Inspiratory resistive breathing induces MMP-9 and MMP-12 expression in the lung

Dimitrios Toumpanakis,∗1 Olga Noussia,∗1 Ioanna Sigala,1 Eleni Litsiou,1 Konstantinos Loverdos,1 Panagiotis Zacharatos,1 Vassiliki Karavana,1 Tatiana Michailidou,1 Christina Magkou,1 Zongmin Zhou,1 Stamatios Theocharis,2 and Theodoros Vassilakopoulos1

1Department of Critical Care and Pulmonary Services and Marianthi Simou Applied Biomedical Research and Training Center, Evangelismos Hospital, University of Athens, Medical School, Athens, Greece; 2First Department of Pathology, University of Athens, Medical School, Athens, Greece; 3Department of Pathology, General Hospital “Evangelismos”, Athens, Greece

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Toumpanakis D, Noussia O, Sigala I, Litsiou E, Loverdos K, Zacharatos P, Karavana V, Michailidou T, Magkou C, Zhou Z, Theocharis S, Vassilakopoulos T. Inspiratory resistive breathing induces MMP-9 and MMP-12 expression in the lung. Am J Physiol Lung Cell Mol Physiol 308: L683–L692, 2015. First published January 16, 2015; doi:10.1152/ajplung.00133.2014.—Inspiratory resistive breathing (IRB) is characterized by large negative intrathoracic pressure and was shown to induce pulmonary inflammation in previously healthy rats. Matrix metalloproteinases (MMP)-9 and -12 are induced by inflammation and mechanical stress in the lung. We hypothesized that IRB induces MMP-9 and -12 in the lung. Anesthetized, tracheostomized rats breathed spontaneously through a two-way valve, connected to an inspiratory resistance, with the tidal inspiratory tracheal pressure set at 50% of the maximum. Quietly breathing animals served as controls. After 3 and 6 h of IRB, respiratory mechanics were measured, bronchoalveolar lavage (BAL) was performed, lung injury score was estimated, and lung MMP-9 was estimated by zymography and ELISA. MMP-9 and MMP-12 immunohistochemistry was performed. Isolated normal alveolar macrophages were incubated with BAL from rats that underwent IRB. After 18 h, MMP-9 and -12 levels were measured in supernatants, and immunocytochemistry was performed. Macrophages were treated with IL-1β, IL-6, or TNF-α, and MMP-9 in supernatants was measured. After 6 h of IRB, leukocytes in BAL increased, and IL-1β and IL-6 levels were elevated. Elasticity and injury score were increased after 3 and 6 h of IRB. Lung MMP-9 levels increased after 6 h of IRB. MMP-9 and MMP-12 were detected in alveolar macrophages and epithelial (bronchial/alveolar) cells after 3 and 6 h of IRB. MMP-9 and MMP-12 were found in supernatants after treatment with 6 h of IRB BAL. Cytosolic immunostaining was detected after treatment with 3 and 6 h of IRB BAL. All cytokines induced MMP-9 in culture supernatants. In conclusion, IRB induces MMP-9 and -12 in the lung of previously healthy rats.

resistive breathing; inflammation; metalloproteinases

RESISTIVE BREATHING IS THE HALLMARK of the pathophysiology of diseases of airway obstruction, such as asthma and chronic obstructive pulmonary disease (COPD), especially during exacerbations (40, 41). Resistive breathing induces cytokine upregulation in the diaphragm (41) in an experimental animal model and provokes systemic inflammation in healthy volunteers (42). Recently, inspiratory resistive breathing (IRB) was found to induce pulmonary inflammation and lung injury, as a consequence of the injurious effect of the large negative intrathoracic pressures, following strenuous contractions of the inspiratory muscles (40).

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes that are released from several lung cell types and inflammatory cells. MMPs are implicated in lung tissue health and disease, including morphogenesis, tissue injury, remodeling, and repair (14, 33). Apart from their well-recognized role in degrading extracellular matrix (26), MMPs have been implicated in inflammatory responses through regulation of leukocyte influx and posttranslational modification of cytokines (34).

