# Up-Regulation of Trypsin and Mesenchymal MMP-8 During Development of Hyperoxic Lung Injury in the Rat

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**ABSTRACT:** Acute lung injury is marked by damage to alveolar-capillary barrier. High pulmonary levels of matrix-degrading serine proteinase trypsin and matrix metalloproteinases (MMP)-2, -8, and -9 have been shown in preterm infants with respiratory distress syndrome (RDS). We studied expression of trypsin and MMP-2, -8, and -9 in rats exposed to >95% oxygen for 24, 48, or 60 h. As demonstrated by zymography and Western immunoblotting, levels of trypsin and MMP-2, -8, and -9 in bronchoalveolar lavage fluid (BALF) sharply increased after 48 h of hyperoxia relative to normoxia controls. This coincided with increase in alveolar-capillary permeability, as indicated by increased protein concentration in BALF. Both neutrophil-derived 80-kD and mesenchymal cell–derived 60-kD MMP-8 isoforms were detected in BALF. Of them, mesenchymal-type MMP-8 predominated. In immunohistochemistry, alveolar epithelium showed strong trypsin expression at 48 and 60 h of hyperoxia, whereas it was predominantly negative in controls. MMP-8 was mostly expressed in macrophages. Marked up-regulation of trypsin and MMP-8 early during hyperoxic lung injury suggests that these enzymes play a role in the pathogenesis of acute lung injury and may therefore be potential targets for therapy of lung injury. (*Pediatr Res* **60:** 395–400, 2006)

The extracellular matrix (ECM) between epithelial and capillary endothelial layers and in particular the basement membranes (BM) of the cell layers are essential in the maintenance of the structural and functional integrity of the alveolar wall (1). Trypsin is a serine proteinase that can efficiently degrade various ECM proteins; at very low concentrations, it is capable of activating proforms of several MMPs, thus having potential to initiate a broad-spectrum proteinase cascade eventually leading to tissue destruction (2,3). Multiple isoforms of trypsin have been demonstrated in human pancreas and in most animal species (4,5). Trypsinogen-1 and -2 were detected for the first time outside the gastrointestinal tract in cyst fluid of human ovarian tumors (6). Later, trypsinogens have been identified in a variety of human extrapancreatic tissues including normal human epithelial cells of the lung, vascular endothelial cells, and various cancers (4,7,8). Recently, ectopic trypsin was identified in rat lung (9).

In the lung, trypsin may induce tissue injury not only through initiating matrix-degrading proteolytic cascade, but also through proinflammatory actions. Trypsin is a potent activator of proteinase-activated receptor 2 ($PAR_2$), a G protein–coupled receptor that is believed to play an important role in airway inflammation (10,11). In preterm infants, high pulmonary trypsinogen-2 early postnatally is associated with the severity of acute lung injury and with the development of bronchopulmonary dysplasia (BPD) (8). Furthermore, in autopsy specimens from preterm infants who died of respiratory distress syndrome (RDS) or BPD, trypsin-2 colocalizes with $PAR_2$ in alveolar and bronchial epithelium (12).

MMPs are a family of zinc-dependent, genetically distinct but structurally related endopeptidases collectively capable of degrading almost all components of ECM. MMP-2 and MMP-9 are type IV collagenases that can disrupt alveolar BM (13). MMP-8 belongs to the collagenase subfamily of MMPs and cleaves collagen types I–III, of which I and III are the major structural components of the pulmonary interstitial ECM. Furthermore, MMP-8 can act on various nonmatrix proteins such as serine proteinase inhibitors (serpins) and chemokines and thus may modulate inflammatory processes in different ways (14,15). Increased levels of MMP-2, MMP-8, and MMP-9 are associated with inflammatory lung diseases such as RDS, BPD, and asthma (13,16–18).

Hyperoxia-induced lung injury (exposure to >95% oxygen), a well-established model of acute lung injury, is characterized by damage to the alveolar-capillary barrier with subsequent increased pulmonary vascular permeability, progressive inflammation, and pulmonary edema (19). In rats exposed to 100% oxygen, the first signs of increased alveolar-capillary permeability are detected at 48 h (20). Severe interstitial and alveolar edema is present at 60 h, leading to death usually within 72 h of exposure (19,20).

