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Validation of the anti-inflammatory properties of small molecule IKK2 inhibitors by comparison to adenoviral-mediated delivery of dominant negative IKK1 and IKK2 in human airways smooth muscle

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d) Abbreviations: COPD, chronic obstructive pulmonary disease, 4', 6'-diamidino-2-phenylinole dihydrochloric hydrate, DAPI; DMEM, Dulbecco's modified eagle medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; HBSS, Hank's balanced salt solution; HASM, human airway smooth muscle; IKK, IκB kinase; MOI, multiplicity of infection; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PMSF, phenylmethylsulfonyl fluoride; .

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Abstract

Asthma and chronic obstructive pulmonary disease (COPD) are characterized by chronic airways inflammation. However, because COPD patients and certain asthmatics show little or no therapeutic benefit from existing corticosteroid therapies there is an urgent need for novel anti-inflammatory strategies. The transcription factor NF-κB is central to inflammation and is necessary for the expression of numerous inflammatory genes. Pro-inflammatory cytokines, including IL-1β and TNFα, activate the IκB kinase (IKK) complex to promote degradation of inhibitory IκB proteins and activate NF-κB. This pathway and, in particular the main IκB kinase, IKK2, are now considered prime targets for novel anti-inflammatory drugs. We have therefore used adenoviral over-expression to demonstrate NF-κB- and IKK2-dependence of key inflammatory genes, including ICAM-1, COX-2, IL-6, IL-8, GM-CSF, RANTES, MCP-1, GROα, NAP-2 and ENA78 in primary human airways smooth muscle cells. As 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, PS-1145 and ML120B, on IL-1β and TNFα-induced expression and this was compared with the corticosteroid dexamethasone. As observed above, ICAM-1, IL-6, IL-8, GM-CSF, RANTES, MCP-1, GROα, NAP-2 and ENA78 expression was reduced by the IKK inhibitors. Furthermore, this inhibition was either as effective, or for ICAM-1, MCP-1, GROα 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, where corticosteroids are ineffective.

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Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are inflammatory diseases of the lung, which are both associated with chronic inflammation of the airways, plus, in the case of COPD, a progressive non-reversible decline in lung function (Barnes, 2004; Barnes and Hansel, 2004). Whilst in the majority of asthma cases, inflammation and disease severity can be controlled by inhaled or oral corticosteroids, there remains a group of patients who's asthma remains poorly controlled due to disease severity or due to insensitivity to corticosteroid treatment (Barnes, 2004; Adcock and Ito, 2004). By 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 agent that target the inflammation associated with COPD and severe and steroid insensitive asthma are desperately needed (Barnes, 2004; Barnes and Hansel, 2004; Wouters, 2004; Adcock and Ito, 2004).

NF-κB is a ubiquitously expressed transcription factor that consists of hetero- or homo-dimers 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-κB signalling pathway is considered to be a potential target for novel anti-inflammatory compounds and indeed a number of pharmaceutical companies are developing compounds which 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 management of inflammatory airways disease (Castro *et al.*, 2003; Karin *et al.*, 2004). In unstimulated or resting cells, NF-κB is localised to the cytoplasm and is associated with a members of a family of

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inhibitory proteins known as IκB (inhibitor of κB) (Hayden and Ghosh, 2004). NF-κB activation is initiated in response to a wide range of stimuli, including the pro-inflammatory cytokines, IL-1β and TNF-α, chemokines, bacterial and viral products (Barnes and Karin, 1997; Li and Verma, 2002; Hayden and Ghosh, 2004). These lead to phosphorylation of IκB by the IκBα kinase (IKK) complex, which in turn leads to IκB polyubiquitination and subsequent degradation by the 26S proteosome (Li and Verma, 2002; Hayden and Ghosh, 2004). As a result, NF-κB proteins are liberated from IκB, usually IκBα, and translocate to the nucleus where they bind to the promoter regions of NF-κB responsive genes and initiate gene transcription (Li and Verma, 2002; Karin *et al.*, 2004; Hayden and Ghosh, 2004). A critical step in the NF-κB pathway is the phosphorylation of IκBα by the IKK complex (Li and Verma, 2002). This complex consists of at least two catalytic subunits, IKK1 (IKKα) and IKK2 (IKKβ) and a regulatory subunit, IKKγ/NEMO, which has no catalytic function, but who's structural role is absolutely required for IKK activation. Although IKK1 and IKK2 are structurally similar, studies in knock-out mice and derived mouse embryonic fibroblasts suggest that IKK2 is the predominant kinase involved in IκBα phosphorylation and hence NF-κB 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-κB pathway in this cell type, we have used primary human airways smooth muscle (HASM) cells and a combination of adenoviral delivery of dominant negative IKK1, IKK2 and dominant IκBα (IκBαΔN) as well as novel small molecule IKK inhibitors. Taken together the data presented in these studies strongly suggest that therapeutic strategies to inhibit NF-κB may be beneficial in airways inflammatory diseases.