MMPs, including MMP-9 and MMP-12, are increased in the lungs of patients with asthma and those with COPD, denoting a role for MMPs in the pathogenesis of obstructive airway diseases (11). In COPD, MMPs are involved in the pathogenesis of emphysema through the protease/antiprotease theory (4). Indeed, mice lacking MMP-12 were protected against smoke-induced pulmonary emphysema (18). Allergen challenge, smoke exposure, activation of inflammatory cells, and the underlying inflammation have been implicated in the regulation of MMP induction in asthma and COPD (10, 14, 22, 29).

Interestingly, pure mechanical stress of lung cells has been found to increase MMP expression. Mechanical stress induced MMP-9 expression from cultured human bronchial epithelial cells in vitro (9, 39). Also cyclic mechanical strain of airway smooth muscle cells led to increased MMP-1, -2, and -3 expression (17). In vivo, ventilation of healthy rats with high tidal volumes was followed by increased MMP-9 protein levels and induction of pulmonary inflammation, compared with low tidal volume (23).

Thus we hypothesized that resistive breathing, through increased mechanical stress and by causing pulmonary inflammation, could lead to increased MMP-9 and -12 expression in previously healthy lungs. To test this hypothesis, we have employed an animal model of IRB in healthy rats. Moreover, to differentiate between the possible mechanisms of resistive breathing-induced MMP expression (mechanical stress vs. inflammatory mediators), normal alveolar macrophages have been incubated with bronchoalveolar lavage (BAL) fluid from animals that underwent resistive breathing.

* D. Toumpanakis and O. Noussia contributed equally to this work.

Address for reprint requests and other correspondence: T. Vassilakopoulos, First Dept. of Critical Care, Univ. of Athens Medical School, Evangelismos Hospital, Ipsilandou 45-47 Str, 10676, Athens, Greece (e-mail: tvassil @med.uoa.gr).
METHODS

**Subjects.** Adult female rats (age 8–12 wk, weight 230 ± 30 g) were used in this study (see figure legends for the number of animals in each experimental group). Animals were purchased from the Hellenic Pasteur Institute and were housed in a 12-h:12-h day/night cycle at the Experimental Surgery Unit of Evangelismos Hospital and were provided with food and water ad libitum. All procedures were in accordance to the European Union Directive for care and use of laboratory animals and were approved by the Greek Veterinary Administration and the ethics committee of Evangelismos Hospital.

**IRB model.** To examine the effects of resistive breathing on the lung, a model of IRB was set, as previously described (40, 41). Briefly, rats were anesthetized with an intraperitoneal (ip) injection of a mixture of ketamine (75 mg/kg) and xylazine (5 mg/kg) and tracheostomized (tracheal cannula, 14G). After a short stabilization period (~15 min), the tracheal cannula was connected to a two-sided nonrebreathing valve (Hans-Rudolf). With the use of a pressure transducer, the tracheal pressure was monitored, and the maximum inspiratory pressure \( P_{\text{imax}} \) was measured during spontaneous breathing efforts through a totally occluded inspiratory port for 10 s ( Direcwin, Raytech Instruments). The inspiratory port was then connected to a tube of small diameter (resistance), and the diameter was adjusted to provide a peak tidal inspiratory pressure \( P_{\text{T}} \) at 50% of maximum (IRB), to mimic severe airway obstruction, as seen in COPD exacerbation and asthma attacks (40). The animals were randomly assigned to 3 or 6 h of IRB. During the procedure, the inspiratory port was connected to a 100% oxygen supply to prevent hypoxemia. Spontaneously breathing animals that breathed 100% oxygen against no load for equal time points served as controls. Supplemental doses of ketamine (30 mg/kg ip) were given during the procedure, as needed.

Following resistive or quiet breathing, the animals received a dose of ketamine and xylazine (75 mg/kg and 10 mg/kg, respectively) and were euthanized by vena cava dissection. Afterward, the thoracic cavity was exposed. The right lung was lavaged with normal saline (2.5 ml × 3 aliquots) and then excised and stored in −80°C for subsequent analysis (gelatin zymography and ELISA).

