

Validation of the Anti-Inflammatory Properties of Small-Molecule I κ B Kinase (IKK)-2 Inhibitors by Comparison with Adenoviral-Mediated Delivery of Dominant-Negative IKK1 and IKK2 in Human Airways Smooth Muscle^[S]

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ABSTRACT

Asthma and chronic obstructive pulmonary disease (COPD) are characterized by chronic airway inflammation. However, because patients with COPD and certain patients with asthma show little or no therapeutic benefit from existing corticosteroid therapies, there is an urgent need for novel anti-inflammatory strategies. The transcription factor nuclear factor- κ B (NF- κ B) is central to inflammation and is necessary for the expression of numerous inflammatory genes. Proinflammatory cytokines, including interleukin (IL)-1 β and tumor necrosis factor (TNF)- α , activate the I κ B kinase complex (IKK) to promote the degradation of inhibitory I κ B proteins and activate NF- κ B. This pathway and, in particular, the main I κ B kinase, IKK2, are now considered prime targets for novel anti-inflammatory drugs. Therefore, we have used adenoviral overexpression to demonstrate NF- κ B and IKK2 dependence of key inflammatory genes, including intercellular adhesion molecule (ICAM)-1, cyclooxygenase-2, IL-6, IL-8, granulocyte macrophage-colony-stimulating factor (GM-CSF), regulated on activation normal T cell expressed and secreted (RANTES), monocyte chemotactic protein-1 (MCP-1), growth-regulated oncogene- α

(GRO α), neutrophil-activating protein-2 (NAP-2), and epithelial neutrophil activating peptide 78 (ENA-78) in primary human airways smooth muscle cells. Because this cell type is central to the pathogenesis of airway inflammatory diseases, these data predict a beneficial effect of IKK2 inhibition. These validated outputs were therefore used to evaluate the novel IKK inhibitors *N*-(6-chloro-9*H*- β -carbolin-8-yl) nicotinamide (PS-1145) and *N*-(6-chloro-7-methoxy-9*H*- β -carbolin-8-yl)-2-methyl-nicotinamide (ML120B) on IL-1 β and TNF α -induced expression, and this was compared with the corticosteroid dexamethasone. As observed above, ICAM-1, IL-6, IL-8, GM-CSF, RANTES, MCP-1, GRO α , NAP-2, and ENA-78 expression was reduced by the IKK inhibitors. Furthermore, this inhibition was either as effective, or for ICAM-1, MCP-1, GRO α , and NAP-2, more effective, than a maximally effective concentration of dexamethasone. We therefore suggest that IKK inhibitors may be of considerable benefit in inflammatory airways diseases, particularly in COPD or severe asthma, in which corticosteroids are ineffective.

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ABBREVIATIONS: COPD, chronic obstructive pulmonary disease; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; HASM, human airway smooth muscle; IKK, I κ B kinase complex; MOI, multiplicity of infection; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; RANTES, regulated on activation normal T cell expressed and secreted; GM-CSF, granulocyte macrophage-colony-stimulating factor; GRO α , growth-regulated oncogene- α ; NAP-2, neutrophil-activating protein-2; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; COX-2, cyclooxygenase-2; NF- κ B, nuclear factor κ B; IL, interleukin; ICAM, intercellular adhesion molecule; TNF α , tumor necrosis factor α ; MCP-1, monocyte chemotactic protein-1; PS-1145, *N*-(6-chloro-9*H*- β -carbolin-8-yl) nicotinamide; ML120B, *N*-(6-chloro-7-methoxy-9*H*- β -carbolin-8-yl)-2-methyl-nicotinamide.

there remains a group of patients whose asthma remains poorly controlled because of disease severity or to insensitivity to corticosteroid treatment (Adcock and Ito, 2004; Barnes, 2004). In contrast, COPD is generally unresponsive to corticosteroid treatment, and apart from smoking cessation, which halts the accelerated decline in lung function, the only genuinely effective treatment is lung transplantation (Barnes and Hansel, 2004; Wouters, 2004). Given the highly invasive nature of this therapy and the shortage of donor tissue, effective treatments such as the development of more specific and potent anti-inflammatory agents that target the inflammation associated with COPD and severe and steroid insensitive asthma are desperately needed (Adcock and Ito, 2004; Barnes, 2004; Barnes and Hansel, 2004; Wouters, 2004).

NF- κ B is a ubiquitously expressed transcription factor that consists of hetero- or homodimers of the Rel family of proteins and regulates the expression of many genes involved in immune and inflammatory responses (Barnes and Karin, 1997; Li and Verma, 2002). Thus, the NF- κ B signaling pathway is considered to be a potential target for novel anti-inflammatory compounds, and indeed, a number of pharmaceutical companies are developing compounds that target this pathway (Barnes and Karin, 1997; Li and Verma, 2002; Karin et al., 2004). A number of these have now been shown to retain anti-inflammatory effects in animal models and could therefore be useful in the therapeutic treatment of inflammatory airways disease (Castro et al., 2003; Karin et al., 2004). In unstimulated or resting cells, NF- κ B is localized to the cytoplasm and is associated with a members of a family of inhibitory proteins known as I κ B (inhibitor of κ B) (Hayden and Ghosh, 2004). NF- κ B activation is initiated in response to a wide range of stimuli, including the pro-inflammatory cytokines, IL-1 β and TNF- α , chemokines, and bacterial and viral products (Barnes and Karin, 1997; Li and Verma, 2002; Hayden and Ghosh, 2004). These lead to phosphorylation of I κ B by the I κ B α kinase (IKK) complex, which in turn leads to I κ B polyubiquitination and subsequent degradation by the 26S proteasome (Li and Verma, 2002; Hayden and Ghosh, 2004). As a result, NF- κ B proteins are liberated from I κ B, usually I κ B α , and translocate to the nucleus, where they bind to the promoter regions of NF- κ B responsive genes and initiate gene transcription (Li and Verma, 2002; Hayden and Ghosh, 2004; Karin et al., 2004). A critical step in the NF- κ B pathway is the phosphorylation of I κ B α by the IKK complex (Li and Verma, 2002). This complex consists of at least two catalytic subunits, IKK1 (IKK α) and IKK2 (IKK β) and a regulatory subunit, IKK γ /NEMO, which has no catalytic function, but whose structural role is absolutely required for IKK activation. Although IKK1 and IKK2 are structurally similar, studies in knockout mice and derived mouse embryonic fibroblasts suggest that IKK2 is the predominant kinase involved in I κ B α phosphorylation and hence NF- κ B activation (Li and Verma, 2002; Hayden and Ghosh, 2004).

