

EFFECT OF INHIBITION OF THE UBIQUITIN-PROTEASOME-SYSTEM AND I κ B KINASE ON AIRWAY INFLAMMATION AND HYPERRESPONSIVENESS IN A MURINE MODEL OF ASTHMA

A.Z. EL-HASHIM¹, W.M. RENNO², H.T. ABDUO¹, S.M. JAFFAL¹,
S. AKHTAR³ and I.F. BENTER³

¹*Department of Applied Therapeutics, Faculty of Pharmacy, Kuwait University;* ²*Department of Anatomy,* ³*Department of Pharmacology, Faculty of Medicine, Kuwait University, Kuwait*

Received June 18, 2010 – Accepted January 26, 2011

The current treatment of asthma is far from optimal and there is a need for novel therapeutic approaches. NF κ B has recently been highlighted as an important pro-inflammatory transcriptional factor and its blockade is believed to represent a new therapeutic approach for asthma. The purpose of this study is to investigate the effects of blocking the actions of NF κ B, through inhibition of the ubiquitin-proteasome system (UPS) or I κ B kinase (IKK), in a murine model of asthma. Treatment with the UPS inhibitor, MG-132 (0.03 and 0.1 mg/kg), did not significantly affect the ovalbumin-induced increase in total and differential cell numbers, histological changes such as perivascular and peribronchial inflammatory cell infiltration, perivascular and peribronchial fibrosis or the increased Penh to methacholine. In contrast, treatment of mice with the IKK inhibitor, BAY 11-7085, (3 and 10 mg/kg) dose-dependently inhibited the ovalbumin-induced increase in airway leukocyte influx and decreased the percentage of airway lymphocytes, neutrophils and eosinophils. Also, BAY 11-7085-treated (10 mg/kg) mice showed a significant decrease in the histologically assessed inflammatory indices as well as a significant reduction in the ovalbumin-induced increase in Penh to inhaled methacholine. Furthermore, BAY 11-7085 significantly inhibited the ovalbumin-induced increase in the level of phosphorylation of I κ B α and extracellular regulated kinases (ERK) 1/2, whilst MG-132 significantly increased the phosphorylation of (ERK) 1/2. These findings confirm the critical role that NF κ B plays in airway inflammation, highlight the importance of IKK in regulating the pro-inflammatory activity of NF κ B and also suggest that UPS may not be a useful drug target for asthma treatment.

NF κ B is a nuclear transcription factor that plays an important role in the induction of several pro-inflammatory genes leading to the increased expression of several cytokines, adhesion molecules, growth factors, mediators and enzymes (1). NF κ B induction in airway epithelium increases lung inflammation in allergen-challenged mice (2) and its

activity in bronchial brush samples is correlated with acute airway obstruction in heaves-affected horses (3). Some studies have also shown enhanced activity of NF κ B in human asthmatic tissue (4) and increased NF κ B p65 expression and I κ B phosphorylation in peripheral blood mononuclear cells from children with moderate asthma (5). Therefore, NF κ B is

Key words: asthma, inflammation, ubiquitin-proteasome system, I κ B kinase and NF κ B

Mailing address: Dr Ahmed El-Hashim,
Dept. of Applied Therapeutics,
Faculty of Pharmacy,
Kuwait University,
P.O. BOX 24923, Safat 13110, Kuwait
Tel: ++965 2498 6056 Fax: ++965 2498 6841
e-mail: ahmed.elhashim@hsc.edu.kw

0394-6320 (2011)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder. Unauthorized reproduction may result in financial and other penalties

considered as an attractive target for treatment of inflammatory diseases such as asthma.

Under basal condition NF κ B is kept sequestered in the cytoplasm bound to I κ B proteins (6). Upon activation by a variety of stimuli, the I κ B proteins are phosphorylated by the serine/ threonine kinase - inhibitor of kappa B kinase (IKK), the phosphorylated I κ Bs are then ubiquitinated and degraded by a 26 S proteasome, unmasking the NF κ B nuclear localization and allowing it to mediate its effects (6). There have therefore been considerable efforts aimed at developing molecules that inhibit this kinase as potential anti-inflammatory drugs (7).

The ubiquitin-proteasome system (UPS) has been receiving considerable attention recently and indeed NF κ B activity is also known to be regulated by this system. The proteasome inhibitor PS-519 has been shown to reduce airway eosinophilia in a rat model of pulmonary eosinophilia (8), therefore, targeting proteasomes may also be a novel approach to the treatment of asthma. The objective of this study is to investigate and compare the effects of blocking the actions of NF κ B, through inhibition of the UPS or IKK in a murine model of ovalbumin-induced airway inflammation and increased Penh to methacholine.