**Immunohistochemistry.** Paraffin-embedded lung sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked by incubation with 3% H2O2 for 5 min. Epitope retrieval was performed by a boiling step for 20 min in 1 mM EDTA, pH 8.0. Afterward, sections were blocked with normal goat serum and incubated overnight at 4°C with either anti-MMP-9 (Santa Cruz Biotechnology) or anti-MMP-12 (Abcam) antibodies. The next day, sections were blocked with normal goat serum and incubated overnight at 4°C with either anti-MMP-9 (Santa Cruz Biotechnology) or anti-MMP-12 (Abcam) antibodies. The next day, sections were blocked with normal goat serum and incubated overnight at 4°C with either anti-MMP-9 (Santa Cruz Biotechnology) or anti-MMP-12 (Abcam) antibodies. The next day, sections were blocked with normal goat serum and incubated overnight at 4°C with either anti-MMP-9 (Santa Cruz Biotechnology) or anti-MMP-12 (Abcam) antibodies. The next day, sections were blocked with normal goat serum and incubated overnight at 4°C with either anti-MMP-9 (Santa Cruz Biotechnology) or anti-MMP-12 (Abcam) antibodies. Afterward, sections were pelleted on glass slides by cytocentrifugation. Differential counts were performed on May-Grunwald-stained cytospins, and percentages of macrophages, monocytes, lymphocytes, and eosinophils were determined when we counted their number in 300 cells.

**MMP-9 protein levels in lung tissue.** Frozen lung sections were homogenized with buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1.5 mM MgCl2, and a cocktail of protease and phosphatase inhibitors at a 1:1,000 concentration. The samples were then centrifuged at 10,000 g for 10 min. The supernatant was collected, and total protein concentration was estimated using a colorimetric protein assay according to the manufacturer (Bio-Rad).

Protein levels of total MMP-9 were determined with ELISA, according to manufacturer’s protocol (Quanti kit ELISA, R&D Systems) and were normalized to total protein content of lung homogenates.

**Isolation and culture of alveolar macrophages.** To differentiate between the possible mechanisms of resistive breathing-induced MMP expression (mechanical stress, inflammatory mediators), incubation of normal alveolar macrophages with BAL fluid from animals that underwent resistive breathing was performed. This allowed for the evaluation of the induction of MMPs from soluble inflammatory
mediators in the BAL fluid independently of the mechanical stressor, as has been previously described in other forms of mechanical stress-induced lung injury (e.g., ventilator-induced lung injury) (13). In detail, alveolar macrophages were isolated from BAL fluid of normal Wistar rats. Following anesthesia (75 mg/kg ketamine and 10 mg/kg xylazine ip) and vena cava dissection, the rats were tracheostomized and lavaged with 10 ml of PBS five times. BAL fluid was centrifuged at 300 g for 10 min, and the cell pellet was resuspended in 1 ml PBS. Over 98% of cells isolated with this technique were macrophages, as determined following cytoospin and May-Grünwald staining. Afterward, 10^6 macrophages per well were put in 96-well plates with DMEM and 10% FCS containing 100 IU/ml penicillin and 1 mg/ml streptomycin and left to adhere for 2 h. The cells were next treated with 5% of pooled (centrifuged-cell free) BAL fluid (added to serum-free culture medium) from quietly breathing animals or animals that underwent 3 and 6 h of IRB (5 animals per group). Samples were loaded in triplicate, and the experiment was repeated three times. In each experiment, wells that contained 5% of BAL fluid in serum-free culture medium (control and 3 and 6 of IRB) but no macrophages were also analyzed.

The cell culture supernatant was collected following 18 h of incubation and stored at −80°C. MMP-9 in macrophage culture supernatants was estimated by gelatin zymography, as described above (20 µl of supernatant was used per sample). Moreover, MMP-12 protein levels in culture supernatants were measured by ELISA, according to manufacturer’s protocol (Uscn Life Science).