Airway smooth muscle cells are a potent source of many cytokines, chemokines, and other mediators and have been implicated in the local amplification of airway inflammatory responses (Howarth et al., 2004). To help evaluate the therapeutic potential of inhibiting the NF- κ B pathway in this cell type, we have used primary human airway smooth muscle (HASM) cells and a combination of adenoviral delivery of dominant-negative IKK1, IKK2, and dominant I κ B α

(I κ B α Δ N), as well as novel small-molecule IKK inhibitors. Taken together, the data presented in these studies strongly suggest that therapeutic strategies to inhibit NF- κ B may be beneficial in airway inflammatory diseases.

Materials and Methods

Reagents. IL-1 β and TNF α were from R&D systems (Abingdon, UK). The IKK-selective inhibitor PS-1145 has been described previously, and the structure has been published elsewhere (Hideshima et al., 2002; Castro et al., 2003). ML120B is a novel ATP binding site IKK2-selective (IC₅₀ at 50 μ M ATP) inhibitor that does not inhibit either IKK1 (EC₅₀ > 100 μ M) or IKK ϵ (EC₅₀ > 100 μ M) or a panel of 28 other kinases at EC₅₀ values of up to 50 μ M (Nagashima et al., 2006; Wen et al., 2006). Both PS-1145 and ML120B were supplied as free bases by Millennium Pharmaceuticals (Cambridge, MA) and were dissolved in dimethyl sulfoxide (DMSO) before dilution in tissue culture medium. All other reagents were from Sigma (Poole, Dorset, UK) unless otherwise stated.

Cell Culture and Adenovirus Infection. Human bronchial tissue was obtained from patients undergoing lung transplantation or surgical resection for carcinoma. The isolation and culture of HASM cells from these tissues has been described previously (Sukkar et al., 2004). The null, green fluorescent protein (Ad5-GFP), dominant I κ B α Δ N, and dominant-negative IKK1(KM), IKK2(KA)-expressing viruses have all been described previously (Krapppmann et al., 1996; Zandi et al., 1997; Catley et al., 2003, 2005). All viruses were titered by endpoint dilution and plaque assay to determine plaque-forming units. Viruses were diluted in Dulbecco's modified Eagle's medium (Sigma) to a multiplicity of infection (MOI) of 30 before infection of HASM cells. This dose of virus showed no significant effect on cell viability as determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) cell viability assay (Fig. 1). Cells were then incubated for 24 h and serum-starved for an additional 24 h before microscopic inspection to confirm normal morphology. The cells were then treated with cytokines or prepared for experimental procedures. Cells grown on glass coverslips were incubated with various MOIs of Ad5-GFP virus before staining with 4',6'-diamidino-2-phenylindole dihydrochloric hydrate and analysis of GFP fluorescence as described previously (Catley et al., 2003). For NF- κ B reporter assays, HASM cells were infected with MOI 30 of an adenovirus carrying an NF- κ B-dependent reporter (Ad-NF- κ B-luc). This construct was obtained by inserting the NF- κ B enhancer [five copies of the classic NF- κ B motif (underlined) 5'-TGG GGA CTT TCC GC-3'], TATA box, and luciferase gene from pNF- κ B-luc (Stratagene, La Jolla, CA) into the Ad5 parent vector. After incubation for 24 h in Dulbecco's modified Eagle's medium, the cells were then changed to fresh serum-free medium and stimulated with IL-1 β or TNF α for 8 h before harvesting for luciferase assay.

IKK Kinase Assay. Kinase assays were performed as described previously with the following modifications (Nasuhara et al., 1999; Catley et al., 2004). Confluent six-well plates were placed in serum-free medium for 24 h before stimulation with IL-1 β or TNF α for 5 min. Cells were immediately put on ice and washed twice with ice-cold Hanks' balanced salt solution. Triplicate wells were then scraped and pooled into one tube before centrifugation and subsequent lysis. Cell lysates were precleared with agarose-conjugated normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h before immunoprecipitation of the IKK complex with an agarose-conjugated IKK γ -specific antibody (Santa Cruz Biotechnology). Antibody target complexes were then collected by centrifugation and washed before resuspension in kinase buffer (20 mM HEPES, pH 7.9, 2 mM MgCl₂, 2 mM MnCl₂, 10 mM β -glycerophosphate, 10 mM NaF, 10 mM 4-nitrophenyl phosphate, 0.5 mM Na₃VO₄, 1 mM benzamide, 0.5 mM phenylmethylsulfonyl fluoride, aprotinin 25 μ g/ml, 10 μ g/ml leupeptin, 2 μ M/ml pepstatin, and 1 mM dithiothreitol). Kinase reactions were performed in kinase buffer using a commer-

cially available I κ B α substrate peptide (Upstate Biotechnology, Lake Placid, NY). For concentration-response experiments, ML120B and PS-1145 were diluted in kinase buffer to the required concentration. The whole reaction mix was run on a 4 to 12% NuPage SDS gel, which was cut at the 22-kDa marker. The bottom portion of each gel was dried for autoradiography, whereas the top portion was subject to Western analysis for IKK γ .

Electromobility Shift Assays and Luciferase Assay. Electromobility shift assays (EMSAs) were performed as described previously (Nasuhara et al., 1999; Catley et al., 2004). Cells for luciferase assay were harvested in 1 \times reporter lysis buffer (Promega) before luciferase assay according to the manufacturers' instructions (Promega) (Nasuhara et al., 1999; Catley et al., 2004).

Western Blotting and Cytokine Release Measurements. Detection of proteins by Western blotting was carried out as described previously (Nasuhara et al., 1999; Holden et al., 2004). Measurement of cytokine release was determined with the SearchLight Proteome Array sandwich ELISAs using the Pierce custom service (Perbio, Woburn, MA).

Cell Viability Assay. Cell viability was assessed colorimetrically by measuring the conversion of MTT by mitochondrial dehydrogenases according to the manufacturer's specification. In brief, the medium of cells to be assayed was replaced with fresh medium containing 1 mg/ml MTT for 15 min at 37°C. Medium was then removed, and the cells were dissolved in DMSO before optical density measurements at 600 nm.

Statistical Analysis. All values are expressed as means \pm S.E.M. Statistical significance was determined using one-way analysis of variance with Dunnett's post test for comparison to the control sample. Significance was taken where $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

Results

Cultured HASM Cells Are Readily Infected by Adenoviral Expression Vectors. HASM cells were infected with various MOIs of a GFP-expressing Ad5 construct. After 4',6'-diamidino-2-phenylindole dihydrochloric hydrate staining, the presence of GFP was analyzed by confocal fluorescence microscopy. An MOI lower than 10 produced only relatively low numbers of GFP-positive cells (data not shown), whereas for MOIs between 10 and 100, a concentration-dependent increase in GFP-positive cells was observed (Supplemental Fig. S1). At an MOI of 30, ~95% of cells were positive for GFP, and this level was used for subsequent experiments.