**Abbreviations: BALF,** bronchoalveolar lavage fluid; **BM,** basement membrane; **BPD,** bronchopulmonary dysplasia; **ECM,** extracellular matrix; **MMP,** matrix metalloproteinase; **MPO,** myeloperoxidase; **$PAR_2$,** proteinase-activated receptor 2; **RDS,** respiratory distress syndrome

Association of high pulmonary levels of trypsin, MMP-2, -8, and -9 with tissue-destructive lung diseases suggests that these proteinases may play an important role in the pathogenesis of lung injury. By use of an experimental animal model, we wanted to determine the cellular localization and chronological increase of these proteinases during development of acute lung injury. Therefore, we characterized trypsin, MMP-2, -8, and -9 in lungs of rats exposed to >95% oxygen for 24, 48, and 60 h.

## METHODS

***Experimental model.*** The study protocol was approved by the Institutional Review Board for Animal Studies of Helsinki University Central Hospital, Helsinki, Finland. Female Wistar rats (Harlan Nederland, Horst, The Netherlands) aged 8 wk were housed for 1 wk before use. The animals were placed in a sealed Plexiglas chamber (85 × 60 × 40 cm) and exposed to >95% $O_2$ for 24 h (*n* = 6), 48 h (*n* = 6), and 60 h (*n* = 6) by administration of pure oxygen at 5 L/min. $O_2$ concentration was monitored with an oximeter (Datex, Helsinki, Finland). Food and water were allowed *ad libitum* and a 12-/12-h light/dark cycle was maintained. Control animals (*n* = 6) were kept in room air. At different exposure times, animals were anesthetized by intraperitoneal injections of ketamine (50 mg/kg) and xylazine (10 mg/kg) and killed by cervical dislocation. Immediately after decapitation, the right lung was removed and cut into two pieces. The pieces were soaked in isotonic saline, snap frozen in liquid nitrogen, and stored at −80°C until determination of myeloperoxidase (MPO) activity. The left lung was removed, fixed in 10% paraformaldehyde for 24 h, embedded in paraffin, and stored at room temperature for subsequent immunohistochemistry.

***Bronchoalveolar lavage (BAL).*** In a parallel experiment, BAL was performed for normoxia controls (*n* = 8) and rats exposed to >95% $O_2$ for 24 h (*n* = 8), 48 h (*n* = 8), and 60 h (*n* = 8). Immediately after killing the rat, the trachea was cannulated, and the lungs were lavaged by flushing with sterile isotonic saline (10 mL) as 10 aliquots of 1 mL each. BALF samples were kept on ice until centrifuged at 500 × *g* for 10 min at 4°C, and the supernatant was stored at −20°C for subsequent analyses. Protein concentration in BALF was measured (21) and used as an indicator of increased vascular permeability and loss of capillary endothelial membrane integrity.

***Gelatin zymography.*** For analysis of gelatinolytic activity by zymography, BALF samples were run on 1.5-mm thick 8%–10% sodium dodecylsulfate (SDS)–polyacrylamide gels impregnated with 1 mg/mL gelatin labeled fluorescently with 2-methoxy-2,4-diphenyl-3(2H)-furanone (Fluka, Sigma Chemical Co.–Aldrich, Buchs SG, Switzerland) as described previously (8). The intensities of gelatinolytic bands were evaluated with Bio-Rad Model GS-700 Imaging Densitometer using Quantity Program, new version of the Molecular Analyst/PC program (Bio-Rad Laboratories, Hercules, CA) (16). Results were expressed as arbitrary units (AU).

***ECL Western blotting.*** In BALF, molecular weight forms of trypsin and MMP-2, -8, and -9 were analyzed by ECL Western immunoblot method using specific antisera as described previously (16). Briefly, the BALF samples were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Schleider & Schuell, Dassel, Germany). Nonspecific binding was blocked with 5% milk powder (Valio, Helsinki, Finland). The membranes were incubated with a 1:500 solution of polyclonal antibodies against human MMP-2 (2), MMP-8 (16), MMP-9 (2), and trypsin (1:500, Chemicon International, Temecula, CA) for 12 h at room temperature, and then with horseradish peroxidase–linked anti–rabbit antibody for 1 h. The proteins were detected by the ECL detection system according to the manufacturer's instructions (Amersham Pharmacia Biotech). The intensities of immunoreacted forms of MMP-8 were evaluated and expressed as arbitrary units as described above (16).

