

Proasthmatic effects and mechanisms of action of the dust mite allergen, Der p 1, in airway smooth muscle

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Background: House dust mite allergen exposure is a key risk factor for the development of allergic asthma. Beyond provoking immune cell-mediated allergic responses, house dust mite allergens were recently shown to exert direct effects on airway structural cells secondary to their intrinsic protease activities.

Objective: This study tested the hypothesis that house dust mite allergen exposure can produce changes in airway responsiveness through a direct effect on airway smooth muscle (ASM).

Methods: Isolated rabbit ASM tissues were exposed to the house dust mite allergen, Der p 1, and induced changes in ASM responsiveness and activation of mitogen-activated protein kinase (MAPK) signaling pathways were examined under different experimental conditions.

Results: The observations demonstrated the following:

(1) Der p 1 exposure elicited enhanced constrictor responses and impaired relaxation responses in the ASM tissues, (2) these proasthmatic-like effects of Der p 1 were attributed to its intrinsic cysteine protease activity, and (3) the induced changes in ASM responsiveness were associated with activation of both the extracellular signal-regulated kinase (ERK) 1/2 and the p38 MAPK signaling pathways.

Additionally, specific blockade of ERK1/2 signaling was found to prevent the Der p 1-induced changes in ASM responsiveness, whereas inhibition of p38 MAPK signaling enhanced the proasthmatic-like action of Der p 1, with the latter effect a result of augmented activation of ERK1/2.

Conclusion: These findings are the first to demonstrate that the dust mite allergen, Der p 1, can directly elicit changes in ASM responsiveness that are associated with activation of MAPK signaling, wherein proasthmatic effects induced by Der p 1 are attributed to activation of ERK1/2, whereas coactivation of p38 MAPK exerts a homeostatic action by negatively regulating ERK1/2 signaling. (*J Allergy Clin Immunol* 2005;116:94-101.)

Key words: Asthma, dust mite allergen, airway smooth muscle, MAP kinases, airway hyperresponsiveness, cysteine protease

Abbreviations used

ASM: Airway smooth muscle
ERK: Extracellular signal-regulated kinase
JNK: c-Jun N-terminal kinase
MAPK: Mitogen-activated protein kinase
MEK: Mitogen-activated protein kinase kinase
PAR: Protease-activating receptor
Rmax: Maximal relaxation
Tmax: Maximal isometric contractile force

Pulmonary exposure to aeroallergens is a key risk factor for the development of allergic airway sensitization and asthma. Although the airway mucosal barrier is specialized in preventing the ingress of foreign proteins, many aeroallergens possess intrinsic protease activity that can disrupt the integrity of the airway epithelium.¹⁻⁵ In this respect, the cysteine protease activity of the dust mite allergen, *Dermatophagoides pteronyssinus* 1 (Der p 1), was found to disrupt the epithelial cell tight junction proteins, ZO-1 and desmoplakin,⁶ thereby likely accounting for reported Der p 1-induced changes in airway microvascular permeability.⁶⁻⁸ Moreover, Der p 1 was found to induce proinflammatory cytokine and chemokine release from respiratory epithelial cells.^{3,5} Given this evidence, together with the important role attributed to dust mite allergens in the development of asthma,⁹⁻¹¹ a disease characterized by altered airway constrictor and relaxant responsiveness, the current study examined whether dust mite allergen also exerts a direct action on airway smooth muscle (ASM) and, hence, potentially contributes to the induction of the proasthmatic phenotype of altered airway responsiveness. The results provide new evidence demonstrating the following: (1) isolated rabbit ASM tissues directly exposed to Der p 1 exhibit altered agonist-mediated constrictor and relaxant responsiveness; (2) these induced proasthmatic-like changes in ASM function are regulated by concomitant activation of the extracellular signal-regulated kinase (ERK) 1/2 and p38 mitogen-activated protein kinase (MAPK) signaling pathways in the Der p 1-exposed ASM; and (3) the latter kinases exert opposing regulatory actions, wherein ERK1/2 activation mediates the Der p 1-induced changes in ASM function, whereas p38 MAPK activation serves a homeostatic role by negatively regulating ERK1/2 activation, thereby limiting its proasthmatic effects. Taken together,

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these findings support the novel concept that exposure to house dust mite allergens can elicit proasthmatic-like changes in airway function that are attributed, at least in part, to a direct action of the allergen on ASM, with the latter resulting in activation of MAPK-dependent signaling mechanisms that regulate ASM responsiveness.

METHODS

Animals

Thirty-four adult New Zealand White rabbits were used in this study, which was approved by the Biosafety and Animal Research Committee of the Joseph Stokes Research Institute at Children's Hospital of Philadelphia. The animals had no signs of respiratory disease for several weeks before the study, and their care and use were in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council.