Following collection of culture supernatant, cells were detached and pelleted on glass slides by cytocentrifugation. Cytospins were then fixed with 4% paraformaldehyde and stored at −80°C. For immunocytochemistry, cytospins were removed from −80°C, air dried, fixed with cold acetone for 10 min at 4°C, and washed with EnVision FLEX wash buffer (DAKO). Permeabilization was performed with 0.1 Triton X-100 in PBS for 30 min at room temperature. EnVision FLEX Peroxidase-Blocking Reagent (DAKO) was applied and left to incubate for 5 min. Cytospins were incubated for 2 h at room temperature with either anti-MMP-9 (EMD Millipore) or anti-MMP-12 (Abcam). Slides were then incubated with EnVision FLEX/horseradish peroxidase (DAKO) for 30 min at room temperature. Immunostaining was visualized with EnVision FLEX DAB+ Chromagen. Counterstain was performed with Harris hematoxylin, and slides were mounted. Negative controls were performed by omitting the primary antibody and incubating the cytopsins with only the antibody diluent.

Cytokine-induced MMP-9 expression by isolated alveolar macrophages. Alveolar macrophages were isolated from BAL fluid of normal Wistar rats. Macrophages were treated with recombinant IL-6, IL-1β, or TNF-α (R&D Systems) at a dose of 0.05, 1.00, 10.00, and 50.00 ng/ml. Macrophages were also treated with a mixture of all three cytokines at a dose of either 1 ng/ml or 10 ng/ml. Exposure to water for injection was used as a control value. Samples were loaded in duplicate, and the experiment was repeated three times. Macrophage culture was performed as described above.

The cell culture supernatant was collected following 18 h of incubation, and MMP-9 protein levels in the supernatant were estimated by ELISA, according to the manufacturer’s protocol (Quantikine ELISA, R&D Systems).

Cytokine levels in BAL fluid samples. Protein levels of IL-1β and IL-6 were determined in BAL fluid samples with ELISA, according to the manufacturer’s protocol (DuoSet ELISA, R&D Systems).

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed with either Student’s t-test or ANOVA, where applicable. When significant, post hoc analysis was performed with Tukey’s test. A P value <0.05 was chosen as statistically significant.

RESULTS

IRB induces pulmonary inflammation and deranges lung mechanics. As previously reported by our group (40), 6 h of resistive breathing was followed by induction of pulmonary inflammation. In detail, following 6 h of IRB, an increase in total cell number in BAL was noticed compared with control and 3 h of IRB (P < 0.001 and P = 0.028, respectively). Both macrophage and neutrophil numbers were elevated, whereas macrophages remained the most abundant cell subpopulation in BAL fluid (see Fig. 1A). Lymphocyte cell count was not altered significantly following IRB ([cells × 10^6/ml] control 3.0 ± 1.06; 3 h of IRB 4.7 ± 1.65; 6 h of IRB 9.05 ± 3.07; ANOVA P nonsignificant; n = 5 per group]. Eosinophil and basophil cell counts were negligible and were not altered after IRB (data not shown). Moreover, compared with control, 6 h of IRB was associated with a twofold increase in protein levels of both IL-1β (P = 0.016) and IL-6 (P = 0.009). A significant increase was also observed compared with 3 h of IRB (Fig. 1, B and D).

Regarding respiratory system mechanics, both 3 and 6 h of IRB were followed by an increase in tissue elasticity compared with control (P = 0.006 and P < 0.001, respectively), whereas, after 6 h of IRB, a further increase in tissue elasticity was noticed compared with 3 h (P = 0.012) (Fig. 1C).

Finally, IRB was associated with an increased total lung injury score, as assessed by histology, compared with control values, both after 3 h (P = 0.04) and after 6 h (P = 0.001) (Fig. 1E). Regarding the independent histological features, the increased total lung injury score was due to increased alveolar and interstitial neutrophil infiltration and alveolar septal thickening. In contrast, neither hyaline membranes nor proteinaceous debris in the airspaces were noticed following IRB.

