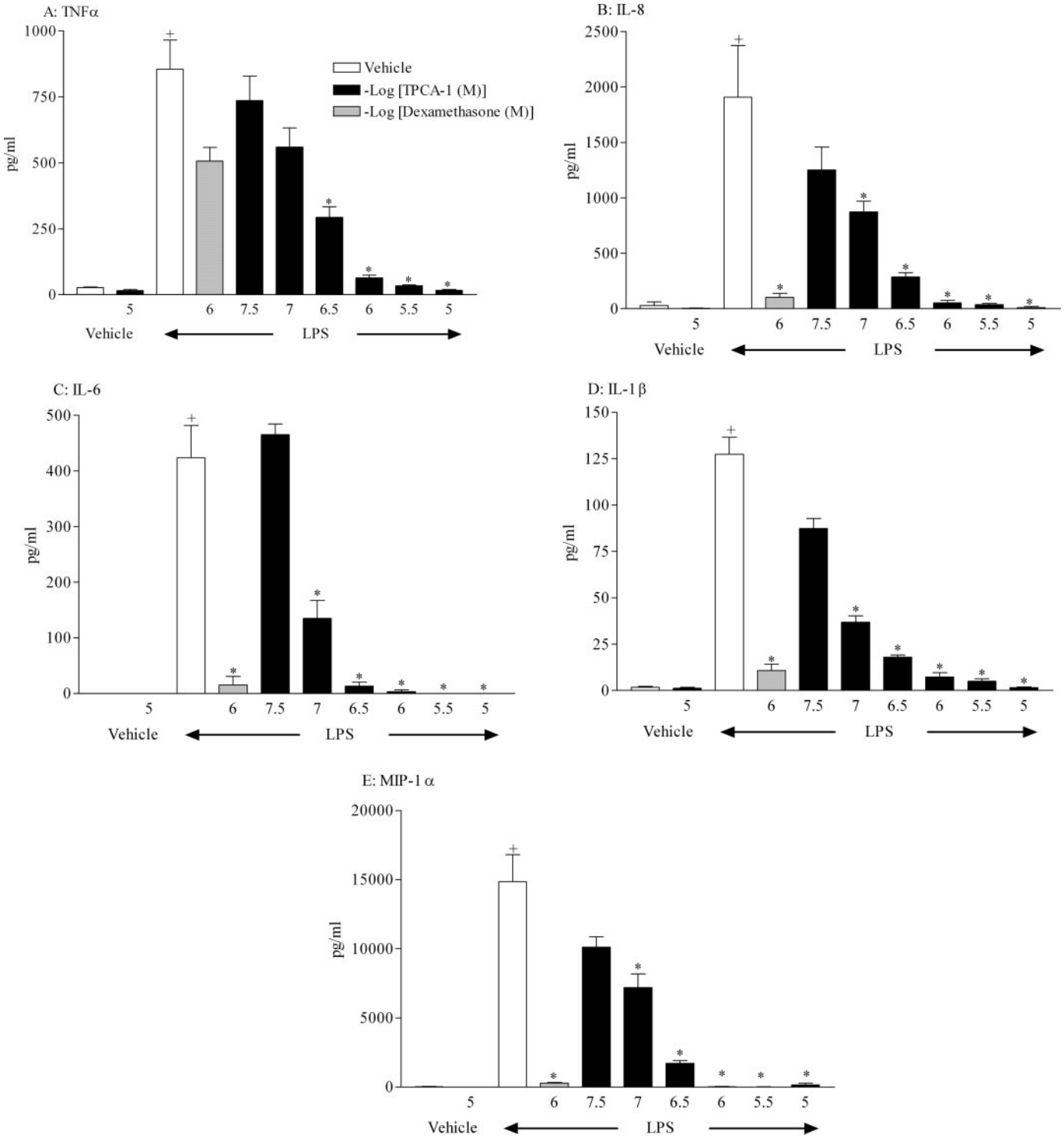

Fig. 1. Effect of IKK-2 inhibitor (shown as the negative log of the molar concentration) on cytokine production in THP-1 cells. Supernatant cytokine levels from cultured human THP-1 cells stimulated with 0.1 μg/ml LPS and treated with TPCA-1 or dexamethasone. A, TNFα; B, IL-8; C, IL-6; D, IL-1β; E, MIP-1α (n = 9). +, significant difference between vehicle stimulated/vehicle treated group and LPS stimulated/vehicle treated group; *, significantly different from LPS stimulated/vehicle treated group ($P < 0.05$).

tion of IKK-2 by TPCA-1, using the dosing regimen stated in the methods, is affecting NF-κB pathway activation an EMSA was carried out. As can be seen from the representative blot, in the steroid-sensitive LPS-driven model, there was an increase in the amount of NF-κB DNA binding, which suggests an increase in NF-κB pathway activation (Fig. 4). The IKK-2 inhibitor caused an inhibition of the LPS-induced increase in NF-κB DNA binding; the profile of inhibition was similar to the impact on inflammatory mediators and cellular burden (Fig. 4). We have demonstrated previously that there is no increase in NF-κB DNA binding over an extensive time course in the PPE-driven, steroid-insensitive model (Birrell et al., 2005c).

In the LPS-driven model, we detected an increase in inflammatory mediators such as TNFα, IL-1β, and MMP-9, which were inhibited by treatment with TPCA-1 and budesonide (Fig. 5). In the PPE-driven model, a similar increase in mediator release was detected, but this was not affected by TPCA-1 or the steroid (Fig. 6). The effect of TPCA-1 on mediator release was mirrored by its effect on inflammatory cell burden in these models in that it was reduced by the IKK-2 inhibitor and budesonide in the LPS model but not in the PPE model (Figs. 7 and 8). Confirmatory evidence for these results is depicted in Figs. 9 and 10, which show the histological profile of the stained lung tissue from both the LPS- and the PPE-driven studies.

When the levels of TNFα, IL-1β, MMP-9, and inducible nitric-oxide synthase gene expression were measured in a time-course experiment performed by Birrell et al. (2005c), a significant increase was observed (Table 1). This would suggest that some transcriptional events are occurring in the elastase-driven model and that the inflammatory mediators measured after PPE insult may be from de novo synthesis.