Preparation and Der p 1 treatment of rabbit ASM tissues

After general anesthesia with xylazine (10 mg/kg) and ketamine (50 mg/kg), rabbits were killed with an overdose of pentobarbital (125 mg/kg). As described previously,¹² the tracheae were removed via open thoracotomy, the loose connective tissue and epithelium were carefully scraped and removed, and the tracheae were divided into ring segments. Each alternate ring was incubated for 24 hours at room temperature in the presence of either vehicle (saline) alone (control), vehicle containing cysteine (5 mmol/L), or varying concentrations of affinity purified Der p 1 allergen (0.1, 0.5, 1.0, 5.0 $\mu\text{g/mL}$) both in the absence and presence of cysteine, because addition of the latter is required to restore the cysteine protease activity of the purified Der p 1. In parallel experiments, 30 minutes before incubation in control or Der p 1-containing medium, ASM segments were treated with the specific cysteine protease inhibitor, E-64 (*trans*-epoxysuccinyl-L-leucyl-amido [4-guanidino]-butane; 1 $\mu\text{mol/L}$); the serine protease inhibitor, leupeptin (1 $\mu\text{mol/L}$); the MAPK kinase (MEK) 1/2-specific inhibitors, U0126 (5 $\mu\text{mol/L}$) or PD98059 (10 $\mu\text{mol/L}$); or the specific p38 MAPK inhibitors, SB202190 (10 $\mu\text{mol/L}$) or SB203580 (10 $\mu\text{mol/L}$). Finally, in separate studies, 1 hour before incubation in control or Der p 1-containing medium, ASM tissues were treated with the potent activator of p38 MAPK, anisomycin, using a concentration (ie, 50 ng/mL) that is significantly less than that required to prevent the known protein translational inhibitory effect of anisomycin.¹³

Pharmacodynamic studies of ASM responsiveness

After incubation, the ASM segments were placed in organ baths containing modified Krebs-Ringer solution aerated with 5% CO_2 in oxygen (pH, 7.35–7.40), and the tissues were attached to force transducers from which isometric tension was continuously monitored on a multichannel recorder, as previously described.¹⁴ Cholinergic contractility was then assessed in the ASM segments by cumulative administration of acetylcholine (10^{-10} to 10^{-3} mol/L). Thereafter, the tissues were repeatedly rinsed with fresh buffer, and subsequent relaxation responses to isoproterenol (10^{-10} to 10^{-4} mol/L) were generated after the tissues were half-maximally contracted with acetylcholine. The constrictor dose-response curves to acetylcholine were analyzed in terms of each tissue's maximal isometric contractile force (T_{max}) to the agonist, the relaxation responses to isoproterenol were assessed in terms of percent maximal relaxation (R_{max}) from the initial level of induced cholinergic contraction, and sensitivity to

the relaxing agent was determined as the corresponding pD50 value (ie, geometric mean ED50 value) associated with 50% of R_{max} .

Immunoblot analysis of MAPK activation

Protein levels of phosphorylated and total ERK1/2 and p38 MAPK were assessed by Western blot analysis of whole cell lysates isolated from rabbit ASM tissues after treatment with either vehicle alone or cysteine-activated Der p 1 (1 $\mu\text{g/mL}$), both in the absence and presence of E-64, SB202190, or anisomycin. The tissues were homogenized and proteins extracted in a buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA (pH 7.4) with 1 mmol/L phenylmethylsulfonyl fluoride, 5 $\mu\text{g/mL}$ aprotinin, and 5 $\mu\text{g/mL}$ leupeptin. After removal of insoluble debris by centrifugation, gel-loading buffer was added to the supernatants, and extracts were then loaded (using 30 μg total protein/sample) on a 10% SDS-PAGE gel for immunoblotting after transferring to a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk and then incubated overnight with monoclonal mouse antihuman phosphotyrosine-threonine (Thr180/Tyr182)-p38 MAPK and p38 MAPK, and antihuman phosphotyrosine-threonine (Thr202/Tyr204)-p44/42 (ERK1/2) and p44/42 primary antibodies. The kinase levels were detected by using enhanced chemiluminescence after a 1-hour incubation with a 1:1000 dilution of horseradish peroxidase-conjugated goat-antimouse secondary antibody, followed by exposure to autoradiography film. Protein band intensities were quantified by using AlphaImager (Alpha Innotech Corp, San Leandro, Calif), and the data were normalized relative to those obtained in untreated ASM samples.