Effect of Adenoviral Overexpression of Dominant I κ B α DN and Both Dominant-Negative IKK1 and IKK2 on NF- κ B Activation. To examine the role of IKK1 and IKK2 in NF- κ B activation, HASM cells were infected

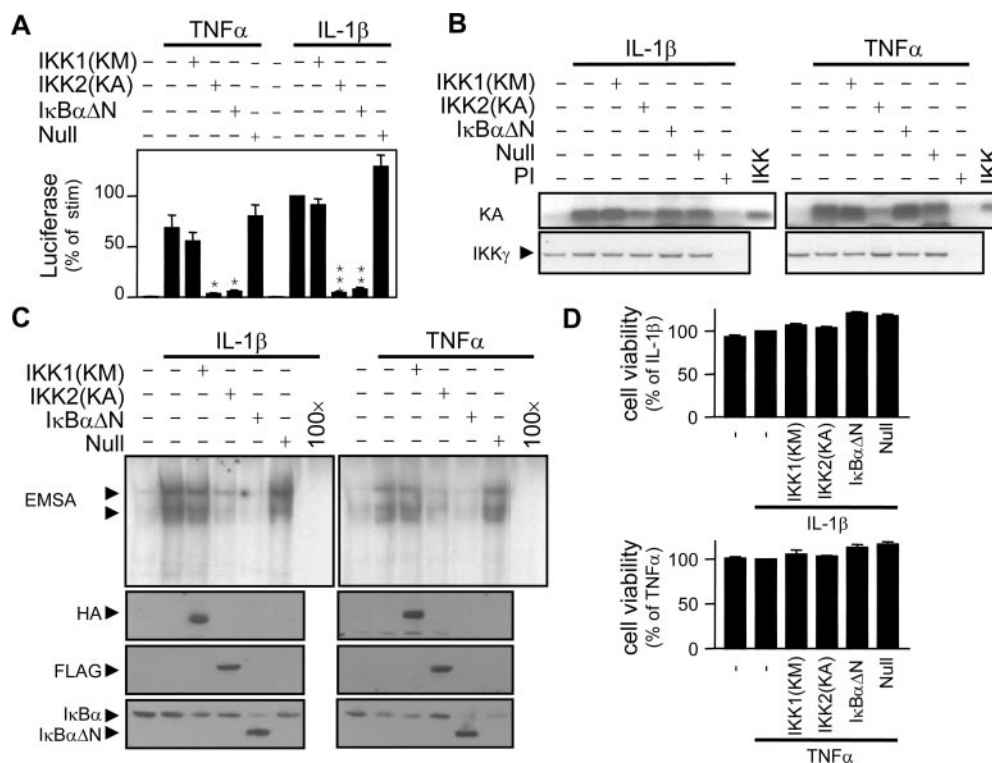


Fig. 1. IKK2 is essential for I κ B kinase activity, NF- κ B activation, and DNA binding. A, HASM cells were infected with an adenovirus (MOI 30) containing the NF- κ B-dependent luciferase reporter (Ad-NF- κ B-luc). The indicated wells were simultaneously coinfecting (MOI 30) with either the null adenovirus or adenoviruses expressing IKK1(KM), IKK2(KA), or I κ B α DN for 24 h before stimulation with IL-1 β (1 ng/ml) or TNF α (10 ng/ml) for a further 8 h and then harvesting for luciferase assay. Data ($n = 4$), expressed as a percentage of the stimulation by IL-1 β , are plotted as means \pm S.E.M. B, HASM cells were infected with the indicated adenovirus (MOI 30) for 48 h before stimulation with IL-1 β (1 ng/ml) or TNF α (10 ng/ml) for 5 min. The IKK complex was then immunoprecipitated and analyzed for I κ B kinase activity (KA) (top). The portion of the gel above the substrate peptide was also analyzed by Western blotting for IKK γ (bottom). Blots and autoradiographs representative of three experiments are shown. C, HASM cells were infected with adenoviral vectors and stimulated as in B above. Cells were harvested for both EMSA and Western blot analysis. Autoradiographs, representative of two such experiments, show NF- κ B DNA binding (top) with parallel Western analysis of cytoplasmic fractions revealing the expression of HA-tagged IKK1(KM) (HA), FLAG-tagged IKK2(KA) (FLAG), and I κ B α (bottom). 100 \times indicates reactions performed in the presence of 100-fold excess of unlabeled probe. D, cells were infected with the indicated viruses for 24 h before being serum-starved for a further 24 h. The cells were then stimulated as indicated for 18 h before MTT assay. Data ($n = 4$) expressed as a percentage of the stimulated samples (IL-1 β or TNF α) are plotted as means \pm S.E.M.

with an adenovirus containing an NF- κ B-dependent luciferase reporter alone or coinfecting with viruses expressing either dominant-negative IKK1 [IKK1(KM)], dominant-negative IKK2 [IKK2(KA)], dominant I κ B α (I κ B α Δ N), or the null virus. Cells infected with the reporter virus alone showed strong induction of luciferase in response to IL-1 β and TNF α (Fig. 1A). This induction of luciferase activity was almost completely reversed by coinfection with IKK2(KA) or I κ B α Δ N, whereas the null virus and dominant-negative IKK1(KM) had no effect on reporter activation (Fig. 1A).

To examine the effect of IKK1(KM) and IKK2(KA) overexpression on IKK complex kinase activity, the complex was immunoprecipitated and assayed for the ability to phosphorylate an I κ B α -derived substrate peptide. IKK activity was strongly induced by both TNF α and IL-1 β (Fig. 1B, top). This response was substantially reduced by overexpression of IKK2(KA), whereas IKK1(KM) or the null virus had no obvious effect. Because I κ B α Δ N acts downstream of the IKK complex, there was no effect on IL-1 β - and TNF α -induced IKK kinase activity. The specificity of the kinase reaction was confirmed by the fact that immunoprecipitation using preimmune sera failed to pull down I κ B α kinase activity or IKK γ , whereas recombinant IKK efficiently phosphorylated

the target. Loading of immunoprecipitated samples was confirmed by blotting for IKK γ (Fig. 1B, bottom).