MATERIALS AND METHODS

Reagents

Chemicals used included ovalbumin (grade V), dexamethasone 21-phosphate disodium salt, halothane, absolute ethanol (Merck KGaA, Germany), formaldehyde (Surechem products LTD, UK), Alu-Gel-S, (SERVA electrophoresis GmbH, Germany), isotone II diluent solution (Beckman Coulter Inc., USA), Zap-OGLOBIN (Coulter electronics LTD, UK), Diff-Quik (Baxter Dade AG, Dudingon, Switzerland), phosphate buffered saline, (Fluka BioChemika, Belgium), Tris-base, NaCl, Na₂P₂O₇, NaF, CaCl₂, MgCl₂, Glycerol, NP₄O, Na₃VO₄, PMSF, protease inhibitor cocktail (Sigma,USA), phosphatase inhibitor Cocktail (Santacruz,USA), acetyl-B-methylcholine bromide, MG-132 and BAY 11-7085 (Tocris, UK and USA), DMSO (BDH, UK), xylene, ethanol (Riedel-de-Haen, USA), hematoxylin, eosin, glacial acetic acid (BDH Laboratory Supplies (Gurr Certistain, UK), Dpx mountant, picric acid, mercuric chloride, phosphomolybdic acid, light green SF yellowish, Schiff's reagent (Fluka Chemical, USA), ferric chloride, HCl

(BDH Laboratory Reagents, UK), acid fuchsin (Gurr microscopy materials, UK) ponceau 2R, (Sigma, USA) periodic acid (BDH Limited, UK), rabbit monoclonal antibodies for total and phosphorylated I κ B α and rabbit polyclonal antibody for phosphorylated ERK1/2 (Cell signaling Technology, Inc, USA), rabbit monoclonal anti-actin antibody (Sigma-Aldrich, St Louis, USA), nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Stock solutions of MG-132 and BAY 11-7085 were made by dissolution in 100% DMSO; subsequent dilutions were made in vehicle containing 10% DMSO, 50% propylene glycol and 40% PBS.

Animals

The male BALB/c mice (6-8 weeks old) used in this study were maintained on an ovalbumin free diet. All experimental protocols were approved by the Animal Welfare Committee and complied with regulations for the use of Laboratory Animals in the Health Sciences Centre, Kuwait University.

Immunization, challenge and drug treatment

BALB/c mice were immunized once by intraperitoneal (i.p.) injection of 10 μ g ovalbumin in 0.2 ml of alu-Gel-S on day 0 (9). Ten days later, mice were challenged intranasally, once a day, over 4 consecutive days, with 20 μ g ovalbumin in 50 μ l PBS. Control animals were similarly immunized with ovalbumin and challenged intranasally with 50 μ l PBS. Nine treatment groups (of 7-10 mice each) were established. Groups 1, 2 and 3 were challenged with PBS and pre-treated with either vehicle, MG-132 (0.1 mg/kg;i.p.) or BAY 11-7085 (10 mg/kg;i.p.), respectively. MG132 is a potent peptide aldehyde proteasome inhibitor, which effectively blocks the proteolytic activity of the 26S proteasome complex (10). BAY 11-7085 is an irreversible inhibitor of I κ B α phosphorylation (11). All groups 4-9 were challenged with ovalbumin. Group 4 was pre-treated with the vehicle. Groups 5 and 6 were pre-treated with MG-132 (0.03 or 0.1 mg/kg;i.p., respectively). Groups 7 and 8 were pretreated with BAY 11-7085 (3 and 10 mg/kg;i.p, respectively), and group 9 was treated with dexamethasone (3 mg/kg; i.p.). For both MG-132 and BAY 11-7085 compounds, higher doses of 0.3 mg/kg and 30 mg/kg, respectively, were studied but these doses were found to have toxic effects (causing death in some animals) and hence these groups were discontinued. The doses and route of administration for both MG-132 and BAY 11-7085 were based on studies that used the same i.p. route and similar doses that showed significant effects for both drugs (12-13). All animals were treated with drugs or vehicle 60 mins before the ovalbumin or PBS intranasal challenge for the 4 days. This dosing

regimen has been classically used in this model with both experimental drugs and the gold standard steroid, dexamethasone and has been shown to be effective (9, 14).

Measurement of (enhanced pause) Penh

An indirect measure of airway function and a correlate of airway responsiveness, enhanced pause (Penh) was measured in response to inhaled methacholine (0.03 – 0.3 M) administration. Penh was assessed 24 h after the last ovalbumin challenge by barometric plethysmography (Buxco Electronics Inc., Troy, New York, USA) using whole body plethysmography (WBP) (9, 14). Penh is a dimensionless value that reflects changes in the waveform of the box pressure signal (breathing pattern) from both inspiration and expiration and combines it with the timing comparison of early and late expiration (Pause). The pressure differences between the main chamber of the body plethysmograph containing the animal and a reference chamber (box pressure signal) were measured. The resulting box pressure signal is caused by volume and resultant pressure changes in the main chamber during the respiratory cycle of the animal. From these box pressure signals, the phases of the respiratory cycle, peak inspiratory pressure (PIP), peak expiratory pressure (PEP), tidal volumes and Penh can be calculated. The response to inhaled methacholine is expressed as the % change in Penh from baseline.

$$\text{Penh} = \text{Pause} \times \frac{\text{PEP}}{\text{PIP}}$$

In our study, changes in temperature or humidity in response to PBS, over the same time period as the methacholine challenge, did not result in any changes in the Penh that are comparable to the changes seen in response to spasmogens such as methacholine.

Bronchoalveolar lavage (BAL) cell counts and differentiation

After completion of the assessment of airway responsiveness, mice were sacrificed with an overdose of halothane following a 30 min recovery time. BAL fluid (BALF) was collected by cannulating the trachea and washing the lungs with saline solution (4 × 0.3 ml each). BALF cells were counted using a particle size counter (Z1 series, Beckman Coulter, Florida, USA) and cytopspins (Shandon Scientific Ltd, Cheshire, U.K.) prepared. Cells were stained with Diff-Quik and a differential count of 200 cells was performed using standard morphological criteria. Results are expressed as both total cell count/ml, total differential count/ml, and as a percentage of macrophages, lymphocytes, neutrophils, and eosinophils in BALF.

