

A murine model of toluene diisocyanate-induced asthma can be treated with matrix metalloproteinase inhibitor

Yong Chul Lee, MD, PhD,^a Chang Ho Song, MD, PhD,^b Heung Bum Lee, MD, PhD,^a
Jong-Lark Oh,^c Yang Keun Rhee, MD, PhD,^a Hae Sim Park, MD, PhD,^d and
Gou Young Koh, MD, PhD^c *Chonju and Suwon, South Korea*

Background: Toluene diisocyanate (TDI) is a leading cause of occupational asthma. TDI-induced asthma is an inflammatory disease of the airways that is associated with airway remodeling. However, there are little data available on the role of matrix metalloproteinases (MMPs) in TDI-induced asthma. **Objective:** We evaluated whether MMP-9 participates in the airway inflammation in TDI-induced asthma. An additional aim of the present study was to determine whether MMP inhibitors could be effective therapeutic agents for TDI-induced asthma.

Methods: We developed a murine model of TDI-induced asthma to examine the involvement of MMPs by performing 2 sensitizations with 3% TDI and 1 challenge with 1% TDI using ultrasonic nebulization.

Results: Murine TDI-induced asthma includes findings of (1) increased inflammatory cells, including neutrophils, lymphocytes, and eosinophils; (2) histologic changes, including infiltration of inflammatory cells around bronchioles, thickened airway epithelium, and accumulation of mucus and debris in the bronchioles; (3) increased MMP-9 activity in inflammatory cells in the airway lumen; and (4) airway hyperresponsiveness. Administration of an MMP inhibitor remarkably reduced all these pathophysiologic findings.

Conclusion: We conclude that TDI-induced occupational asthma is associated with the induction of MMP-9 in inflammatory cells, and the inhibition of MMP-9 may be a good therapeutic strategy. (*J Allergy Clin Immunol* 2001;108:1021-6.)

Key words: Toluene diisocyanate, matrix metalloproteinase, asthma, airway hyperresponsiveness

Abbreviations used

BAL: Bronchoalveolar lavage
MMP: Matrix metalloproteinase
MMPI: Matrix metalloproteinase inhibitor
Penh: Enhanced pause
TDI: Toluene diisocyanate

Toluene diisocyanate (TDI), a low-molecular-weight compound widely used in the production of polyurethane foams, automobile paints, varnishes, and related products, is a leading cause of occupational asthma.^{1,2} TDI-induced asthma is characterized by hyperresponsiveness and inflammation of the airways.^{2,3} This inflammation is associated with infiltration of lymphocytes, eosinophils, and neutrophils into the bronchial lumen.^{3,4} Airway remodeling, which often occurs in the bronchial tree, probably arises as a consequence of inflammatory processes.

The extracellular matrix is a dynamic structure, and its homeostasis depends on an equilibrium between synthesis and degradation of extracellular matrix components. A balance between the expression of proteases and antiproteases, particularly the matrix metalloproteinases (MMPs) and their inhibitors, maintains this homeostasis under normal conditions. The expression of these same proteases and antiproteases is critical in tissue repair and remodeling in some pulmonary inflammatory diseases.⁵⁻⁸ In allergic asthma, the mechanisms of remodeling of the bronchial tree caused by extracellular matrix deposition are well characterized. Of the MMP family, MMP-9 is the major proteinase that induces bronchial remodeling in asthma.^{9,10} In addition, MMP-9 induces the migration of eosinophils and neutrophils across basement membranes.^{11,12} Recently, MMPs were reported to play a crucial role in the infiltration of ovalbumin-induced airway inflammatory cells and the induction of airway hyperresponsiveness in a murine model of asthma.¹³ However, there are little data available on the role of MMPs in TDI-induced asthma.

In the present study we developed a murine model of TDI-induced asthma to examine the involvement of

From the Departments of ^aInternal Medicine and ^bAnatomy and ^cthe National Creative Research Initiatives Center for Endothelial Cells, Chonbuk National University Medical School, Chonju; and ^dthe Department of Allergy and Clinical Immunology, Ajou University Hospital, Suwon.