EMSA was performed to examine the effect of I κ B α Δ N and both IKK1(KM) and IKK2(KA) on the induction of NF- κ B DNA binding. As expected, both IL-1 β and TNF α strongly induced NF- κ B DNA binding, and in each case, the addition of excess unlabeled probe competed out the DNA binding complexes indicating specificity to the NF- κ B probe (Fig. 1C). Over-expression of IKK2(KA) and I κ B α Δ N completely prevented this induction, whereas IKK1(KM) and the null virus revealed no effect. Cytoplasmic fractions from samples assayed by EMSA were analyzed in parallel by Western blotting to confirm the overexpression of HA-tagged IKK1(KM), FLAG-tagged IKK2(KA), and truncated I κ B α Δ N (Fig. 1C, bottom). Taken together, these data clearly demonstrate that NF- κ B-dependent transcription, I κ B kinase activity, and NF- κ B nuclear translocation and DNA binding are dependent on the activity of IKK2, not IKK1, in HASM cells.

Effect of Adenoviral Infection on HASM Cell Viability. To confirm that the above effects on NF- κ B activation were not due to HASM cell death, MTT cell viability assays were performed after infection with the four viruses. In each case, none of the viruses showed any obvious effect on cell viability (Fig. 1D).

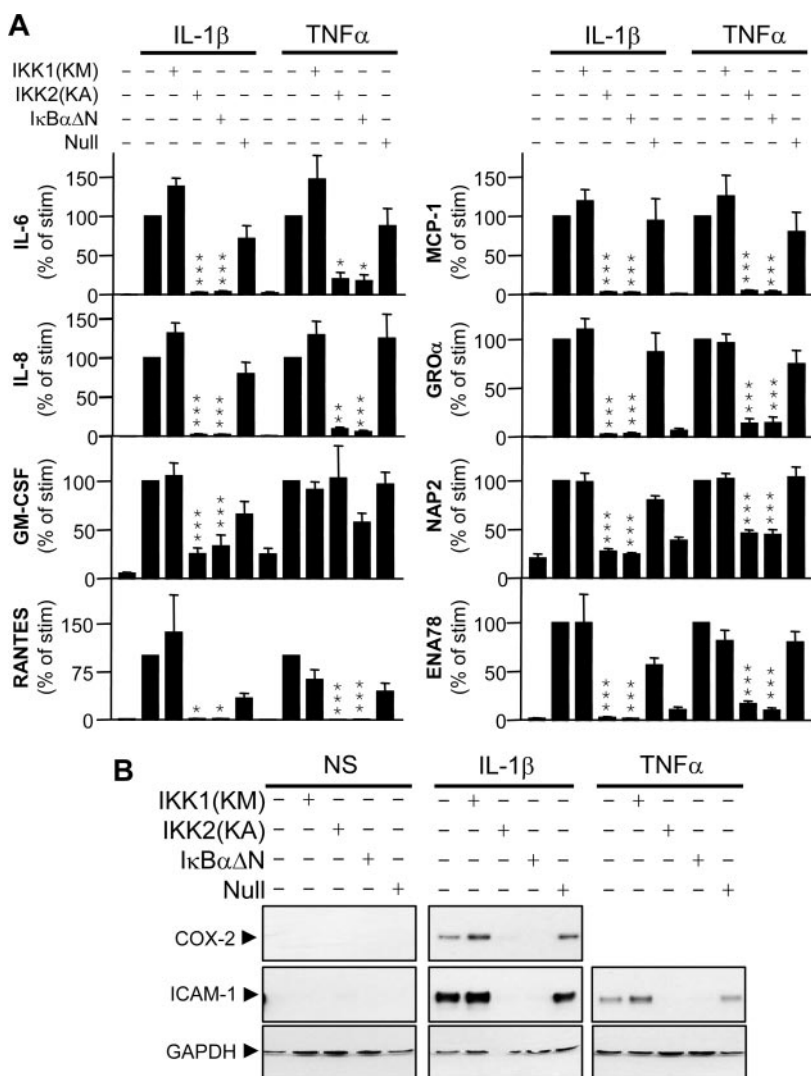


Fig. 2. The effect of IKK and NF- κ B inhibition on the production of inflammatory mediators. HASM cells were infected with the indicated viruses (MOI 30) for 48 h before stimulation with IL-1 β (1 ng/ml) or TNF α (10 ng/ml) for a further 18 h. Culture medium and cell lysates were then harvested for analysis of cytokine release by Pierce SearchLight Multiplex ELISA Assay System (A) or Western blot analysis (B), respectively. A, data ($n = 4$ to 6) are expressed as a percentage of the stimulation (stim) and are plotted as means \pm S.E.M. B, representative blots from the six experiments is shown.

Repression of Inflammatory Genes Expression by $I\kappa B\alpha\Delta N$ and IKK2(KA). To examine the role of IKK1, IKK2, and NF- κ B, the expression of inflammatory mediators and genes was examined by SearchLight Proteome ELISA Arrays (Perbio) and Western blotting. The SearchLight arrays demonstrated that IL-6, IL-8, GM-CSF, RANTES, MCP-1, GRO α , NAP-2, and epithelial neutrophil activating peptide 78 expression is strongly increased by both IL-1 β and TNF α (Fig. 2 and Supplemental Table S1). This increase, with the exception of TNF α -induced GM-CSF, was profoundly inhibited by the IKK2(KA) and $I\kappa B\alpha\Delta N$ adenoviruses. In contrast, the null virus and overexpression of IKK1(KM) had little or no effect on the expression of these cytokines.

In preliminary studies, both IL-1 β and TNF α potently induced ICAM-1 expression. In contrast and consistent with previous reports (Pang and Knox, 1997), only IL-1 β and not TNF α produced an increase in COX-2 protein (data not shown). Western blotting was therefore used to monitor the expression of IL-1 β -induced COX-2 and IL-1 β - and TNF α -induced ICAM-1 expression in the presence of the above adenoviruses (Fig. 2B). Consistent with the cytokine data above, IKK2(KA) and $I\kappa B\alpha\Delta N$ near completely inhibited IL-

1 β - and TNF-induced ICAM-1 expression and IL-1 β -induced COX-2 expression, whereas dominant-negative IKK1 and the null virus showed no obvious effect (Fig. 2B). These data clearly indicate that targeted inhibition of NF- κ B and IKK2 prevents the expression of numerous inflammatory genes and suggests that inhibition of this pathway could be beneficial in the treatment of inflammatory airway diseases.

