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Revised

Inhibition of p38-MAPK-dependent bronchial contraction after ozone by corticosteroids

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Running Title: Impaired corticosteroid function in MKP-1$^{-/-}$ mice

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Abstract

Background: We determined the role of p38 mitogen-activated protein kinase (MAPK) in the increased airway smooth muscle contractile responses following ozone and modulation by corticosteroids.

Methods: Mice were exposed to air or ozone (3ppm; 3 hours) and isometric contractile responses of bronchial rings to acetylcholine (ACh) were measured using a myograph, in the presence of p38 MAPK inhibitor, SB239063(10^{-6}M) or dexamethasone (10^{-6}M). Because MAPK-phosphatase-1(MKP-1) is a negative regulator of p38MAPK, we also studied these effects in MKP-$1^{-/-}$ mice.

Results: Bronchial rings from ozone-exposed wild-type and MKP-$1^{-/-}$ mice showed increased contractile responses, with a leftward shift of the dose-response curve in MKP-$1^{-/-}$ mice. SB239063 equally inhibited bronchial contraction in air- and ozone-exposed C57/BL6 and MKP-$1^{-/-}$ mice. Dexamethasone inhibited ACh-induced bronchial contraction in both air- and ozone-exposed C57/BL6 mice, but not in air- or ozone-exposed MKP-$1^{-/-}$ mice. ACh stimulated p38 MAPK and HSP27 phosphorylation as measured by Western blot, effects suppressed by SB239063 in C57/BL6 and MKP-$1^{-/-}$ mice, but not by dexamethasone in either air- or ozone-exposed MKP-$1^{-/-}$ mice.

Conclusions: p38 MAPK plays a role in maximal ACh-induced isometric contractile responses and in increased contractility induced by ozone. Dexamethasone inhibits ACh-induced airway smooth muscle contraction through phosphorylation of p38 MAPK and HSP27.

Key words: p38 MAPK, MKP-1, dexamethasone, HSP-27, Acetylcholine, Airway smooth muscle.

INTRODUCTION

The mitogen-activated protein kinases (MAPKs) are serine/threonine family of kinases that consist of three members, p38 MAPK, c-Jun N-terminal kinases (JNK) and extracellular signal-regulated protein kinase (ERK). MAPKs are activated by phosphorylation of specific tyrosine and threonine residues in their active domains. p38 MAPK is activated by inflammatory cytokines and cellular stresses including oxidative stress, and involved in cellular processes such as cell proliferation, apoptosis and inflammation[1;2]. Its role in airway smooth muscle contractile responses is less clear. P38 MAPK is expressed in airway smooth muscle and in other smooth muscles such as in the gastrointestinal smooth muscle and becomes activated during acetylcholine-induced contraction [3-5]. Subsequent to this p38 MAPK activation is the downstream activation of MAPKAP kinase-2, which in turn leads to the phosphorylation of heat shock protein (HSP)27. HSP27 may associate with proteins such as actomyosin and tropomyosin involved in ASM contraction [5;6]. Inhibition of p38 MAPK has been reported to increase force-fluctuation-induced re-lengthening in maximally activated shortened bovine tracheal smooth muscle strips, an effect that involves the stabilisation of cytoskeletal remodelling [7]. In addition, p38 MAPK appears to be important in underlying the bronchial hyperresponsiveness induced by the oxidant, ozone[8], or by allergic inflammation [9;10]. The process of bronchial hyperresponsiveness could be secondary to enhanced airway smooth muscle contractile responses, particularly after ozone exposure[11]. This is similar to the direct effect of inflammatory cytokines IL-1β and TNF-α in increasing ASM contractility to

acetylcholine[12;13], changes that were also prevented by pre-exposure to the glucocorticoid, dexamethasone[13].

The inactivation of MAPKs such as p38 is partly dependent on MAP kinase phosphatases (MKPs), also referred to as dual specificity phosphatases (DUSPs), particularly by MKP-1, which also deactivates other MAPK members including p38 and JNK [14;15]. MKP-1 is up-regulated by the environmental pollutant, ozone, and by pro-inflammatory cytokines, thereby limiting MAPK activation through a feedback mechanism. In addition, corticosteroids also up-regulate very rapidly the expression of MKP-1 in inflammatory cells and also in airway smooth muscle[16-18], raising the possibility that MKP-1 could mediate relaxant effects of ASM contractile responses dependent on MAPK activation, particularly p38.

In the present study, we examined the role of p38 MAPK in the enhanced isometric contractile responses of bronchi induced by exposure to ozone and the effect of corticosteroids by studying the effect of p38 MAPK inhibitors and of knocking-down MKP-1 as in MKP-1$^{-/-}$ mice. Our data indicate that both baseline and enhanced maximal isometric contractile cholinergic responses are dependent on p38 MAPK activation and that the relaxant effect of corticosteroids may be mediated through MKP-1.

METHODS

Reagents

Acetylcholine (ACh), 5-hydroxytryptamine (5-HT), indomethacin, SB 239063 (p38 MAPK inhibitor), PD 98059 (ERK inhibitor), SP 600125 (JNK inhibitor) and dexamethasone were obtained from Sigma Aldrich (Dorset, UK). SD 282, another p38 MAPK inhibitor, was a gift from Scios Inc (Fremont, CA, USA). Rabbit anti-phospho-p38 MAPK and anti-p38MAPK, and rabbit anti-phospho-HSP27 and anti-HSP27 were obtained from Cell Signalling Technology (Beverly, MA, USA). Horseradish-peroxidase conjugated anti-rabbit immunoglobulin was obtained from Dakocytomaton (Glostrup, Denmark). ECL Plus was obtained from Amersham, GE healthcare (Buckinghamshire, UK) and Re-Blot plus mild solution from Millipore (Billerica, MA, USA)

Mice

Pathogen-free, male 10-14 weeks old MKP-$1^{-/-}$ mice, obtained from Kennedy Institute of Rheumatology Division, Imperial College London were used. MKP-$1^{-/-}$ mice were on a mixed C57/BL6-129/Sv genetic background and were backcrossed over nine generations, then intercrossed heterozygotes[19]. MKP-$1^{-/-}$ mice were identified by PCR-based screen of genomic DNA from tail tips. Pathogen-free, male C57/BL6 mice of 10-14 weeks old purchased from Harlan (Oxon, UK) were used as wild type control. The animals were housed in BioSciences facilities of Imperial College, under controlled temperature (20°C) and humidity (40-60%), in a 12 hour light/12 hour dark cycle with food and water supplied *ad libitum*. The protocols and procedures in the study were approved by the Animal Ethics Committee of Imperial College in compliance with UK Home Office regulations.