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Reprint requests: Yong Chul Lee, MD, PhD, Department of Internal Medicine, Chonbuk National University Medical School, 634-18, Keumam-dong, Chonju, 561-712, South Korea.

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MMPs. We found that MMP-9 derived from airway inflammatory cells is a major determinant of TDI-induced asthma. In addition, we found evidence that MMP inhibitors (MMPi) may be effective therapeutic agents for TDI-induced asthma.

METHODS

Generation of a murine TDI-induced asthma model

Specific pathogen-free female BALB/c mice were purchased from the Korean Research Institute of Chemistry Technology (Daejeon, Korea). Mice were housed throughout the experiments in a laminar flow cabinet and maintained on standard laboratory chow ad libitum. All mice were 8 to 10 weeks of age. This study was approved by the Ethics Committee for Animal Experiments of the Chonbuk National University Medical School. Standard guidelines for laboratory animal care were followed.¹⁴ Mice were sensitized by means of intranasal administration of 20 μ L of 3% TDI dissolved in ethyl acetate/olive oil (1:4) after achievement of light anesthesia (sodium pentobarbitone, 30 mg/kg, administered intraperitoneally) once daily for 5 consecutive days according to the method of Scheerens et al,¹⁵ with some modifications. After 3 weeks, these animals were further sensitized with the same reagent for 5 consecutive days. On day 7 after the second sensitization (day 38), mice were individually placed in a horizontal cylindrical chamber and challenged through the airways with 1% TDI dissolved in ethyl acetate/olive oil (1:4) by means of ultrasonic nebulization (NE-U12; Omron, Tokyo, Japan). As a control, mice were sensitized and challenged by the same procedures with the same amount of TDI solvent (ethyl acetate/olive oil 1:4).

Sampling and cellular analysis of bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) was performed at 0, 4, 24, 48, and 72 hours after the 1% TDI challenge. At the time of lavage, the mice were killed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg body weight, administered intraperitoneally). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated, and the catheter was secured with ligatures. Prewarmed 0.9% NaCl solution (0.7 mL) was slowly infused into the lung, and approximately 0.4 mL of instilled fluid was consistently recovered from each mouse. These BAL fluids were pooled and then centrifuged at 4°C. The supernatants were kept at -70°C. Total cell numbers were counted with a hemocytometer. Smears of BAL cells prepared with a Cytospin II (Shandon, Runcorn, United Kingdom) were stained with Diff-Quik solution (Dade Diagnostics of PR, Inc, Aguada, Puerto Rico) for differential cell counting. Parts of slides were used for immunocytochemistry of MMP-9. Two independent blinded investigators counted the cells with a microscope. Approximately 400 cells were counted in each of 4 different random locations. Interinvestigator variation was less than 5%. The mean number from the 2 investigators was used to estimate the cell differentials.

Measurements of MMPs in BAL fluids

The proforms of the MMPs (pro-MMPs) were quantitated by means of enzyme immunoassays according to the manufacturer's protocol (Fuji Chemical Industries, Toyama, Japan). The hydrolytic activity of MMP-9 was measured by means of gelatin zymography. Zymography was performed as previously described.^{9,16} Samples containing the same amount of protein (10 μ g) were mixed with 5 $\times$ sample buffer (0.4 mol/L TRIS-HCl [pH 6.8], 5% SDS, 20% glycerol, and 0.1% bromophenol blue) and were applied to a 10% SDS-PAGE containing 0.1% gelatin. Reference standards were MMP-2

and MMP-9 (Chemicon International, Inc, Temecula, Calif). After running, gels were incubated in 2.5% Triton X-100 for 1 hour and incubated in the enzyme buffer (0.05 mol/L TRIS-HCl [pH 7.5], 0.02 mol/L NaCl, 5 mmol/L CaCl₂, and 0.02% Brij-35) for 24 hours at 37°C. The gels were stained with 0.5% Coomassie brilliant blue 250 solution and destained with several changes of 30% methanol and 10% acetic acid.