Discussion

It is currently believed that chronic inflammation is not only central to the pathogenesis of many respiratory diseases but that it is also partly responsible for many of the symptoms. Hence, a great deal of effort has been (and continues to be) made to find anti-inflammatory therapies to treat these diseases. Inhibition of NF-κB pathway activation is a popular target for novel anti-inflammatory therapies. Activation of the NF-κB pathway is believed to play a key role in the induction of pro-inflammatory gene expression, leading to the synthesis of inflammatory mediators and recruitment/activation of inflammatory cells (Baldwin, 2001). As discussed in the introduction, our group has preliminary data to suggest that a similar profile of airway inflammation, characterized by airway neutrophilia, can be induced by NF-κB-dependent or -independent mechanisms. The data obtained previously demonstrated that NF-κB pathway activation was present in the LPS-driven model and that the inflammatory response was blocked by steroid treatment (Birrell et al., 2005b). Others have also shown, in a similar murine model, a prominent role for NF-κB activation in LPS-induced airway

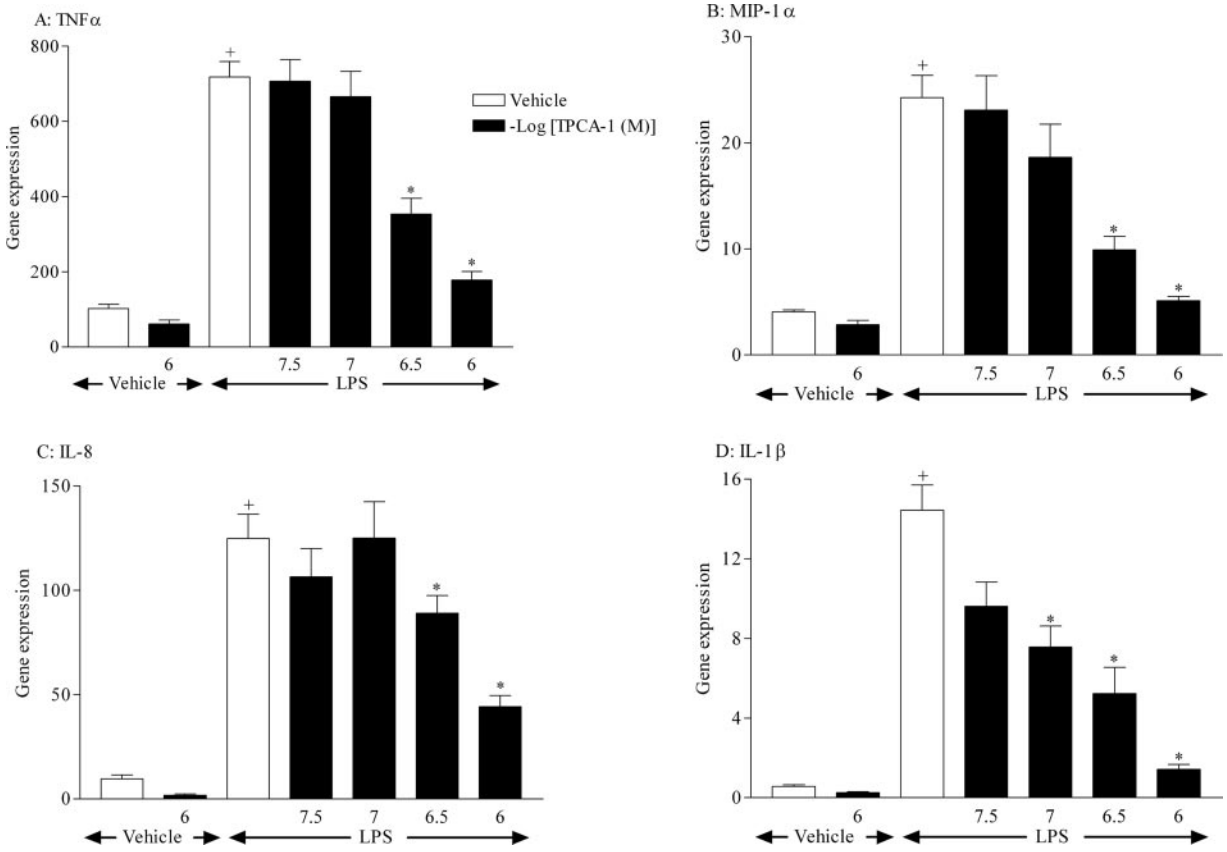

Fig. 2. Effect of IKK-2 inhibitor (shown as the negative log of the molar concentration) on cytokine gene expression in THP-1 cells. Gene expression $[2^{-\Delta ct} (\times 10^6)]$ levels in 0.1 μg/ml LPS-stimulated human THP-1 cells and treated with TPCA-1 (A, TNFα; B, MIP-1α; C, IL-8; and D, IL-1β ($n = 9$). +, significant difference between vehicle stimulated/vehicle treated group and LPS stimulated /vehicle treated group; ∗, significantly different from LPS stimulated/vehicle treated group ($P < 0.05$).

inflammation (Poynter et al., 2003). In the elastase-driven model, a similar inflammatory response was observed despite the fact that an increase in NF-κB pathway activation was not observed (Birrell et al., 2005c). Steroid therapy is believed to affect the actions of NF-κB, and in the elastase-driven model, the inflammatory response proved resistant to treatment with a steroid (Birrell et al., 2005c), and so perhaps the lack of effect of an IKK-2 inhibitor in this model could have been expected. However, although some groups have reported an inhibitory action of glucocorticoids on NF-κB translocation and DNA binding (Mukaida et al., 1994; Cazes et al., 2001), we have evidence to suggest that steroids do not inhibit NF-κB translocation and binding, and only partially affect NF-κB reporter gene assays (Newton et al., 1998; Birrell et al., 2005a). It is not clear why these discrepancies exist, but it may be due to the different cell types and/or stimulus employed in these studies and suggests that controversy still surrounds the action of steroids on NF-κB pathway activation. In our previous study, we were unable to demonstrate an increase in NF-κB pathway activation in the elastase model when looking at gross changes in pathway activation in lung tissue samples at different time points after elastase administration (Birrell et al., 2005c). Even though the results of this study are compelling, it may be possible that, despite the extensive time course being studied, we may not have chosen the correct time point to demonstrate NF-κB pathway activation. In addition, it may be possible that local increases in NF-κB DNA binding were lost when the whole lung tissue was assessed. One way in which to address this question would be to perform immunohistochemical studies to search for discrete areas of NF-κB pathway activation (e.g., by determining nuclear localization of p65). However, in this study, we have adopted a pharmacological approach by using a selective inhibitor of this signaling pathway. We have shown previously, in human cell based assays, that an IKK-2 inhibitor has a more comprehensive anti-inflammatory profile and different mechanism compared with that of a steroid (Birrell et al., 2005a). Therefore, the aim of this study was to compare the impact of an IKK-2 inhibitor with that of a steroid in the proposed NF-κB-depen-