Ozone exposure

Mice were exposed to filtered air or to ozone generated from an Ozoniser (Model 500 Sander Ozoniser, Germany), mixed with filtered air at a concentration of 3 parts per million in a sealed Perspex container, as described before [20]. Ozone concentration was continually monitored with an ozone probe (ATi Technologies, Oldham, UK) placed within the box.

Bronchial ring preparation and myography

At 24 h after exposure, mice were sacrificed by cervical dislocation. Lungs were rapidly taken out of the chest and immersed in physiological salt solution (PSS) composed of NaCl, 119 mM; KCl, 4.7 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.17 mM; $NaHCO_3$, 25 mM; KH_2PO_4, 1.18 mM; EDTA, 0.027 mM; and glucose, 5.5 mM). Intrapulmonary bronchi from the left lobe were dissected under the microscope, parenchymal and connective tissues were carefully removed [21]. Segments of bronchi, 200–400 μm in diameter, 2mm in length, were mounted to metal prongs of wire myograph 610M (Danish Myo Technology, Aarhus, Denmark), suspended in an organ bath, filled with 5ml of PSS, bubbled with 95% oxygen and maintained at 37°C. The isometric tension was recorded and analysed using Chart software (AD Instruments Ltd., Hastings, U.K.). The optimal length for each bronchial ring taken at the point at which increased stretch ceased to increase active tension was obtained from active length-tension responses. In brief, bronchi were first stretched to 0.5mN and then were sequentially stretched by 200 μm increments in length (passive tension) and stimulated to contract actively (active tension) with 124mM potassium PSS (KPSS). Optimal length was taken at the point at which increased stretch ceased to increase active tension. Bronchi were then allowed to

equilibrate for 30 min in PSS and 3 μM indomethacin was added into the organ bath to inhibit epithelial prostaglandin release. The first bronchial contractile response was generated with 10^{-9}M to 10^{-3}M of ACh or 10^{-9}M to 10^{-4}M of 5-HT. The effect of certain compounds on the bronchial contractile response was assessed in bronchi from air- and ozone-exposed mice. Bronchi were washed four times with PSS, incubated with the p38 MAPK inhibitor SB239063 or SD282, or ERK inhibitor PD98059, or JNK inhibitor SP600125 or dexamethasone for 1 h and then a second contractile response was performed. We also studied an inhibitor of MAPK-activated protein kinase 2 (MK2), a direct downstream substrate of p38 kinsae, PF-3644022[22]. The concentration-response curves were fitted by nonlinear regression and with Hill equation (GraphPad Prism 4.03, San Diego, CA, USA) to provide an estimated maximal contraction (E_{max}) and the negative logarithm of the effective concentration to cause 50% of the maximal contractile response (pEC_{50}).

Western blot of p38 MAPK and HSP27 phosphorylation, and of MKP-1

Intrapulmonary bronchi were dissected, mounted onto two metal prongs and immersed in PSS. After reaching optimal tension with KPSS, each bronchus was incubated with SB 239063 (10^{-6}M) or dexamethasone (10^{-6}M) or neither for 1 hour in the organ bath, and then 10^{-3}M of ACh was added for 5 minutes. Bronchial samples were snap-frozen in liquid nitrogen. The frozen samples were homogenised in extraction buffer (50 mM Tris buffer, 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, 20 mM Na_3VO_4 and protease inhibitor cocktail) by sonication on ice for seconds. After sonication and centrifugation (15,000 rpm; 15 min), the supernatants were collected. Equal amounts of protein (10

μg) per lane were loaded onto 10% Bis-Tris gel (NuPAGE, Invitrogen, Paisley, UK). The gel was run for 45min and transferred to nitrocellulose membrane with dry blotting system (iBlot, Invitrogen, Paisley, UK). The membrane was blocked with 5% non-fat dry milk for 1 h at room temperature and incubated with rabbit anti-phospho-p38 MAPK or anti-phospho-HSP27 antibodies (1:1000) overnight at 4°C. Following incubation, the membrane was washed and incubated with horseradish peroxidise conjugated anti-rabbit immunoglobulins for 2 h at room temperature, followed by ECL Plus. Band densities were quantified using a densitometer with Grab-It and Gel Work Software (UVP, Cambridge, UK). The membrane was stripped with Re-Blot plus mild solution (Millipore, Billerica,USA) and re-probed with anti-p38 MAPK or anti-HSP27 antibodies (1:1000). Densitometric results for p38 MAPK or HSP27 phosphorylation were expressed as a ratio of phosphorylated p38MAPK or HSP27 to non-phosphorylated p38 MAPK or HSP27, respectively.

To probe for MKP-1, we used a rabbit anti-MKP-1(V-15) (Santa Cruz Biotechnology. Inc,CA,USA ,1:1000) and incubated the membranes overnight at 4°C. The membrane was then stripped with Re-Blot plus mild solution (Millipore, Billerica,USA) and reprobed with α-tubulin rabbit-antibody (Cell signalling Technology, Beverly, USA, Catalogue # 2125, 1:1000). Densitometric results for MKP-1 were expressed as a ratio of MKP-1 to α-tubulin.

Data analysis

All results were expressed as mean ± SEM. t-test and two-way analysis of variance (ANOVA) with Bonferroni correction were used to make a comparison between different groups as appropriate. $P<0.05$ was regarded as statistically significant.

RESULTS

ACh-induced contractile response in ozone-exposed MKP-1$^{-/-}$ mice

Bronchial contractile responses to ACh 2 hours apart in C57/BL6 and MKP-1$^{-/-}$ mice were identical (data not shown). In ozone-exposed C57/BL6 mice, E_{max} was increased compared to air-exposed mice (7.73±0.31 mN to 10.06± 0.37 mN; $p < 0.05$) but pEC_{50} values remained unchanged. In ozone-exposed MKP-1$^{-/-}$ mice, there was a similar enhancement of E_{max} (7.76±0.27 mN to 9.62± 0.40 mN), with a shift of the dose-response curve to the left (pEC_{50}: 5.69 ± 0.14 vs 5.25 ± 0.11, $p<0.05$) (Fig 1).

