Published in : Toxicology (2005)
Status : Posprint (Author's version)

Repeated cadmium nebulizations induce pulmonary MMP-2 and MMP-9 production and enphysema in rats

Nathalie Kirschvink[a,1], Grégoire Vincke[a,1], Laurence Fiévez[a], Cécile Onclinx[a], Delphine Wirth[a,] Michèle Belleflamme[a,] Renaud Louis[b,] Didier Cataldo[b,] Michael J. Peck[c], Pascal Gustin[a]

[a] *Department for Functional Sciences B41, Section of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Veterinary Medicine, University of Liège, Liège 4000, Belgium*
[b] *Center for Biomedical Integrative Genoproteomics (CBIG), University of Liège, Liège 4000, Belgium*
[c] *UCB Pharma, Br aine l'Alleud, Belgium*

Abstract

This study describes induction of pulmonary inflammation, production of matrix metalloprotease of type 2 (MMP-2) and type 9 (MMP-9), and emphysema in cadmium (Cd)-exposed rats. Sprague-Dawley rats were randomly distributed into two groups: one placebo-exposed group undergoing saline (NaCl 0.9%) inhalation (n = 30) and one Cd-exposed group undergoing cadmium ($CdCl_2$ 0.1%) inhalation (n = 30). The animals of the placebo- and Cd-exposed groups were divided in five subgroups (n = 6). Subgroups underwent either a single exposure of 1 h or repeated exposures three times weekly for 1 h during 3 weeks (3W), 5 weeks (5 W), 5 weeks followed by 2 weeks without exposure (5 W + 2) or 5 weeks followed by 4 weeks without exposure (5 W + 4). Each animal underwent determination of enhanced pause (Penh) as index of airflow limitation prior to the first exposure as well as before sacrifice. The animals were sacrificed the day after their last exposure. The left lung was fixed for histomorphometric analysis (determination of median interwall distance (MIWD)), whilst bronchoalveolar lavage fluid (BALF) was collected from the right lung. BALF was analyzed cytologically, and MMP-2 and MMP-9 levels were determined by gelatine zymography. Twelve rats previously instilled with pancreatic elastase were used as positive emphysema controls and underwent the same investigations. Cd-exposure induced a significant increase of BALF macrophages, neutrophils and MMP-9 up to 5 W + 4, whereas MMP-2 gelatinolytic activity returned to baseline levels within 5W. MIWD was significantly increased in all repeatedly Cd-exposed groups and elastase-treated rats. Penh was increased in Cd-exposed rats after a single exposure and after 3W. MMP gelatinolytic activity was significantly correlated with macrophages, neutrophils and Penh. In repeatedly exposed rats, MIWD was positively and significantly correlated with MMP gelatinolytic activity, suggesting that increased MMP-2 and MMP-9 production favours the development of emphysema.

Keywords : Animal model ; emphysema ; metalloproteases ; cadmium

1. Introduction

Pulmonary emphysema is characterised by abnormal enlargement of the respiratory regions of the lung, distal to terminal bronchioles, and is accompanied by destruction of alveolar walls (Heard et al., 1979). In humans, emphysema predominantly develops in patients exposed to tobacco smoke and is associated with chronic obstructive pulmonary disease (COPD), defined as the progressive development of poorly reversible airflow limitation, and as an abnormal inflammatory response of the lungs to noxious particles and gases (Pauwels et al., 2001; Barnes et al., 2003). Repeated exposure to cigarette smoke is the major inducing factor of COPD and subsequent emphysema, but others such as air pollution, under-nutrition and occupational exposure might also lead to emphysema (Barnes et al., 2003; Coxson et al., 2004).

The pathogenesis of smoking-induced emphysema is under intensive investigation and recent research focuses on the proteases-antiprotease equilibrium, based on the imbalance between proteases produced by inflammatory cells in the lower respiratory tract and the antiproteolytic defences of the lung, inducing lung matrix degradation

[1] These authors contributed equally to the study.

by excessive activity of proteolytic enzymes (Barnes et al., 2003; Cataldo et al., 2003; Van den Steen et al., 2002). Matrix metallo-proteinases (MMPs) belong to a family of zinc- and calcium-dependent enzymes, which are implied in numerous physiological and pathological processes (for review see Van den Steen et al., 2002). Among the MMPs, gelatinases A (or MMP-2) and B (MMP-9) are believed to play a predominant role in lung tissue remodelling and repair through degradation of collagen and different matrix proteins including elastase (Atkinson and Senior, 2003; Cataldo et al., 2003). In humans suffering from COPD, an increased secretion and/or activity of MMP-2 and MMP-9 have been demonstrated in bronchoalveolar lavage fluid (BALF) (Betsuyaku et al., 1999), induced sputum (Cataldo et al., 2000), lung tissue (Ohnishi et al., 1998), alveolar macrophages, neutrophils and lymphocytes (for review see Atkinson and Senior, 2003) and peripheral blood (Mao et al., 2003), suggesting that the proteolytic activity of MMPs is related to pulmonary inflammation and might favour development of emphysema.