Histology and immunochemistry

Lung tissues were fixed with 10% (vol/vol) neutral-buffered formalin. Paraffin tissue blocks were sectioned at 4 μ m. Cells obtained from BAL fluids by means of centrifugation were fixed with 4% paraformaldehyde for 5 minutes. Tissues and cells were incubated with anti-MMP-9 antibody (Chemicon International, Inc) at 4°C overnight, and signals were visualized with the EnVision system (DAKO, Copenhagen, Denmark). The sections were counterstained with Meyer hematoxylin.

Administration of MMPi

MMPi-I (inhibitory concentration at 50% on MMP-9, 150 μ mol/L, 4-Abz-Gly-Pro-D-Leu-D-Ala-NHOH; Calbiochem, San Diego, Calif) was used to inhibit MMP-9 activity. MMPi-I was administered intraperitoneally (10 or 20 mg/kg body weight) 3 times at 24-hour intervals, beginning 30 minutes before TDI challenge.

Measurement of airway responsiveness

Airway responsiveness was measured in mice 3 days after TDI challenge (day 41). Experiments were conducted with mice in an unrestrained and conscious state according to the method of Hamelmann et al.¹⁷ Mice were placed in a barometric plethysmographic chamber (All Medicus Co, Seoul, Korea). Aerosolized methacholine in increasing concentrations (2.5-50 mg/mL) was nebulized through an inlet of the main chamber for 3 minutes. Readings were taken and averaged for 3 minutes after each nebulization. The bronchopulmonary resistance was expressed as enhanced pause (Penh), which was calculated as follows, according to the manufacturer's protocol:

$$\text{Penh} = \frac{(\text{Expiratory time/Relaxation time} - 1) \times (\text{Peak expiratory flow/Peak inspiratory flow})}{1}$$

Results are expressed as the percentage increase in Penh after challenge with each concentration of methacholine, where baseline Penh (after saline challenge) is expressed as 100%.

Statistical analysis

Data are expressed as means $\pm$ SD. Statistical comparisons were performed with 1-way ANOVA, followed by the Fisher exact test. Correlation was calculated with the Spearman rank test. Statistical significance was set at a *P* value of less than .05.

RESULTS

Increased numbers of inflammatory cells in BAL fluids of mice sensitized and challenged with TDI

Cellular analysis revealed that macrophages were the major cellular component in the BAL fluid obtained during the prechallenge period. The number of macrophages did not change significantly during the period after TDI inhalation. At 24 hours after TDI inhalation, neutrophils markedly increased in the BAL fluid. The increased number of neutrophils continued up to 72 hours after TDI inhalation. The numbers of lymphocytes and eosinophils were increased signifi-