***MPO activity.*** MPO activity was measured as a marker of pulmonary neutrophil accumulation, as previously described (22). Briefly, lung samples were thawed and homogenized in phosphate-buffered saline (PBS). After centrifugation, the pellet was resuspended in 1 mL of 50 mmol/L potassium phosphate containing 0.5 g/100 mL centrimonium bromide. Resuspended pellets were frozen at −70°C until the MPO assay was performed. Frozen samples were thawed, sonicated for 90 s, incubated in a 60°C water bath for 2 h. After centrifugation, 33 μL of supernatant was combined with 957 μL of 50 mmol/L potassium phosphate buffer, pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 5 × $10^{-4}$ M hydrogen peroxide. Change in absorbance was measured in a Lambda Bio UV/VIS Spectrometer (Perkin Elmer, Boston, MA) at 460 nm absorbance. MPO activity was expressed as mU/mg protein. Protein concentration was quantified by the method of Bradford (21).

***Immunohistochemistry.*** In lung tissue, trypsin immunoreactivity was visualized with monoclonal anti–trypsin antibody, MAB 1482 (Chemicon International), and MMP-8 immunoreactivity with rabbit polyclonal antibody against human MMP-8 (23). Four-micrometer sections were deparaffinized and rehydrated. To enhance antigen retrieval, sections were pretreated with microwave heat. The sections were then treated with 0.5% hydrogen peroxide in methanol for 30 min and blocked with either normal horse serum (trypsin) or normal goat serum (MMP-8) (both 1:20) for 15 min. Primary antibody to trypsin (diluted 1:1000) or to MMP-8 (diluted 1:250) was added, and the sections were incubated overnight at room temperature. Bound antibody was visualized by the avidin-biotin complex immunoperoxidase technique (ABC) (Elite ABC Kit, Vectastain, Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. The sections were incubated with the biotinylated second layer antibody and the peroxidase-labeled avidin-biotin complex for 30 min each. Peroxidase activity was developed with 3-amino-9-ethyl-carbazole (A-5754, Sigma Chemical Co.), and finally the sections were stained with hematoxylin. Sections that were treated with PBS served as negative controls. Rat pancreatic and skin specimens were used as positive controls for trypsin and MMP-8 immunohistochemistry (24).

The immunoreactivities of trypsin and MMP-8 were analyzed independently by two investigators (K.S. and K.C.) in a blinded fashion. In each case, the entire section of lung tissue (approximately 1 cm²) was evaluated. The level of trypsin immunoreactivity was scored in a semiquantitative manner according to the following method: absent = 0, low = 1, moderate = 2, or strong = 3. Separate scores were given for bronchial and bronchiolar epithelium, alveolar epithelium, and vascular smooth muscle.

***Statistical analysis.*** Data are expressed as box and whisker plots, representing median, interquartile range, and 10th and 90th percentiles. Comparisons between controls and hyperoxia groups were performed with the nonparametric multiple comparison Kruskal-Wallis test (StatView 5.0.1, Abacus Concepts Inc., Berkeley, CA). The Dunn's test was used for the *post hoc* comparisons. *p* Values <0.05 were considered statistically significant.

## RESULTS

No evident signs of respiratory distress or macroanatomic changes could be detected up to 48 h of exposure to >95% $O_2$. At 60 h, pleural effusions were found on opening the thoracic cavity and the lungs looked hemorrhagic and consolidated. Protein concentration in BALF was stable until 48 h, when it was significantly increased, and after 60 h of exposure a 10-fold increase was detected as compared with controls (Fig. 1*A*). Increased MPO activity in lung tissue was first observed at 48 h, and the MPO activity further increased by 60 h (Fig. 1*B*).