Among the animal models of emphysema, those based on the administration of elasto-lytic enzymes such as elastase or papain (for review see Snider et al., 1986) or those based on calorie restriction (for review see Snider et al., 1986; Massaro et al., 2004) do not develop airspace enlargement in response to an inflammatory process. On the opposite, models based on inhalation of cadmium or cigarette smoke have been shown to induce an inflammation within the lung, leading in certain models to emphysematous lesions (for review see Snider et al., 1986; Tuder et al., 2003; Wright and Churg, 2002; Selman et al., 2003). The implication of MMPs and their inhibitors (tissue inhibitor of matrix proteases (TIMP)) in development of emphysema has been demonstrated in several animal models exposed to cigarette smoke (Hautamaki et al., 1997; Shapiro, 2000; Tuder et al., 2003; Selman et al., 2003; Churg et al., 2004; Xu et al., 2004), but to the best of the authors' knowledge, MMPs have not yet been investigated in an animal model of pulmonary cadmium exposure.

As cadmium is one of the numerous components of tobacco smoke (Nandi et al., 1969) and accumulates in man during the exposure to tobacco (Paakko et al., 1989; Grasseschi et al., 2003), we hypothesized that cadmium could have a direct effect on the occurrence of tobacco-related emphysema in humans. The objective of the present study was then to determine whether cadmium plays a role in emphysema, lung inflammation, and metalloprotease production. Elastase-treated rats were used as positive emphysema controls, whilst placebo rats were exposed to saline and served as negative controls.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (n = 72) weighing between 440 and 520 g at the start of the study were used. The animals were obtained from the University's animal breeding unit. The rats were weighed weekly throughout the study. They were housed in small groups (n = 2-3) in appropriate cages on wood shavings, and received food and water ad libitum. The cages were cleaned twice weekly, and water was changed every 2 days. The animals were kept at 21 °C with a 12-h light-dark cycle. The study was approved by the Animal Ethical Committee of the University of Liège.

2.2. Study design

2.2.1. Cadmium-induced emphysema

The animals were randomly distributed into two groups: one placebo-exposed group undergoing saline (NaCl 0.9%) inhalation (n = 30) and one cadmium-exposed group undergoing cadmium ($CdCl_2$ 0.1%) inhalation (n = 30). The animals of the placebo- and cadmium-exposed groups were further divided in five subgroups of six animals each. Each cadmium-exposed group was matched with a placebo-exposed group. The different subgroups underwent either a single exposure (D2) of 1 h or repeated exposures three times weekly for 1 h during 3 weeks (3W), 5 weeks (5W), 5 weeks followed by 2 weeks without exposure (5 W + 2) or 5 weeks followedby 4 weeks without exposure (5W + 4). Each animal underwent measurements by B WBP prior to the first exposure as well as before euthanasia. The animals were sacrificed the day aftertheir last exposure. Bronchoalveolar lavage and subsequent BALF analyses were performed on the right lung, whereas the left lung was fixed for histomorphometric analysis.

2.2.2. Elastase-induced emphysema

Twelve rats were used as positive emphysema controls 8 weeks after a single intra-tracheal elastase administration. Their lungs were prepared as described here above.

2.3. Repeated cadmium or placebo exposure

Cadmium chloride (0.1% $CdCl_2$ prepared in sterile saline (NaCl 0.9%), Sigma-Aldrich, Brussels, Belgium) was nebulized using an ultrasonic nebulizer (Ultraneb 2000, 1.63 MHz, Devilbiss, Somerset, USA) known to generate particles of a median diameter of 3 µm (ranging from 0.5 to 5 µm). The aerosol output was 6 mL/min, which were propelled by the airflow of 30L/min into a glass chamber (dimensions (length × width × height): 58 cm × 38 cm × 34 cm) where groups of six rats were allowed to move freely during exposure. Two lateral openings (diameter of 10 mm) in the chamber ensured a regular dispersion of the aerosol. The whole system was placed within an extractor hood ensuring a safe aerosol evacuation. Each group was nebulized three times weekly during 1 h. The placebo-exposed groups were nebulized with sterile saline using the same chamber, which was carefully cleaned in order to avoid cross contamination.

2.4. Barometric whole body plethysmography measurements

Respiratory variables were measured during 30 min before the first exposure and at the end of the study, 24 h after the last exposure. In order to acclimatize the rats to the procedure, the animal were trained on three consecutive days preceding the measurements by placing them for 30 min into the plethysmograph chamber. A system of BWBP for unrestrained rodents was used (Emka Technologies, France). The plethys-mograph was ventilated by a continuous bias flow of 2 L/min (Bias Flow Regulator, Vent2, Emka Technologies, France). A differential pressure transducer (Emka Technologies, France) was connected on one pole to the main chamber and on the second pole to a reference chamber equilibrated with atmospheric pressure by a small channel (1.5 mm). Transduced signals were amplified, digitised and sampled at 100 Hz by use of the IOX software version 1.530, which provided a breath-by-breath analysis of pressure signals and allowed the calculation of Penh, a unit-less estimator of airflow limitation (Hamelmann et al., 1997; Kirschvink et al., 2005). The chamber pressure signal was calibrated by dynamic injection of 5 mL of room air via a syringe into the main chamber of the plethysmograph.

2.5. Intra-tracheal elastase administration

Rats were anesthetised by an i.p. injection of ke-tamine (50mg/kg; Imalgène 1000, Merial, Belgium, Bruxelles, Belgium) and xylazine (5mg/kg; Xyl-M 2%, VMD, Berendonk, Belgium). Elastase (porcine pancreatic elastase, Roche Diagnostics, Mannheim, Germany) was diluted in sterile saline and 75IU/100 g body weight was instilled (~ 100 µL) by use of a flexible tube (outer diameter: 2 mm) via the oral cavity and the larynx into the distal part of the trachea. The animals were placed during anaesthesia recovery in dorsal recumbence; the head and thorax slightly elevated.