Statistical analysis

The results are expressed as means $\pm$ SE values. Comparisons between groups were made by using the Student *t* test (2-tailed) or ANOVA with the Tukey posttest analysis, where appropriate. A probability of $<.05$ was considered statistically significant. Statistical analyses were conducted by using the Prism computer program by GraphPad Software Inc (San Diego, Calif).

Reagents

All chemicals were purchased from Sigma (St Louis, Mo) unless otherwise indicated. Der p 1 was obtained from Indoor Biotechnologies (Charlottesville, Va). The MEK and p38 MAPK inhibitors were purchased from EMD Biosciences Inc (San Diego, Calif). The primary antibodies for Western blotting were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif), and the enhanced chemiluminescence reagents and goat-antimouse secondary antibody were from Amersham Pharmacia Biotech (Piscataway, NJ). All drug concentrations are expressed as final bath concentrations. Isoproterenol and acetylcholine were made fresh for each experiment and were dissolved in normal saline to prepare 10^{-3} mol/L stock solutions.

RESULTS

Effects of Der p 1 on rabbit ASM responsiveness

To assess the effects of Der p 1 on ASM responsiveness, agonist-mediated constrictor and relaxation responses were compared in control and Der p 1-exposed isolated rabbit ASM segments, both in the absence and presence of E-64 (1 $\mu\text{mol/L}$). As shown in Fig 1, relative to control tissues, ASM segments exposed to a maximally effective dose of Der p 1 (ie, 1 $\mu\text{g/mL}$) exhibited increased constrictor responses to exogenously administered acetylcholine. Accordingly, relative to the mean $\pm$ SE maximal

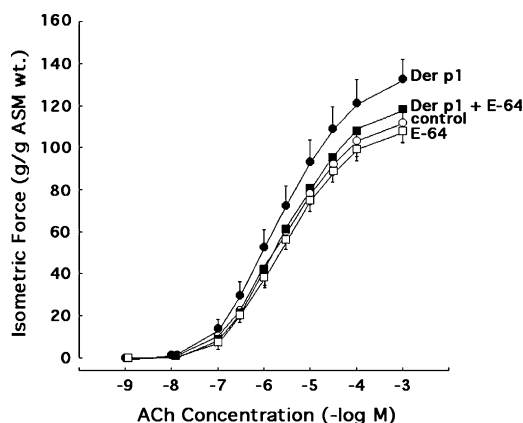


FIG 1. Comparison of constrictor responses to acetylcholine (ACh) in rabbit ASM tissues exposed for 24 hours to Der p 1 in the absence and presence of E-64. Data represent means $\pm$ SE values from 8 experiments.

constrictor response (Tmax) obtained in control ASM, which amounted to 111.4 ± 7.4 g/g ASM weight (wt.), the mean Tmax response was significantly increased at 133.7 ± 8.4 g/g ASM wt. in the Der p 1-treated ASM tissues ($P < .05$). This increased constrictor responsiveness to acetylcholine was largely abrogated in Der p 1-exposed ASM pretreated with E-64, whereas E-64 had no effect on the constrictor responses to acetylcholine in tissues that were not exposed to Der p 1.

Under the same treatment conditions, during subsequent sustained half-maximal contraction with acetylcholine, administration of the β -adrenoceptor agonist, isoproterenol, produced cumulative dose-dependent relaxation of the precontracted ASM segments. As depicted in Fig 2, relative to control ASM, the relaxation responses to isoproterenol were significantly attenuated in the Der p 1-exposed ASM. Accordingly, the mean $\pm$ SE Rmax values obtained in the Der p 1-treated tissues and control ASM tissues amounted to $37.9\% \pm 4.9\%$ and $57.4\% \pm 4.0\%$, respectively ($P < .01$), and the corresponding sensitivities to isoproterenol (ie, pD50 values) averaged 5.99 ± 0.04 and 6.22 ± 0.03 -log M, respectively ($P < .05$). This impaired relaxation responsiveness to isoproterenol was prevented in Der p 1-exposed tissues pretreated with E-64, whereas E-64 had no effect in tissues that were not exposed to Der p 1.

In relation to these observations, it should be noted that, in comparable experiments conducted on ASM tissues exposed to Der p 1 that were not preactivated with cysteine (ie, required to restore the cysteine protease activity of the purified allergen), we found no effect of the inactivated Der p 1 on the tissues' constrictor or relaxant responsiveness (data not shown). Moreover, contrasting these effects of cysteine protease inhibition with E-64, we found that the induced changes in constrictor and relaxant responsiveness elicited in ASM exposed to cysteine-activated Der p 1 were unaffected by pretreating the tissues with the serine protease inhibitor, leupeptin ($1 \mu\text{mol/L}$; data not shown).