Lung tissue preparation for histopathology

Lungs from all mice groups were resected, fixed in 10% formalin and embedded in paraffin wax. Sections (5 µm) were stained with H&E and examined for pathological changes under light microscopy. Sections were also stained with Masson's Trichrome stains to evaluate fibrillar collagen and connective tissue matrix proteins. Three-micrometer serial sections were mounted on each slide. Mucus and mucus containing goblet cells in the bronchial epithelium were stained with a Periodic Acid-Schiff (PAS) staining. All the slides were independently scored by three different observers to assess intra-observer variation. The average of these three scores was calculated for all the mice used in this study. Ten sections from each mouse were assessed in this study using a Zeiss 40 microscope.

Western blot analyses of IκB-α and ERK1/2

SDS-PAGE gel electrophoresis and Western Blotting for phosphorylation of total and phosphorylated forms of IκB-α and ERK1/2 was performed. Briefly, after completion of the BALF procedure, the animals were sacrificed by an overdose of halothane. The lungs were removed, washed with phosphate buffered saline (pH 7.4) at 4°C and snap-frozen in liquid nitrogen and stored at -80°C. The tissue samples were then pulverized whilst frozen and then transferred to lysis buffer (pH 7.6) containing 10 mM Tris-base, 140 mM NaCl, 10 mM Na₄P₂O₇, 1 mM NaF, 1 mM CaCl₂, 1 mM MgCl₂, 10% Glycerol, 1% NP40, 2 mM Na₃VO₄, 1 mM PMSF, protease inhibitor cocktail, phosphatase inhibitor cocktail. The tissues were then homogenised using Polytron PT 4000 (Switzerland) and the samples were left to lyse completely by incubation on an ice-cold shaker for 30 minutes, centrifuged at 14,000 rpm for 20 min at 4°C to collect supernatants. Protein concentrations were then measured by Lowry's Protein Assay. Aliquots containing equal amounts of protein were subjected to SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were then incubated with the indicated antibodies and subsequently with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were detected with SuperSignal chemiluminescent substrate (Pierce, UK) using Kodak autoradiography film (G.R.I., Rayne, U.K.). To ensure equal loading of proteins, β-actin levels were detected using primary rabbit anti-human β-actin antibody followed by the secondary anti-rabbit IgG horse-radish peroxidase conjugated antibody. Images were analysed and quantified by densitometry (Biorad, USA).

Statistics

All numerical values are expressed as means+S.E.M.

Total cell counts represent the number of BALF cells/ml. Differential cell counts represent both the absolute number of each cell type and % of each cell type counted in 200 cells. Percentage change in Penh was used as an index of the airway responsiveness to methacholine. A Kruskal Wallis analysis of variance was used to compare mean differences between individual groups in Penh, total and differential cell data. The mean difference was considered as significant at a probability level of less than 0.05. Analysis was performed with SPSS for Windows (V.17; SPSS Inc. Evanston, Illinois, USA). For the histopathology, a semi-quantitative 5-level lung pathology score was used to grade the extent of abnormalities in each microscopic field at 200X.

The grading scale is shown below.

Grade Degree Description:

- 0 Normal histology.
- +
- ++ Moderate changes.
- +++ Marked changes.
- ++++ Severe changes.

RESULTS

Effect of MG-132 and BAY 11-7085 on total and differential BALF cell count

Ovalbumin challenge induced a significant increase in BALF total leukocytes, compared with PBS, ($7.9 \pm 1.5 \times 10^5$ vs $2.4 \pm 0.2 \times 10^5$ /ml, Fig. 1A; $P < 0.05$), and also increased lymphocytes (absolute, $0.20 \pm 0.05 \times 10^5$ /ml vs $0.01 \pm 0.004 \times 10^5$ /ml and also %, $3.3 \pm 1.1\%$ vs $0.3 \pm 0.2\%$; $P < 0.05$), neutrophils (absolute, $0.56 \pm 0.15 \times 10^5$ /ml vs $0.03 \pm 0.01 \times 10^5$ /ml and also %, $7.0 \pm 0.7\%$ vs $1.2 \pm 0.3\%$; $P < 0.05$) and eosinophils (absolute, $3.43 \pm 1.04 \times 10^5$ /ml vs $0.06 \pm 0.02 \times 10^5$ /ml and also %, 40.4 ± 6.2 vs $2.4 \pm 0.8\%$; $P < 0.05$), while causing a significant decrease in the % of macrophages ($49.3 \pm 5.9\%$ vs $96.0 \pm 0.8\%$, Fig. 1B; $P < 0.05$). Pretreatment with MG-132, at both 0.03 or 0.1 mg/kg, did not inhibit the ovalbumin-induced increase in total or differential

Table I

	PVICI	PBICI	GCHP & MOP	PVF	PBF
PBS challenged	0	0	0	0	0
OVA challenged	(++++)	(++++)	(++++)	(++++)	(++++)
OVA challenged + MG-132 (0.1 mg/kg)	(++++)	(++++)	(+++)	(+++)	(+++)
OVA challenged + BAY 11-7085 (10 mg/kg)	(+)	(+)	(+)	(+)	(+)
OVA challenged + DEX (3 mg/kg)	(++)	(++)	(++)	(++)	(++)
Grading scale:	(0) Normal histology	(+) Minimal changes	(++) Moderate changes	(+++ Marked changes	(++++ Severe changes