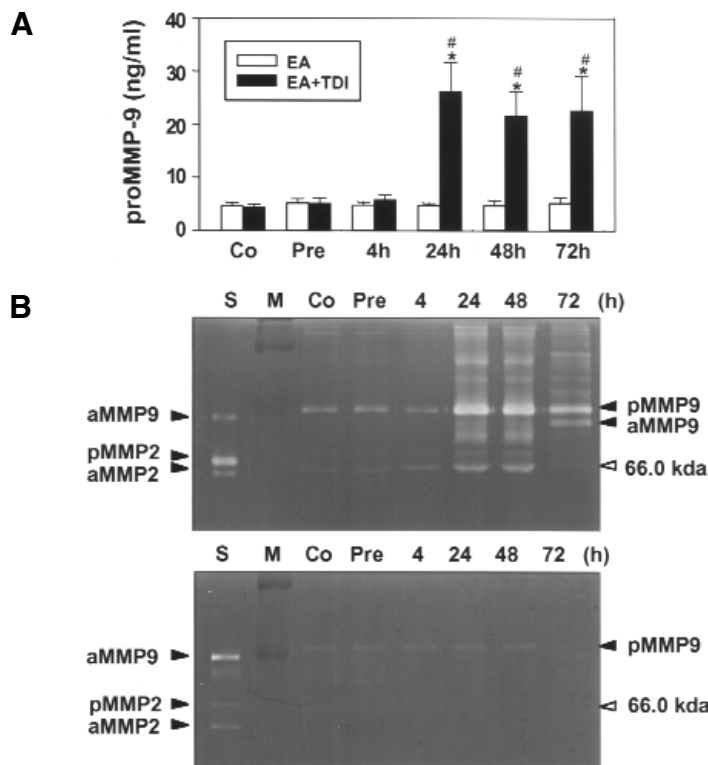


FIG 1. Effect of TDI challenge on MMP secretions in BAL fluids: **A**, enzyme immunoassay of pro-MMP-9; **B**, gelatin zymography. Animals were challenged with TDI in ethyl acetate/olive oil (*upper panel*) or ethyl acetate/olive oil alone (*lower panel*). Lane S contains standards: the proform of MMP-9 (pMMP9), the active form of MMP-2 (aMMP2), and the proform of MMP-2 (pMMP2). Molecular weight markers (open arrowheads on right) were used to estimate molecular masses. EA, Ethyl acetate/olive oil; Co, no treatment; Pre, before challenge. * $P < .05$ versus before challenge; # $P < .05$ versus ethyl acetate/olive oil.

cantly from 4 to 72 hours after TDI inhalation. Overall, the total number of cells was significantly greater from 24 to 72 hours after TDI inhalation. In contrast, no significant changes in the cellular components were observed after inhalation of the control agent, ethyl acetate/olive oil.

MMP-9 in the BAL fluid of mice sensitized and challenged with TDI

Enzyme immunoassay revealed that pro-MMP-9 was the major MMP in the BAL fluid obtained from the prechallenge period. In TDI-exposed mice, pro-MMP-9 increased approximately 5.7-fold at 24 hours after TDI inhalation and continued at almost the same level up to 72 hours (Fig 1, A). In contrast, no significant changes in the pro-MMP-9 level were observed after inhalation of the control agent, ethyl acetate/olive oil (Fig 1, A). Pro-MMP-1, pro-MMP-2, and pro-MMP-3 were not detectable in any of the BAL fluids obtained during the experimental periods (data not shown).

Profiles of MMP-9 in the BAL fluids were semiquantitatively assayed by means of gelatin zymography. Consistent with the results obtained from the enzyme immunoassays, gelatin zymography revealed that the 92-kD pro-MMP-9 band was predominant after challenge with TDI (Fig 1, B). The levels of pro-MMP-9 increased

approximately 3.1-, 2.6-, and 2.3-fold at 24, 48, and 72 hours after TDI inhalation, respectively, compared with the prechallenge period (Fig 1, B). Active MMP-9 (approximately 84 kD) was observed most prominently at 72 hours (Fig 1, B). Interestingly, several unidentified gelatinolytic bands were observed at 24 and 48 hours after TDI inhalation (Fig 1, B). In contrast, no significant changes in the levels of MMP-9 or the unidentified gelatinolytic bands were observed after inhalation of the control agent, ethyl acetate/olive oil (Fig 1, B). In addition, we counted the neutrophils and eosinophils in the BAL fluids at the same time points. There were significant correlations between the level of pro-MMP-9 and the number of neutrophils ($r = 0.699$, $P < .01$) or eosinophils ($r = 0.727$, $P < .01$).