***Characterization of trypsin and MMPs in BALF.*** In gelatin zymograms, three gelatinolytic proteinases of molecular weights 28, 68, and 90 kD were clearly detected at 48 h of
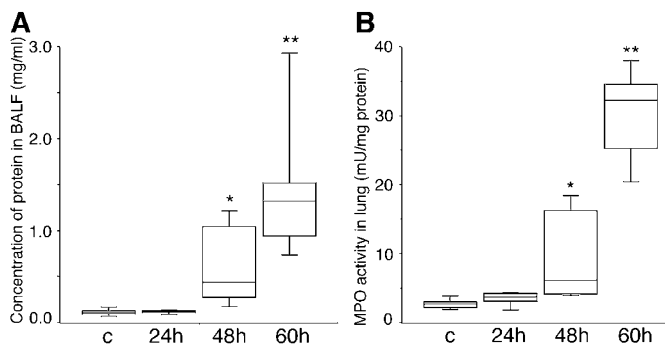


**Figure 1.** Protein concentration in BALF samples (*n* = 8 per group) (*A*) and MPO activity in lung homogenates (*n* = 6 per group) (*B*) from controls and rats exposed to >95% oxygen for 24, 48, and 60 h. Data shown as box and whisker plots, representing median, interquartile range, and 10th and 90th percentiles. **p* < 0.05; ***p* < 0.0005 *vs* controls.

exposure, and the gelatinolytic activity was further increased at 60 h (Fig. 2A). In contrast, very faint gelatinolytic activity was detected in BALF samples from controls or rats exposed to 24 h of hyperoxia (Fig. 2A). By ECL Western blotting, the 28-kD species was identified as trypsin (Fig. 2B), and the 68- and 90-kD species as proenzyme forms of MMP-2 and -9, respectively (data not shown). At 48 and 60 h of exposure, Western blot analysis of BALF samples with anti-MMP-8 revealed the presence of both neutrophil-derived 80-kD MMP-8 and 60-kD mesenchymal cell–derived MMP-8 species, of which the 60-kD mesenchymal cell–derived MMP-8 species predominated. No MMP-8 immunoreactivity could be demonstrated in controls or in rats exposed to 24 h of hyperoxia (Fig. 2C). Densitometric analysis demonstrated that BALF levels of trypsin and MMP-2 were significantly increased by 48 h of hyperoxia (Fig. 3A,B). At 60 h, the levels of these enzymes were further increased, and a significant increase was also detected in levels of MMP-9 (Fig. 3), as well as in levels of both neutrophil-derived and mesenchymal cell–derived MMP-8 (Fig. 4).

*Trypsin immunohistochemistry.* In normoxia controls, low trypsin immunoreactivity was detected in bronchial and bronchiolar epithelial cells, whereas alveolar epithelium was predominantly negative (Fig. 5A). After 24 h of hyperoxia, there was a tendency for increased trypsin immunoreactivity in bronchial and bronchiolar epithelium when compared with controls (Figs. 5B and 6B). At 48 and 60 h, immunohistochemistry revealed moderate to strong expression of trypsin in alveolar epithelium, whereas in bronchial and bronchiolar epithelium, the trypsin level remained low (Fig. 5C and D). When compared with controls, semiquantitative scoring showed a significant increase in the level of trypsin immunoreactivity in alveolar epithelium of rats exposed to 48 and 60 h of hyperoxia ($p < 0.05$ and $p < 0.005$, respectively)
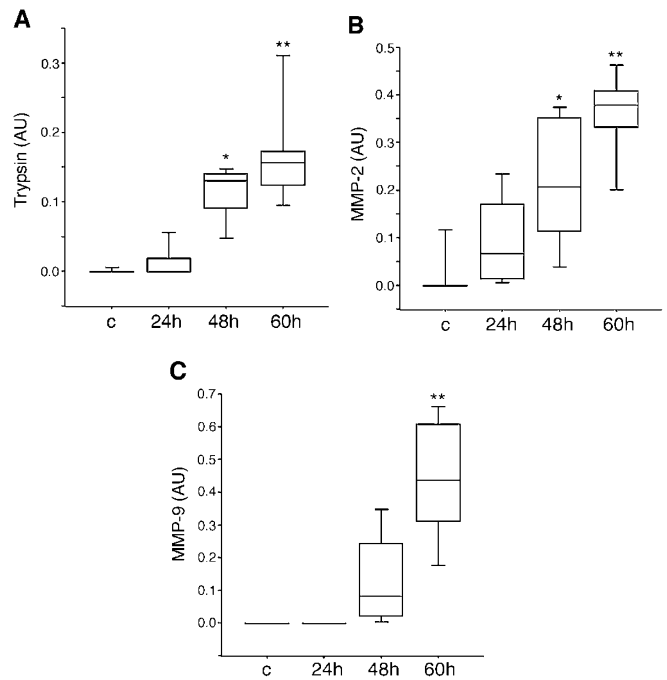