PVICI = Perivascular Inflammatory Cell Infiltrate

PBICI = Peribronchial Inflammatory Cell Infiltrate

GCHP & MOP = Goblet Cell Hyper/metaplasia & Mucus Overproduction

PVF = Perivascular Fibrosis

PBF = Peribronchial Fibrosis

Fig 1A

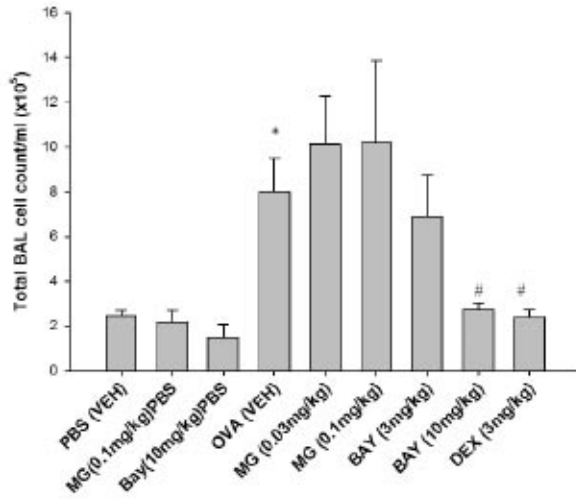


Fig 1B

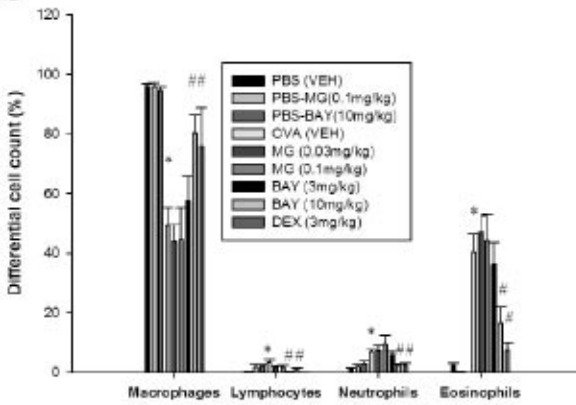


Fig. 1. Effect of MG-132 (0.03 and 0.1 mg/kg), BAY 11-7085 (3 and 10 mg/kg) and dexamethasone (3 mg/kg) on ovalbumin-induced increase in total (A) and differential (B) BALF cell count. Results are expressed as means $\pm$ S.E.M. ($n=7-10$). * $P<0.05$ vs time matched PBS challenged mice. # $P<0.05$ vs time matched ovalbumin-challenged mice.

cell numbers (Fig. 1, A and B; $P>0.05$). Indeed, there was a tendency for an increase in total, neutrophils and eosinophils cell counts in this treatment group (Fig. 1, A and IB). In contrast, treatment with BAY 11-7085 (3 and 10 mg/kg) dose-dependently decreased the influx of ovalbumin-induced leukocytes compared to the control group ($6.8 \pm 0.3 \times 10^5$ and $2.7 \pm 0.3 \times 10^5$ vs $7.9 \pm 1.5 \times 10^5$ /ml, respectively, Fig. 1A) and this was statistically significant at the 10 mg/kg ($P<0.05$).

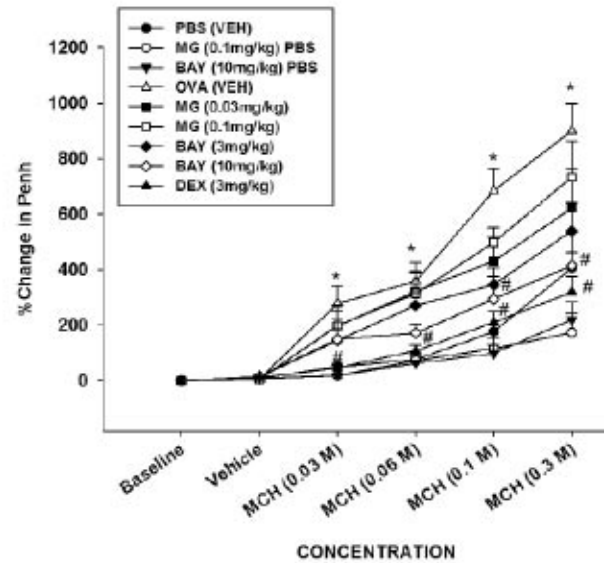


Fig. 2. Effect of MG-132 (0.03 and 0.1 mg/kg), BAY 11-7085 (3 and 10 mg/kg) and dexamethasone (3 mg/kg) on ovalbumin-induced increase in Penh to inhaled methacholine. Results are expressed as means $\pm$ S.E.M. ($n=7-10$). * $P<0.05$ vs time matched PBS-challenged mice. # $P<0.05$ vs time matched ovalbumin-challenged mice.