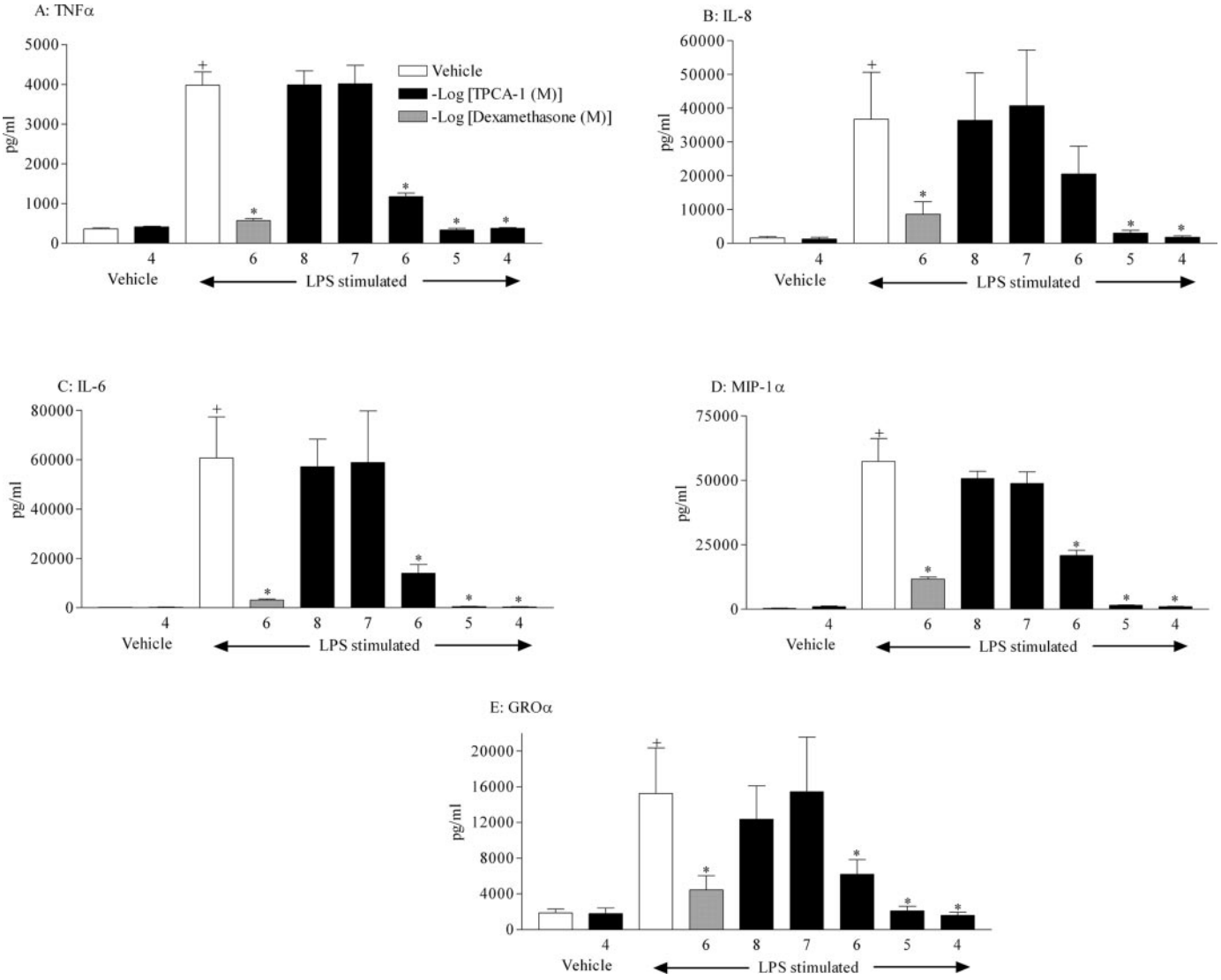

Fig. 3. Effect of IKK-2 inhibitor (shown as the negative log of the molar concentration) on cytokine production in macrophages. Supernatant cytokine levels from primary human lung tissue macrophages stimulated with LPS (0.1 μg/ml) and treated with TPCA-1 or dexamethasone. A, TNFα; B, IL-8; C, IL-6; D, MIP-1α; E, GROα ($n = 5$). +, significant difference between vehicle-stimulated/vehicle-treated group and LPS-stimulated/vehicle-treated group; ∗, significantly different from LPS-stimulated/vehicle-treated group ($P < 0.05$).

dent and -independent models. Although it is not the subject of this publication, it would be interesting to determine whether the combination of an IKK-2 inhibitor with a steroid resulted in any additive or synergistic effects.

The IKK-2 inhibitor blocked the release of a range of cytokines from LPS stimulated THP-1 cells (Fig. 1) and human lung tissue macrophages (Fig. 3). Assessment of gene expression (Fig. 2) suggested that TPCA-1 affected the transcriptional level (i.e., inhibition of TNFα/MIP-1α/IL-1β/IL-8 at the protein and gene levels with 300 nM TPCA-1 was 68 versus 59%, 89 versus 75%, 67 versus 87%, and 30 versus 86%, respectively), although post-transcriptional events cannot be ruled out. The reduction in inflammatory cytokine production observed in this cell system is similar to that seen in another study performed by our group, using the same compound, in which we demonstrated that TPCA-1 blocked IL-1β-induced NF-κB reporter assay activity and mediator release in human airway smooth muscle cells (IC_{50} between 100 and 1000 nM) (Birrell et al., 2005a).