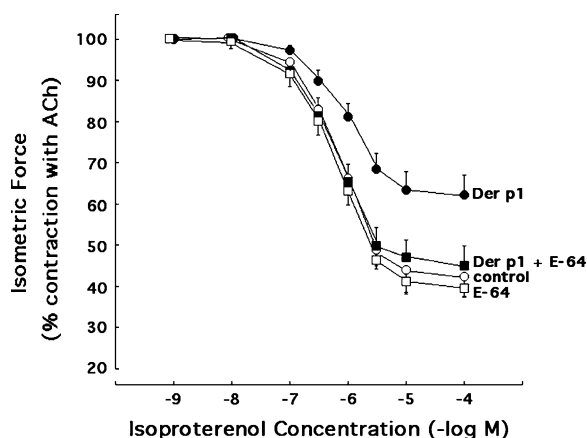


FIG 2. Comparison of relaxation responses to isoproterenol in rabbit ASM tissues exposed for 24 hours to Der p 1 in the absence and presence of E-64. Data represent means $\pm$ SE values from 8 experiments. ACh, Acetylcholine.

MAPK activation in Der p 1-exposed ASM

Previous studies have implicated activation of MAPKs in mediating the changes in airway responsiveness in animal models of allergic asthma^{15,16} and the induced release of various cytokines and chemokines by stimulated ASM cells.¹⁷⁻²¹ Given this evidence, we examined the role of MAPK activation in regulating these Der p 1-induced changes in ASM responsiveness. MAPK activation was assessed by Western blot analysis, using antibodies specific for the phosphorylated forms of ERK1/2 and p38 MAPKs, as well as antibodies specific for total ERK and p38 proteins. As shown in Fig 3, A (left panel), enhanced expression of phosphorylated ERK1/2 was detected as early as 15 minutes, with progressively increased expression for as long as 60 minutes after Der p 1 exposure. Enhanced expression of phosphorylated p38 MAPK appeared within 5 minutes, and was transiently increased for as long as 30 minutes thereafter, but was distinctly reduced by 60 minutes after Der p 1 exposure (Fig 3, B, left panel). On the basis of densitometric analysis of the results obtained in 4 experiments, the magnitudes of maximal enhanced expression of phosphorylated ERK1/2 and p38 MAPK detected in Der p 1-exposed tissues averaged 5.8-fold and 3.2-fold above the corresponding basal (pretreatment) levels, respectively. Contrasting these observations, the levels of neither phosphorylated ERK1/2 nor p38 MAPK were appreciably altered in response to Der p 1 exposure in tissues that were pretreated with E-64 (Fig 3, A and B, right panels). Moreover, neither total ERK nor p38 MAPK protein levels were systematically affected by Der p 1 exposure in either the absence or presence of E-64.

Role of MAPKs in regulating Der p 1-induced changes in ASM responsiveness

In light of these results, we next investigated the potential regulatory roles of MAPK activation by assessing the effects of selective inhibitors of the MEK-ERK1/2 and p38 MAPK pathways on Der p 1-induced changes in

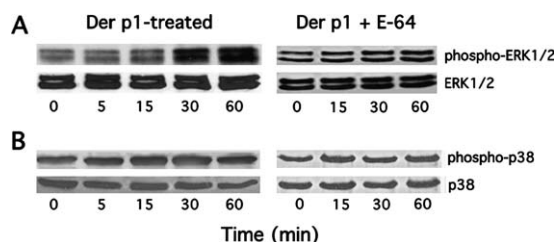


FIG 3. Der p 1–induced activation of ERK1/2 and p38 MAPK in rabbit ASM tissues in the absence and presence of the cysteine protease inhibitor, E-64. Tissue lysates were prepared at the indicated time points for immunoblotting using antibodies specific for phosphorylated and total ERK1/2 and p38 MAPK (see Methods).

ASM responsiveness. As shown in Fig 4, relative to control tissues, ASM exposed to Der p 1 exhibited enhanced maximal constrictor responses to acetylcholine, providing a mean $\pm$ SE Tmax value of 123.8 ± 6.5 g/g ASM wt., relative to the value of 109.9 ± 8.7 g/g ASM wt. obtained in control ASM ($P < .05$). Interestingly, this enhanced constrictor responsiveness was further augmented in Der p 1–exposed tissues that were pretreated with the specific p38 MAPK inhibitor, SB202190 (10 μ mol/L), yielding an average Tmax value (ie, 136.2 ± 6.2 g/g ASM wt.) that was significantly greater ($P < .05$) than that obtained in the Der p 1–exposed tissues not receiving the inhibitor. In marked contrast with the latter effect of p38 MAPK inhibition, the changes in ASM constrictor responsiveness to acetylcholine were completely abrogated in Der p 1–exposed tissues pretreated with the specific MEK1/2 inhibitor, U0126 (5 μ mol/L), providing Tmax values similar to those generated in control ASM segments.