Histologic findings and expression of MMP-9 in the airway

Histologic analyses revealed typical pathologic findings of asthma in the TDI-exposed mice. Numerous inflammatory cells were infiltrated around the bronchioles, the airway epithelium was thickened, and there was accumulation of mucus and debris in the lumen of the bronchioles (Fig 2, B). Immunohistochemical analyses of lung tissue of TDI-exposed mice revealed immunoreactive MMP-9 on inflammatory cells and extracellular

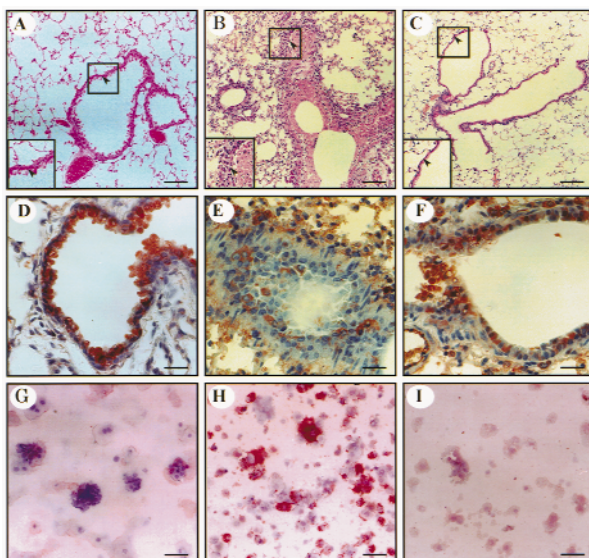


FIG 2. Effect of TDI challenge and MMPI-I on MMP-9 secretions in the bronchioles and BAL fluids in the mice sensitized and challenged with TDI. Sampling was performed at 72 hours after ethyl acetate/olive oil alone (**A**, **D**, and **G**), TDI plus ethyl acetate/olive oil (**B**, **E**, and **H**), or TDI plus ethyl acetate/olive oil plus MMPI (**C**, **F**, and **I**). Representative hematoxylin and eosin-stained sections of the lungs (**A**, **B**, and **C**): arrowheads indicate airway wall thickness. Representative light microscopy of MMP-9-positive cells in the bronchioles (**D**, **E**, and **F**): dark-brown color indicates MMP-9-positive cells. Representative light microscopy of MMP-9-positive cells in the BAL fluids (**G**, **H**, and **I**): brownish pink color indicates MMP-9-positive cells. Bar indicates scale of 50 μ m (**A-C** and **G-I**) or 5 μ m (**D-F**).

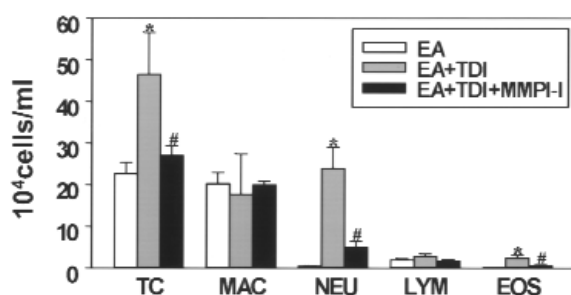


FIG 3. Effect of MMPI-I on total and differential cellular components of BAL fluids in TDI-sensitized mice. EA, Ethyl acetate/olive oil; TC, total cell; MAC, macrophage; NEU, neutrophil; LYM, lymphocyte; EOS, eosinophil. * $P < .05$ versus ethyl acetate/olive oil; # $P < .05$ versus EA, ethyl acetate/olive oil plus TDI.

matrix around bronchioles and on inflammatory cells and debris in the airway lumen (Fig 2, *E*). In the control mice positive MMP-9 staining was seen only in the airway epithelium (Fig 2, *D*). Immunocytologic analyses in the BAL fluid of TDI-exposed mice revealed that the immunoreactive MMP-9 was localized on BAL cells, including cells that resembled macrophages, neutrophils, and eosinophils (Fig 2, *H*). In the control mice almost no MMP-9-positive cells were detected (Fig 2, *G*).