MMP-9 lung tissue activity in gelatin zymography. The gelatinolytic activity of MMP-9 was increased in tissue homogenates from animals that underwent 6 h of IRB, compared with control and 3 h of IRB (P < 0.001), indicating increased levels of MMP-9 in lung tissue following 6 h of resistive breathing (Fig. 2A). No difference was observed between quietly breathing control animals for 3 and 6 h, and these animals were pooled into one group (data not shown).

MMP-9 protein levels in lung tissue. Protein levels of MMP-9 increased after 6 h of IRB, compared with control values (P < 0.001), as assessed by ELISA (Fig. 2B). In contrast, following 3 h of IRB, the levels of MMP-9 did not differ from control values.

MMP cellular location by immunohistochemistry. As shown in Fig. 3, MMP-9 was found in a higher degree in lung tissue sections of animals that underwent both 3 and 6 h of IRB, compared with control. Indeed, the main cell types that were found to express MMP-9 were the alveolar macrophages (Fig. 3H) and the alveolar epithelial cells, yet to a lesser degree. MMP-12 was also found to be upregulated following 3 and 6 h of IRB (Fig. 4). Again, the alveolar macrophages were the main source of MMP-12 (Fig. 4H), with the alveolar epithelial cells to a lesser degree. Both MMP-9 and MMP-12 were also expressed in airway epithelial cells after 3 and 6 h of IRB, compared with quietly breathing animals (Figs. 3F and 4F, respectively).

Culture of alveolar macrophages with BAL fluid. Incubation of normal alveolar macrophages with 5% of BAL fluid from
6 h of inspiratory resistive breathing (IRB) induces pulmonary inflammation. A: increased bronchoalveolar lavage (BAL) fluid cellularity was noticed after 6 h of IRB, including raised counts of both macrophages and neutrophils, compared with quietly breathing animals. Ctr, control. B and D: protein levels of IL-1β and IL-6 in BAL fluid were increased after 6 h of IRB, compared with animals of both control and 3 h of IRB. C: both after 3 and 6 h of IRB, an increase in tissue elasticity was noticed, indicating the presence of lung injury. E: increased total lung injury score was noticed after 3 and 6 h of IRB compared with control. F–H: representative figures of hematoxylin and eosin-stained lung sections of ctr, 3 h of IRB, and 6 h of IRB, respectively. Data are presented as means ± SE, n = 5–8 per group; *P < 0.05 to ctr, #P < 0.05 to 3 h IRB; open bar, control; shaded bar, 3 h of IRB; solid bar, 6 h of IRB.
animals that underwent 6 h of resistive breathing resulted in a fivefold induction of MMP-9 gelatinolytic activity of cell culture supernatants ($P < 0.002$) compared with 5% of BAL fluid alone (Fig. 5). In contrast, BAL fluid from neither control animals nor animals with 3 h of resistive breathing had an effect on MMP-9 levels in macrophage culture supernatants. Moreover, immunocytochemical analysis showed increased intracellular MMP-9 levels in macrophages treated with BAL fluid from both 3 and 6 h of IRB animals, compared with control (Fig. 5B).

Regarding MMP-12, increased protein levels in macrophage culture supernatant were found after treatment with BAL fluid from animals that underwent 6 h of IRB, compared with control and 3 h of IRB ($P = 0.04$) (Fig. 6A). By immunocytochemical analysis, increased MMP-12 staining was detected in alveolar macrophages treated with BAL fluid from animals that underwent both 3 and 6 h of IRB, compared with control (Fig. 6B).

**Cytokine-induced MMP-9 expression by isolated alveolar macrophages.** MMP-9 in macrophage culture supernatant was significantly increased following exposure to IL-6, IL-1$\beta$, or TNF-$\alpha$, at the highest dose used (50 ng/ml) (IL-6 1.7-fold over control, $P = 0.03$; IL-1$\beta$ 2.3-fold over control, $P = 0.005$; TNF-$\alpha$ 5.25-fold over control, $P = 0.003$). Interestingly, a mixture of all three cytokines at the lower dose of 10 ng/ml was also able to upregulate MMP-9 expression from alveolar macrophages almost fivefold over control values ($P < 0.001$) (Fig. 7).