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Materials and Methods

Reagents. IL-1β and TNFα were from R&D systems (Abingdon, UK). The IKK selective inhibitor, PS-1145 (N-(6-chloro-9*H*-β-carbolin-8-ly) nicotinamide), has previously been described and the structure published (Hideshima *et al.*, 2002; Castro *et al.*, 2003). ML120B (*N*-(6-chloro-7-methoxy-9*H*-β-carbolin-8-yl)-2-methyl-nicotinamide) is a novel ATP binding site IKK2-selective (IC_{50} at 50 μM ATP) inhibitor that does not inhibit either IKK1 (EC_{50} > 100 μM) or IKKε (EC_{50} > 100 μM) or a panel of 28 other kinases at EC_{50}s of up to 50 μM (Nagashima *et al.*, 2006; Wen *et al.*, 2006). Both PS-1145 and ML120B were supplied as free bases by Millennium Pharmaceuticals and were dissolved in dimethyl sulfoxide (DMSO) prior to dilution in tissue culture medium. All other reagents were from Sigma (Poole, 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 IκBαΔN, and dominant negative IKK1(KM), IKK2(KA), expressing viruses have all been previously been described (Catley *et al.*, 2003; Catley *et al.*, 2005; Krappmann *et al.*, 1996; Zandi *et al.*, 1997). All viruses were tittered by end point dilution and plaque assay to determine plaque forming units. Viruses were diluted in Dulbecco's modified eagle medium (DMEM) (Sigma, Poole, UK) to a multiplicity of infection (MOI) of 30 prior to 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 hours and serum starved for an additional 24 hours prior to 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

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MOIs of Ad5-GFP virus prior to staining with 4', 6'-diamidino-2-phenylinole dihydrochloric hydrate (DAPI) and analysis of GFP fluorescence as previously described (Catley *et al.*, 2003). For NF-κB reporter assays, HASM cells were infected with MOI 30 of an adenovirus carrying a NF-κB-dependent reporter (Ad-NF-κB-luc). This construct was obtained by inserting the NF-κB enhancer (5 copies of the classical NF-κB motif (underlined) 5' - TGG GGA CTT TCC GC -3'), TATA box and luciferase gene from pNF-κB-luc (Stratagene, La Jolla, CA) into the Ad5 parent vector. After incubation for 24 h in DMEM, the cells were then changed to fresh serum free medium and stimulated with IL-1β or TNFα for 8 hours prior to harvesting for luciferase assay.

IKK kinase assay. Kinase assays were performed as previously described with the following modifications (Catley *et al.*, 2004; Nasuhara *et al.*, 1999). Confluent 6 well plates were placed in serum free medium for 24 hours prior to stimulation with IL-1β or TNFα for 5 minutes. Cells were immediately put on ice and washed twice with ice cold Hanks balanced salt solution (HBSS). Triplicate wells were then scraped and pooled into one tube before centrifugation and subsequent lysis. Cell lysates were pre-cleared with agarose-conjugated normal rabbit IgG (Santa Cruz, Santa Cruz, CA) for 1 hour prior to immunoprecipitation of the IKK complex with an agarose-conjugated IKKγ specific antibody (Santa Cruz). Antibody target complexes were then collected by centrifugation and washed prior to resuspension in kinase buffer (20 mM HEPES pH 7.9, 2 mM MgCl, 2 mM MnCl, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM 4-nitrophenyl phosphate, 0.5 mM Na_3VO_4, 1 mM benzemidine, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin 25 μg/ml, leupeptin 10 μg/ml, pepstatin 2 μm/ml and dithiothreitol (DTT) 1 mM). Kinase reactions were performed in kinase buffer using a commercially available IκBα 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 4-12% NuPage SDS gel, which was cut at

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the 22 kDa marker. The bottom portion of each gel was dried for autoradiography, whereas the top portion was subject to western analysis for IKKγ.