**Figure 2.** BALF samples from controls and rats exposed to >95% oxygen for 24, 48, and 60 h. Gelatin zymogram (A); ECL Western immunoblotting for trypsin (B). Lane 1: BALF sample from rat exposed to hyperoxia for 60 h, lane 2: rat pancreatic trypsin, lane 3: human tumor-associated trypsin-2. *Arrowheads* indicate 28 kD trypsin. ECL Western immunoblotting for MMP-8 (C). Both neutrophil-derived (PMN) and mesenchymal cell-derived (mes) isoforms of MMP-8 are detected. Positions of molecular weight standards indicated by kD.



**Figure 3.** Levels of trypsin (A), MMP-2 (B), and MMP-9 (C) in BALF from controls and rats exposed to >95% oxygen for 24, 48, and 60 h as evaluated by densitometric analysis of gelatinolytic bands. Data shown as in Fig. 1. AU, arbitrary unit; *$p < 0.01$; **$p < 0.0005$ *vs* controls.
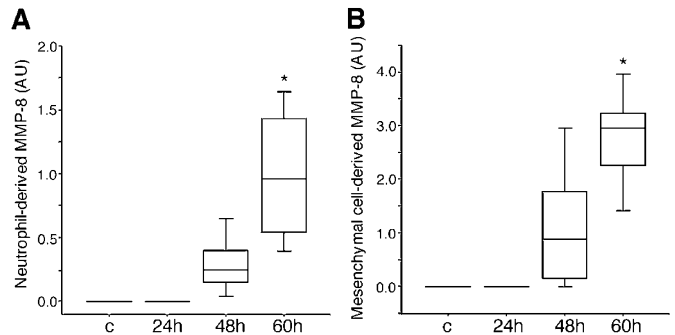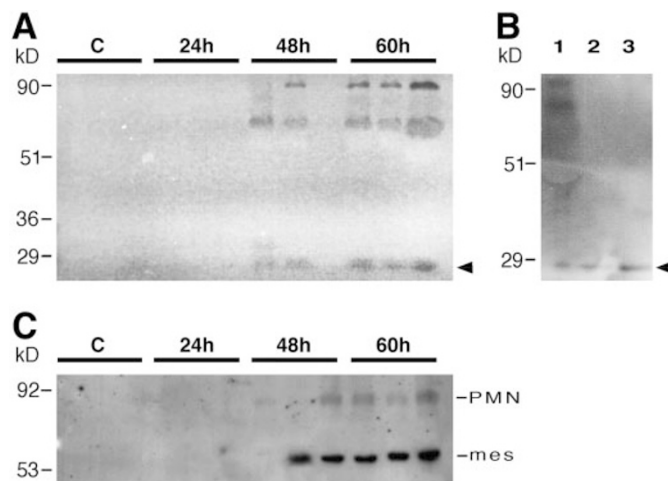


**Figure 4.** Levels of neutrophil-derived latent MMP-8 (A) and mesenchymal cell–derived latent MMP-8 (B) in BALF from controls and rats exposed to >95% oxygen for 24, 48, and 60 h. Evaluated by densitometric analysis of immunoreactive bands in Western immunoblots. Data shown as in Fig. 1. AU, arbitrary unit; *$p < 0.0005$ *vs* controls.

(Fig. 6A). In four of the six rats exposed to 48 or 60 h of hyperoxia, moderate or strong trypsin immunoreactivity could be detected in vascular smooth muscle of large arteries, whereas smooth muscle of smaller arteries was negative. In addition, trypsin immunoreactivity was detected in hyaline membranes and occasionally in intra-alveolar macrophages.