In parallel with these observations, relative to control tissues, the relaxation responses to isoproterenol were significantly attenuated in Der p 1–exposed ASM tissues (Fig 5), providing Rmax values that averaged $48.8\% \pm 4.7\%$, compared with the mean Rmax of $62.6\% \pm 4.6\%$ obtained in control tissues ($P < .05$). This attenuated relaxant responsiveness was further significantly impaired ($P < .05$) in Der p 1–exposed tissues pretreated with SB202190, wherein the mean Rmax amounted to $37.6\% \pm 7.1\%$ ($P < .01$). By comparison, the reduced relaxation responsiveness to isoproterenol was largely ablated in Der p 1–exposed ASM tissues pretreated with U0126, providing Rmax values that were not significantly different from those obtained in control ASM segments.

As with the above inhibitors, in separate experiments we found qualitatively comparable opposing effects of p38 and ERK1/2 inhibition on ASM responsiveness in Der p 1–exposed tissues pretreated with other selective MAPK inhibitors, including the p38 MAPK inhibitor, SB203580, and the MEK-ERK1/2 inhibitor, PD98059 (data not shown). Moreover, contrasting with their effects in Der p 1–exposed tissues, neither of these inhibitors produced a significant change in ASM constrictor or relaxant responsiveness when administered to tissues that were not exposed to Der p 1 (data not shown).

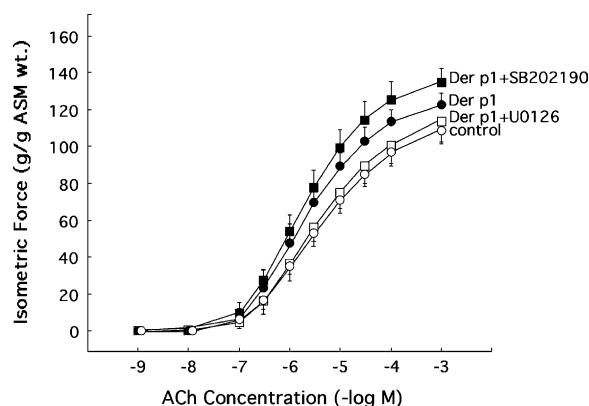


FIG 4. Comparison of constrictor responses to acetylcholine (ACh) in rabbit ASM tissues exposed for 24 hours to Der p 1 in the absence and presence of SB202190 or U0126. Data represent means $\pm$ SE values.

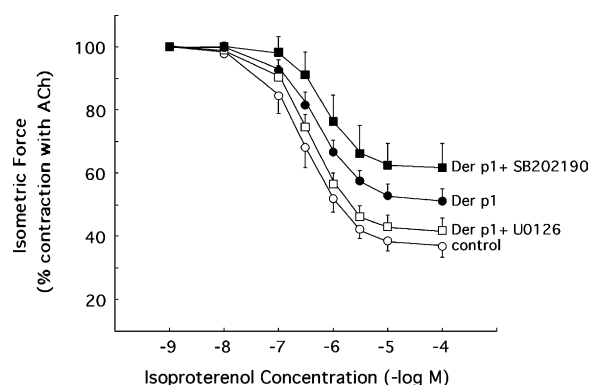


FIG 5. Comparison of relaxation responses to isoproterenol in rabbit ASM tissues exposed for 24 hours to Der p 1 in the absence and presence of SB202190 or U0126. Data represent means $\pm$ SE values. ACh, Acetylcholine.

Modulatory effect of p38 MAPK on ERK1/2 activation in Der p 1–exposed ASM

These observations suggested that the Der p 1–induced changes in ASM responsiveness were mediated by activation of the MEK-ERK1/2 signaling pathway, whereas concomitant activation of p38 MAPK exerted an opposing regulatory effect. This consideration appeared consistent with the findings in recent reports that demonstrated a negative regulatory interaction between the ERK1/2 and p38 MAPK signaling pathways, wherein p38 MAPK activation was found to inhibit ERK1/2 signaling²² and, conversely, inhibition of p38 MAPK was shown to activate ERK1/2.^{23–26} To address the latter mechanism herein, we examined the separate effects of inhibition and stimulation of p38 MAPK on Der p 1–induced activation of ERK1/2. As exemplified by the Western blots depicted in Fig 6 (top panel), relative to tissues that were not pretreated with the p38 MAPK inhibitor, Der p 1–exposed ASM tissues pretreated with SB202190 displayed distinctly enhanced time-related