In addition, pretreatment with BAY 11-7085, at 10 mg/kg, compared to ovalbumin challenged mice, significantly decreased the airway lymphocytes (absolute, 0.01 ± 0.01 vs $0.20 \pm 0.05 \times 10^5$ /ml and also %, 0.4 ± 0.3 vs 3.3 ± 1.1 , $P<0.05$), neutrophils (absolute, 0.08 ± 0.02 vs $0.56 \pm 0.15 \times 10^5$ /ml and also %, 2.4 ± 0.4 vs 7.0 ± 0.7 , $P<0.05$), eosinophils (absolute, 0.59 ± 0.24 vs $3.43 \pm 1.04 \times 10^5$ /ml and also %, 16.6 ± 5.4 vs $40.4 \pm 6.2\%$, $P<0.05$; Fig. 1B). Pretreatment with BAY 11-7085, at 10 mg/kg, also significantly inhibited the ovalbumin - induced decrease in % of macrophages (80.3 ± 5.6 vs $49.3 \pm 5.9\%$, $P<0.05$; Fig. 1B). Dexamethasone (3 mg/kg) significantly reduced total cell number, % of lymphocyte, neutrophil, eosinophil influx and also inhibited the ovalbumin-induced decrease in airway macrophages ($P<0.05$; Fig. 1, A and B). Treatment of PBS challenged mice with MG-132 (0.1 mg/kg) or BAY 11-7085 (10 mg/kg) did not have significant effects on baseline total cell count or differential % cell numbers (Fig. 1, A and B).

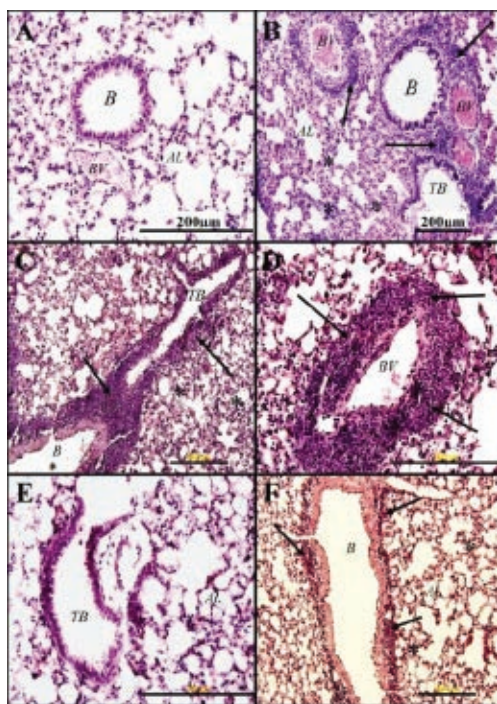


Fig. 3. Representative light photomicrographs display Hematoxylin and Eosin (H/E) staining of lung sections from PBS challenged-vehicle treated mice (A), ovalbumin challenged-vehicle treated mice (B), MG-132 (0.1mg/kg) (C and D), BAY 11-7085 (10mg/kg) (E) and DEX (3mg/kg) (F) treated groups. AL: alveoli, B: bronchioles, BV: blood vessels, TB: terminal bronchioles. arrow = increase in peribronchial and perivascular cellular inflammation

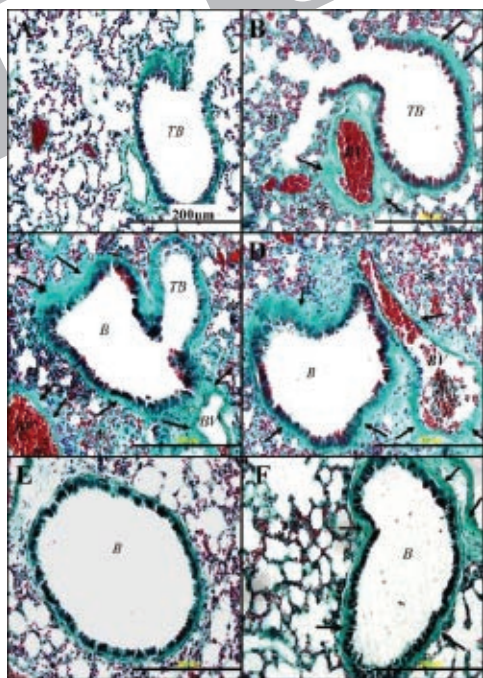


Fig. 4. Histological examination of Periodic Acid Schiff (PAS) stain of PBS challenged-vehicle treated mice (A), lung sections of mice challenged with ovalbumin (B), mice treated with MG-132 (0.1mg/kg) (C and D), BAY 11-7085 (10mg/kg) treated mice lungs (E) and DEX (3mg/kg) treated mice (F). B: bronchioles, BV: blood vessels, TB: terminal bronchioles. arrows = increase in bronchial mucus production and goblet cell hyper/metaplasia compared to the PBS challenged group. arrow heads = represent a remarkable reduction in bronchiole mucus production and goblet cell hyper-metaplasia and was comparable to the PBS challenged group.