Electromobility shift assays (EMSA) and luciferase assay. EMSA was performed as previously described (Nasuhara *et al.*, 1999; Catley *et al.*, 2004). Cells for luciferase assay were harvested in 1 × reporter lysis buffer (Promega) prior to 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 previously described (Nasuhara *et al.*, 1999; Holden *et al.*, 2004). Measurement of cytokine release 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 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases according to the manufacturers' specification. In brief, the medium of cells to be assayed was replaced with fresh medium containing 1 mg/ml MTT for 15 minutes at 37°C. Medium was then removed and the cells dissolved in DMSO prior to optical density measurements at 600 nm.

Statistical analysis. All values are expressed as means plus or minus the standard error of the mean (SEM). 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. Following DAPI staining, the presence of GFP was analysed by confocal fluorescence microscopy. Below a MOI of 10, only relatively low

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numbers of GFP-positive cells were observed (data not shown), whereas between MOIs of 10 and 100 a concentration-dependent increase in GFP-positive cells was observed (Supplemental data, Fig. 1). At a MOI of 30, ~95% of cells were positive for GFP and this level was used for subsequent experiments.

Effect of adenoviral over-expression of dominant IκBαΔN and both dominant negative IKK1 and IKK2 on NF-κB activation. To examine the role of IKK1 and IKK2 in NF-κB activation, HASM were infected with an adenovirus containing an NF-κB-dependent luciferase reporter alone or co-infected viruses expressing either dominant negative IKK1 (IKK1(KM)), dominant negative IKK2 (IKK2(KA)), dominant IκBα (IκBαΔN) or the null virus. Cells infected with the reporter virus alone showed strong induction of luciferase in response to IL-1β and TNFα (Fig. 1A). This induction of luciferase activity was almost completely reversed by co-infection with IKK2(KA) or IκBαΔN, whilst 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) over-expression on IKK complex kinase activity, the complex was immunoprecipitated and assayed for the ability to phosphorylate an IκBα-derived substrate peptide. IKK activity was strongly induced by both TNFα and IL-1β (Fig. 1B, upper panels). This response was substantially reduced by over-expression of IKK2(KA), whereas IKK1(KM) or the null virus had no obvious effect. Since IκBαΔN acts downstream of the IKK complex, there was no effect on IL-1β- and TNFα-induced IKK kinase activity. The specificity of the kinase reaction was confirmed by the fact that immunoprecipitation using pre-immune sera failed to pull-down IκBα kinase activity or IKKγ, whereas recombinant IKK efficiently phosphorylated the target. Loading of immunoprecipitated samples was confirmed by blotting for IKKγ (Fig. 1B, lower panels).

EMSA was performed to examine the effect of IκBαΔN and both IKK1(KM) and IKK2(KA) on the induction of NF-κB DNA binding. As expected both IL-1β and TNFα strongly induced NF-κB DNA binding and in each case the addition of excess cold probe competed out the DNA binding complexes

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indicating specificity to the NF-κB probe (Fig. 1C). Over-expression of IKK2(KA) and IκBαΔN completely prevented this induction, whereas, IKK1(KM) and the null virus revealed no effect. Cytoplasmic fractions from samples assayed by EMSA were analysed in parallel by western blotting to confirm the over-expression of HA tagged IKK1(KM), FLAG-tagged IKK2(KA) and truncated IκBαΔN (Fig. 1C, lower panels). Taken together these data clearly demonstrate that NF-κB-dependent transcription, IκB kinase activity and NF-κB nuclear translocation and DNA binding are dependent on the activity of IKK2, not IKK1 in HASM.

Effect of adenoviral infection on HASM cell viability. To confirm that the above effects on NF-κB 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)

Repression of inflammatory genes expression by IκBαΔN and IKK2(KA). To examine the role of IKK1, IKK2 and NF-κB, 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, GROα, NAP2 and ENA78 expression is strongly increased by both IL-1β and TNFα (Fig. 2 & Supplemental data, Table 1). This increase, with the exception of TNFα-induced GM-CSF, was profoundly inhibited by the IKK2(KA) and IκBαΔN adenoviruses. In contrast, the null virus and over expression of IKK1(KM) had little, or no, effect on the expression of these cytokines.