In the preclinical rodent model of LPS-induced airway inflammation, we observed an increase in NF-κB pathway activation, as measured by an increase in NK-κB DNA binding (Fig. 4). Treatment with TPCA-1 inhibited this increase in NF-κB–DNA binding, suggesting that the compound is affecting NF-κB pathway activation (Fig. 4). The profile of inhibition by TPCA-1 on the NF-κB pathway was very similar to the impact on inflammatory cytokines, MMP-9 levels, and cellular recruitment, suggesting a causative role (Figs. 5, 7, and 9). However, it is possible that some of the impact of the IKK-2 inhibitor is not through inhibition of NF-κB pathway activation. Data have been published suggesting a role for IKK-2 in NF-κB-independent events. For example, Sizemore et al. (2004) have demonstrated an NF-κB-independent role for IKK-2 in IFNγ stimulated gene expression.

It has been demonstrated previously by our group that there is no increase in NF-κB DNA binding in the steroid-insensitive, elastase-driven model (Birrell et al., 2005c). In this model, the IKK-2 inhibitor did not affect the airway inflammatory response, in contrast to the inhibitory effect observed in the LPS model (Figs. 6, 8, and 10). This result, by itself, does not prove that elastase-induced inflammation is NF-κB-independent; IKK-2 is now believed not to be central to all NF-κB pathway activation (Hayden and Ghosh, 2004).

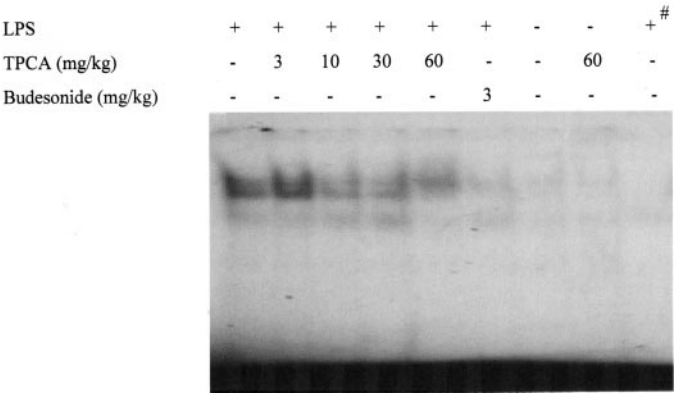

Fig. 4. NF-κB pathway activation in the lung tissue. A representative autoradiograph from EMSA analysis of NF-κB DNA binding in the nuclear fraction extracted from the lung collected from the LPS study. To demonstrate specificity, 100-fold excess of unlabeled competitor consensus oligonucleotides was added to a reaction (indicated with #).

However, these data, in conjunction with the absence of an increase in NF-κB DNA binding and the lack of impact of a steroid, make for a persuasive argument. In fact, these data may suggest that, along with NF-κB–dependent inflamma-

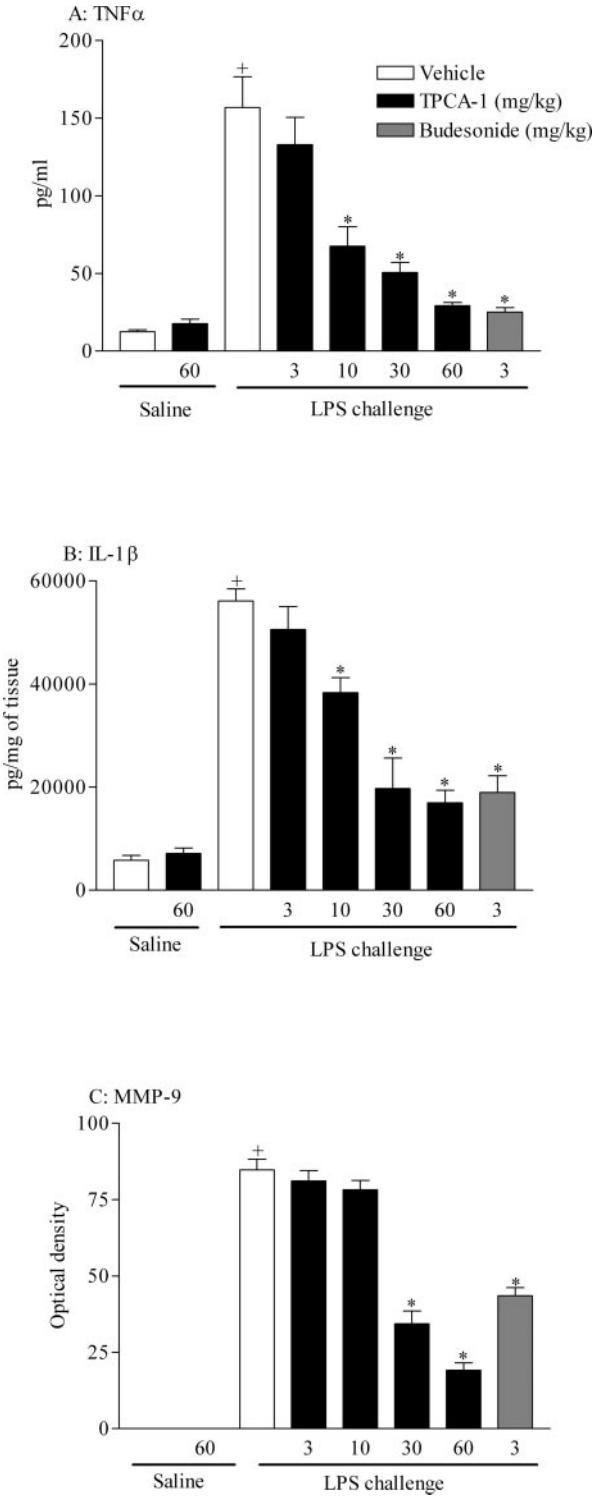

Fig. 5. Effect of IKK-2 inhibitor on LPS induced mediator release in the rat lung. Impact of TPCA-1 or budesonide on lung levels of inflammatory mediators 6 h after exposure to aerosolized LPS (1 mg/ml for 30 min). A, BAL fluid TNFα levels; B, lung tissue IL-1β levels; C, BAL fluid MMP-9 levels. +, significantly different from saline-challenged/vehicle-treated group; *, significantly different from LPS challenged/vehicle-treated group ($P < 0.05$).