Fig. 5. Masson's Trichrome staining of lung sections from PBS challenged mice (A), ovalbumin challenged - vehicle treated mice (B), MG-132 (0.1mg/kg) treated group (C and D), BAY 11-7085 (10mg/kg) treated mice (E) and DEX (3mg/kg) treated group (F). B: bronchioles, BV: blood vessels, TB: terminal bronchioles. asterisk = increase in inflammatory cells infiltration. arrow = marked increase in peribronchial and perivascular fibrosis

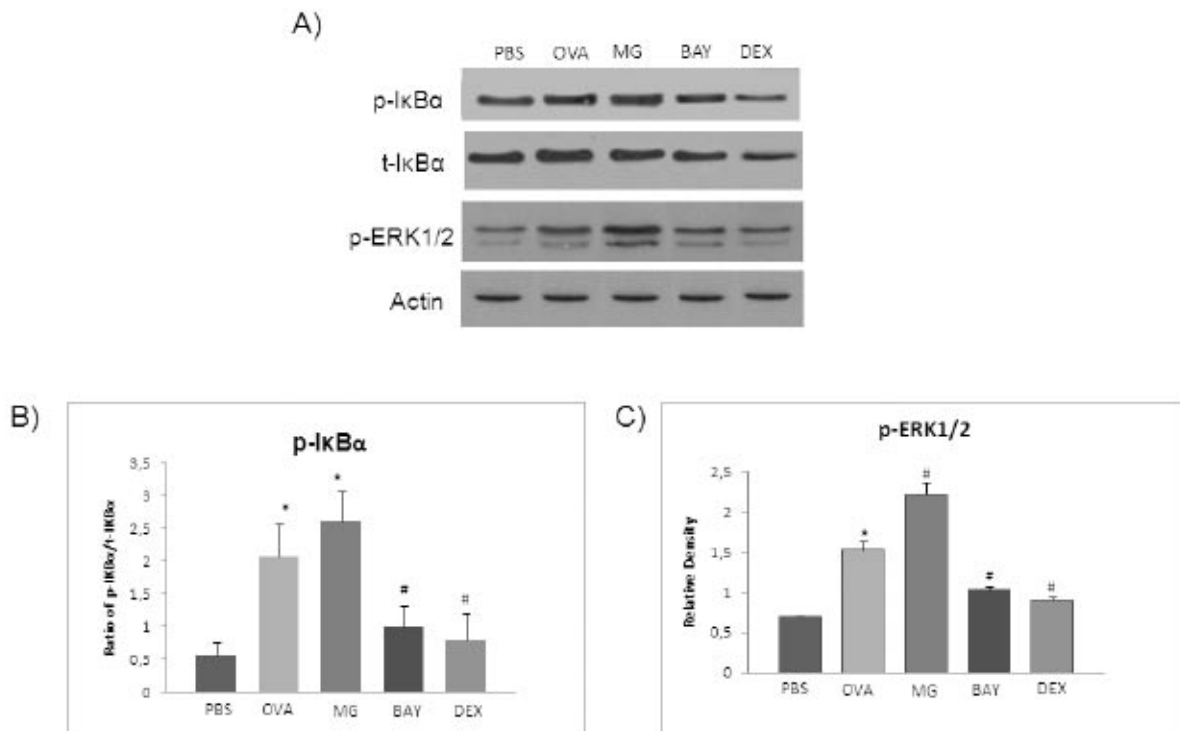


Fig. 6. Western blot analysis of total (t-) and phosphorylated (p)-I κ B α and phosphorylated (p) – ERK1/2 protein levels from lungs of PBS challenged mice pretreated with vehicle (PBS), and also from OVA challenged pretreated with vehicle (OVA), MG-132 at 0.1 mg/kg (MG) BAY 11-7085 at 10mg/kg (BAY) and DEX at 3mg/kg (DEX), respectively. **A)** Representative blots and densitometric quantification of at least three independent experiments ($n=3$) showing **B)**, relative levels of p- I κ B α (data normalized to β -actin shown as a ratio of phosphorylated to total I κ B α protein) and **C)**, relative levels of p-ERK1/2 (normalized to β -actin). Data shown represent the mean $\pm$ SEM.

*Statistically significant difference ($p<0.05$) compared with PBS challenged and vehicle pre-treated mice.

Statistically significant difference ($p<0.05$) compared with OVA challenged and vehicle pre-treated mice.

Effect of MG-132 and BAY 11-7085 on the ovalbumin-induced increase in Penh to methacholine

Ovalbumin challenge induced a significant increase in Penh compared to control animals which demonstrated a shallow dose-dependent increase in Penh in response to inhaled methacholine. This increase in Penh was noted at all of the methacholine doses used, 0.03 – 0.3 M, ($P<0.05$; Fig.2). Treatment with MG-132 at either 0.03 or 0.1 mg/kg did not alter the ovalbumin-induced increase in % Penh at any of the doses of methacholine. In contrast, treatment with BAY 11-7085, at 10 but not 3 mg/kg, significantly reduced the ovalbumin-induced increase in Penh to methacholine both at 0.1 M (683.7 ± 81.4 vs 294.4 ± 80.9) and 0.3 M methacholine (899.6 ± 99.2 vs 414.4 ± 120.2) ($P<0.05$; Fig. 2). As expected, treatment with dexamethasone significantly reduced the ovalbumin-induced increase in Penh at all the doses

of methacholine used ($P<0.05$; Fig. 2). Treatment of PBS challenged mice with MG-132 (0.1 mg/kg) or BAY 11-7085 (10 mg/kg) did not have significant effects on baseline Penh responsiveness (Fig. 2).