In preliminary studies, both IL-1β and TNFα potently induced ICAM-1 expression. By contrast, and consistent with previous reports (Pang and Knox, 1997), only IL-1β, and not TNFα, produced an increase in COX-2 protein (data not shown). Western blotting was therefore employed to monitor the expression of IL-1β-induced COX-2 and IL-1β- and TNFα-induced ICAM-1 expression in the presence of the above adenoviruses (Fig. 2B). Consistent with the cytokine data above, IKK2(KA) and IκBαΔN

near completely inhibited IL-1β- and TNF-induced ICAM-1 expression and IL-1β-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 NF-κB 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-κB 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 immunoprecipiated IKK complex from IL-1β- or TNFα-treated cells, confirmed the efficacy of PS-1145 and ML120B by preventing phosphorylation of the IκB substrate (Fig. 3A). In the case of PS-1145, and consistent with previous reports that quote IC_{50} values in the region of 0.1 - 0.15 μM (Castro *et al.*, 2003; Hideshima *et al.*, 2002), IKK kinase activity was reduced to or near basal levels at concentrations over 1 μM, whereas ML120B produced maximal effects at 10 to 30 μM. By comparison pre-treatment of cells with 1 μM of the anti-inflammatory glucocorticoid, dexamethasone, appeared to have no effect on immunoprecipitated IKK activity (Fig. 3A).

To test the effect of PS-1145 and ML120B on NF-κB-dependent transcription, HASM cells were infected with the adenoviral NF-κB-dependent luciferase reporter, Ad-NF-κB-luc, and treated with various concentrations of PS-1145 or ML120B prior to stimulation with IL-1β or TNFα. PS-1145 produced a concentration-dependent reduction in NF-κB-dependent transcription, which was maximal at 10 μM for both IL-1β and TNFα with resultant EC_{50} values of around 0.3 μM (Fig 3B). Likewise, ML120B concentration-dependently reduced IL-1β-and TNFα-induced NF-κB-dependent transcription (Fig. 3B). In this case, the effect was maximal at 30 μM and the EC_{50} was around 1 μM (Fig. 3B). For

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comparison, dexamethasone inhibited IL-1β- and TNFα-induced NF-κB-activation by only 47% and 45%.

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

Effect of PS-1145 and ML120B on HASM 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 following stimulation with TNFα (Fig. 3D). However, whilst PS-1145 had no effect on viability after IL-1β stimulation, ML120B resulted in a significant ~30% loss of cell viability following IL-1β stimulation. Whilst this effect could exaggerate the apparent effect of ML120B on IL-1β-induced NF-κB activation, it is not sufficient to explain the 90% reduction in NF-κB-dependent transcription seen on the reporter assay. Thus, the above data demonstrates that the ML120B and PS-1145 both inhibit NF-κB dependent transcription, IκB kinase activity and NF-κB 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-κB responsive in Fig 2. As before, the expression of IL-6, IL-8, GM-CSF, RANTES, MCP-1, GROα, NAP-2 and ENA-78 were all increased by IL-1β. Pre-treatment with PS-1145 or ML120B significantly inhibited the release of all the cytokines (Fig. 4 & Supplemental data, Table 2).

Similarly, TNFα also induced the expression of IL-6, IL-8, GM-CSF, RANTES, MCP-1, GROα, NAP-2, and ENA-78. Whereas the expression of IL-6, IL-8, RANTES, MCP-1, GROα and ENA-78 were significantly reduced by ML120B and PS-1145, the repression of NAP-2 was only significant at

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the highest concentration of ML120B (Fig. 4). By 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-1β and induction of ICAM-1 by TNFα (Fig. 5). In the case of ICAM-1, this effect was significantly reduced by both PS-1145 and ML120B, 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 maximally effective concentration of dexamethasone (Ammit *et al.*, 2002). IL-1β-induced IL-6, IL-8, RANTES and ENA-78 and TNFα-induced IL-6, IL-8 and RANTES were strongly inhibited by dexamethasone and in each case this was to a similar level as 10 μM of PS-1145 or ML120B (Fig. 4 & Supplemental data, Table 2). By contrast, IL-1β-induced MCP-1 showed a significant ~58% reduction by dexamethasone that was considerable less that the 83 and 94% inhibition produced by PS-1145 and ML120B respectively. Similarly TNFα-induced MCP-1 was inhibited by only 49% by dexamethasone whereas PS-1145 and ML120B produced effects of 67 and 84% respectively (Fig. 4 & Supplemental data, Table 2). Likewise, dexamethasone was a poor inhibitor of ICAM-1 expression, whereas both PS-1145 and ML120B were considerably more effective (Fig. 5). By contrast, the effect of the IKK inhibitors on COX-2 expression was less clear cut, yet dexamethasone totally prevented IL-1β-induced expression (Fig. 5). Since the expression of all these genes was measured in parallel, the efficacy of 1 μM 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 this data that these IKK inhibitors are either more, or, as effective as dexamethasone at preventing inflammatory expression of the genes tested.