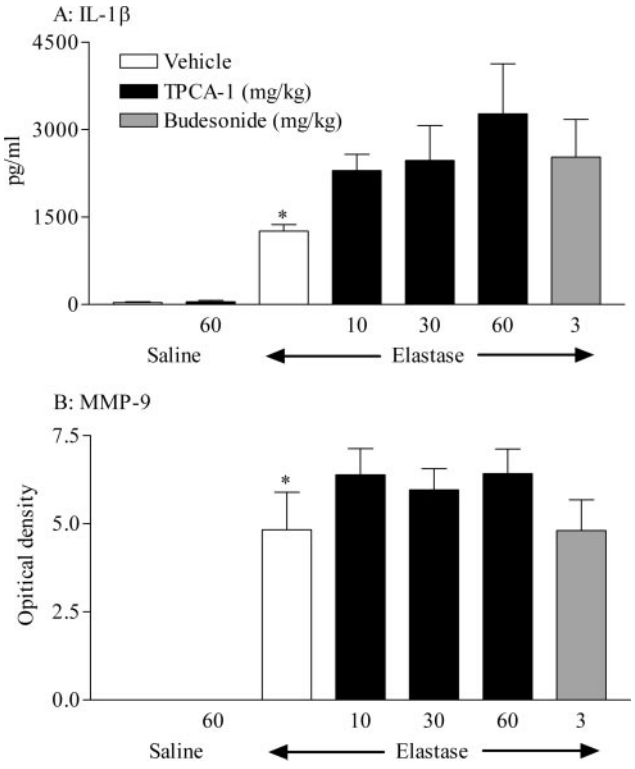

Fig. 6. Effect of IKK-2 inhibitor on elastase-induced mediator release in the rat lung. Impact of TPCA-1 or budesonide on lung levels of inflammatory mediators 48 h after exposure to PPE (120 U/kg, intratracheal). A, BAL IL-1β levels; B, BAL fluid MMP-9 levels. +, significantly different from saline-challenged/vehicle-treated group; ∗, significantly different from PPE-challenged/vehicle-treated group ($P < 0.05$).

tion, aspects of the inflammatory profile in diseased patients could be independent of the NF-κB transcription factor. It is possible that this NF-κB–independent inflammation could be the reason for the limited impact of glucocorticoid therapies in respiratory diseases such as COPD and severe asthma, especially because these compounds are thought to achieve efficacy through influencing aspects of the NF-κB pathway. One may question the clinical relevance of the inflammation observed in the elastase model, especially considering the publications in which an increase in NF-κB DNA binding has been shown in patients with COPD (Caramori et al., 2003; Di Stefano et al., 2004) and severe asthma (Gagliardo et al., 2003). However, we speculate that these two alternative inflammatory pathways, as in the results we have generated in these animal models, may coexist in the human disease. Indeed, Caramori et al. (2003) have published data demonstrating an increase in nuclear p65 staining in macrophages from COPD patients but not in neutrophils from the same patients. In addition, Di Stefano et al. (2004) demonstrated an increase in the expression of NF-κB in the lungs of "healthy" smokers, suggesting that some of the increase observed may be related directly to smoking cigarettes rather than a facet of disease per se. Furthermore, a recent publication described no significant difference in nuclear localization of NF-κB in bronchial biopsies from healthy smokers compared with patients with COPD. Indeed, in the same study BAL fluid leukocytes from healthy smokers showed a significant reduction in NF-κB DNA binding compared with nonsmokers, whereas nuclear translocation of NF-κB during exacerbations of COPD did not differ from that in nonsmokers (Drost et al., 2005). However, regardless of whether this