2.6. Bronchoalveolar lavage and lung fixation

The rats were sacrificed by a lethal i.p. injection of 200 mg/kg pentobarbital (Dolethal, Vetoquinol, France). The chest wall was opened and the cardiopulmonary tract including the lower part of the trachea was carefully extracted from the rib cage. The heart was withdrawn and the entire lung was carefully manipulated in order to avoid pleural rupture and blood contamination of the airways. A catheter was introduced via the trachea into the right lung and a ligature was placed on the main right bronchus. The right lung was lavaged by two successive instillations of 8 mL saline (NaCl 0.9%), which were recovered by gentle suction. Both fractions were pooled. BALF recovery ranged from 81 to 96 %, and did not differ between rat groups. The right lung was discarded after the lavage.

A second catheter was introduced into the left main bronchus and also ligatured. The left lung was immediately formalin-fixed (4%) by a connexion of the bronchial catheter to a formalin circuit kept at 4 °C and allowing a constant pulmonary pressure of 25 cm H_2O for at least 24 h. Once fixed at a constant pressure, the left lung was kept in 4% formalin until being processed for histology.

2.7. Cytological analysis of bronchoalveolar lavage fluid

One hundred microlitres of BALF were mixed with 400 µL of Tiirck solution, and a total cell count using a Thomas cell was performed within 4 h after collection. Four counts were performed for each BALF sample. One hundred and fifty microlitres of BALF were used for cytospin centrifugation (Shandon, Pittsburgh, PA, USA), and were Giemsa stained for differential cell count. Hundred cells were systematically counted; epithelial cells were very rare and were not taken into account.

2.8. Determination of protein content in bronchoalveolar lavage fluid

One millilitre of BAL fluid was kept at -80 °C for the determination of protein concentration. Protein levels were determined using a spectrophotometric assay (microprotein, Elitech Diagnostics, France). A standard curve was run, and each sample was analysed in duplicate. Working reagent (1mL) was mixed with 20 µL of BAL fluid, and absorbance was read at 598 nm after 5 min of incubation. Coefficients of variation were lower than 5%.

2.9. Determination of BALF matrix metalloprotease activity

Five millilitre of BALF were centrifuged for 15 min at 4 °C (450 × *g*), and the supernatant was fractioned into 500 µL samples, which were kept at -80 °C until analysis. The activity of MMP-2 and MMP-9 was measured by gelatin zymography (Cataldo et al., 2000). Standards of MMP-9 (human pro-enzyme MMP-9; oncogene, San Diego, CA, USA) and MMP-2 (human pro-enzyme MMP-2; oncogene, San Diego, CA, USA) and BALF samples diluted in non-reducing sample buffer were loaded onto gelatin gels and were subjected to electrophoresis on 36% acrylamide SDS gels containing porcine skin gelatine (1%, Sigma, St. Louis, MO, USA). After electrophoresis the gels were washed twice in Triton-X-100 2% for 30 min, and incubated at 37 °C for 24 h in an activation buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$. Following incubation the gels were rinsed and stained for 30 min with Coomassie blue, and destained for ~2 h in a solution of 10% acetic acid and 20% methanol (v/v). Gelati-nolytic activity appeared as unstained zones against a blue background. Gels were scanned (Epson Perfection 2450 Photo, Seiko Epson Corp., Japan) and converted to numeric images for quantification using NIH imaging software, and were analysed with Scion imaging analysis program (Scion Corporation, Frederick, MA, USA). Results were expressed as average arbitrary units (AU) corresponding to pixel density × mm^2 for the bands of proteolysis normalized by the same value calculated for a known amount of control.

2.10. Lung histomorphometry

After fixation, lungs were embedded in paraffin and 2-3 µm sagittal slices were cut in the medial lung portion and stained with haematoxylin-eosin and Masson's trichrome. Histomorphometry was performed on haematoxylin-eosin stained slices, whereas Masson's trichrome stained slices were used to illustrate the development of collagen deposits. The lung field selected for histomorphometry was arbitrarily chosen in the dorsal part of the lung where emphysematous lesions, when they were present, were most prominent. Care was taken to avoid regions containing pleura or large bronchi. The lung field (magnification: 100 ×) was dig-italised using a numeric camera (3 CCD Sony XP007P, Japan) and Image-Pro Plus software (MediaCybernet-ics, Silver Spring, MD, USA). A grid with seven horizontal and vertical lines was superposed on the field, and the distances between alveolar structures that were crossed by the gridlines were measured using Scion Image (Scion Corporation, Frederick, MD, USA). This method gave around 200 individual measurements per lung field allowing the calculation of the median inter-wall distance (MIWD) for each animal, used as index of the diameter of the alveoli and the alveolar ducts (Gevenois et al., 1995). Shrinkage was not taken into consideration.

2.11. Statistical analysis

All data except MIWD data were normally distributed and presented as mean ± S.D., and were analysed by a two-way analysis of variance (ANOVA), followed by PLSD-Fishertests, when appropriate. MIWD data were shown as medians and were analysed by Kruskall-Wallis and Mann-Whitney tests. Correlation analysis was performed by linear regression. The *p*-value lower than 0.05 was considered as statistically significant.