**DISCUSSION**

The main findings of our study are that IRB results in elevated protein levels of MMP-9 and MMP-12 in the lung and that BAL fluid from animals that underwent 6 h of IRB increases MMP-9 and MMP-12 protein levels in culture supernatant of normal alveolar macrophages.

Resistive breathing is the hallmark of the pathophysiology of diseases of airway obstruction, characterized by increased...
negative intrathoracic pressures, attributable to strenuous con-
tractions of the inspiratory muscles. As previously found (40),
6 h of IRB was followed by an inflammatory response in the
lung, as indicated by the increased BAL fluid cellularity, the
presence of lung injury in lung tissue sections, and the raised
levels of IL-1β and IL-6 in BAL fluid. Interestingly, in our
model, increased levels of both MMP-9 and MMP-12 were
detected by immunohistochemistry as early as 3 h of IRB. The
ey 3 h of IRB. The early detection of MMP-9 and MMP-12 after 3 h of IRB,
despite the absence of detectable inflammation in our model at
this time point, raises the possibility that a direct mechanical
stimulus exists for the early expression of MMPs. Indeed,
mechanical stress may induce MMP-9 expression from cul-
tured human bronchial epithelial cells in vitro (9, 39). In our
study, immunohistochemistry revealed an increased MMP-9
and MMP-12 presence in alveolar and bronchial epithelial
cells, which are subjected to increased negative intrathoracic
pressures (mechanical stress) following IRB and are expected
to sense mechanical stimuli. Increased MMP expression has
been reported in other states of high mechanical stress imposed
on the lung; both MMP-8 and MMP-9 are upregulated follow-
ing high tidal volume ventilation, and the absence of MMP-8
gene or inhibition of MMP-9 protects against ventilator-in-
duced lung injury (1, 23). Moreover, positive staining of

Fig. 4. Detection of MMP-12 in lung tissue sections by immunohistochemistry.
Representative images of MMP-12 immunohistochemistry in lung of control
(B), 3 h of IRB (C), and 6 h of IRB (D) are presented. As shown also for
MMP-9, increased levels of MMP-12 were detected mainly in alveolar macro-
phages and to a lesser degree in lung alveolar epithelial cells, after 3 and 6
h of IRB. Moreover, increased MMP-12 expression was also noticed in airway
epithelial cells both after 3 h (E) and 6 h (G) of IRB, compared with control
(E). Brown denotes the presence of MMP-12. A: negative control staining form
tissue section of 3 h of IRB. H: higher magnification image (×600) of alveolar
macrophages after 6 h of IRB. n = 8 per group, brown denotes positive
staining for MMP-12, black arrows, alveolar macrophages.

Fig. 5. MMP-9 is induced in culture supernatants of isolated normal alveolar
macrophages incubated with BAL fluid from animals that underwent 6 h of
IRB. A, top: representative image of gelatin zymography from 3 experiments.
Increased MMP-9 activity is noticed (right) in culture supernatants of normal
alveolar macrophages when incubated with 5% of BAL fluid from animals that
underwent 6 h of IRB, compared with control and 3 h of IRB. No significant
gelatinolytic activity is noticed in absence of alveolar macrophages. Bottom:
quantification of gelatin zymography reveals an ~5-fold induction of MMP-9
gelatinolytic activity of alveolar macrophages culture supernatants when in-
cubated with 5% of BAL fluid from animals that underwent 6 h of IRB,
compared with culture medium and 5% of BAL fluid alone. No significant
effect of 5% of BAL fluid from either control animals or those that underwent
3 h of IRB is noticed. B: representative images of MMP-9 immunocytochem-
istry in alveolar macrophages. As denoted by positive (brown) staining,
increased cytoplasmic levels of MMP-9 were detected in alveolar macrophages
after incubation with BAL fluid from animals that underwent 3 and 6 h of IRB,
compared with control. Data are presented as means ± SE, n = 3 experiments
(containing samples in triplicates) per group; *P < 0.05 to culture medium
plus 5% of BAL fluid 6 h of IRB, #P < 0.05 to 10^5 macrophages plus 5%
of BAL fluid from ctr and 3 h of IRB. Open bar, control; shaded bar, 3 h of
IRB; solid bar, 6 h of IRB.
alveolar macrophages at 3 h of IRB raises the possibility that direct activation of alveolar macrophages by mechanical stress may also occur during resistive breathing. Mechanical deformation has been shown to activate alveolar macrophages in vitro, inducing the expression of inflammatory cytokines and MMP-9 (35).