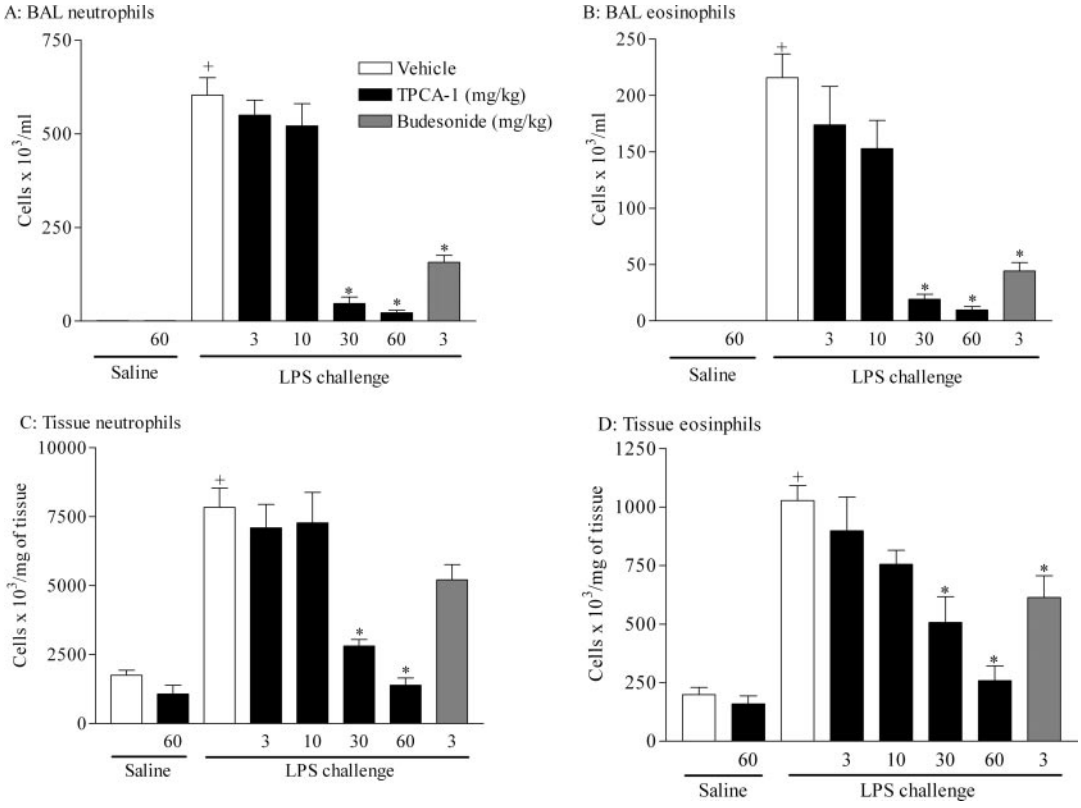

Fig. 7. Effect of IKK-2 inhibitor on LPS-induced cellular recruitment in the rat lung. Number of neutrophils (A) and eosinophils (B) in the lavage fluid and neutrophils (C) and eosinophils (D) in the lung tissue after exposure to aerosolized LPS and treatment with vehicle, TPCA-1, or budesonide. +, significantly different from saline-challenged/vehicle-treated group; ∗, significantly different from LPS challenged/vehicle-treated group ($P < 0.05$).

model represents the clinical situation at the very simplest level, it provides us with a robust model for dissecting the signaling pathways behind this NF-κB-independent, steroid-resistant inflammatory response.

At the moment, the molecular mechanism(s) responsible for the inflammation observed in the elastase-driven model is unclear. If the mechanism(s) could be elucidated and could be shown to be relevant to human diseases, it might highlight potential novel anti-inflammatory targets for the treatment of steroid-resistant diseases. It is possible that the inflammation observed in the elastase model is downstream of IKK-2/NF-κB (i.e., that it is driven by pre-existing mediators that are activated directly or indirectly by the elastase). The increase in gene expression (Table 1), however, suggests that transcriptional events are occurring that are independent of IKK-2 and increased NF-κB–DNA binding and are not sensitive to treatment with a glucocorticoid. It is also possible that the increase in inflammatory mediator gene expression is due to changes in the basal transcriptional machinery (i.e., altered chroma-

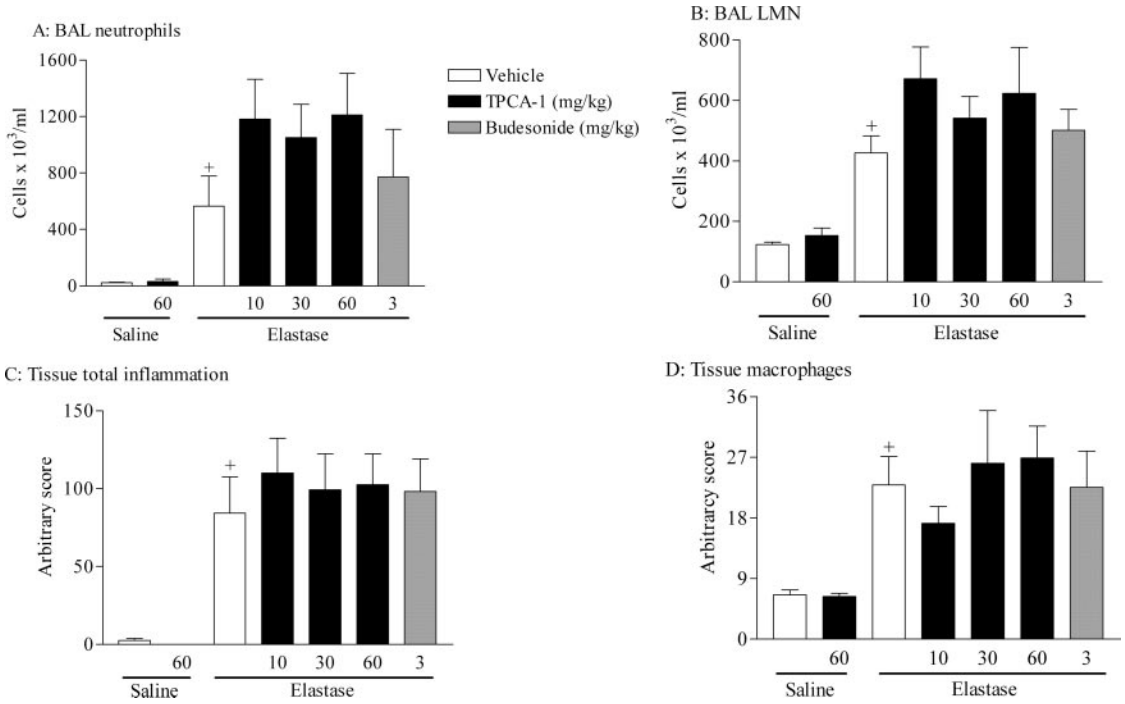

Fig. 8. Effect of IKK-2 inhibitor on elastase-induced cellular recruitment in the rat lung. Number of neutrophils (A) and lymphomononuclear cells (B) in the lavage fluid and total inflammatory cell burden (C) and macrophage (D) in the lung tissue after exposure to PPE (120 U/kg, i.t.) and treatment with vehicle, TPCA-1, or budesonide. +, significantly different from saline-challenged/vehicle-treated group; ∗, significantly different from PPE-challenged/vehicle-treated group ($P < 0.05$).