SB 230963 and dexamethasone on ACh-induced bronchial contraction

We used SB 239063 to investigate the role of p38 MAPK in the increased contractile response after ozone. SB 239063 inhibited the maximal contractile tension in a concentration-dependent fashion with an inhibition of 9.8±3.6% at 10^{-8}M, 20.7±7.4% at 10^{-7}M (data not shown). SB 239063(10^{-6}M) inhibited bronchial contraction in air-exposed C57/BL6 mice by 29.1± 4.0%, with no change in pEC_{50} ($p<0.01$, Fig 2A). Because indomethacin was present in the bathing solution, we determined whether it could have influence the modulation of the contractile response by SB239063. However, we obtained the same inhibitory effect of SB239063 in the absence of indomethacin in the bathing solution, indicating lack of influence of prostaglandins (data not shown). This inhibition by SB239063 (10^{-6}M) was also observed after contraction with another receptor-mediated constrictor, 5-HT, of the order of 30.9 ± 5.0% ($p<0.01$, Fig 3A). Using another p38 MAPK inhibitor, SD282 (10^{-}

^{6}M), maximal response to ACh was also decreased by 23.6±5.3% ($p<0.01$, Fig 3B).

SB 239063 also reduced bronchial contraction of bronchi from ozone-exposed C57/BL6 mice by 24.4± 3.8% ($p<0.01$, Fig 2B). SB239063 also lowered E_{max} by 25.8 ± 3.1% ($p<0.01$) and by 21.8± 3.9% ($p<0.01$) in air- and ozone-exposed MKP-1$^{-/-}$mice respectively (Fig 2C &D).

We also investigated the role of ERK and JNK in ACh-induced bronchial contraction by using ERK inhibitor PD 98059 (10^{-6}M) and JNK inhibitor SP 600125 (10^{-6}M). Neither PD 98059 nor SP 600125 inhibited the maximal bronchial response (Fig 3C&D). In addition, we investigated whether an inhibitor of MAPK-activated protein kinase 2 (MK2), a direct downstream substrate of p38 kinsae, PF-3644022[22], was also involved. PF-3644022 (10^{-7}M to 10^{-5}M) dose-dependently also inhibited the maximal ACh-induced bronchial contraction (Fig 3E).

Dexamethasone inhibited the bronchial contraction at 10^{-6}M by 21.6±4.0% in air-exposed mice ($p<0.01$, Fig 4A), while at 10^{-8} M and 10^{-7}M there was no effect (data not shown). Dexamethasone also reduced E_{max} by 19.9±3.9% in ozone-exposed C57/BL6 mice ($p<0.01$, Fig 4B). However, in neither air- nor ozone-exposed MKP-1$^{-/-}$ mice, was there any significant attenuation by dexamethasone (8.9 ± 2.5% and 5.9 ± 1.6 % respectively, NS; Fig 4C & D).

p38MAPK and HSP27 activation by ACh

Air-exposed mice. In bronchial preparations from air-exposed C57/BL6 mice, ACh increased p38 MAPK phosphorylation (Thr^{180}/Tyr^{182}) and HSP27 phosphorylation(Ser^{82}), measured as the ratio of phosphorylated p38 MAPK

and HSP27 to total p38 MAPK and HSP27, respectively (Fig 5A & 6A). Both SB239063 and dexamethasone inhibited p38 MAPK and HSP27 phosphorylation in C57/BL6 mice. In air-exposed MKP-$1^{-/-}$ mice, the ratio of phosphorylated p38 MAPK to total p38 MAPK and of phosphorylated HSP27 to total HSP27 increased in ACh-stimulated bronchial preparations ($p<0.05$ & $p<0.01$ respectively; Fig 5). SB239063, but not dexamethasone, inhibited ACh-induced p38 MAPK and HSP27 phosphorylation in MKP-$1^{-/-}$mice (Fig 5).

Ozone-exposed mice. The baseline levels of p38 MAPK and HSP27 phosphorylation were not significantly different between the air-exposed and the ozone-exposed mice. p38 MAPK and HSP27 phosphorylation were increased in ACh-stimulated bronchial preparations in ozone-exposed C57/BL6 mice ($p<0.01$ & $p<0.05$ respectively; Fig 6), although the increase was not different from that observed in air-exposed C57/BL6 mice. SB239063 and dexamethasone reduced p38 MAPK and HSP27 activation. ACh-stimulated p38 MAPK and HSP27 phosphorylation in bronchial preparations in ozone-exposed MKP-$1^{-/-}$ mice ($p<0.01$ & $p<0.05$ respectively; Fig 6). In ozone-exposed MKP-$1^{-/-}$ mice, while SB239063 reduced the p38 MAPK and HSP27 phosphorylation ($p<0.01$), dexamethasone did not (Fig 6).

Effect of dexamethasone on MKP-1 expression

We determined whether dexamethasone treatment of bronchial tissues would cause any changes in MKP-1 expression. We found that incubation of bronchial tissues from wild type mice with acetylcholine (10^{-3} M) caused no significant change in MKP-1 expression. However, after incubation with dexamethasone (10^{-6}M) and acetylcholine (10^{-3}M) for 1 hr, there was a

significant increase in expression of MKP-1 (Fig 7). We found similar results in bronchial tissues obtained from ozone-exposed mice.

DISCUSSION

We have shown that the maximal isometric contractile response to ACh in isolated murine bronchi was inhibited by p38 MAPK inhibitors, indicating that p38 MAPK activation contribute to maximal ASM isometric contraction. Measurement of p38 phosphorylation confirmed the increase in activation of p38 MAPK. The other MAPKs, JNK and ERK, do not appear to be involved in this response. Corticosteroids inhibited maximal contractile responses, an effect that was absent in MKP-$1^{-/-}$ mice, where p38 MAPK activation would not be modulated by MKP-1 which is upregulated by corticosteroids. Indeed, dexamethasone reduced the degree of p38 MAPK activation induced by ACh, but, in MKP-$1^{-/-}$ mice, there was no effect of dexamethasone on p38 MAPK activation. These results support the concept that the effect of CS on contractile responses resulted from the modulation of MKP-1 expression.

We also demonstrated that in ozone-induced enhancement of the cholinergic contractile responses that a similar situation exists. This enhanced response was inhibited by a p38 MAPK inhibitor, and the suppressive effect of CS may also be due to an increase in MKP-1 expression, since the ozone-enhanced contractile responses observed in MKP-$1^{-/-}$ mice was not affected by corticosteroids. In MKP-$1^{-/-}$ mice, ozone exposure not only caused an enhancement of the maximal contractile response, but also caused a significant leftward shift of the acetylcholine dose-response curve indicating an enhanced sensitivity of the contractile response. This indicates that there may be other kinases apart from p38 MAPK under the control of MKP-1 that could determine the sensitivity of the isometric contractile response.