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Discussion

The activation pathway leading to the transcription factor NF-κB is currently considered a rational target for novel anti-inflammatory therapies (Barnes and Karin, 1997; Karin *et al.*, 2004). In the current study, over-expression of dominant negative IKK2 and dominant IκBαΔN prevented various parameters of NF-κB 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-1β- and TNFα-mediated induction of NF-κB activity in HASM cells (see (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-κB-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-κB and IKK2-dependent. In contrast, IL-1β-induced GM-CSF expression was only partly prevented by dominant IκBα and dominant negative IKK2, whilst there was no significant effect on TNFα-induced GM-CSF. These data therefore suggest a lesser role for NF-κB in the induction of GM-CSF in HASM. In addition, our data also demonstrate strong NF-κB and IKK2 dependence for RANTES, MCP-1, GROα, NAP-2 and ENA78. In terms of the role of NF-κB in the induction of these inflammatory genes, this is likely to be direct, via NF-κB 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-κB 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 (see Fig. 2). This effect, whilst not significant, may relate to a role for IKK1 in limiting NF-κB activation (Lawrence *et al.*, 2005).

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Having characterized and validated the above responses as both NF-κB and IKK2-dependent in primary HASM, 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-κB activation. This indicates a potential utility of these compounds in preventing inflammatory gene expression in HASM. This expectation was bourn out by the finding that the IKK inhibitors significantly reduced the IL-1β-induced expression of all the inflammatory genes tested, with the exception of COX-2. Whilst the ability of PS-1145 and ML120B to reduce TNFα-induced GM-CSF and PS-1145 to reduce NAP-2 was not significant, this may in part due to the reduced NF-κB-dependence of these genes, in particular GM-CSF, as suggested by the adenoviral studies. Finally, we would like to point out that genes such as COX-2 and GM-CSF are primary response genes whose expression is tightly regulated by feedback control (Newton *et al.*, 2001; Newton *et al.*, 1997). Importantly, such feedback processes appear to reply on new gene synthesis as is evidenced by the phenomena of mRNA superinduction by protein synthesis inhibitors (Newton *et al.*, 2001; Newton *et al.*, 1997). It is possible that NF-κB-dependent genes are involved in these feedback control mechanisms (Newton *et al.*, 2001). Thus given a greater residual NF-κB 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-κB 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-κB is widely established as being anti-apoptotic in most cells and the hope is that the inhibition of NF-κB 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-κB activity. This was even true following, the more

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generally pro-apoptotic, TNFα stimulation and suggests that IKK2-induced NF-κB activity is not required to prevent HASM entry into apoptosis. Similarly, PS-1145 also showed no effect on host cell apoptosis, whereas ML120B produced a minor reduction in cell viability after IL-1β stimulation. Whilst this effect was too small to account for the observed changes in gene expression, this was never-the-less unexpected. Given that dominant negative IKK2 and dominant IκBαΔN as well as PS-1145 produced no effect on apoptosis, it seems unlikely that the apoptotic effect of ML120B is due to inhibition of NF-κB. 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 documents that NF-κB and IKK2 inhibition results in reduced activation of HASM cells. Thus impaired expression of ICAM-1, and presumably other NF-κB-dependent adhesion molecules (Pahl, 1999), will tend to reduce recruitment of inflammatory cells to the muscle. Furthermore the panel of inflammatory chemokines examined in this study re-enforces the important role of the HASM in all the above inflammatory processes (Howarth *et al.*, 2004). Thus the expression of chemokines including, MCP-1, ENA-78, NAP-2, GROα, RANTES and IL-8 were all inhibited by preventing NF-κB or IKK2 activity. Since 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 their inhibition would also have a large impact on the airway inflammatory cell infiltrates that are seen in asthma and COPD (Traves *et al.*, 2002; Traves *et al.*, 2004; Barnes, 2004; Barnes and Hansel, 2004; Howarth *et al.*, 2004). Finally, despite clear NF-κB-dependence in other cell types and inhibition by the IκBαΔN and the dominant negative IKK2 adenoviruses in HASM, the failure of the IKK inhibitors to completely prevent the expression of COX-2 suggests that basal or low level NF-κB activity may be sufficient for expression (Pahl, 1999; Catley

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et al., 2005). However, given the existence of aspirin-sensitive asthma, this effect may not be undesirable (Jawien, 2002). Furthermore as prostaglandin E_2 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 may be beneficial to the resolution of inflammation (Belvisi *et al.*, 1997; Lazzeri *et al.*, 2001; Clarke *et al.*, 2004; Gilroy *et al.*, 1999).