Effect of MG-132 and BAY 11-7085 on the ovalbumin-induced histological changes

Table I summarizes all the histopathological findings in the control, ovalbumin challenged-vehicle treated and ovalbumin challenged-drug treated mice. In general, lung sections from control mice (immunized with ovalbumin and challenged intranasally with PBS) showed consistently normal histology with the various stains used (Figs. 3A, 4A, 5A and Table I). However, lung sections from mice that were challenged intranasally with ovalbumin showed severe perivascular and peribronchial inflammatory cell infiltrate (Fig. 3B, Table I), severe goblet cell

hyper/metaplasia (Fig. 4B, Table I) and severe perivascular and peribronchial fibrosis suggesting airway remodeling, (Fig. 5B, Table I). Lung sections from mice that were pretreated with MG-132 (0.1mg/kg) similarly had an overall pronounced degree of airway inflammation. There was severe perivascular and peribronchial inflammatory cell infiltrate (Fig. 3 C and D and Table I), and marked goblet cell hyper/metaplasia (Fig. 4 C and D and Table I). In addition, MG-132 pretreated mice also had a marked perivascular and peribronchial fibrosis (Fig. 5C and Table I). In contrast, the BAY 11-7085-treated (10 mg/kg) mice lungs showed remarkable improvement in all the histopathological parameters assessed and these were comparable to the dexamethasone-treated mice in almost all aspects. In BAY 11-7085-treated mice, there was a noticeable decrease in the perivascular and peribronchial inflammatory cell infiltration, goblet cell hyper/metaplasia and perivascular and peribronchial fibrosis (Figs. 3E, 4E, 5E and Table I) with a graded score of only minimal changes compared to ovalbumin challenged mice. These changes were similar to the histological changes noted in the dexamethasone-treated mice, which were scored mainly as moderate changes (Figs. 3F, 4F, 5F and Table I).

Effect of MG-132 and BAY 11-7085 on the ovalbumin-induced increase in p-IkBa and p-ERK1/2 in the lungs

To assess whether MG-132 and BAY 11-7085 are impacting on NFkB activation, we studied the effect of these two drugs on the levels of p-IkBa and p-ERK1/2 expression. Ovalbumin challenge significantly increased the levels of phosphorylated-IkBa compared to the PBS challenged control animals (Fig. 6, A and B). Pretreatment with BAY 11-7085 and dexamethasone reduced the level of phosphorylated-IkBa (Fig. 6, A and B). In contrast, treatment with MG-132 tended to increase the levels of phosphorylated-IkBa compared to that of the ovalbumin challenged group, although this was not significant (Fig. 6, A and B). Moreover, ovalbumin challenge also significantly increased the levels of phosphorylated-ERK1/2 compared to the PBS challenged control animals (Fig. 6, A and C). Pretreatment with BAY 11-7085 and dexamethasone significantly reduced the level of

ERK1/2 phosphorylation compared to the ovalbumin challenged animals. Interestingly however, MG-132 significantly increased the level of phosphorylated-ERK1/2 above that seen in the ovalbumin challenged group (Fig. 6, A and C).

DISCUSSION

NFkB plays an important role in inflammatory diseases such as asthma and COPD. One pathway that has recently attracted increasing attention is the UPS which plays a critical role in the selective degradation of intracellular proteins including the NFkB inhibitory molecule IkBa (15). Our findings show, for the first time, that in a murine model of inflammation, the UPS inhibitor MG-132 did not significantly affect the ovalbumin-induced asthma-like changes such as an increase in total and differential cell numbers, perivascular and peribronchial inflammation and fibrosis, goblet cell hyper/metaplasia, airway remodeling or the increased Penh to methacholine. Indeed, MG-132 significantly increased ERK1/2 phosphorylation suggesting, if anything, that inhibition of the UPS enhances airway inflammation. This finding also confirms the observation noted in the cytology data where the MG-132-treated group tended to have a higher total, neutrophil and eosinophil cell count. The enhanced phosphorylation of ERK1/2 in the MG-132-treated group, an inhibitor of UPS, is suggestive of a pro-inflammatory activity. This may reflect the rather complex nature of the UPS and its regulatory role and clearly requires further study. Thus, our finding is in contrast to data from a previous *in vivo* study showing that the UPS inhibitor, PS-519, was effective in reducing allergen-induced pulmonary eosinophilia when administered directly into the airways as well as other *in vitro* studies showing that MG-132 has anti-inflammatory activity (8, 12).

Interestingly however, several other *in vitro* and *in vivo* studies have also reported that inhibition of the UPS can result in an increase in inflammatory cytokines and symptoms. For example, a study has reported that treatment of BALB/c mice, infected with respiratory syncytial virus, with the UPS inhibitor, bortezomib, resulted in increased inflammation (16).

In this study, we also assessed the effect of the

inhibition of IKK on the ovalbumin-induced airway inflammation and the increased Penh to methacholine. Our data show that, in contrast to UPS inhibition, inhibition of the IKK complex with BAY 11-7085 dose-dependently inhibited the ovalbumin-induced total and differential inflammatory cellular influx, the ovalbumin-induced histopathological changes and the increased Penh to methacholine, suggesting a critical role for this enzyme in regulating the NF κ B mediated inflammation. These findings are in line with data from both *in vitro* and *in vivo* studies which show that inhibition of this kinase is associated with significant reduction in indices of inflammation (17-19). This effect was also associated with inhibition of both p-I κ B α and p-ERK1/2, which are likely to be the underlying mechanisms for the BAY 11-7085 action. Our data also demonstrate that BAY 11-7085 significantly inhibited the ovalbumin-induced increase in Penh to methacholine. It is likely that the inhibition of the increased Penh to methacholine is secondary to the inhibition of the ovalbumin-induced inflammation (20).