Our studies are related to an understanding of the mechanisms by which ozone exposure can lead to airways hyperresponsiveness *in vivo*. They indicate that an increase in the airway smooth muscle contractile response may be an important contributory factor. This may occur through the activation of oxidative stress since in a previous study we have shown that pre-treatment of mice with an antioxidant, N-acetylcysteine, prior to ozone exposure prevented the increased isometric contractile response (unpublished). Furthermore, our current study shows that p38 MAPK may contribute to the enhanced contractile response as demonstrated by the increased activation of p38 in airway smooth muscle and by the inhibitory effect of a p38 MAPK inhibitor. It must be noted that in the baseline state, there is already activation of p38 MAPK that is important in regulating the maximal isometric contractile response to receptor-mediated contraction induced by acetylcholine or 5-hydroxytryptamine.

The mechanisms by which p38 MAPK could regulate airway smooth muscle contractile response are unclear. This activation is observed in the baseline state when the muscle is maximally contracted by acetylcholine and also by other receptor-mediated constrictors such as 5-hydroxytryptamine. This would support the notion that the contractile response itself may activate p38 MAPK pathways. An enhanced maximal contractile response to acetylcholine was observed after exposure of mice to ozone, and p38 MAPK activation underlied this augmentation. HSP27 could be one of the downstream effectors of p38 MAPK since the p38 MAPK inhibitor we used inhibited HSP27 phosphorylation. In studies of vascular smooth muscle, p38 modulated actin organisation, and HSP27 was shown to inhibit actin

polymerisation in a phosphorylated-dependent manner and mediated the re-arrangement of actin[23]. Phosphorylation of HSP27 in airway smooth muscle in response to the cholinergic agonist, carbachol, has been previously shown [24], together with tyrosine phosphorylation of p38 MAPK. p38 MAPK can also phosphorylate other non-muscle proteins such as the actin and myosin-binding protein, caldesmon, which is important in endothelial cytoskeletal remodelling and migration[25] and in urokinase-induced smooth muscle cell migration[26]. Interestingly, the idea that p38 MAPK may be important in promoting actin filament capping and shortening of actin filaments by non-phosphorylated HSP27 has been proposed [7]. Thus, p38 MAPK has been related to airway smooth muscle function, including tissue mechanics, cell migration and proliferation, and gene expression[27].

Activated p38 kinase directly phosphorylates and activates the mitogen-activated protein kinase-activated protein (MAPKAP) kinases MK2, MK3, and MK5 [28]. We now demonstrate that downstream of p38 MAPK at least MK-2 can regulate the contractile response of the airways to acetylcholine using a selective inhibitor of MK2. Similar results have been demonstrated in the inhibitory effect of lipopolysaccharide-induced TNFa production by monocytes[22].

The inhibition of phosphorylation of p38 by SB239063 that we have demonstrated has been shown previously by other groups[29;30]. Additional studies have demonstrated that p38α can autophosphorylate[31] and can *trans*phosphorylate [32], and finally, another p38 MAPK inhibitor SB203508 inhibits the enzymatic activity of both activated and unactivated forms of p38α[33].

Corticosteroids have been shown to have multiple effects on smooth muscle function such as uncoupling of H1-histamine receptors[34], or a reduction in muscarinic receptor expression[35]; in addition, they could increase airway smooth muscle relaxation by interfering with β_2-adrenoceptor pathways[36]. In this study, dexamethasone inhibited the maximal isometric contractile response to acetylcholine, both in the baseline state or after exposure to an oxidant stress in wild type mice, through a p38 MAPK-dependent pathway. We showed that dexamethasone could upregulate MKP-1 in the bronchial rings within one hour of incubation, which may in turn regulate p38-MAPK activity. However, dexamethasone was ineffective in inhibiting bronchial smooth muscle contraction in MKP-$1^{-/-}$ mice, supporting the concept that p38 MAPK could be an important mechanism by which corticosteroids could be inhibiting the contractile responses. The reason may be due to the failure of dexamethasone to induce the expression of MKP-1 in bronchial smooth muscle from MKP-$1^{-/-}$ mice. By promoting the expression of MKP-1 in both air- and ozone-exposed mice, dexamethasone can inhibit the activity of p38 MAPK. We confirmed that dexamethasone inhibited p38 MAPK phosphorylation in air and ozone- exposed wild type mice, but not in air and ozone-exposed MKP-$1^{-/-}$ mice. This could also explain why dexamethasone could not inhibit HSP27 phosphorylation, which is associated with smooth muscle contraction. The potential direct effect of corticosteroids has also been demonstrated in canine airway smooth muscle cells where corticosteroids increased force fluctuation-induced re-lengthening through an inhibition of p38 MAPK and enhancement of MKP-1[37].

In summary, the inhibition of p38 MAPK activation by SB239063 and by dexamethasone could lead to a reduction of the maximal airway smooth muscle isometric contraction due to acetylcholine, and this was dependent on the expression of MKP-1. p38 MAPK phosphorylation of certain muscle and non-muscle proteins may contribute to the maximal contractile response.

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FIGURE LEGENDS

Figure 1. Acetylcholine(ACh)-induced isometric bronchial contractile tension in air- and ozone-exposed C57/BL6 mice (Panel A, n=12 in air-exposed group, n=9 in ozone-exposed group) and MKP-$1^{-/-}$ mice (Panel B, n=11 in air-exposed group, n=9 in ozone-exposed group). Data expressed as mean ± S.E.M. *p<0.05, **p<0.01, compared with air-exposed groups.