As previously mentioned, whilst corticosteroids are part of the first line therapy in the clinical management of asthma, there are certain patients who's asthma is poorly controlled by such drugs and use in COPD is largely without benefit (Barnes, 2004; Adcock and Ito, 2004; Barnes and Hansel, 2004; Wouters, 2004). In the current study, a maximally effect concentration of dexamethasone was therefore compared to 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-1β- and TNFα-induced GROα, NAP-2 and MCP-1 appeared 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-κB-dependent and prevented by the both PS-1145 and ML120B, was largely unaffected by dexamethasone. Such findings may be highly significant in the context of COPD patients where peripheral blood monocytes show increased chemotactic responses to GROα and NAP-2, and MCP-1 levels are elevated in the BAL fluid of smokers, (Traves *et al.*, 2002; Traves *et al.*, 2004). Thus relative steroid insensitive of these chemokines and ICAM-1 may explain some of the steroid insensitivity observed in COPD. Importantly these data highlight the possibility that IKK2 inhibitors may prove to be more effective anti-inflammatory compounds when compared to corticosteroids for the treatment of COPD.

In conclusion, this study demonstrates that the inhibition of NF-κB by targeting IKK2 in HASM has an inhibitory effect on the expression of a number of inflammatory genes. Based on the biological

activities of these genes and 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. Importantly, our data also indicates 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-κB-inhibitors, may provide effective anti-inflammatory benefits in patients with difficult asthma or in COPD where corticosteroids have proved to be ineffective.

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Footnotes

a)

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b)

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Legends for figures

Fig. 1. IKK2 is essential for IκB kinase activity, NF-κB activation and DNA binding. A, HASM cells were infected with an adenovirus (MOI 30) containing the NF-κB-dependent luciferase reporter (Ad-NF-κB-luc). Simultaneously, the indicated wells were co-infected (MOI 30) with either the null adenovirus or adenoviruses expressing IKK1(KM), IKK2(KA) or IκBαΔN for 24 hours prior to stimulation with IL-1β (1 ng/ml) or TNFα (10 ng/ml) for a further 8 hours and then harvesting for luciferase assay. Data (n = 4), expressed as a percentage of the stimulation by IL-1β, are plotted as means ± SEM. B, HASM cells were infected with the indicated adenovirus (MOI 30) for 48 hours prior to stimulation with IL-1β (1 ng/ml) or TNFα (10 ng/ml) for 5 minutes. The IKK complex was then immunoprecipitated and analysed for IκB kinase activity (KA) (upper panels). The portion of the gel above the substrate peptide was also analysed by western blotting for IKKγ (lower panels). Blots and autoradiographs representative of 3 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-κB DNA binding (upper panels) with parallel western analysis of cytoplasmic fractions revealing expression of HA tagged IKK1(KM) (HA), FLAG tagged IKK2(KA) (FLAG) and IκBα (lower panels). 100X indicates reactions performed in the presence of 100 fold excess of unlabelled probe. D, Cells were infected with the indicated viruses for 24 hours prior to being serum starved for a further 24 hours. The cells were then simulated as indicated for 18 hours prior to MTT assay. Data (n = 4), expressed as a percentage of the stimulated samples (IL-1β or TNFα), are plotted as means ± SEM.