In conclusion, our data show that inhibition of the UPS system with MG-132 does not reduce the ovalbumin-induced airway inflammation or the increased Penh to methacholine, two typical features of asthma, in a murine model of inflammation. In contrast, BAY 11-7085 inhibited the ovalbumin-induced airway inflammation and the increased Penh to methacholine. These findings show that NF κ B activated pathways are central to the pathogenesis of allergic diseases, such as asthma, and inhibition of this molecule via inhibition of IKK may be a more viable therapeutic approach than the inhibition of the UPS.

ACKNOWLEDGEMENTS

The authors are thankful to the support of Mini Mathew and Sunny Okojo from the Animal Resources Center of Health Sciences Center, and Ms Bindu Chandrasekhar, Department of Pharmacology and Toxicology, for her excellent assistance with the Western blots.

REFERENCES

- Holgate ST. Pathogenesis of asthma. Clin Exp Allergy 2008; 38:872-97.
- Sheller JR, Polosukhin VV, Mitchell D, Cheng DS, Peebles RS, Blackwell TS. Nuclear factor kappa B induction in airway epithelium increases lung inflammation in allergen-challenged mice. Exp Lung Res 2009; 35:883-895.
- Bureau F, Bonizzi G, Kirschvink N, Delhalle S, Desmecht D, Merville MP, Bours V, Lekeux P. Correlation between nuclear factor-kappaB activity in bronchial brushing samples and lung dysfunction in an animal model of asthma. Am J Respir Crit Care Med 2000; 161:1314-21.
- Hart LA, Krishnan VL, Adcock IM, Barnes PJ, Chung KF. Activation and localization of transcription factor, nuclear factor-kappaB, in asthma. Am J Respir Crit Care Med 1998; 158:1585-92.
- La Grutta S, Gagliardo R, Mirabella F, Pajno GB, Bonsignore G, Bousquet J, Bellia V, Vignola AM. Clinical and biological heterogeneity in children with moderate asthma. Am J Respir Crit Care Med 2003; 167:1490-5.
- Janssen-Heininger YM, Poynter ME, Aesif SW, et al. Nuclear factor kappaB, airway epithelium, and asthma: avenues for redox control. Proc Am Thorac Soc 2009; 6:249-255.
- Strnad J, Burke JR. IkappaB kinase inhibitors for treating autoimmune and inflammatory disorders: potential and challenges. Trends Pharmacol Sci 2007; 28:142-8.
- Elliott PJ, Pien CS, McCormack TA, Chapman ID, Adams J. Proteasome inhibition: A novel mechanism to combat asthma. J Allergy Clin Immunol 1999; 104:294-300.
- El-Hashim A, Yousefi S, Edafiogho I, Raghupathy R, Yousif M, Simon HU. Anti-inflammatory and immunosuppressive effects of the enaminone E121. Eur J Pharmacol 2010; 632:73-78.
- Han YH, Moon H J, You BR, Park WH. The effect of MG132, a proteasome inhibitor on HeLa cells in relation to cell growth, reactive oxygen species and GSH. Oncol Rep 2009; 1:215-21.
- Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME. Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects *in vivo*. J Biol Chem 1997; 1;272:21096-103.
- Meiners S, Hoche B, Weller A, et al. Downregulation

- of matrix metalloproteinases and collagens and suppression of cardiac fibrosis by inhibition of the proteasome. *Hypertension* 2004; 44:471-7.
13. Yang G, Abate A, George AG, Weng YH, Dennerly PA. Maturation differences in lung NF-kappaB activation and their role in tolerance to hyperoxia. *J Clin Invest* 2004; 114:669-78.
 14. El-Hashim AZ, Wyss D, Zuany-Amorim C. Kinetics of the increased Penh to methacholine and airway eosinophilia in BALB/c mice and their modulation by different dexamethasone treatment regimens. *Pulm Pharmacol Ther* 2002; 15:467-75.
 15. Wang J, Maldonado MA. The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Mol Immunol* 2006; 3:255-261.
 16. Lupfer C, Patton KM, Pastey MK. Treatment of human respiratory syncytial virus infected BALB/c mice with the proteasome inhibitor bortezomib (Velcade, PS-341) results in increased inflammation and mortality. *Toxicology* 2010; 268:25-30.
 17. Birrell MA, Wong S, Hardaker EL, Catley MC, McCluskie K, Collins M, Haj-Yahia S, Belvisi MG. IkappaB kinase-2-independent and -dependent inflammation in airway disease models: relevance of IKK-2 inhibition to the clinic. *Mol Pharmacol* 2006; 69:1791-800.
 18. Ziegelbauer K, Gantner F, Lukacs NW, et al. A selective novel low-molecular-weight inhibitor of IkappaB kinase-beta (IKK-beta) prevents pulmonary inflammation and shows broad anti-inflammatory activity. *Br J Pharmacol* 2005; 145:178-92.
 19. Sommers CD, Thompson JM, Guzova JA, et al. Novel tight-binding inhibitory factor-kappaB kinase (IKK-2) inhibitors demonstrate target-specific anti-inflammatory activities in cellular assays and following oral and local delivery in an *in vivo* model of airway inflammation. *J Pharmacol Exp Ther* 2009; 330:377-88.
 20. Berend N, Salome CM, King GG. Mechanisms of airway responsiveness in asthma. *Respirology* 2008; 13:624-31.