IKK Inhibitors PS-1145 and ML120B Are Potent Inhibitors of NF- κ B Activation. To further explore the relationship between IKK inhibition and expression of inflammatory mediators, the effect of the two small-molecule IKK inhibitors PS-1145 and ML120B was examined (Hideshima et al., 2002; Castro et al., 2003; Wen et al., 2006). Kinase assays, performed using immunoprecipitated IKK complex from IL-1 β - or TNF α -treated cells, confirmed the efficacy of PS-1145 and ML120B by preventing phosphorylation of the $I\kappa B$ substrate (Fig. 3A). In the case of PS-1145 and consistent with previous reports that quote IC₅₀ values in the region of 0.1 to 0.15 μ M (Hideshima et al., 2002; Castro et al., 2003), IKK kinase activity was reduced to or near basal levels at concentrations greater than 1 μ M, whereas ML120B produced maximal effects at 10 to 30 μ M. In comparison, pretreatment of cells with 1 μ M concentration of the anti-inflam-

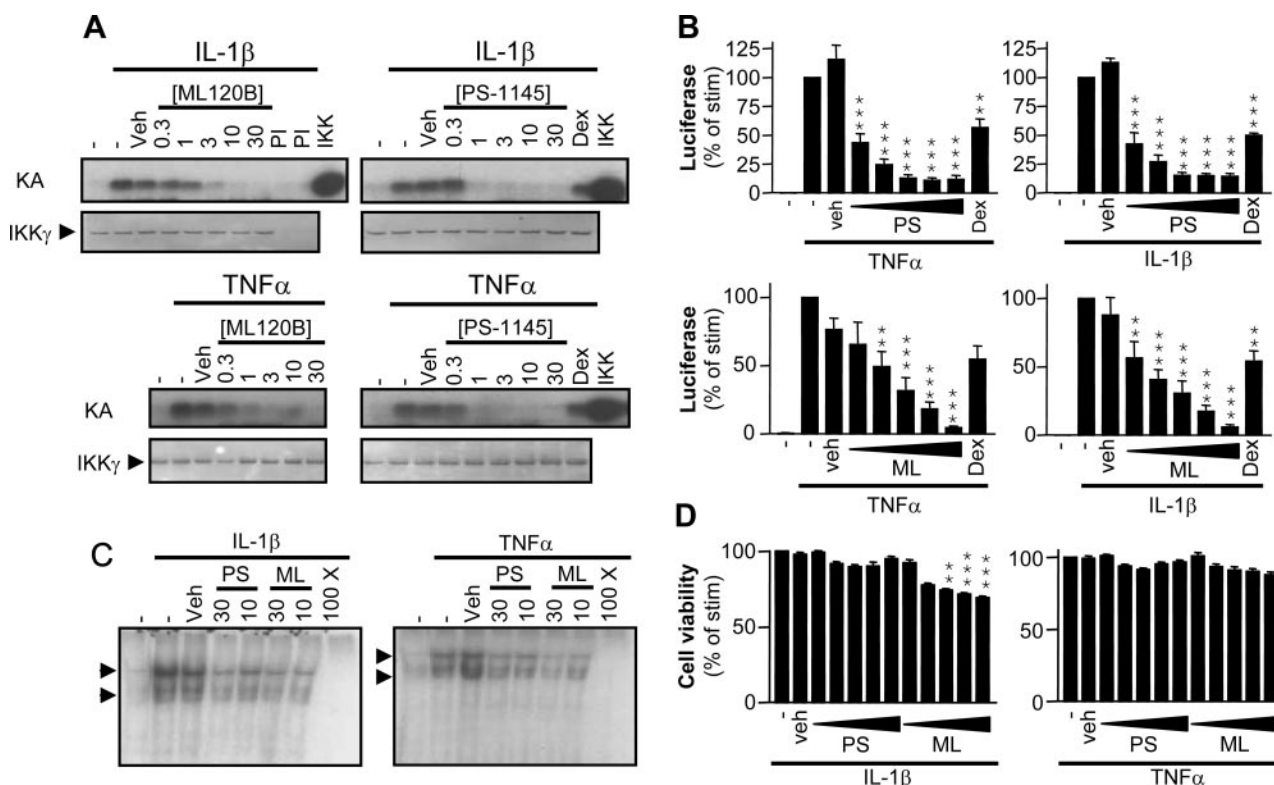


Fig. 3. Effect of PS-1145 and ML120B on NF- κ B activation. A, IKK complex was immunoprecipitated from HASM cells that had been stimulated with IL-1 β (1 ng/ml) or TNF α (10 ng/ml) for 5 min. $I\kappa B$ kinase activity was assayed in the presence of the indicated concentration of PS-1145 or ML120B. After kinase assay (KA), reaction products were size-fractionated, and the lower parts of gels were exposed for autoradiography, whereas the upper portions were subjected to Western blot analysis for IKK γ . Autoradiographs and blots are representative of two such experiments. IKK indicates recombinant active IKK2, PI indicates reactions performed on immunoprecipitates using preimmune sera, and Dex indicates HASM cells that were pretreated for 90 min with 1 μ M dexamethasone before stimulation and harvesting. Veh indicates reactions performed in the presence of DMSO at the highest concentration used. B, HASM cells were infected with Ad-NF- κ B-luc (MOI 30) as before. Cells were pretreated with increasing concentrations (0.3, 1, 3, 10, and 30 μ M) of PS-1145 (PS), ML120B (ML), or dexamethasone for 90 min before stimulation with IL-1 β (1 ng/ml) or TNF α (10 ng/ml) for 8 h. Cells were then harvested for luciferase assay. Data ($n = 4$) are expressed as a percentage of the stimulation and are plotted as means \pm S.E.M. C, HASM cells were treated with the indicated concentration of PS-1145 and ML120B for 90 min before stimulation with IL-1 β (1 ng/ml) and TNF α (10 ng/ml) for 5 min. Cells were then harvested for nuclear proteins, which were analyzed for NF- κ B DNA binding by EMSA. Autoradiographs representative of two such experiments are shown. D, cells were serum-starved for 24 h before pretreatment with drugs as indicated. The cells were then stimulated as indicated for 18 h before MTT assay. Data ($n = 4$) expressed as a percentage of the stimulated samples (IL-1 β or TNF α) are plotted as means \pm S.E.M.

matory glucocorticoid dexamethasone seemed to have no effect on immunoprecipitated IKK activity (Fig. 3A).

To test the effect of PS-1145 and ML120B on NF- κ B-dependent transcription, HASM cells were infected with the adenoviral NF- κ B-dependent luciferase reporter Ad-NF- κ B-luc and treated with various concentrations of PS-1145 or ML120B before stimulation with IL-1 β or TNF α . PS-1145 produced a concentration-dependent reduction in NF- κ B-dependent transcription, which was maximal at 10 μ M for both IL-1 β and TNF α , with resultant EC₅₀ values of approximately 0.3 μ M (Fig. 3B). Likewise, ML120B concentration-dependently reduced IL-1 β - and TNF α -induced NF- κ B-dependent transcription (Fig. 3B). In this case, the effect was maximal at 30 μ M, and the EC₅₀ was approximately 1 μ M (Fig. 3B). For comparison, dexamethasone inhibited IL-1 β - and TNF α -induced NF- κ B-activation by only 47 and 45%.