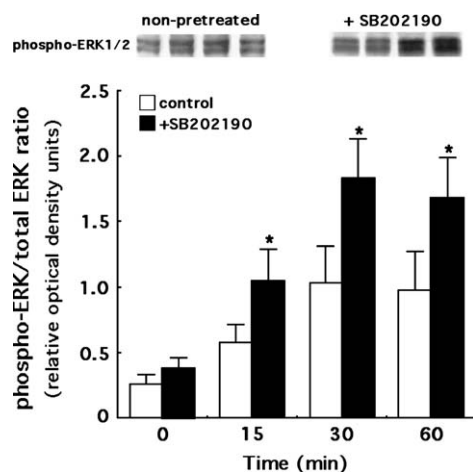


FIG 6. Inhibition of p38 MAPK with SB202190 enhances Der p 1–induced ERK1/2 phosphorylation. *Upper panel*, Representative Western blots depicting Der p 1–induced temporal changes in phosphorylated ERK1/2 expression in control (nonpretreated) and SB202190-pretreated tissues. *Lower panel*, Scanned densitometric results in which the intensity of each phosphorylated ERK signal is normalized to its corresponding total ERK signal. Data represent means $\pm$ SE values from 3 experiments (* $P < .05$).

expression of phosphorylated ERK1/2. Total ERK protein levels were not systematically affected by SB202190, and hence, the time-specific ratios of the levels of phosphorylated to total ERK protein were significantly increased in the Der p 1–exposed tissues pretreated with SB202190 (Fig 6, *bottom panel*). These observations implicating a negative regulatory effect of p38 MAPK on Der p 1–induced ERK1/2 activation were further substantiated in separate experiments wherein the effects of anisomycin, a potent activator of p38 MAPK, were compared in control and Der p 1–exposed ASM tissues. As shown by the immunoblots in Fig 7, relative to control (unstimulated) ASM, tissues exposed for 30 minutes to either anisomycin (50 ng/mL), Der p 1 alone, or both in combination exhibited increased expression of phosphorylated p38 MAPK. Correspondingly, treatment with anisomycin had essentially no effect on phosphorylated ERK1/2 expression in unstimulated ASM; however, the induced enhanced expression of phosphorylated ERK1/2 exhibited by Der p 1–exposed ASM was largely abrogated when Der p 1–exposed tissues were cotreated with anisomycin. Of note, the levels of neither total p38 MAPK nor ERK1/2 protein were systematically altered by these different treatment conditions (data not shown). Thus, taken together, these observations support the concept that activation of p38 MAPK in Der p 1–exposed ASM serves, at least in part, to limit the magnitude of Der p 1–stimulated coactivation of ERK1/2.

Effect of p38 MAPK activation on Der p 1–induced changes in ASM responsiveness

Given these results, we next examined whether the negative regulatory effect of p38 MAPK on ERK1/2 activation is reflected by a protective action of p38 MAPK

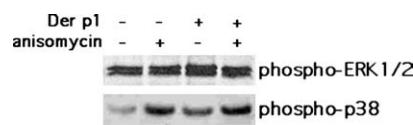


FIG 7. Activation of p38 MAPK with anisomycin inhibits Der p 1–induced enhanced phosphorylation of ERK1/2. Lysates for immunoblotting were prepared by using control (unstimulated) ASM and tissues treated with anisomycin or Der p 1 alone and in combination.

activation on Der p 1–induced changes in ASM responsiveness. Accordingly, constrictor and relaxation responses were compared in control and Der p 1–exposed tissues in both the absence and presence of pretreatment of the tissues with anisomycin (50 ng/mL). As depicted in Fig 8, relative to control ASM segments (*open circles*), Der p 1–exposed tissues exhibited increased constrictor responses to acetylcholine (*filled circles*), wherein the mean $\pm$ SE Tmax value amounted to 119.0 ± 8.2 g/g ASM wt., compared with the corresponding value of 107.4 ± 6.6 g/g ASM wt. obtained in the control ASM ($P < .05$). This enhanced constrictor responsiveness to acetylcholine was completely abrogated in Der p 1–exposed ASM tissues pretreated with anisomycin (*filled squares*), whereas anisomycin had no effect in control (vehicle-exposed) ASM segments (*open squares*). In addition, the impaired relaxation responsiveness to isoproterenol exhibited in Der p 1–exposed ASM segments was also largely prevented in tissues pretreated with anisomycin, whereas anisomycin had no significant effect in control ASM (Fig 9). Furthermore, in relation to these observations, given that anisomycin is known to activate both p38 MAPK and the stress-activated protein kinase, c-Jun N-terminal kinase (JNK),¹³ in extended experiments, we examined whether the effects of anisomycin on Der p 1–induced changes in ASM responsiveness were attributed, at least in part, to coactivation of JNK. The results demonstrated that the protective effect of anisomycin on Der p 1–induced changes in ASM responsiveness was not appreciably affected in Der p 1–exposed tissues copretreated with the JNK inhibitor, SP600125 (1 μ mol/L; data not shown).