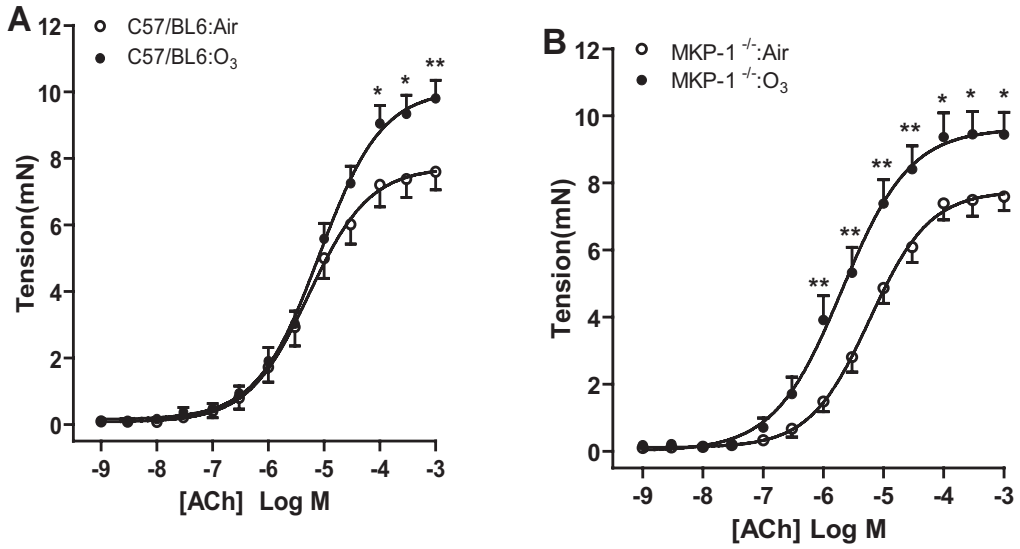

Figure 2. Effect of SB 239063 (10^{-6}M) on ACh-induced bronchial contractile responses in air-exposed C57/BL6 mice (Panel A, n=12) and MKP-$1^{-/-}$ mice (Panel B, n=11), and in ozone-exposed C57/BL6 mice (Panel C, n=9) and MKP-$1^{-/-}$ mice (Panel D, n=9). Data expressed as mean ±S.E.M. *p<0.05, **p<0.01, compared with untreated ones.

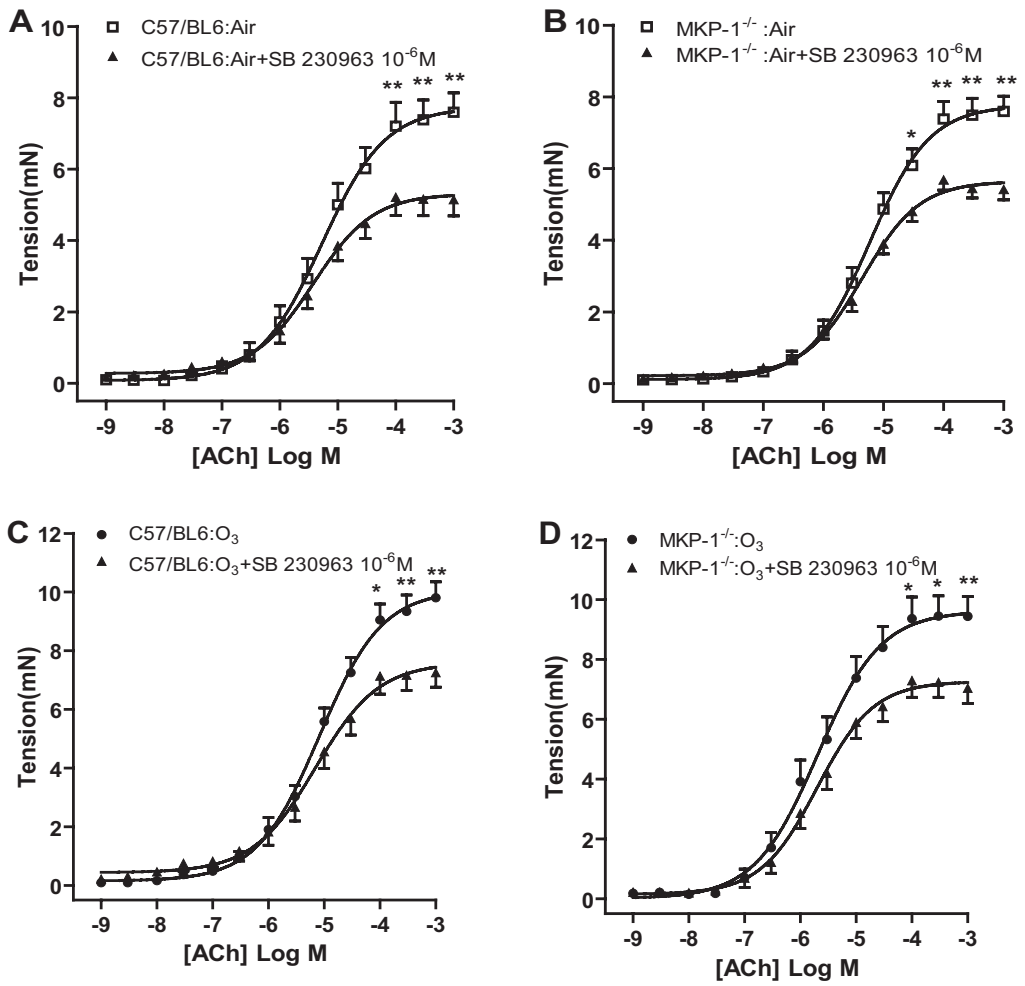

Figure 3. Inhibition of 5-hydroxytryptamine (5-HT)-induced bronchial contractile tension by p38 MAPK inhibitor SB 239063 (10^{-6}M) in air-exposed C57/BL6 mice (**Panel A**, n=6). **Panel B** shows the inhibition of ACh-induced bronchial contraction by p38 MAPK inhibitor, SD 282 (10^{-6}M), in air-exposed mice (n=3) while the effects of ERK inhibitor, PD 98059 (10^{-6}M) and of JNK inhibitor, SP600125 (10^{-6}M) are shown in **Panels C & D** in air-exposed C57/BL6 mice, n=6 in each group. Panel E shows the effect of an MK2 inhibitor, PF3644022, on ACh induced bronchial contractile tension in air-exposed C57/BL6 mice. Data points are expressed as mean ±S.E.M.*p<0.05, **p<0.01, compared with untreated ones.