*MMP-8 immunohistochemistry.* Almost no immunoreactivity for MMP-8 could be demonstrated until 48 h of hyperoxia, when positive cells with a large cytoplasm obviously representing recruited macrophages could be detected in edematic perivascular space (Fig. 7A–C). At 60 h, a large number of macrophages strongly positive for MMP-8 were detected in alveoli as well as in interstitium (Fig. 7D and E). Although MMP-8 was predominantly localized in macrophages, at 48 h, positive immunostaining was also found in intravascular and
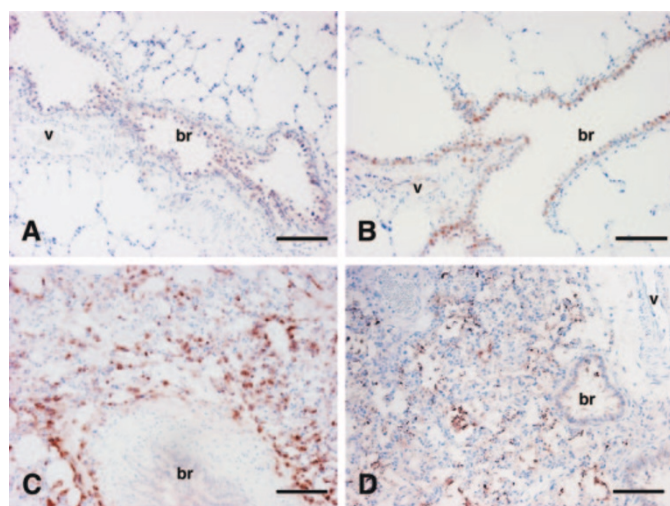
**Figure 5.** Immunohistochemical localization of trypsin in hyperoxia-injured and control rat lung. In controls (*A*) and rats exposed to >95% oxygen for 24 h (*B*), low trypsin immunoreactivity is observed in bronchial epithelium, whereas alveolar epithelium is predominantly negative. After exposure to >95% oxygen for 48 h (*C*) and 60 h (*D*), strong trypsin immunoreactivity is detected in alveolar epithelium, whereas immunoreactivity in bronchial epithelium remains low. br, bronchiole; v, blood vessel. Scale bars = 100 $\mu$m.
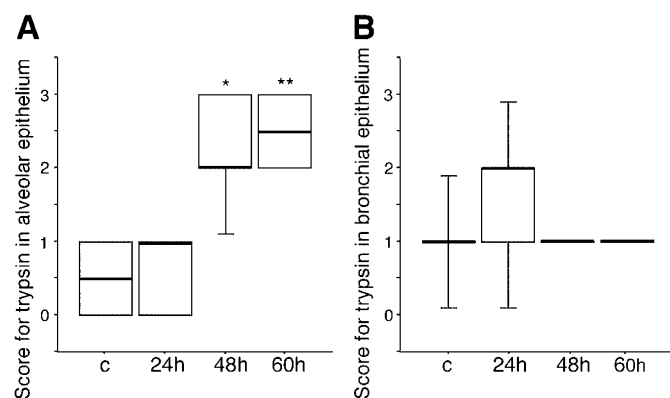


**Figure 6.** Box plot of semiquantitative analysis of trypsin immunoreactivity in alveolar (*A*) and bronchial (*B*) epithelium in normoxia controls and rats exposed to >95% oxygen for 24, 48, and 60 h (*n* = 6 per group). Semiquantitative scoring of trypsin immunoreactivity was assessed as follows: absent = 0, low = 1, moderate = 2, or strong = 3. Data shown as in Fig. 1. *$p < 0.05$; **$p < 0.005$ *vs* controls.



**Figure 7.** Immunohistochemical localization of MMP-8 in hyperoxia-injured and control rat lung. Lung samples from control (*A*) and rat exposed to >95% oxygen for 24 h (*B*) show no MMP-8 immunoreactivity. In rats exposed to >95% oxygen for 48 h(*C*) and 60 h (*D*), marked MMP-8 immunoreactivity is detected in recruited macrophages (*arrows*) and neutrophils (*arrowheads*). (*E*) Positive intra-alveolar macrophage; (*F*) positive intravascular neutrophils. br, bronchiole; v, blood vessel. Scale bars: *A–D* = 100 $\mu$m; *E* and *F* = 10 $\mu$m.

perivascular neutrophils and, in addition, at 60 h in neutrophils located in the interstitium and intra-alveolar space (Fig. 7*F*).