DISCUSSION

To our knowledge, the results of the current study are the first to demonstrate that the ubiquitous aeroallergen, Der p 1, can directly exert proasthmatic-like effects on ASM function that are characterized by enhanced agonist-mediated ASM contractility and impaired β -adrenoceptor-mediated ASM relaxation. Our observed actions of purified Der p 1 on ASM function were attributed to its intrinsic cysteine protease activity, because the induced changes in ASM responsiveness were largely prevented in Der p 1–exposed ASM cells and tissues pretreated with the cysteine protease inhibitor, E-64. Additionally, the specificity of action of Der p 1 as a cysteine protease was substantiated by the finding that ASM responsiveness was

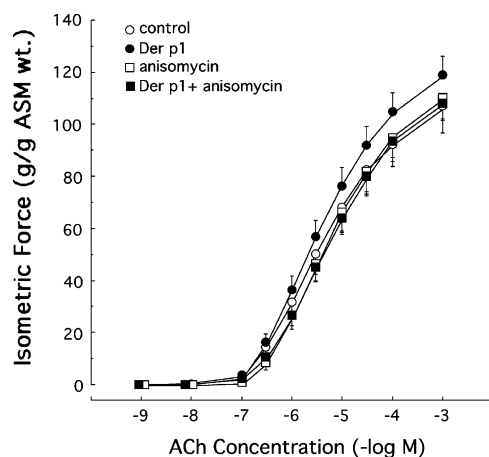


FIG 8. Comparison of constrictor responses to acetylcholine (ACh) in rabbit ASM tissues exposed for 24 hours to Der p 1, both in the absence and presence of pretreatment of the tissues with anisomycin (50 ng/mL). Data represent means $\pm$ SE values.

unaltered in tissues exposed to Der p 1 that was not preactivated with cysteine; cysteine is required to restore the cysteine protease activity of the purified allergen. In relation to these observations, it is relevant to note that the reported cellular responses to the proteolytic activities of Der p 1 and other dust mite allergens have been attributed to activation of 1 or more members of a group cell surface receptors comprising the protease-activating receptor (PAR) family. Cell surface expression of PARs has been identified in the airways of different animal species, including human beings,^{27,28} and among the PARs, particular attention has been given to PAR-2, given that this receptor subtype has been implicated in mediating the evoked release of cytokines from respiratory epithelial cells exposed to Der p 1 and other Der p allergens,^{5,29} as well as to cockroach extract, which is associated with ERK activation.³⁰ In addition, PAR-2 activation has been shown to induce the release of COX products from epithelial and ASM cells and to provoke enhanced contractility in isolated human bronchial preparations.³¹⁻³⁶ These earlier findings suggest that the effects observed here of Der p 1 in ASM were likely caused by PAR-2 activation, although the involvement of PAR-2 and/or other PARs in mediating our observed effects of Der p 1 on ASM responsiveness remains to be investigated systematically.

Recent evidence implicates a crucial role for activation of the MAPK signaling cascade in regulating cytokine synthesis by stimulated ASM cells, most notably including the ERK1/2 and p38 MAPK pathways.¹⁷⁻²¹ Our current observations extend these earlier findings by demonstrating that the induced changes in ASM responsiveness in Der p 1-exposed ASM are also associated with activation of both the MEK-ERK1/2 and p38 MAPK pathways (Fig 3). Moreover, in examining the roles of these signaling pathways, we found that pretreatment with a MEK1/2 inhibitor, U0126 or PD98059, largely prevented the Der p 1-induced changes in ASM constrictor and relaxant responsiveness (Figs 4 and 5). These

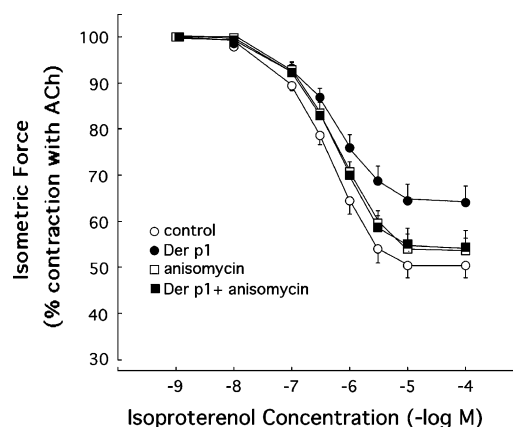


FIG 9. Comparison of relaxation responses to isoproterenol in rabbit ASM tissues exposed for 24 hours to Der p 1, both in the absence and presence of pretreatment of the tissues with anisomycin. Data represent means $\pm$ SE values. ACh, Acetylcholine.