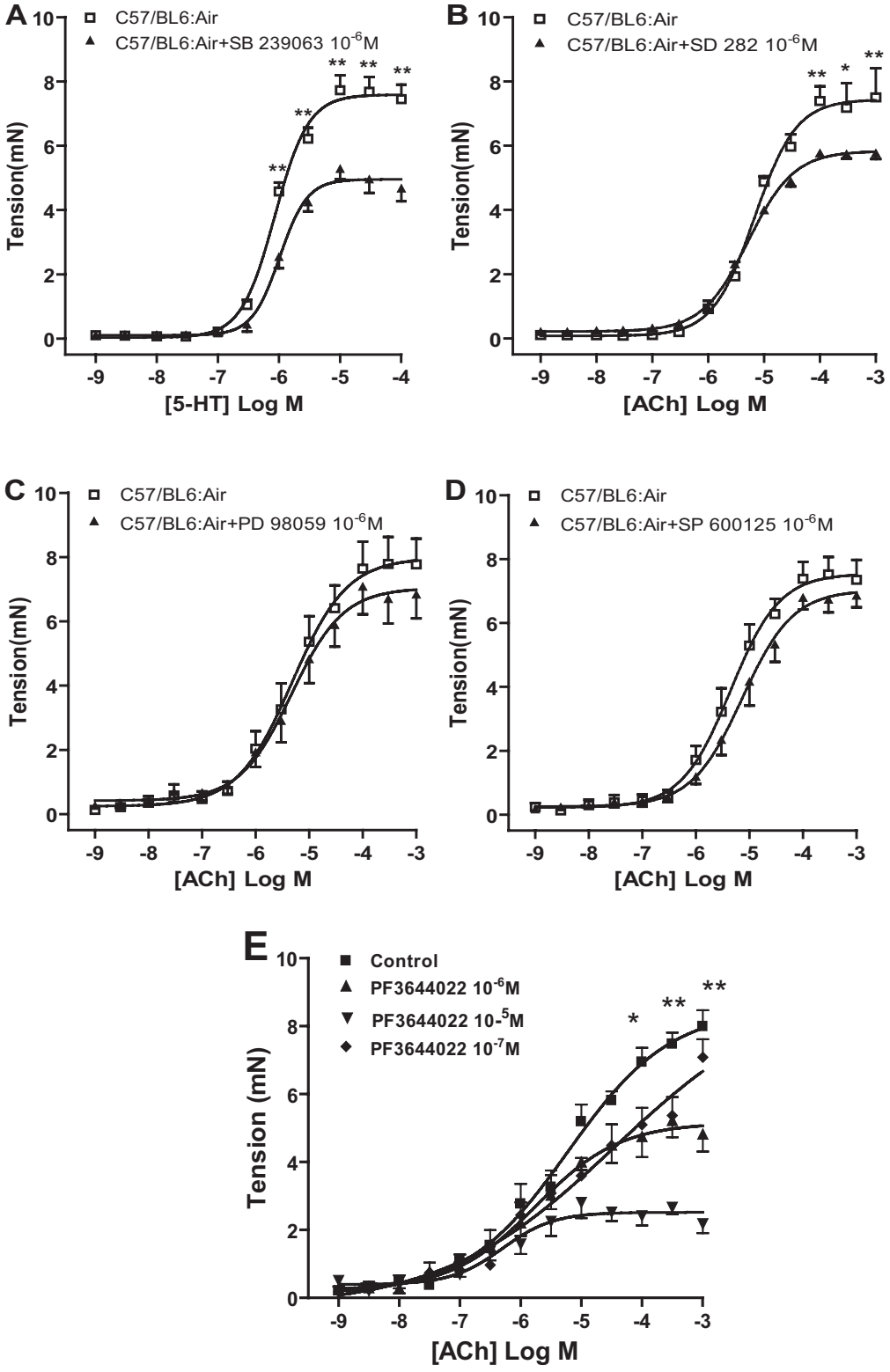
A
C57/BL6:Air
C57/BL6:Air+SB 239063 10-6M
Tension(mN)
[5-HT] Log M
B
C57/BL6:Air
C57/BL6:Air+SD 282 10-6M
Tension(mN)
[ACh] Log M
C
C57/BL6:Air
C57/BL6:Air+PD 98059 10-6M
Tension(mN)
[ACh] Log M
D
C57/BL6:Air
C57/BL6:Air+SP 600125 10-6M
Tension(mN)
[ACh] Log M
E
Control
PF3644022 10-6M
PF3644022 10-5M
PF3644022 10-7M
Tension (mN)
[ACh] Log M

Fig 3

Figure 4. Effect of dexamethasone (Dex; 10^{-6}M) on inhibition of ACh-induced bronchial contractile tension in both air-exposed C57/BL6 mice (**Panel A**, n=11) and MKP-$1^{-/-}$ mice (**Panel B**, n=11) and in ozone-exposed C57/BL6 mice (**Panel C**, n=9) and MKP-$1^{-/-}$ mice (**Panel D**, n=9). Data points are expressed as mean ±S.E.M. *p<0.05, **p<0.01, compared with untreated ones.