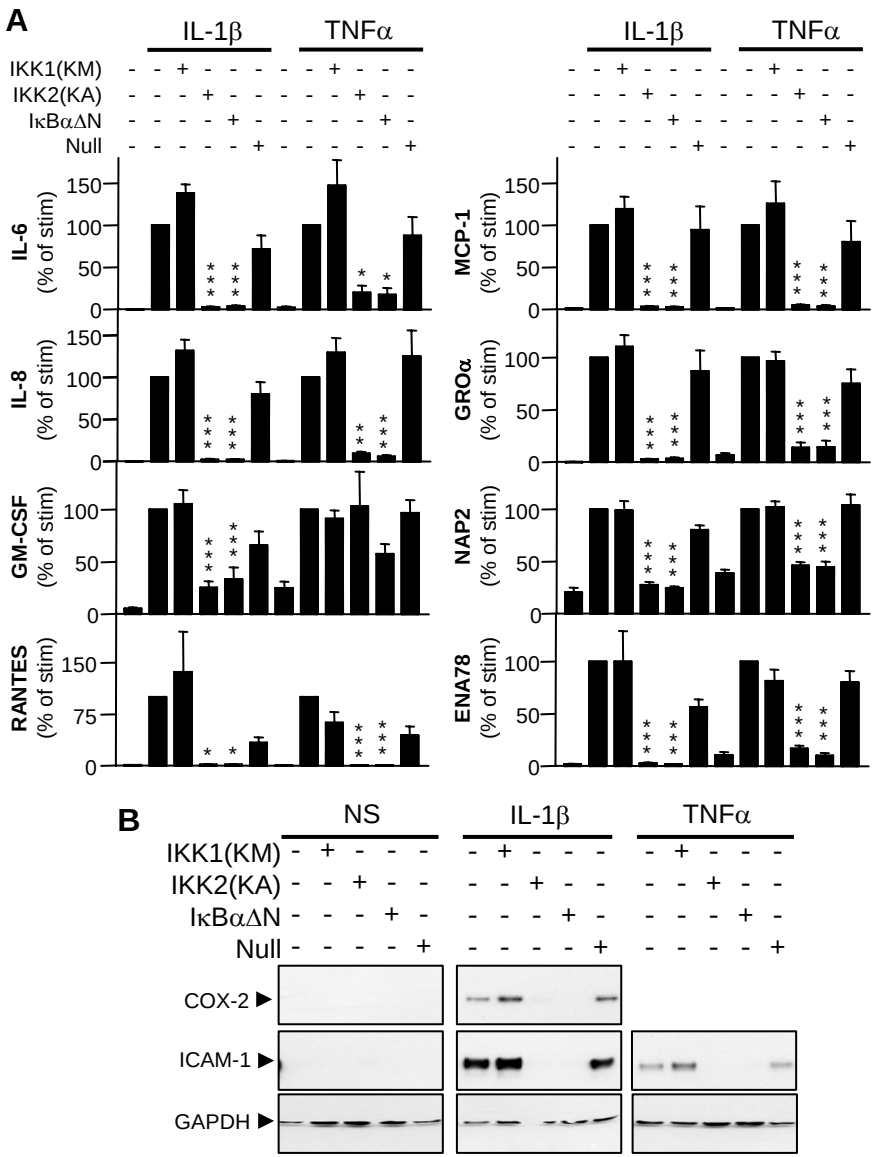
Fig. 2. The effect of IKK and NF-κB inhibition on the production of inflammatory mediators. HASM cells were infected with the indicated viruses (MOI 30) for 48 hours prior to stimulation with IL-1β (1 ng/ml) or TNFα (10 ng/ml) for a further 18 hours. Culture medium and cell lysates were then harvested

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 percentage of the stimulation (stim) and are splotted as means ± SEM. B, Representative blots from the 6 experiments is shown.

Fig. 3. Effect of PS-1145 and ML120B on NF-κB activation. A, IKK complex was immunoprecipitated from HASM cells that had been stimulated with IL-1β (1 ng/ml) or TNFα (10 ng/ml) for 5 minutes. IκB kinase activity was assayed in the presence of the indicated concentration of PS-1145 or ML120B. Following kinase assay (KA), reaction products were size fractionated and the lower parts of gels exposed for autoradiograpgy, whilst the upper portions were subject to western blot analysis for IKKγ. Autoradiographs and blots are representative of 2 such experiments. IKK indicates recombinant active IKK2, PI indicates reactions performed on immunoprecipates using pre-immune sera and Dex indicates HASM that cells were pre-treated for 90 minutes with 1 μM dexamethasone prior to stimulation and harvesting. Veh indicates reactions performed in the presence of DMSO at the top concentration used. B, HASM cells were infected with Ad-NF-κB-luc (MOI 30) as before. Cells were pre-treated with increasing concentrations (0.3, 1, 3, 10, 30 μM) of PS-1145 (PS), ML120B (ML) or dexamethasone for 90 minutes prior to stimulation with IL-1β (1 ng/ml) or TNFα (10 ng/ml) for 8 hours. Cells were then harvested for luciferase assay. Data (n = 4) is expressed as a percentage of the stimulation and is plotted means ± SEM. C, HASM cells were treated with the indicated concentration of PS-1145 and ML120B for 90 minutes prior to stimulation IL-1β (1 ng/ml) and TNFα (10 ng/ml) for 5 minutes. Cells were then harvested for nuclear proteins, which were analysed for NF-κB DNA binding by EMSA. Autoradiographs representative of two such experiments are shown. D, Cells were serum starved for 24 hours prior to pre-treatment with drugs as indicated. The cells were then simulated as indicated for 18 hours prior to MTT assay. Data (n = 4), expressed as a percentage of the stimulated samples (IL-1β or TNFα) are plotted as means ± SEM.