3. Results

3.1. Evolution of body weight

Body weight of the saline- and cadmium-exposed animals constantly increased over time ($p < 0.01$) but differed between groups ($p < 0.005$); the weight of the cadmium-exposed rats (D2: 470 ± 21g; W3: 470 ± 38g; W5: 505 ± 64g; W5 + 2: 519 ± 46g; W5 + 4: 524 ± 46 g) being lower than that of placebo-exposed rats (D2: 481 ± 21g; W3: 494 ± 38g; W5: 541 ± 28g; 540 ± 34g; W5 + 2: 540 ± 34d; W5 + 4: 541 ± 45g). Elastase-instilled rats showed normal growth in comparison to controls. No mortality due to cadmium or elastase exposure was noted.

3.2. Barometric whole body plethysmography

By comparison to their respective control groups, Penh of rats nebulized once or during 3 weeks with cadmium was significantly increased (Fig. 1a). Respiratory rate tended to be increased in rats after a single cadmium-exposure (data not shown). Penh remained unchanged in elastase-instilled rats (Fig. 1b).

Fig. 1. *(a) Penh recorded in rats (six rats per group) before placebo or cadmium exposure, and 24 h after the last exposure with either placebo (NaCl 0.9%) or cadmium. D2, single exposure; W3 and W5, three exposures per week during 3 and 5 weeks, respectively; W5 + 2 and W5 + 4, three exposures per week during 5 weeks with 2 and 4 weeks of recovery, respectively, (b) Penh recorded in elastase-treated rats (n=12) before instillation and 8 weeks after instillation. (*) Significantly different from respective pre-exposure value, ($) significantly different from all other values, p < 0.01.*

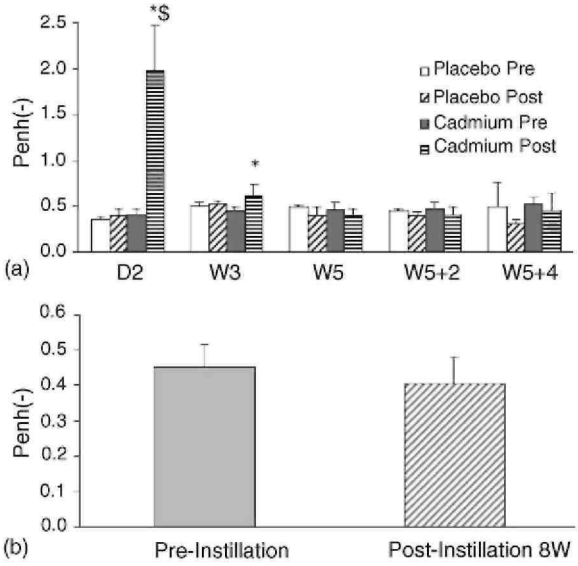


Fig. 2. *(a) BALF macrophage; and (b) neutrophil count in placebo-and cadmium-exposed rats (n = 6 per group) as well as in elastase-treated rats (n = 12). Refer to legend of (a) forx-axis abbreviations. (*) Significantly different from respective placebo-group (* p < 0.05, **p < 0.01; ***p < 0.005), ($) significantly different from respective D2-group, p < 0.05.*

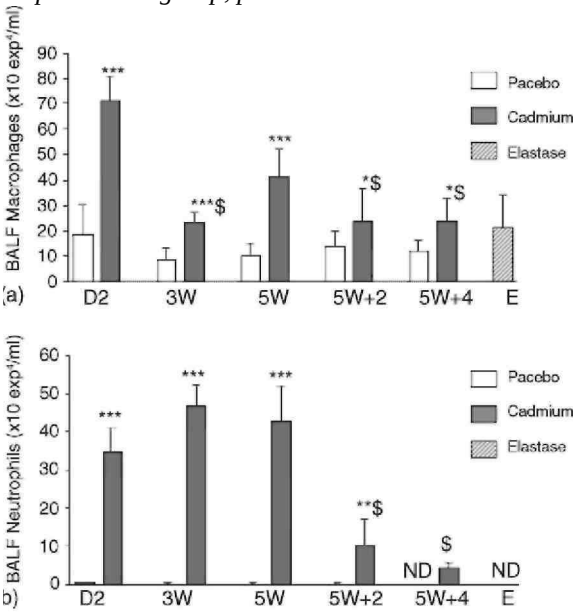


Table 1 *Relative changes of bronchoalveolar cytology in placebo- and cadmium-exposed rats*

Time point	Group	Macrophage (%)	PMN (%)	Lymphocyte (%)
D2	Placebo (n = 6)	98 ± 2	1 ± 1	1 ± 1
	Cadmium (n = 6)	67 ± 4*	32 ± 3*	1 ± 1
W3	Placebo (n = 6)	98 ± 1	1 ± 1	0.2 ± 0.4
	Cadmium (n = 6)	33 ± 4*	67 ± 4*	0.8 ± 0.8
W5	Placebo (n = 6)	98 ± 2	1 ± 1	1 ± 1
	Cadmium (n = 6)	48 ± 5*	51 ± 6*	0.7 ± 1
W5 + 2	Placebo (n = 6)	97 ± 1	1 ± 1	1.3 ± 1.7
	Cadmium (n = 6)	71 ± 5*	27 ± 5*	0.7 ± 1
W5 + 4	Placebo (n = 6)	99 ± 1	0.5 ± 0.5	0.5 ± 0.5
	Cadmium (n = 6)	85 ± 5*	14 ± 4*	0.3 ± 0.4
	Elastase (n=12)	99 ± 1	0.1 ± 0.3	0.5 ± 0.6

Data are shown as mean ± S.D.
Rat groups were sacrificed after a single exposure (D2), after 3 (W3) or 5 (W5) weeks of exposure, after 5 weeks of exposure followed by 2 (W5 + 2) or 4 (W5 + 4) weeks of recovery. (*) Significantly different from respective placebo-value ($p < 0.001$).