observations fundamentally agree with those reported in recent studies that have demonstrated an important role for ERK1/2 activation in mediating the *in vitro* changes in bronchial contractility observed in ovalbumin-sensitized guinea pigs,¹⁵ as well the *in vivo* airway hyperresponsiveness seen in ovalbumin-sensitized mice.¹⁶ In the latter study, treatment with the MEK1/2 inhibitor, U0126, was found to inhibit significantly the airway hyperresponsiveness and elevated intrapulmonary eosinophil counts and cytokine levels in ovalbumin-sensitized mice.¹⁶

Contrasting with the role of ERK activation, our current observations demonstrated that coactivation of p38 MAPK exerts a homeostatic (ie, protective) action in Der p 1-exposed ASM by attenuating the proasthmatic effects mediated by ERK1/2 activation. Accordingly, we found that, along with induced potentiation of Der p 1-induced changes in ASM responsiveness (Figs 4 and 5), inhibition of p38 MAPK elicited enhanced expression of phosphorylated ERK1/2 in Der p 1-exposed ASM (Fig 6). The latter finding concurs with earlier observations made in other cell systems wherein inhibition of p38 MAPK was also found to enhance ERK1/2 phosphorylation and thereby augment ERK1/2-mediated cellular responses.²³⁻²⁶ These previous findings, together with our current observations, are consistent with the concept that p38 MAPK negatively regulates the ERK1/2 signaling pathway. Supporting this concept, a direct 1-way cross-talk between p38 MAPK and ERK1/2 was recently demonstrated, wherein phosphorylated p38 α was found to couple with ERK1/2 and thereby sterically block ERK1/2 phosphorylation by MEK1/2,²² and possibly also act via a protein kinase that lies upstream of MEK1/2.²³ Our results herein provide evidence that concurs with this cross-talk mechanism, given the observations that activation of p38 MAPK with anisomycin largely prevented both the Der p 1-induced enhanced phosphorylation of ERK1/2 (Fig 7) and changes in ASM responsiveness (Figs 8 and 9), whereas anisomycin had no effect in control ASM

preparations. These findings concur with those previously reported using HL-60 leukemia cells showing that anisomycin inhibits stimulated ERK activity but has no effect on basal ERK activity in unstimulated cells.³⁷

In evaluating the findings of the current study, it must be emphasized that the results pertain to experiments conducted using isolated rabbit ASM tissues. Thus, the extent to which our observations pertain to the *in vivo* state, and the applicability of our findings to the human condition, are open to speculation. In this regard, it is relevant to note that our observed changes in constrictor and relaxant responsiveness in the Der p 1–exposed rabbit ASM tissues mimicked the perturbations in airway function that characterize the human asthmatic phenotype, including enhanced bronchoconstrictor responsiveness and impaired β -adrenoceptor–mediated airway relaxation.^{38,39} Although this consideration and the well established role of dust mite allergen exposure in the development of asthma support the notion that our current observations may be relevant to the human *in vivo* condition, it remains to be established whether Der p 1 exposure directly elicits comparable changes in responsiveness in isolated human ASM tissues and altered airway responsiveness in the *in vivo* state.

In conclusion, the current study investigated the role and mechanism of action of the dust mite allergen, Der p 1, in regulating ASM responsiveness. The results provided new evidence demonstrating the following: (1) Der p 1 exposure provokes proasthmatic-like changes in constrictor and relaxant responsiveness in isolated rabbit ASM tissues; (2) these effects of Der p 1 are attributed to its intrinsic cysteine protease activity, which elicits activation of both the ERK1/2 and p38 MAPK intracellular signaling pathways; (3) activation of ERK1/2 is responsible for mediating the Der p 1–induced changes in ASM responsiveness; and (4) contrasting this effect, coactivation of p38 MAPK serves to limit homeostatically the magnitude of Der p 1–induced changes in ASM responsiveness by downregulating ERK1/2 activity and, hence, its proasthmatic-like effects. Collectively, these observations provide new information demonstrating that exposure to the dust mite allergen, Der p 1, can directly elicit proasthmatic-like changes in ASM function. Accordingly, the findings support the novel concept that ASM may play an important innate role in contributing to the acquisition of dust mite allergen–induced airway sensitization and asthma.

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