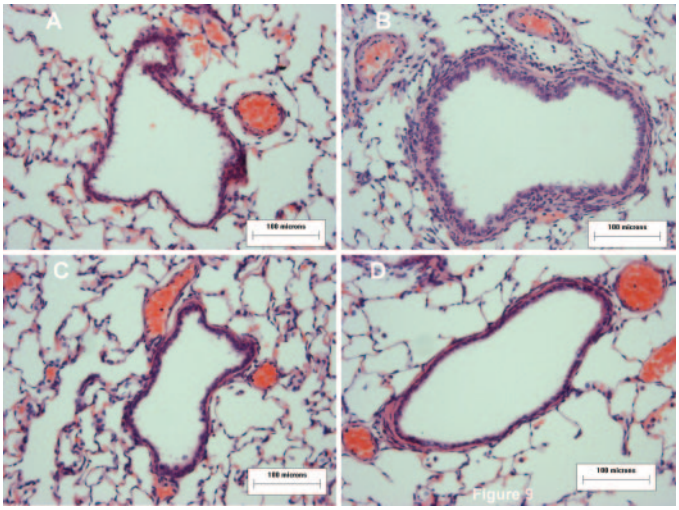

Fig. 9. Effect of IKK-2 inhibitor on cellular inflammation after LPS challenge. Paraffin sections were cut from tissue taken from vehicle-challenged/vehicle-dosed (A); LPS-challenged/vehicle-dosed (B); LPS-challenged/TPCA-1 (60 mg/kg) dosed (C); and LPS-challenged/budesonide (3 mg/kg) dosed groups (D). These were then stained with Mayer's hematoxylin and eosin for demonstration of cellular inflammation.

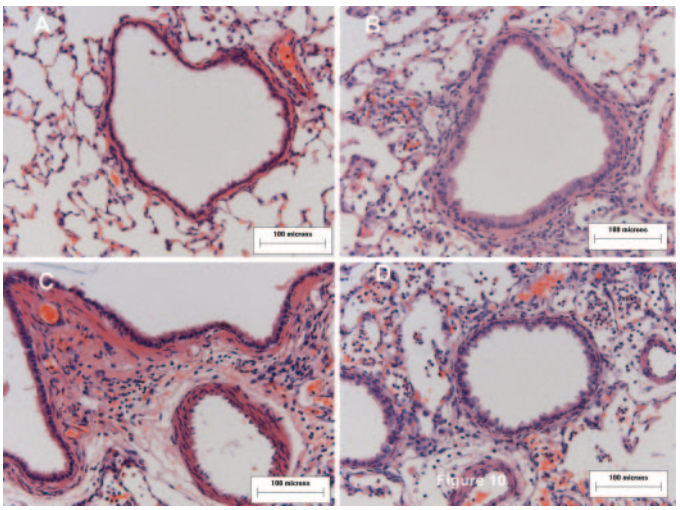

Fig. 10. Effect of IKK-2 inhibitor on cellular inflammation after PPE challenge. Paraffin sections were cut from tissue taken from vehicle-challenged/vehicle-dosed (A); PPE-challenged/vehicle-dosed (B); PPE-challenged/60 mg/kg TPCA-1–dosed (C); PPE-challenged/3 mg/kg budesonide-dosed (C) groups. These were then stained with Mayer's hematoxylin and eosin for demonstration of cellular inflammation.

TABLE 1

Data from the elastase driven model time course

Data are expressed as -fold difference from time-matched, vehicle-dosed control groups.

	Time after Challenge			
	2 h	6 h	24 h	48 h
TNFα	1.2 ± 0.1	2.2 ± 0.6	2.2 ± 0.8	4.5 ± 1.6
IL-1β	1.4 ± 0.4	3.2 ± 0.7	3.3 ± 0.9	3.3 ± 0.7
MMP-9	1.7 ± 0.6	1.7 ± 0.5	3.2 ± 0.9	5.2 ± 1.0
iNOS	1.9 ± 0.4	5.3 ± 2.2	4.2 ± 1.3	15.0 ± 10.9

tin configuration) or post-transcriptional modification (i.e., increase in mRNA stability).

In summary, we have demonstrated that similar profiles of airway inflammation, induced by different stimuli, can be NF-κB-dependent or -independent and IKK-2 inhibitor/steroid-sensitive or -insensitive. Elucidation of the molecular mechanisms behind this apparent NF-κB-independent inflammation could lead to the discovery of novel anti-inflammatory targets and the development of inhibitors that may be effective in diseases that are less sensitive to steroids, such as COPD and severe asthma.

Acknowledgments

We thank GlaxoSmithKline for the generous donation of TPCA-1 and acknowledge the chemists involved in the synthesis: Stefano Livia, James Callahan, Yue Li, and Robert Blade. We would also like to thank Dr. J. De Alba for help capturing the images of the stained histological sections; D. Hele for help in proofreading the manuscript, and for S. Sham for help with the preparation of the manuscript.

References

Baldwin AS Jr (2001) Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Investig* **107:**3–6.

Barnes PJ (2001) Cytokine modulators as novel therapies for airway disease. *Eur Respir J Suppl* **34:**67–77.

Barnes PJ and Adcock IM (1997) NF-kappa B: a pivotal role in asthma and a new target for therapy. *Trends Pharmacol Sci* **18:**46–50.

Belvisi MG, Wicks SL, Battram CH, Bottoms SE, Redford JE, Woodman P, Brown TJ, Webber SE, and Foster ML (2001) Therapeutic benefit of a dissociated glucocorticoid and the relevance of in vitro separation of transrepression from transactivation activity. *J Immunol* **166:**1975–1982.

Beyaert R, Heyninck K, and Van Huffel S (2000) A20 and A20-binding proteins as cellular inhibitors of nuclear factor-kappa B-dependent gene expression and apoptosis. *Biochem Pharmacol* **60:**1143–1151.

Birrell MA, Hardaker E, Wong S, McCluskie K, Catley M, De Alba J, Newton R, Haj-Yahia S, Tao Pun K, Watts CJ, et al. (2005a) Iκ-B kinase-2 inhibitor blocks inflammation in human airway smooth muscle and a rat model of asthma. *Am J Respir Crit Care Med* **172:**962–971.

Birrell MA, McCluskie K, Wong S, Donnelly LE, Barnes PJ, and Belvisi MG (2005b) Resveratrol, an extract of red wine, inhibits lipopolysaccharide induced airway neutrophilia and inflammatory mediators through an NF-kappaB-independent mechanism. *FASEB J* **19:**840–841.

Birrell MA, Wong S, Hele DJ, McCluskie K, Hardaker E, and Belvisi MG (2005) (2005c) Steroid resistant inflammation in a rat model of COPD is associated with a lack of NF-{kappa}B activation. *Am J Respir Crit Care Med* **172:**74–84.