3.3. Broncho alveolar lavage cytology

Cytology of BALF revealed a significant increase of total cells in all cadmium-exposed groups, whereas elastase-instilled animals were similar to saline-exposed rats. This increase in total cells was due to significant changes in macrophages (Fig. 2a) and neutrophils (Fig. 2b), which both persisted up to 4 weeks after the end of cadmium-exposures. Lymphocyte counts did not change significantly (data not shown). Table 1 illustrates relative changes of BALF cytology.

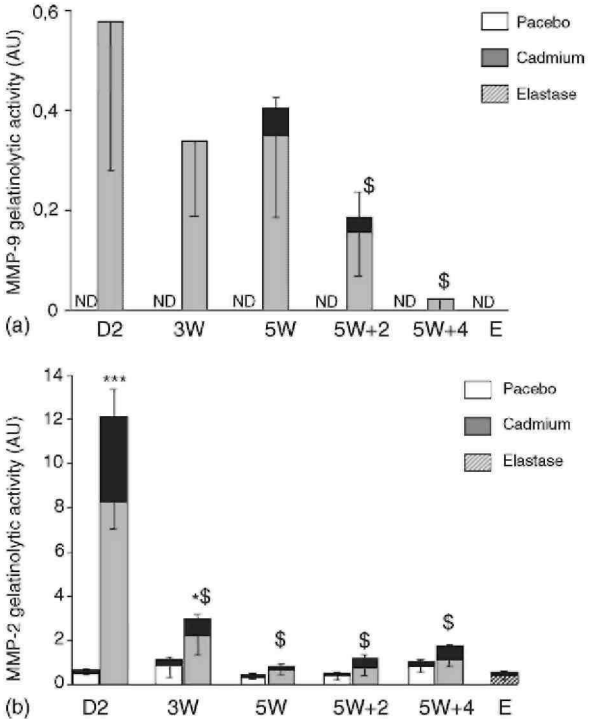
3.4. Protein concentration of broncho alveolar lavage fluid

A dramatic increase of BALF protein concentration was noted after a single cadmium exposure (1003 ± 702 mg/L) in comparison to placebo-exposed rats (103 ± 18mg/L, $p < 0.0001$). There were no time-related differences in placebo-exposed rats (data not shown). Cadmium-exposed rats of group 3W, 5W, 5W + 2 and 5W + 4 did not differ from time-matched controls (data not shown). Elastase-instilled rats were also similar to placebo-exposed animals.

3.5. Zymography

A significant increase of Pro-MMP-9-related gelati-nolytic activity was detected in BALF of all cadmium-exposed rats, whereas no activity could be detected in saline-exposed and elastase-instilled rats (Fig. 3a). Pro-MMP-9 gelatinolytic activity was highest after a single cadmium-exposure, and decreased progressively. Nevertheless, even after 4 weeks of exposure cessation, Pro-MMP-9 activity remained detectable. Activated MMP-9 forms were rarely present. In contrast to MMP-9, some MMP-2-related gelatinolytic activity (mainly Pro-MMP2) was detected in BALF of saline-exposed and elastase-instilled rats. Similarly to MMP-9, MMP-2 levels were also highest after a single cadmium exposure, but returned to control values within 5 weeks of exposure (Fig. 3b). Activated form of MMP-2 was also significantly increased after a single cadmium-exposure as well as in animals that underwent 3 weeks of exposure.

Fig. 3. *(a) BALF MMP-9; and (b) MMP-2 gelatinolytic activity in placebo- and cadmium-exposed rats (n = 6 per group) as well as in elastase-treated rats (n= 12). Gelatinolytic activity of pro-forms are shown in white and grey for placebo- and cadmium-exposed animals, respectively. Gelatinolytic activity of activated forms are shown in black. Refer to legend of Fig. 2a for x-axis abbreviations. ND: no gelatinolytic activity detected. (*) Significantly different from respective placebo-group (*p < 0.05, ***p < 0.005); ($) significantly different from respective D2-group, p < 0.05.*



3.6. Histomorphometry

Whilst some elastase-treated rats showed macroscopic signs of emphysema (subpleural bullae) at lung dissection, no macroscopic lesions were seen in cadmium-exposed rats.

Light microscopy revealed moderate alveolar filling with oedema fluid and increased numbers of macrophages and neutrophils within the alveoli of rats of the cadmium group D2. Bronchial walls were thickened whilst no mucus was seen within the airways. After 3 and 5 weeks of exposure, distal airspace enlargement appeared and was most homogenous in the dorsal part of the lung. Alveolar oedema was no more present and only few macrophages, neutrophils and lymphocytes were present within the alveolar spaces. Multifocal parenchymal and peri-bronchial accumulation of inflammatory cells was noted within in the whole lung. In groups 5W + 2 and 5W + 4, alveolar accumulation of inflammatory cells further decreased, whereas peribronchial and multifocal inflammatory nodules persisted. Airspace enlargement was further detectable, but appeared as to be more heterogeneous. In elastase-instilled rats, no signs of alveolar or bronchial inflammation were detected, but large regions of dramatically increased alveoli were seen. In comparison to cadmium-exposed rats, airspace enlargement was less homogenous within the lung, but lesions were more pronounced. Representative slides for saline-, cadmium- and elastase-exposed rats are shown in Fig. 4.