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, 30 μM) of PS-1145 (PS), ML120B (ML) or dexamethasone (1 μM) for 90 minutes prior stimulation with IL-1β (1 ng/ml) or TNFα (10 ng/ml) for 18 hours. 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) is expressed as a percentage of stimulated (stim) and are plotted as means ± SEM. Veh indicates HASM incubated in the presence of DMSO at the top concentration used.

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-1β (A) and TNFα (B) stimulations. Data (n = 6) was analysed by densitometry and after normalisation to GAPDH expressed as a percentage of stimulation (stim) and plotted as mean ± SEM. Representative blots are shown. Veh indicates HASM incubated in the presence of DMSO at the top concentration used.



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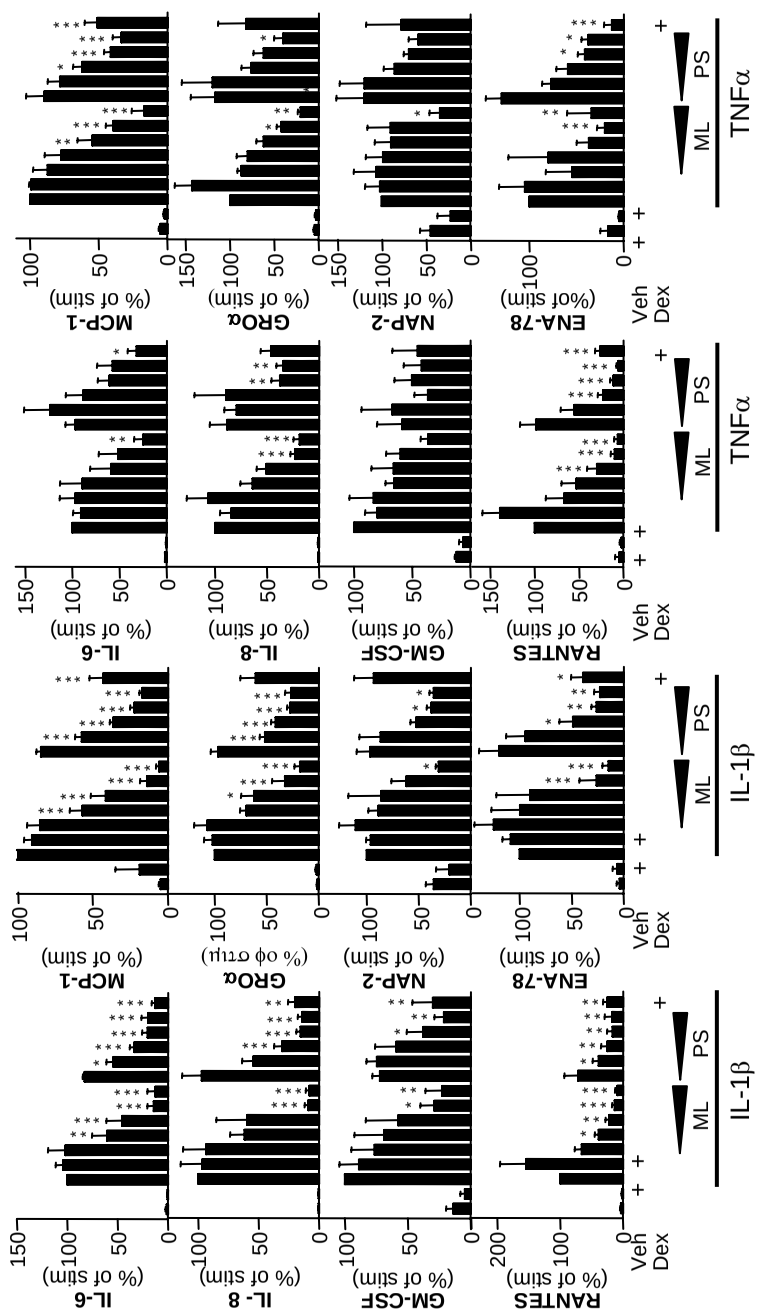

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