EMSA was performed to determine whether PS-1145 and ML120B prevent NF- κ B nuclear translocation and DNA binding. These experiments revealed increased DNA binding in both IL-1 β - and TNF α -treated samples, and in each case, this response was reduced by both PS-1145 and ML120B at 10 and 30 μ M (Fig. 3C).

Effect of PS-1145 and ML120B on HASM Cell Viability. MTT assays were performed to examine the effect of PS-1145 and ML120B on cell viability. No significant effect was observed after treatment with ML120B or PS-1145 after stimulation with TNF α (Fig. 3D). However, whereas PS-1145 had no effect on viability after IL-1 β stimulation, ML120B resulted in a significant ~30% loss of cell viability after IL-1 β stimulation. Whereas this effect could exaggerate the apparent effect of ML120B on IL-1 β -induced NF- κ B activation, it is

not sufficient to explain the 90% reduction in NF- κ B-dependent transcription seen on the reporter assay. Thus, the above data demonstrate that the ML120B and PS-1145 both inhibit NF- κ B dependent transcription, I κ B kinase activity, and NF- κ B nuclear translocation and DNA binding.

PS-1145 and ML120B Inhibit Inflammatory Gene Expression. The effect of these inhibitors on inflammatory gene expression was tested using the panel of inflammatory mediators demonstrated to be NF- κ B-responsive in Fig. 2. As before, the expression of IL-6, IL-8, GM-CSF, RANTES, MCP-1, GRO α , NAP-2, and ENA-78 were all increased by IL-1 β . Pretreatment with PS-1145 or ML120B significantly inhibited the release of all of the cytokines (Fig. 4 and Supplemental Table S2).

Likewise, TNF α also induced the expression of IL-6, IL-8, GM-CSF, RANTES, MCP-1, GRO α , NAP-2, and ENA-78. Whereas the expression of IL-6, IL-8, RANTES, MCP-1, GRO α , and ENA-78 were significantly reduced by ML120B and PS-1145, the repression of NAP-2 was only significant at the highest concentration of ML120B (Fig. 4). In contrast, the repression of GM-CSF was only partial and failed to reach a level of significance (Fig. 4).

Parallel Western blot analysis of these samples again revealed a strong induction of ICAM-1 and COX-2 by IL-1 β and induction of ICAM-1 by TNF α (Fig. 5). In the case of ICAM-1, both PS-1145 and ML120B significantly reduced this effect, whereas the inhibition of COX-2 expression did not reach significance.

Comparison with Dexamethasone. To directly compare the effects of the IKK inhibitors with the effectiveness of a known corticosteroid, HASM cells were also treated with a

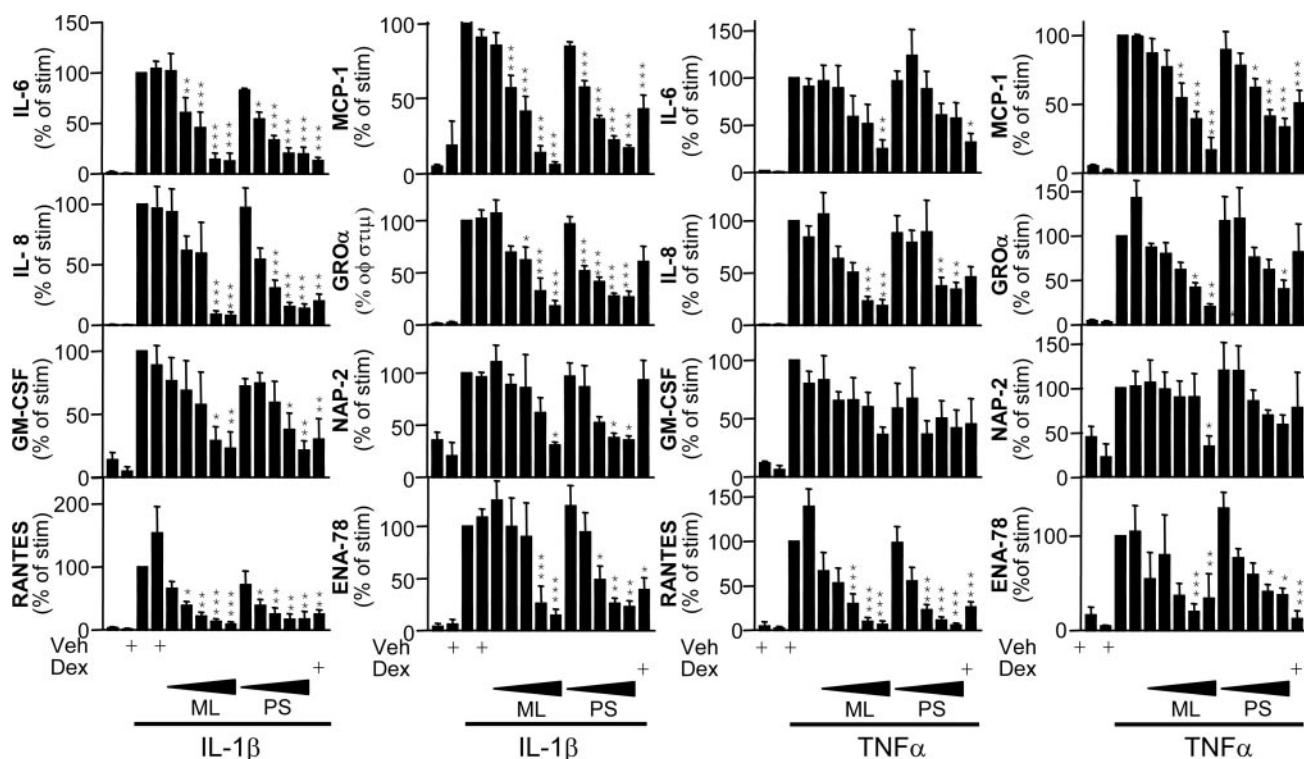


Fig. 4. Effect of PS-1145 and ML120B on cytokine and chemokine expression. HASM cells were treated as indicated with increasing concentrations (0.3, 1, 3, 10, and 30 μ M) of PS-1145 (PS), ML120B (ML), or dexamethasone (1 μ M) for 90 min before stimulation with IL-1 β (1 ng/ml) or TNF α (10 ng/ml) for 18 h. Cell culture medium and cell lysates were then harvested for analysis of cytokine release by Pierce SearchLight Multiplex ELISA Assay System. Data ($n = 5$ to 6) are expressed as a percentage of stimulated (stim) and are plotted as means \pm S.E.M. Veh indicates HASM cells incubated in the presence of DMSO at the highest concentration used.