Masson's trichrome stains showed that peribronchial collagen deposition increased in rats after repeated (at least 3 weeks) cadmium exposure (Fig. 5). Placebo-exposed rats showed only very mild peribronchial collagen deposit within the large bronchi, whereas collagen could be detected in distal airways and - to a less extent - in alveolar interstitium, in cadmium-exposed rats.

The histomorphometric analysis indicated that rats undergoing repeated cadmium-exposure had a significantly increased MIWD in comparison to their respective controls (Fig. 6). Except for the cadmium group D2, where MIWD was significantly decreased, no differences for MIWD between cadmium groups were noted. The MIWD of saline-exposed groups remained unchanged throughout exposures, even if a larger variability was seen in the groups 5W + 2 and 5W + 4. Elastase-instilled animals had the highest MIWD, which was significantly different

from that of saline- and cadmium-exposed rats.

***Fig. 4**. (a) Histological examination of the left lung sections of rats following saline exposure; (b) unique cadmium exposure; (c) 5-week cadmium exposure; and (d) elastase treatment. Haematoxylin and eosin stain. Magnification 100 ×. Note the apparent alveolar oedema and inflammation after a single cadmium exposure and airspace enlargement after 3-week cadmium exposure and elastase administration.*

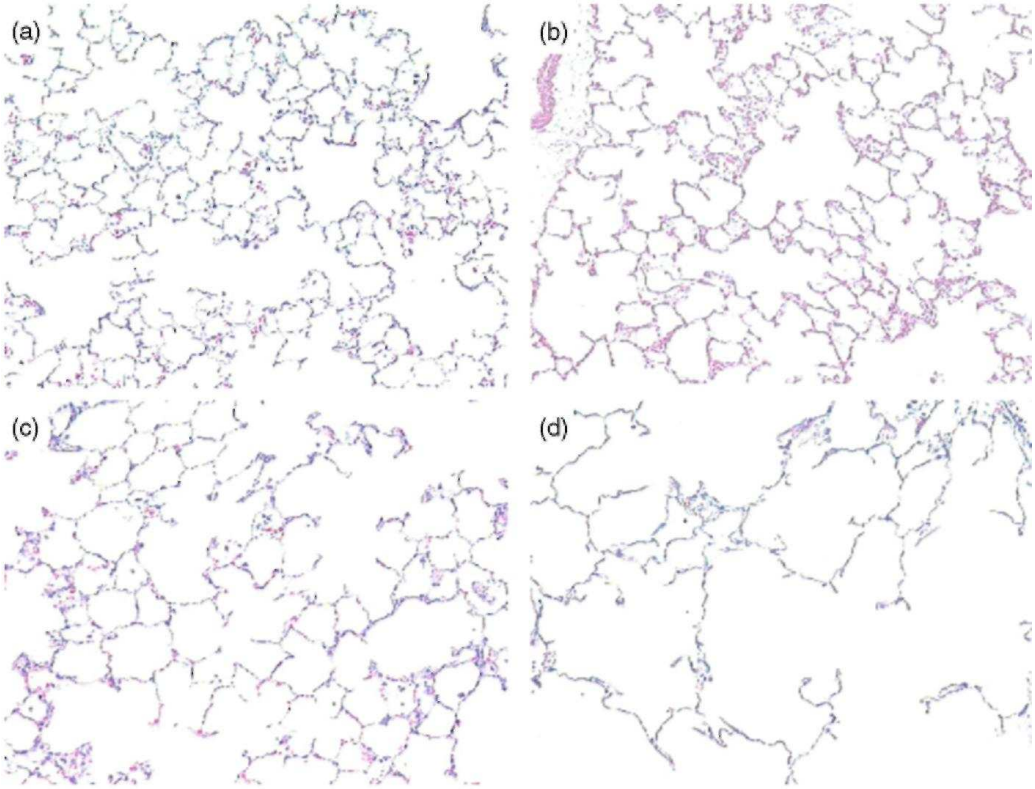


***Fig. 5.** (a) Histological examination of the left lung sections of rats following saline exposure; and (b) a 5-week cadmium exposure. Masson's trichrome stain. Magnification l00x. Note the apparent peri-bronchial collagen deposit in a cadmium-exposed rat.*