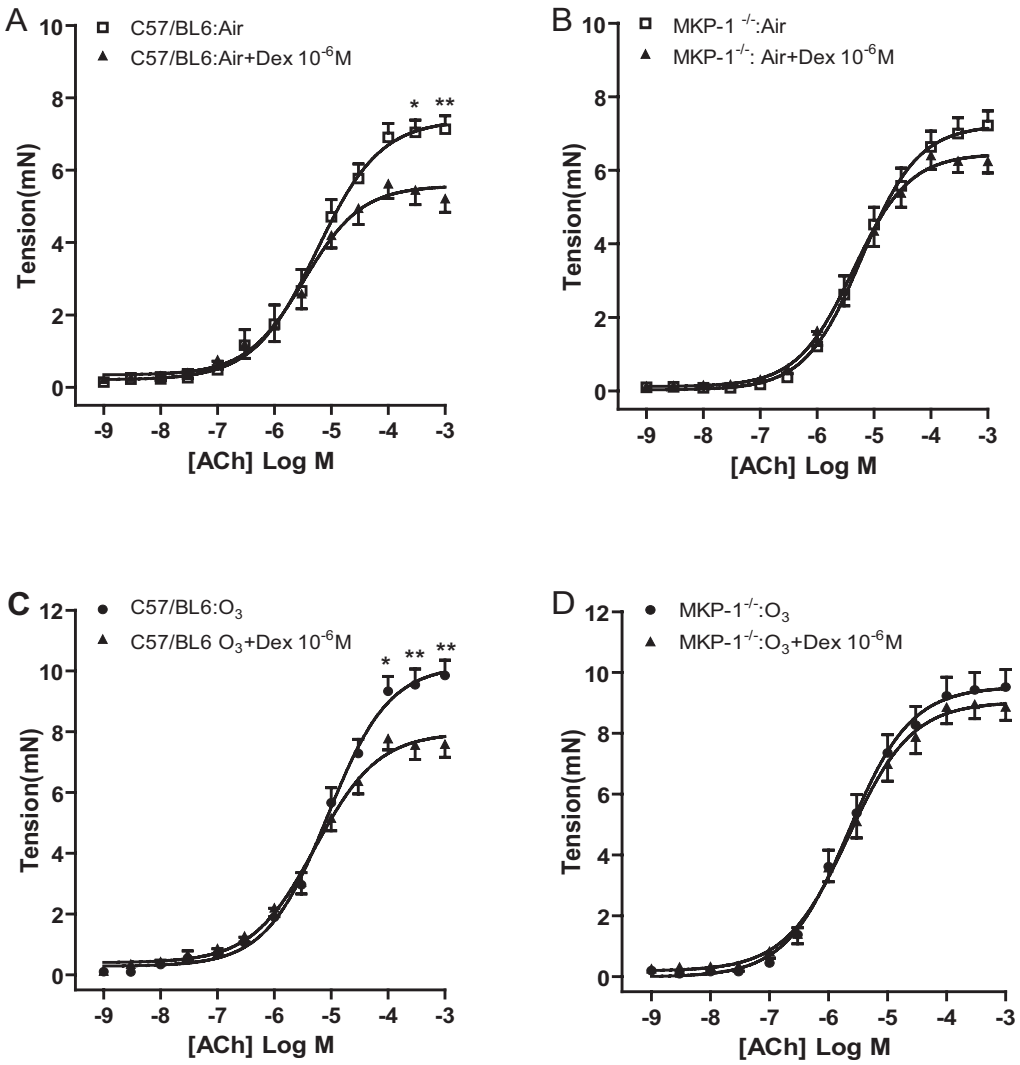

Figure 5. Western blot analysis of ratio of phosphorylated p-p38 MAPK to non-phosphoryated p38 MAPK (**Panels A & B**) and of ratio of phosphorylated HSP27 to non-phosphoryated HSP27 (**Panels C & D**) in bronchial

preparations which were un-stimulated (con), ACh-stimulated and pre-treated with SB 239063 (10^{-6}M) or dexamethasone (Dex; 10^{-6}M) from air-exposed C57/BL6 and MKP-$1^{-/-}$ mice. Each panel shows representative Western blots of phosphorylated p38 MAPK and non-phosphoryated p38 MAPK, and phosphorylated HSP27 to non-phosphoryated HSP27, with individual results of n=6-7 in each group. *p<0.05, **p<0.01, compared with ACh-stimulated ones.

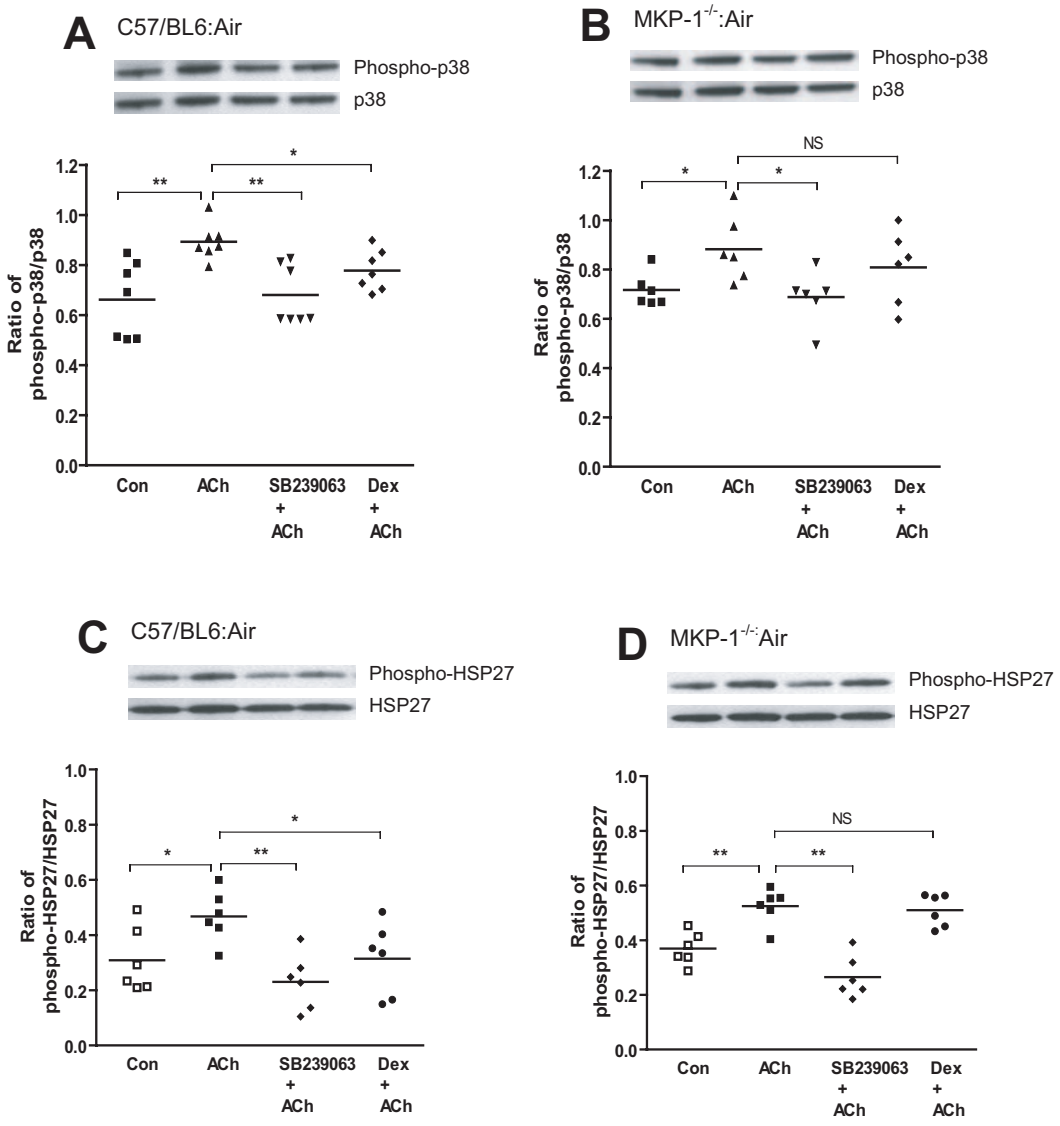

Fig 5

Figure 6. Western blot analysis of ratio of phosphorylated p-p38 MAPK to non-phosphoryated p38 MAPK (**Panels A & B**) and of ratio of phosphorylated HSP27 to non-phosphoryated HSP27 (**Panels C & D**) in bronchial preparations which were un-stimulated (con), ACh-stimulated and pre-treated with SB 239063 (10^{-6}M) or dexamethasone (Dex; 10^{-6}M) from ozone-exposed C57/BL6 and MKP-$1^{-/-}$ mice. Each panel shows representative Western blots of phosphorylated HSP27 and non-phosphoryated HSP27, and phosphorylated HSP27 to non-phosphoryated HSP27, with individual results of n=6-7 in each group. *$p<0.05$, **$p<0.01$, compared with ACh-stimulated ones.