maximally effective concentration of dexamethasone (Ammit et al., 2002). IL-1 β -induced IL-6, IL-8, RANTES, and ENA-78 and TNF α -induced IL-6, IL-8, and RANTES were strongly inhibited by dexamethasone, and in each case, this was to a similar level as 10 μ M concentrations of PS-1145 or ML120B (Fig. 4 and Supplemental Table S2). In contrast, IL-1 β -induced MCP-1 showed a significant \sim 58% reduction by dexamethasone that was considerably less than the 83 and 94% inhibition produced by PS-1145 and ML120B, respectively. Likewise, TNF α -induced MCP-1 was inhibited by only 49% by dexamethasone, whereas PS-1145 and ML120B produced effects of 67 and 84%, respectively (Fig. 4 and Supplemental Table S2). Likewise, dexamethasone was a poor inhibitor of ICAM-1 expression, whereas both PS-1145 and ML120B were considerably more effective (Fig. 5). In contrast, the effect of the IKK inhibitors on COX-2 expression was less clearcut, yet dexamethasone totally prevented IL-1 β -induced expression (Fig. 5). Because the expression of all of these genes was measured in parallel, the efficacy of 1 μ M dexamethasone is shown by the near total repression of COX-2, IL-6, IL-8, and RANTES. Thus, these data clearly demonstrate varying degrees of dexamethasone sensitivity and show that this does not correlate with the sensitivity to IKK inhibitors. Furthermore, with the exception of COX-2, it is clear from these data that these IKK inhibitors are either

more or as effective as dexamethasone at preventing inflammatory expression of the genes tested.

Discussion

The activation pathway leading to the transcription factor NF- κ B is currently considered a rational target for novel anti-inflammatory therapies (Barnes and Karin, 1997; Karin et al., 2004). In the current study, overexpression of dominant-negative IKK2 and dominant I κ B α Δ N prevented various parameters of NF- κ B activation, whereas dominant-negative IKK1 had little or no effect. This finding is consistent with data from embryonic fibroblasts cultured from IKK1- and IKK2-deficient mice and suggests that, in common with other systems, IKK2 is the major IKK responsible for IL-1 β - and TNF α -mediated induction of NF- κ B activity in HASM cells (Hayden and Ghosh, 2004). Previous studies in human pulmonary epithelial cells have shown that genes such as COX-2, IL-8, GM-CSF, and ICAM-1 are highly NF- κ B-dependent and that this required IKK2 activity (Catley et al., 2005). Therefore, the expression of a panel of inflammatory genes was also examined in the current HASM study. As occurred in the epithelial cells, the expression of COX-2, ICAM-1, and IL-8 was up-regulated by pro-inflammatory stimulation, and in each case, these were NF- κ B- and IKK2-dependent. In contrast, IL-1 β -induced GM-CSF expression was only partly prevented by dominant I κ B α and dominant-negative IKK2, whereas there was no significant effect on TNF α -induced GM-CSF. These data therefore suggest a lesser role for NF- κ B in the induction of GM-CSF in HASM cells. In addition, our data also demonstrate strong NF- κ B- and IKK2 dependence for RANTES, MCP-1, GRO α , NAP-2, and epithelial neutrophil activating peptide 78. In terms of the role of NF- κ B in the induction of these inflammatory genes, this is likely to be direct, via NF- κ B binding to gene promoters and causing transcriptional up-regulation. However, the above studies do not exclude the formal possibility of an indirect effect. Thus, NF- κ B could induce a factor or factors that are necessary for gene induction. In addition, IKK1(KM) tended to enhance the expression of genes such as IL-6, IL-8, COX-2, and possibly ICAM-1 (Fig. 2). This effect, although not significant, may relate to a role for IKK1 in limiting NF- κ B activation (Lawrence et al., 2005).

Having characterized and validated the above responses as both NF- κ B- and IKK2-dependent in primary HASM cells, this system was used to evaluate the effect of small-molecule IKK inhibitors. Consistent with the adenovirus data, PS-1145 and the more IKK2-selective ML120B (Nagashima et al., 2006; Wen et al., 2006) also impaired all parameters of NF- κ B activation. This indicates a potential utility of these compounds in preventing inflammatory gene expression in HASM cells. This expectation was borne out by the finding that the IKK inhibitors significantly reduced the IL-1 β -induced expression of all the inflammatory genes tested, with the exception of COX-2. Although the ability of PS-1145 and ML120B to reduce TNF α -induced GM-CSF and PS-1145 to reduce NAP-2 was not significant, this may be due in part to the reduced NF- κ B-dependence of these genes, particularly GM-CSF, as suggested by the adenoviral studies. Finally, it is worth noting that genes such as COX-2 and GM-CSF are primary response genes whose expression is tightly regulated by feedback control (Newton et al., 1997, 2001). It is

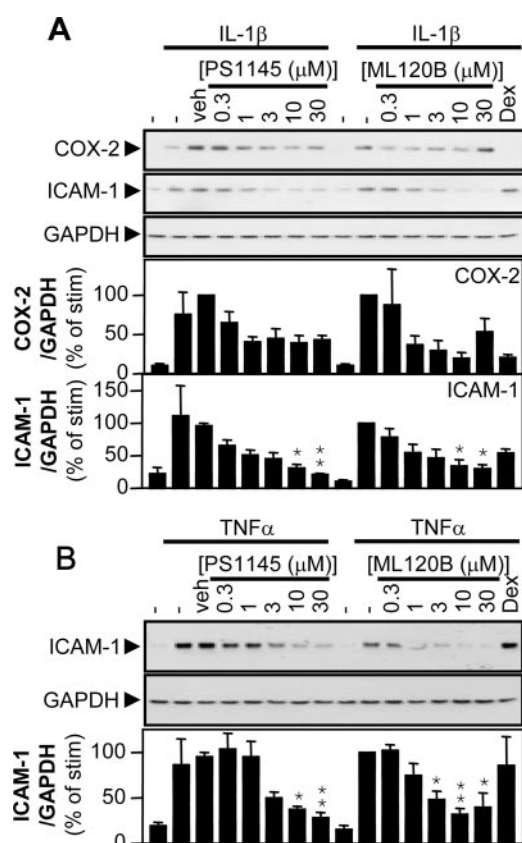


Fig. 5. Effect of PS-1145 and ML120B on COX-2 and ICAM-1. Cell lysates from experiments in Fig. 4 were harvested for Western blot analysis of COX-2 and ICAM-1 for both IL-1 β (A) and TNF α (B) stimulations. Data ($n = 6$) were analyzed by densitometry and after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were expressed as a percentage of stimulation (stim) and plotted as mean \pm S.E.M. Representative blots are shown. Veh indicates HASM cells incubated in the presence of DMSO at the highest concentration used.