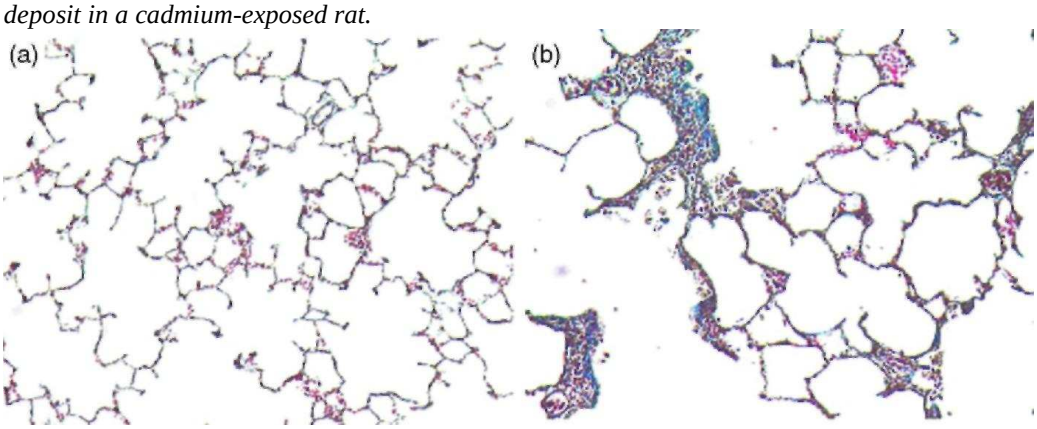
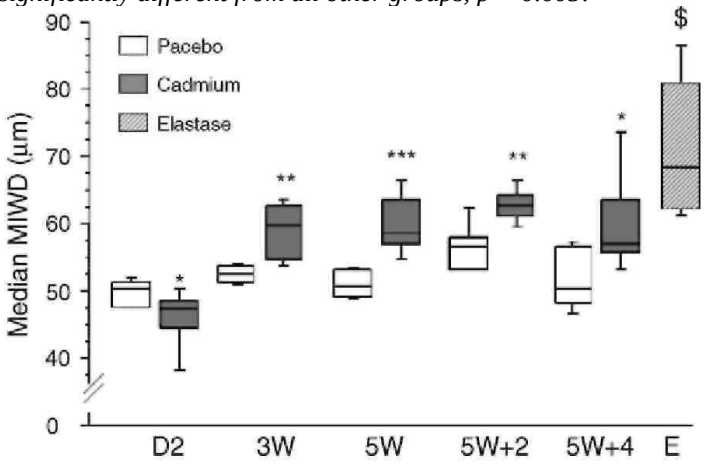


Fig. 6**. Median interwall distance (MIWD) in placebo- (empty boxes) and cadmium-exposed (dark grey boxes) rat, as well as in elastase-treated rats (shaded box) (n = 12). Refer to legend of Fig. 2a for x-axis abbreviations. The line within each box represents the median value, the upper and lower lines of the box represent 75th and 25th percentiles, respectively, and the upper and lower whiskers represent the 90th and 10th centiles, respectively. (*) Significantly different from respective placebo-group, *p < 0.05, **p < 0.01, p < 0.005; ($) significantly different from all other groups, p < 0.005.*



3.7. Correlation analyses

As shown in Table 1, BALF inflammatory cells were positively correlated with BALF MMP-9 and MMP-2 gelatinolytic activity. MMP-9-related gelati-nolytic activity was strongly correlated with neutrophils (r = 0.79) and macrophages (r = 0.72). Interestingly, Penh, as in vivo index of airflow limitation, was also significantly correlated with MMP-9 (r = 0.4) and MMP-2 (r = 0.79) activity as well as with BALF total cell count (r = 0.48). When the rats that were sacrificed after a single cadmium exposure were included in the correlation analysis, MIWD was negatively correlated with MMP-2 activity (r = -0.53) and macrophages (r = -0.27). If only repeatedly exposed animals were considered, MIWD was positively correlated with MMP-9 (r = 0.41) and MMP-2 (r = 0.33) activity as well as with BALF total cell counts (r = 0.37), macrophages (r = 0.3) and neutrophils (r = 0.36). Regarding the other variables, exclusion of D2-datasets did not strongly affect the results given in Table 2 (data not shown).

***Table 2** Coefficients of correlation between histomorphometric, inflammatory and functional variables of saline- and cadmium-exposed rats (n = 60)*

	MIWD	MMP-9	MMP-2	Total cells	Macrophages	Neutrophils	Lymphocytes	Penh
MIWD	-	NS	-0.53**	NS	-0.27*	NS	NS	NS
MMP-9	NS	-	0.67***	0.83***	0.72***	0.79***	0.37**	0.40**
MMP-2	-0.53**	0.67***	-	0.67***	0.74***	0.48***	0.27*	0.79***
Total cells	NS	0.83***	0.67***	-	0.91***	0.91***	0.52***	0.48**
Macrophages	-0.27*	0.72***	0.74***	0.91***	-	0.66***	0.56***	NS
Neutrophils	NS	0.79***	0.48***	0.91***	0.66***	-	0.36**	NS
Lymphocytes	NS	0.37**	0.27*	0.56***	0.56***	0.36**	-	NS
Penh	NS	0.40**	0.79***	0.48**	NS	NS	NS	-
MIWD (n= 54)	-	*0.41"*	*0.33**	*0.37"*	*0.30**	*0.36***	*NS*	*NS*

MIWD, median interwall distance; Penh, enhanced pause determined before sacrifice; NS, non significant. * p < 0.05, ** p < 0.005, *** p < 0.001. Bottom row (italic): coefficients of correlation when data of acute cadmium-exposure (single exposure; n = 6) was not taken into account (n = 54).

4. Discussion

This study describes, in a rat model of cadmium-induced emphysema, an early increase of pulmonary MMP-9 and MMP-2 related gelatinolytic activities that correlate with neutrophilic and macrophagic lung inflammation.

In comparison with an elastase-induced model of emphysema, airspace enlargement observed in cadmium-nebulized rats is less pronounced but correlates with pulmonary inflammation.