## DISCUSSION

Acute lung injury is marked by destruction of the alveolar-capillary barrier. We show a striking increase in the pulmonary expression of the potent matrix-degrading serine proteinase trypsin during the development of hyperoxic lung injury in rat. After 48 h of exposure to >95% oxygen, immunohistochemical analysis demonstrated strong expression of trypsin in alveolar epithelium, which, in contrast, showed almost no immunoreactivity for trypsin under normoxic conditions.

Trypsin can directly attack various important structural components of the pulmonary ECM such as native collagen types I and IV (3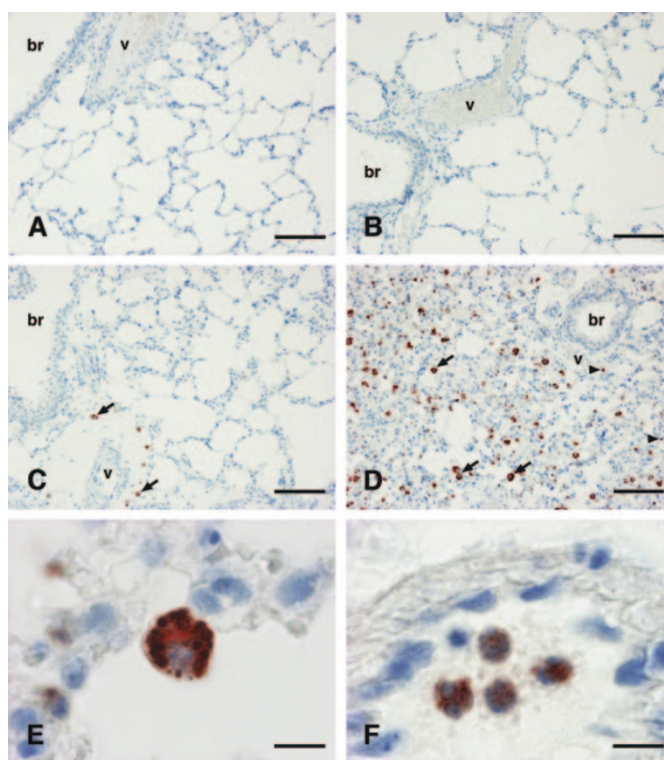,25), of which collagen IV is the major component of the alveolar-capillary BM. In addition to trypsin, zymography of BALF samples from rats exposed to 48 and 60 h of hyperoxia demonstrated the up-regulation of two other gelatinolytic enzymes that were identified as MMP-2 and -9. Similarly to trypsin, MMP-2, and MMP-9 can efficiently degrade BM structures (2,13). The marked up-regulation of trypsin, MMP-2, and MMP-9 in BALF coincided with an increase in the alveolar-capillary permeability, as indicated by increased protein concentration in BALF, suggesting that they may play a role in the degradation of alveolar-capillary BM associated with the development of hyperoxic lung injury. In accordance with our results, an earlier study showed increased expression of pulmonary MMP-2 and -9 in rats exposed to 100% oxygen for 60 h (26). The expression of MMP-2 was localized in interstitial cells and MMP-9 in intra-alveolar macrophages (26). In our study, the markedly increased expression of trypsin in alveolar epithelium corroborates an important role for alveolar epithelium in the pathogenesis of oxidant injury leading to increased permeability and subsequent pulmonary edema (19,20).

Trypsin efficiently activates various pro-MMPs, including MMP-8 and -9, at very low concentrations by cleaving off the activation propeptide, which concomitantly results in diminished molecular weight of the target MMP (2,3). In this study, most of the MMP-2, -8, and -9 detected in BALF samples were in nonconverted proforms as demonstrated by Western

immunoblotting. This is in line with the findings of our previous study, in which we characterized the presence and the molecular weight forms of MMP-2, -8, and -9 in tracheal aspirate fluid samples from preterm infants with RDS (16). It is noteworthy that some of the nonconverted forms may represent oxidatively activated forms of these MMPs *in vivo* since oxidative activation of MMP-8 or -9 does not necessarily involve changes in their molecular sizes (27,28).