noteworthy that such feedback processes seem to rely on new gene synthesis, as is evidenced by the phenomenon of mRNA superinduction by protein synthesis inhibitors (Newton et al., 1997, 2001). It is possible that NF- κ B-dependent genes are involved in these feedback-control mechanisms (Newton et al., 2001). Thus, given a greater residual NF- κ B activity with the small-molecule inhibitors, the competing effects of both positive and negative control processes may explain the variable effects, especially on COX-2 expression, between the viral and pharmacological inhibitors used in the current study.

In pulmonary epithelial cells, inhibition of IKK2 and prevention of NF- κ B activity reduced cell viability, and activation of poly(ADP-ribose) polymerase-1 and caspase-3 cleavage indicated an induction of apoptosis (Catley et al., 2005). Indeed, NF- κ B is widely established as being antiapoptotic in most cells, and the hope is that the inhibition of NF- κ B in the context of cancer may prove beneficial (Aggarwal, 2004). However, in our hands, HASM cells showed no significant reduction in viability in response to adenoviral-mediated inhibition of NF- κ B activity. This was even true after the more generally proapoptotic TNF α stimulation and suggests that IKK2-induced NF- κ B activity is not required to prevent HASM cell entry into apoptosis. Likewise, PS-1145 also showed no effect on host cell apoptosis, whereas ML120B produced a minor reduction in cell viability after IL-1 β stimulation. Although this effect was too small to account for the observed changes in gene expression, this was nevertheless unexpected. Given that dominant-negative IKK2, dominant I κ B Δ N, and PS-1145 produced no effect on apoptosis, it seems unlikely that the apoptotic effect of ML120B is due to inhibition of NF- κ B. Therefore, this compound may have off-target effects on pathways that regulate apoptosis.

To be effective, anti-inflammatory treatments need to prevent the activation of structural cells, such as smooth muscle and epithelial cells, in addition to preventing the migration, differentiation, survival, and activation of infiltrating inflammatory cells. The data reported here clearly document that NF- κ B and IKK2 inhibition results in reduced activation of HASM cells. Thus, impaired expression of ICAM-1 and presumably other NF- κ B-dependent adhesion molecules (Pahl, 1999) will tend to reduce the recruitment of inflammatory cells to the muscle. Furthermore, the panel of inflammatory chemokines examined in this study reinforces the important role of the HASM cells in all of the above inflammatory processes (Howarth et al., 2004). Thus, the expression of chemokines, including MCP-1, ENA-78, NAP-2, GRO α , RANTES, and IL-8, were all inhibited by preventing NF- κ B or IKK2 activity. Because many of these are known to be up-regulated in inflammatory diseases of the airways and are important in chemotactic and migratory responses, it is likely that their inhibition would also have a large affect on the airway inflammatory cell infiltrates that are seen in asthma and COPD (Traves et al., 2002, 2004; Barnes, 2004; Barnes and Hansel, 2004; Howarth et al., 2004). Finally, despite clear NF- κ B dependence in other cell types and inhibition by the I κ B Δ N and the dominant-negative IKK2 adenoviruses in HASM cells, the failure of the IKK inhibitors to completely prevent the expression of COX-2 suggests that basal or low-level NF- κ B activity may be sufficient for expression (Pahl, 1999; Catley et al., 2005). However, given the existence of aspirin-sensitive asthma, this effect may not be

undesirable (Jawien, 2002). Furthermore, because prostaglandin E₂ is the predominant prostanoid produced by these cells, and this both induces cAMP and represses the expression of various inflammatory genes, including GM-CSF and RANTES, it seems likely that the continued expression of COX-2 in HASM cells may be beneficial to the resolution of inflammation (Belvisi et al., 1997; Gilroy et al., 1999; Lazzeri et al., 2001; Clarke et al., 2004).

As previously mentioned, although corticosteroids are part of the first-line therapy in the clinical treatment of asthma, there are certain patients whose asthma is poorly controlled by such drugs, and their use in the treatment of COPD is largely without benefit (Adcock and Ito, 2004; Barnes, 2004; Barnes and Hansel, 2004; Wouters, 2004). In the current study, a maximally effective concentration of dexamethasone was therefore compared with the effect of IKK inhibition by PS-1145 and ML120B (Ammit et al., 2002). In the case of IL-6, IL-8, GM-CSF, RANTES, and ENA-78, the inhibition by ML120B was at least as effective as dexamethasone. However, certain genes such as IL-1 β - and TNF α -induced GRO α , NAP-2, and MCP-1 seemed to be less sensitive to inhibition by dexamethasone than by the IKK inhibitor. Furthermore, in this study, ICAM-1 expression, which was both highly NF- κ B-dependent and prevented by both PS-1145 and ML120B, was largely unaffected by dexamethasone. Such findings may be highly significant in the context of patients with COPD, in whom peripheral blood monocytes show increased chemotactic responses to GRO α and NAP-2, and MCP-1 levels are elevated in the bronchoalveolar lavage fluid of smokers (Traves et al., 2002, 2004). Thus, a relative steroid insensitive of these chemokines and ICAM-1 may explain some of the steroid insensitivity observed in COPD. It is important to note that these data highlight the possibility that IKK2 inhibitors may prove to be more effective anti-inflammatory compounds compared with corticosteroids for the treatment of COPD.

In conclusion, this study demonstrates that the inhibition of NF- κ B by targeting IKK2 in HASM cells has an inhibitory effect on the expression of a number of inflammatory genes. Based on the biological activities of these genes and the key role of HASM in the pathogenesis of both asthma and COPD, we suggest that IKK inhibitors may prove beneficial to the therapeutic management of these diseases. It is noteworthy that our data also indicate that IKK inhibition is at least as effective as inhibition by corticosteroids. Furthermore, in the case of certain genes that were relatively resistant to corticosteroids, we show a profound effect of IKK inhibitors. Therefore, we suggest that IKK inhibitors and other NF- κ B inhibitors may provide effective anti-inflammatory benefits in patients with difficult asthma or in COPD where corticosteroids have proven to be ineffective.

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