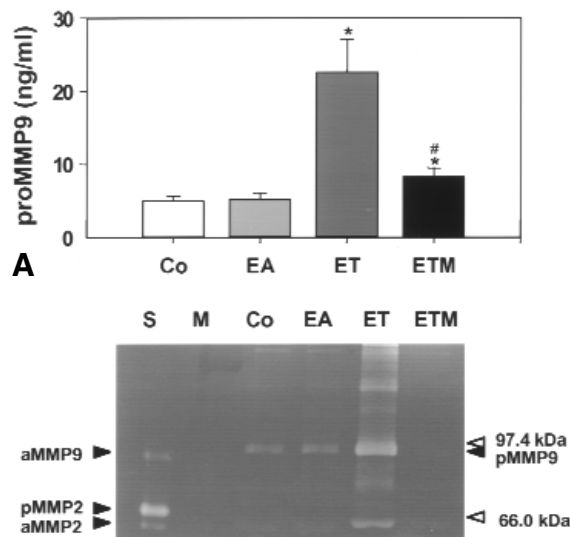


FIG 4. Effect of MMPI-I on MMP secretions in the BAL fluids of mice sensitized and challenged with TDI. **A**, Enzyme immunoassay of pro-MMP-9. **B**, Gelatin-zymography. Lane S contains standards: the active form of MMP-9 (*aMMP9*), the active form of MMP-2 (*aMMP2*), and the proform of MMP-2 (*pMMP2*). Lane M contains standard molecular weight markers (*open arrowheads* on right). Co, No treatment; EA, ethyl acetate/olive oil; ET, ethyl acetate/olive oil plus TDI; ETM, ethyl acetate/olive oil plus TDI plus MMPI-I. * $P < .05$ versus EA, ethyl acetate/olive oil; # $P < .05$ versus ethyl acetate/olive oil plus TDI.

Effect of MMPI-I on the cytopathologic changes in mice sensitized and challenged with TDI

Daily administration of MMPI-I (20 mg/kg body weight administered intraperitoneally) remarkably reduced the increase in the numbers of total cells, neutrophils, and eosinophils in the BAL fluid at 72 hours after TDI inhalation (Fig 3). In addition, daily administration of MMPI-I reduced the level and activity of MMP-9 in the BAL fluid at 72 hours after TDI inhalation compared with that seen in control mice (Fig 4, *A* and *B*). Furthermore, MMPI-I-treated mice showed a marked reduction in the thickening of airway epithelium, infiltration of inflammatory cells in the peribronchiolar regions, and number of inflammatory cells and debris in the airway lumen (Fig 2, *C*). Immunocytologic analyses in the BAL fluid revealed that the immunoreactive MMP-9 was markedly reduced on BAL cells from MMPI-I-treated mice (Fig 2, *F*). These results suggest that the migration of inflammatory cells, especially, MMP-9-containing neutrophils and eosinophils, into the bronchial lumens was strongly inhibited by MMPI-I.

MMPI-I reduced TDI-induced airway hyperresponsiveness

In the mice sensitized and challenged with TDI, the dose-response curve of the percentage of Penh shifted to the left compared with that of the control mice. In addition, the percentage increase in Penh produced by methacholine (from 2.5-50 mg/mL) administration was signif-

icantly greater in the mice sensitized and challenged with TDI compared with in the control mice (Fig 5). Thus mice sensitized and challenged with TDI exhibit airway hyperresponsiveness. Daily administration of MMPI-I (10 and 20 mg/kg body weight administered intraperitoneally) reduced TDI-induced airway hyperresponsiveness. In response to increasing doses of methacholine, MMPI-I-treated mice showed a rightward shift in the dose-response curve of the percentage increase of Penh compared with that seen in untreated mice (Fig 5). The shift was dose dependent.