Interestingly, the major source of MMPs following resistive breathing was found by immunohistochemistry to be the alveolar macrophages. Alveolar macrophages are known sources of mechanical stressors, which are known modulators of inflammatory processes through matrix degradation, regulation of inflammatory cell influx, and cytokine/chemokine activation (28, 34). For example, MMP-12 (found increased in our study), is reported to induce MMP expression in vitro in mouse peritoneal macrophages and human macrophages, respectively (24, 37). Our results also indicate that all three cytokines are able to induce MMP-9 expression in cultured alveolar macrophages and that they exhibit a synergistic effect. It should be noticed that the concentration of cytokines found to be effective in stimulating MMP expression is larger than the concentration of cytokines found in the BAL fluid of animals undergoing IRB, indicating that cytokines may in part contribute to MMP-9 upregulation and act possibly in synergy with other mediators.

Interestingly, although MMP-9 and MMP-12 levels were not increased in supernatants after incubation with BAL fluid from animals that underwent 3 h of resistive breathing, positive cytosolic staining of macrophages for MMP-9 and MMP-12 was detected by immunocytochemistry at this time point. We have no clear explanation for this. It is possible that BAL fluid from 3 h of IRB may contain proinflammatory mediators to induce MMP expression but that the stimulation is not adequate to result in secretion of large amounts of MMPs in the supernatant. This is also in accordance with our results in lung tissue sections, where by immunohistochemistry MMP-9 is detected in lung tissue after 3 h of IRB, but not by ELISA or zymography, which showed increased levels only after 6 h of IRB. It is also possible that differences between techniques may account for this discrepancy, and positive cytosolic staining at 3 h, as shown by immunohistochemistry, may reflect an initial phase of MMP production before secretion of greater amounts of molecules that would be detected with zymography or ELISA in lung homogenates.

The early detection of MMPs (after 3 h of IRB) indicates a possible role of MMP-9 and MMP-12 in the initiation of the inflammatory response following IRB. Indeed, MMPs are known modulators of inflammatory processes through matrix degradation, regulation of inflammatory cell influx, and cytokine/chemokine activation (28, 34). For example, MMP-12 (found increased in our study), is reported to mediate both macrophage and neutrophil influx in cigarette smoke-mediated (18) and immune complex-mediated (46) lung injury, respectively, cell types that are both recruited in the lung following IRB.

The choice to study MMP-9 and MMP-12 in our model of mechanical airway obstruction is due to the importance of these molecules in the pathogenesis of obstructive lung diseases, including both asthma and COPD. MMP-9 and MMP-12 are implicated in the complex inflammatory processes leading to airway remodeling and lung tissue destruction in COPD (10). Increased levels of both MMP-9 and MMP-12 are found in induced sputum of patients with COPD (12, 44). Interestingly, polymorphisms of the MMP-12 gene are correlated to the risk of COPD in adult smokers (21), and, in experimental settings, knockout mice for MMP-12 were protected against smoke-induced pulmonary emphysema (18). MMPs are also considered to contribute to the structural changes of the airways seen in severe asthma (22). In mildly asthmatic patients,

have also shown that TNF-α is also induced in the BAL fluid following 6 h of IRB (40). Cytokines are known regulators of MMP expression (14). Indeed, activation of alveolar macrophages in vitro by IL-1β upregulated the expression of MMP-9 (36), and increased levels of both MMP-9 and MMP-12 were found in the lung of IL-1β-overexpressing conditional transgenic mice (25). Moreover, both IL-6 and TNF-α have been reported to induce MMP-9 expression in vitro in mouse peritoneal macrophages and human macrophages, respectively (24, 37).