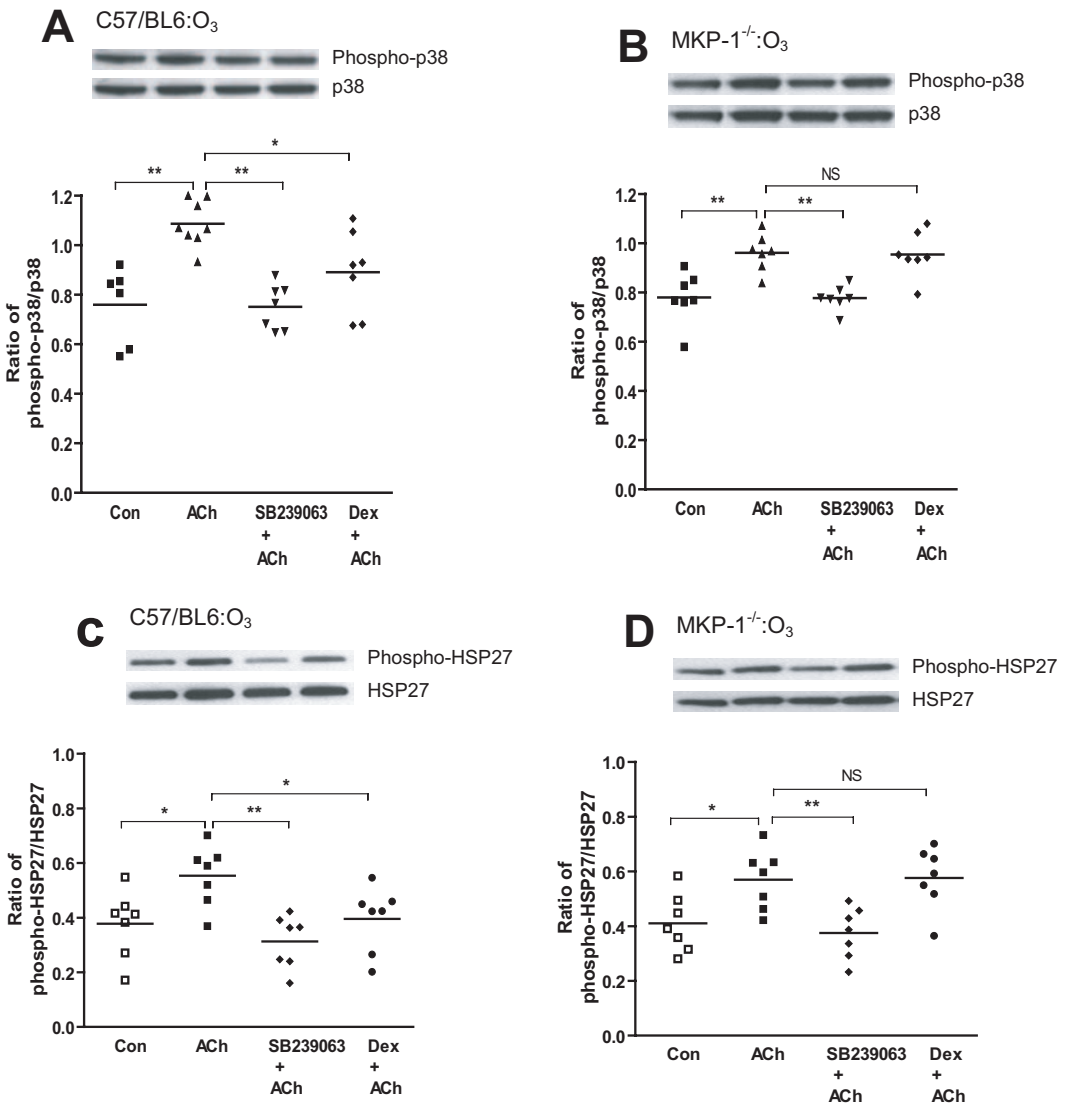

Fig 6

Figure 7. Panel A: Representative Western blot analysis of MKP-1 and α-tubulin under control conditions, after addition of acetylcholine (10^{-3}M), and after acetylcholine (10^{-3}M) and dexamethasone (10^{-6}M) to bronchial tissues from air-exposed mice. Lower part of panel shows the individual data-points of the expression of MKP-1 and α-tubulin measured as densitometric ratio of

MKP-1 to α-tubulin in bronchial tissues. **Panel B**: Similar data is shown for bronchial tissues from ozone-exposed mice.

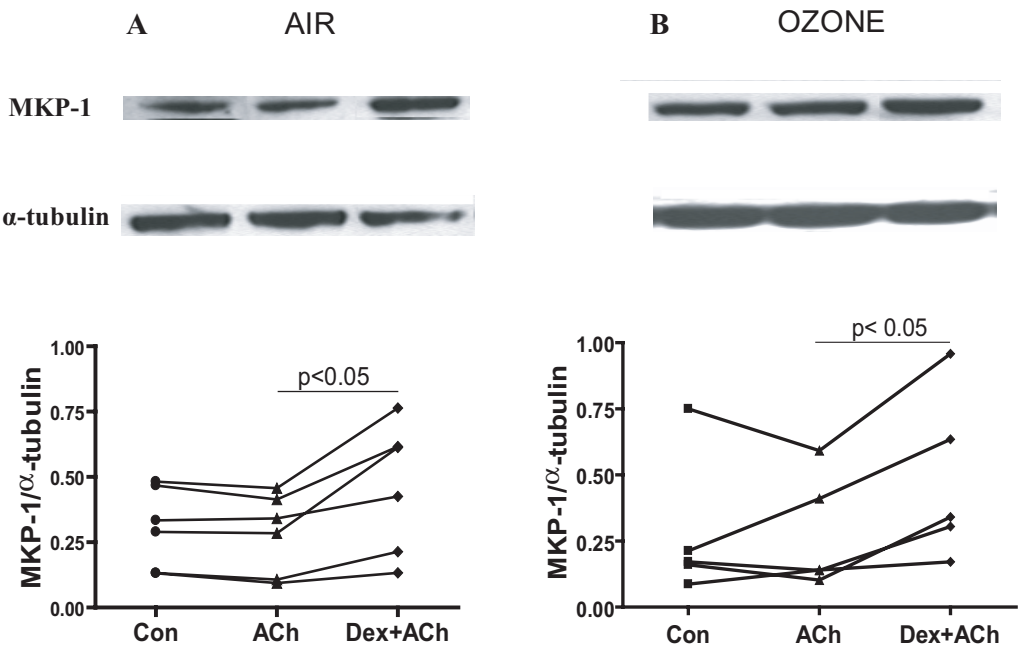

Fig 7