DISCUSSION

Isocyanate chemicals, including TDI, are currently the most common causes of occupational asthma in developed countries.^{1,2} Although considerable controversy remains regarding its pathogenesis, TDI-induced asthma is characterized by airway hyperresponsiveness to TDI. In addition, an important pathophysiologic feature of TDI-induced asthma is airway inflammation. In this study we developed a murine model of TDI-induced asthma. These mice have the following typical pathophysiologic features of human TDI-induced asthma: airway hyperresponsiveness; infiltration of inflammatory cells, especially neutrophils, lymphocytes, and eosinophils, around the bronchioles; thickened airway epithelium; inflammatory cells and debris in the airway lumen; and increased expression of MMP-9 in the airway. These findings are consistent with pathophysiologic changes found in TDI-induced asthma in human subjects.^{3,4,18,19} Thus we believe that this model will be very useful for further investigations into TDI-induced asthma.

In this study we found that the release of MMP-9 was upregulated in association with the accumulation of inflammatory cells to airways in a murine model of TDI-induced asthma. Accordingly, administration of MMPI-I, which has an inhibitory effect on MMP-9, almost completely prevented the pathophysiologic findings. Airway inflammation in TDI-induced asthma is characterized with the accumulation of inflammatory cells in airway lumen, and MMP-9 may play a crucial role in facilitating the migration of these cells to the airways. This idea may explain the correlation between the increased levels of MMP-9 and the number of neutrophils and eosinophils in the BAL fluid. Therefore we conclude that MMP-9 may have an important role in inducing and maintaining TDI-induced asthma.

Several studies have provided evidence of involvement of neutrophils in the pathogenesis of TDI-induced asthma.^{3,4,20} In TDI-induced asthma in human subjects, neutrophilia in BAL fluid has been found in association with asthmatic reactions induced by isocyanate challenge.²¹ Neutrophils could be an important source of proinflammatory cytokines and proteolytic enzymes.^{22,23} Sustained release of these inflammatory products enhances neutrophil-induced inflammation in the airway and subsequent airway hyperactivity.^{24,25} As we previously reported,²⁰ neutrophils may contribute to bronchoconstriction induced by TDI. Furthermore, the num-

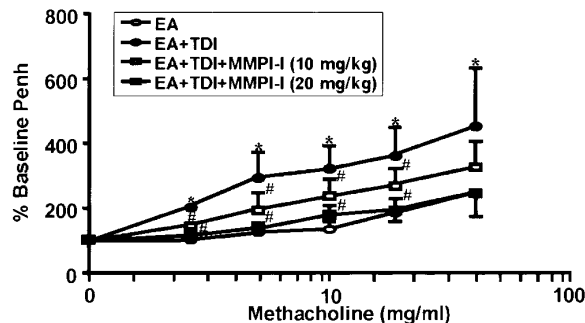


FIG 5. Effect of TDI challenge and MMPI-I on airway responsiveness in mice sensitized and challenged with TDI. EA, Ethyl acetate/olive oil. * $P < .05$ versus ethyl acetate/olive oil; # $P < .05$ versus ethyl acetate/olive oil plus TDI.

ber of eosinophils in the BAL fluid increased in our TDI-induced asthma model, although the magnitude was less than that seen with neutrophils. Eosinophils release toxic granule proteins during their activation.²⁶ These products can also cause airway hyperresponsiveness.

On the basis of the marked increase in MMP-9 in the airway luminal cells and fluids, we administered MMPI-I to examine its therapeutic effect. MMPI-I was dramatically effective on all pathophysiologic findings examined. One likely mechanism for the effectiveness of MMPI-I is that MMP-9 alters the extracellular matrix to allow the migration of inflammatory cells, including neutrophils and eosinophils, to the airways. In this study the administration of MMPI inhibits not only the MMP-9 activity in the contents of bronchial lumen but also the migration of inflammatory cells through the endothelial and epithelial basement membrane. Without these inflammatory cells, the other pathologic changes do not occur. Thus the MMPI abolished airway hyperresponsiveness. Our results strongly indicate that MMPs are potentially powerful therapeutic agents for TDI-induced asthma.

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