Our results also indicate that all three cytokines are able to induce MMP-9 expression in cultured alveolar macrophages and that they exhibit a synergistic effect. It should be noticed that the concentration of cytokines found to be effective in stimulating MMP expression is larger than the concentration of cytokines found in the BAL fluid of animals undergoing IRB, indicating that cytokines may in part contribute to MMP-9 upregulation and act possibly in synergy with other mediators.

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6 h after allergen challenge, an increase in sputum MMP-9 levels was detected, an effect correlated with the fall in FEV$_1$ during the late asthmatic response, i.e., the severity of airway obstruction (5). As in our study, alveolar macrophages are among the major sources of MMP-9 and MMP-12 in both asthma and COPD (11). The regulation of MMP expression in diseases of airway obstruction is multifactorial because many regulators including allergen challenge, smoke exposure, activation of inflammatory cells, and the underlying inflammation have been implicated (10, 14, 22, 29).

A limitation of our study is the use of 100% oxygen during resistive breathing to prevent hypoxemia. Although both lung injury (31) in general and elevated MMP expression in particular (6, 8, 15) have been reported following hyperoxia, these studies employed much longer time periods than the ones used in our study. Therefore, we believe that hyperoxia had a minimal role in our observations. More importantly, animals in the control group also received 100% oxygen during spontaneous breathing.

Clinical implications. Our results raise the possibility that severe resistive breathing could account, as a pathogenetic mechanism per se, independently of the underlying inflammation, for the progression of obstructive airway diseases during exacerbations. The resistive load in our model was chosen to reflect the loads encountered in severe airway obstruction, especially during exacerbations of both COPD and asthma (38, 40, 43). MMP-9 is found increased in the induced sputum of patients with COPD exacerbation (32). Moreover, Viglio et al. (45) reported increased urine levels of desmosine during COPD exacerbations compared with stable patients, reflecting augmented elastin breakdown in the lung during exacerbations. Thus the mechanical deformation induced by resistive breathing could act as a stimulus for MMP expression, together and/or independently from other inflammatory stimuli, such as the microbiome. MMP activation could then lead to elastin degradation and thus disease progression. Moreover, elastin fragments, derived through MMP-12 proteolysis, have been found to exert chemotactic activity for monocytes, contributing to emphysema progression (20). Thus our data may suggest a possible mechanistic explanation (resistive breathing-induced MMP activation) to the interesting observation that patients with frequent exacerbations of COPD (where severe resistive breathing is noted) exhibit more rapid decline in lung function (7, 27). Moreover, although the impact of asthma exacerbations on disease progression is much less conceivable, recurrent asthma attacks have been also shown to correlate to a more rapid lung function decline in asthmatics (3). These frequent exacerbations could lead to repeated bouts of resistive breathing-induced MMP activation and thus elastin degradation, accounting for the more rapid decline in lung function. Of course, it should be acknowledged that our results cannot be directly extrapolated in clinical states, such as asthma and COPD, where an underlying inflammation already exists and where acute resistive breathing might have different consequences in the release of MMPs and other inflammatory mediators.

In conclusion, the results of our study show that IRB induces MMP expression in the lungs of previously healthy animals, providing an interesting novel pathogenetic mechanism for the progression of diseases of airway obstruction during exacerbations.

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DISCLOSURES
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REFERENCES


19. Houghton AM, Quintero PA, Perkins DL, Kobayashi DK, Kelley DG, Marconcini LA, Mecham RP, Senior RM, Shapiro SD. Elastin frag-