Cazes E, Giron-Michel J, Baouz S, Doucet C, Cagnoni F, Oddera S, Korner M, Dasic G, Testi R, Azzarone B, et al. (2001) Novel anti-inflammatory effects of the inhaled corticosteroid fluticasone propionate during lung myofibroblastic differentiation. *J Immunol* **167:**5329–5337.

Caramori G, Romagnoli M, Casolari P, Bellettato C, Casoni G, Boschetto P, Chung KF, Barnes PJ, Adcock IM, Ciaccia A, et al. (2003) Nuclear localisation of p65 in sputum macrophages but not in sputum neutrophils during COPD exacerbations. *Thorax* **58:**348–351.

Courtois G, Smahi A, and Israel A (2001) NEMO/IKK gamma: linking NF-kappa B to human disease. *Trends Mol Med* **7:**427–430.

Di Stefano A, Caramori G, Ricciardolo FL, Capelli A, Adcock IM, and Donner CF (2004) Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. *Clin Exp Allergy* **34:**1156–1167.

Drost EM, Skwarski KM, Sauleda J, Soler N, Roca J, Agusti A, and MacNee W (2005) Oxidative stress and airway inflammation in severe exacerbations of COPD. *Thorax* **60:**293–300.

Gagliardo R, Chanez P, Mathieu M, Bruno A, Costanzo G, Gougat C, Vachier I, Bousquet J, Bonsignore G, and Vignola AM (2003) Persistent activation of nuclear factor-kappaB signaling pathway in severe uncontrolled asthma. *Am J Respir Crit Care Med* **168:**1190–1198.

Hayden MS and Ghosh S (2004) Signaling to NF-kappaB. *Genes Dev* **18:**2195–2224.

Hu Y, Baud V, Delhase M, Zhang P, Deerinck T, Ellisman M, Johnson R, and Karin M (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science (Wash DC)* **284:**316–320.

Karin M (1999) The beginning of the end: IκB kinase (IKK) and NF-κB activation. *J Biol Chem* **274:**27339–27342.

Karin M and Lin A (2002) NF-kappaB at the crossroads of life and death. *Nat Immunol* **3:**221–227.

Li L, Xia Y, Nguyen A, Lai YH, Feng L, Mosmann TR, and Lo D (1999a) Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J Immunol* **162:**2477–2487.

Li Q, Lu Q, Hwang JY, Buscher D, Lee KF, Izpisua-Belmonte JC, and Verma IM (1999b) IKK1-deficient mice exhibit abnormal development of skin and skeleton. *Genes Dev* **13:**1322–1328.

Li Q, Van Antwerp D, Mercurio F, Lee KF, and Verma IM (1999c) Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. *Science (Wash DC)* **284:**321–325.

Li Q and Verma IM (2002) NF-kappaB regulation in the immune system. *Nat Rev Immunol* **2:**725–734.

Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, et al. (1997) IKK-1 and IKK2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science (Wash DC)* **278:**860–866.

Mukaida N, Morita M, Ishikawa Y, Rice N, Okamoto S, Kasahara T, and Matsushima K (1994) Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-κ B is target for glucocorticoid-mediated interleukin 8 gene repression. *J Biol Chem* **269:**13289–13295.

Newton R, Hart LA, Stevens DA, Bergmann M, Donnelly LE, Adcock IM, and Barnes PJ (1998) Effect of dexamethasone on interleukin-1beta-(IL-1beta)-induced nuclear factor-kappaB (NF-kappaB) and kappaB-dependent transcription in epithelial cells. *Eur J Biochem* **254:**81–89.

Podolin PL, Callahan JF, Bolognese BJ, Li YH, Carlson K, Davis TG, Mellor GW, Evans C, and Roshak AK (2005) Attenuation of murine collagen-induced arthritis by a novel, potent and selective small molecule inhibitor of IκB kinase 2, TPCA-1, occurs via reduction of pro-inflammatory cytokines and antigen-induced T cell proliferation. *J Pharmacol Exp Ther* **312:**373–381.

Poynter ME, Irvin CG, and Janssen-Heininger YM (2003) A prominent role for airway epithelial NF-kappa B activation in lipopolysaccharide-induced airway inflammation. *J Immunol* **170:**6257–6265.

Rothwarf DM and Karin M (1999) The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. *Sci STKE* **5:**RE1.

Sakurai H, Suzuki S, Kawasaki N, Nakano H, Okazaki T, Chino A, Doi T, and Saiki I (2003) Tumor necrosis factor-alpha-induced IKK phosphorylation of NF-κB p65 on serine 536 is mediated through the TRAF2, TRAF5 and TAK1 signaling pathway. *J Biol Chem* **278:**36916–36923.

Scheidereit C (1998) Signal transduction. Docking IkappaB kinases. *Nature (Lond)* **395:**225–226.

Sizemore N, Agarwal A, Das K, Lerner N, Sulak M, Rani S, Ransohoff R, Shultz D, and Stark GR (2004) Inhibitor of kappaB kinase is required to activate a subset of interferon gamma-stimulated genes. *Proc Natl Acad Sci USA* **101:**7994–7998.

Takeda K, Takeuchi O, Tsujimura T, Itami S, Adachi O, Kawai T, Sanjo H, Yoshikawa K, Terada N, and Akira S (1999) Limb and skin abnormalities in mice lacking IKKalpha. *Science (Wash DC)* **284:**313–316.

Underwood SL, Raeburn D, Lawrence C, Foster M, Webber S, and Karlsson JA (1997) RPR 106541, a novel, airways-selective glucocorticoid: effects against antigen-induced CD4+ T lymphocyte accumulation and cytokine gene expression in the Brown Norway rat lung. *Br J Pharmacol* **122:**439–446.

Address correspondence to: Professor Maria G. Belvisi, Head Respiratory Pharmacology Group, Airway Diseases, Faculty of Medicine, National Heart and Lung Institute, Imperial College, Dovehouse Street, London, SW3 6LY, UK. E-mail: m.belvisi@imperial.ac.uk