Besides its capacity to degrade ECM and BM structures, trypsin can cause tissue damage by inducing inflammation (11,29,30). In a rat model of pancreatitis-associated lung injury, infusion of trypsin or trypsinogen causes an acute dose-dependent pulmonary injury characterized by perivascular edema and hemorrhage (29). This lung injury is neutrophil dependent and possibly mediated by the ability of trypsin to up-regulate pulmonary intercellular adhesion molecule-1, a key vascular endothelial adhesion molecule necessary for transport of leukocytes from intravascular space into inflamed tissues (30). The proinflammatory effects of trypsin may also be mediated through activation of $PAR_2$, a G protein–coupled receptor that is believed to play an important role in inflammation (10,11). $PAR_2$ is markedly up-regulated by inflammatory agents and widely expressed in lungs (11). Activation of $PAR_2$ increases lung vascular and epithelial permeability to protein and causes pulmonary edema in a dose-dependent manner (10). In preterm infants with RDS, trypsin-2 colocalizes with $PAR_2$ in airway epithelium (12).

However, increased trypsin in alveolar epithelium may also play a protective role in the development of lung injury. In the injured lung, active transepithelial transport of $Na^+$ limits alveolar edema (31). Trypsin increases ion transport across rat alveolar type II cells and paracellular resistance, indicating that it may play an important role in the clearance of alveolar fluid (32). In fluid-filled lungs in a rat model, inhibition of trypsin by intratracheal soyabean trypsin inhibitor or $\alpha_1$-antitrypsin decreases amiloride-sensitive lung fluid clearance, and the effect is partially restored by instillation of trypsin (32).

In control rats, we detected low trypsin expression in bronchial epithelium. This is in accordance with earlier studies showing expression of trypsin in bronchial epithelium of human adults and of term newborn infants without pulmonary pathology and supports the hypothesis that trypsin also participates in normal cellular functions in the lung (7,8).

We observed a marked up-regulation of MMP-8 after 48 of exposure to hyperoxia. MMP-8 (collagenase-2) was previously regarded solely as a neutrophil-specific MMP that is stored in granules and released on degranulation (33). However, certain activated mesenchymal cells and macrophages also express MMP-8 (18,23). In our study, immunoblotting of BALF samples demonstrated the presence of both neutrophil-derived 80-kD MMP-8 and 60-kD mesenchymal cell–derived MMP-8 species, of which the mesenchymal cell–derived MMP-8 species clearly predominated. Immunohistochemical analysis confirmed that in the hyperoxic lung, MMP-8 was mostly expressed in recruited macrophages, which at 48 h were detected in perivascular space and at 60 h in alveoli and interstitium. In inflammation, MMP-8 is believed to have a

pivotal role in the degradation of collagen Type I. Recently, an unexpected anti-inflammatory role was evidenced for MMP-8 in the lung (15,34). One possible mechanism is the regulation of inflammatory cell apoptosis, as demonstrated by reduced neutrophil apoptosis in MMP-8$-/-$ mice during allergen-induced lung inflammation (34). During the development of hyperoxic lung injury, a large number of infiltrating inflammatory cells is found in the rat lung (35). Whether the role of MMP-8 in hyperoxic lung injury is anti-inflammatory possibly by regulating inflammatory cell apoptosis remains a subject for future research.

In summary, in a rat model of hyperoxic lung injury, we have demonstrated a striking increase in pulmonary expression of the potent matrix-degrading serine proteinase trypsin after 48 h of hyperoxia. Trypsin was localized in alveolar epithelium, which in controls was predominantly negative. In line with trypsin, the expression of especially mesenchymal cell–derived MMP-8 was clearly increased during the course of hyperoxic lung injury and was localized in intra-alveolar and interstitial macrophages. Although we cannot directly draw a parallel from our results in the hyperoxic lung injury model to earlier findings in preterm infants with RDS, these results support the hypothesis that high pulmonary levels of trypsin and MMP-8 are injurious in the lung. Further exploration of the roles of trypsin and MMP-8 in acute lung injury may offer new targets for therapeutic intervention.

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