One drawback of this study is the lack of precise information about the particle size and cadmium concentration of the $CdCl_2$ aerosols used. Indeed, particle size plays a considerable role in inhalation toxicity, such as shown in the study of Cassée et al. (2002) where pulmonary toxicity and deposition of $CdCl_2$ aerosols were shown to increase with decreasing particle diameter (1495 nm versus 637 nm, 170 nm and 33nm).

In rats, neutrophils and macrophages have been shown to release MMP-9 and MMP-2 in response to different stimuli in vitro (Gibbs et al., 1999; Warner et al., 2000). Both proteases are upregulated in human prostatic epithelial cells in vitro in response to cadmium (Achanzar et al., 2001) and in the lung during in vivo endotoxin challenges in rats (Kohno et al., 2004). Accordingly, inflammatory cells could account for the increase of MMP-9 and MMP-2 levels observed in response to the cadmium exposure. In comparison with placebo-exposed rats, a unique nebulization with cadmium induced an important and significant increase of BALF MMP-9 and MMP-2 amounts. Comparatively, MMP-9 activity levels were lower than that of MMP-2 and were undetectable in placebo-exposed animals, whereas a residual MMP-2 activity levels were found after placebo exposure. By repeating neb-ulizations, MMP-9 levels remained increased up to W5 + 4, whereas MMP-2 was similar to that of controls within 5 weeks. Both proteases were positively correlated with BALF macrophage and neutrophil counts. However, MMP-9, which is reported to be predominantly released by neutrophils (Warner et al., 2000; Atkinson and Senior, 2003; Churg et al., 2004), was strongly correlated with this cell type, whereas MMP-2 showed a better correlation with macrophage count.

The inflammatory response observed in cadmium-exposed rats was predominated by neutrophils and macrophages and similar to that reported in earlier studies (Paterson, 1947; Snider et al., 1973; Dervan and Hayes, 1979; Hart, 1986). As the main objective of our model was the assessment of the effect of chronic cadmium exposure, only a single group of rats was investigated after a unique exposure, allowing confirmation that cadmium induced the expected acute inflammatory reaction (Thurlbeck and Foley, 1963; Snider et al., 1973; Dervan and Hayes, 1979). By repeating cadmium exposure, BALF macrophages slightly decreased, whereas BALF neutrophils remained elevated. After cessation of cadmium nebu-lizations, BALF macrophages and neutrophils progressively decreased but remained different from controls for at least 4 weeks. These results suggest that a persisting inflammation had been induced and resemble to findings made in human smokers with persistent bronchial inflammation even after smoking cessation (Barnes et al., 2003). Similar observations have been made in cigarette smoke exposed mice that developed chronic pulmonary inflammation associated with increased MMP activity (Seagrave et al., 2004).

All cadmium-exposed rats that underwent at least three weeks of nebulizations showed a significant increase of their MIWD, reflecting the development of emphysema. Four weeks after exposure cessation, individual MIWD values were more variable but still significantly different from respective controls, which either could be due to tissue regeneration or to weight-related changes (Massaro et al., 2004). Age-matched rats that had been exposed to placebo showed similar MIWD values throughout the nebulization period as well as throughout the follow-up period. Their growth-related increase of body weight was significantly more important than that of cadmium-exposed rats, suggesting that cadmium exposure and subsequent pulmonary inflammation interfered with growth. Poor body condition or starvation favours development of emphysema in man and in rodents (Coxson et al., 2004; Massaro et al., 2004). Accordingly, the emphysematous changes observed in our rats might have been amplified when differences in body weight were most important, i.e. at 3W, 5W and 5W + 2. As the weight difference between placebo- and cadmium-exposed rats decreased at 5W + 4, it might be hypothesized that the differences in MIWD were less affected by body condition than at earlier time points. In order to exclude the potential contribution of growth, it would be interesting to perform this study in adult rats with a stabilized weight at the beginning of the study. Indeed, data obtained in placebo-exposed rats indicate that weight stabilized at 5W, whereas cadmium-exposed rats had not yet reached a plateau at 5W + 4.

In comparison with earlier studies investigating the effect of respiratory cadmium exposure, a lower extent of fibrosis was detected. Nevertheless, placebo-exposed animals did only show peri-bronchial collagen deposit at the level of large bronchi, whereas collagen was detected in distal airways in cadmium-exposed animals. Consequently, it seems unlikely that the emphysematous lesions detected in cadmium-exposed rats were exclusively due to alveolar wall distortion, as supposed in other studies (Snider et al., 1973; Dervan and Hayes, 1979; Kononov etal., 2001).

Intra-tracheal elastase instillation was used as well-established method for emphysema induction (for review see Snider et al., 1986; Damon et al., 1982). Indeed, MIWD values obtained in these rats were significantly higher

than those recorded in placebo-and in cadmium-exposed rats. According to literature, elastase-treated rats are most often sacrificed 8 weeks after instillation for histomorphometry (Snider et al., 1986), which explains why intermediate data on BALF cytology or MMP-activity were not obtained in this study. Our data nevertheless indicate that elastase administration led to emphysema without significant pulmonary inflammation or MMP-9 and MMP-2 activation at the time of sacrifice.

Interestingly, MIWD recorded in rats undergoing placebo or cadmium exposure for at least 3 weeks was significantly and positively correlated with BALF MMP-9 and MMP-2 levels as well as with BALF macrophages and neutrophils. These results are in agreement with the studies performed in human COPD patients (Di Stefano et al., 1998), and allow hypothesizing that cadmium-induced emphysema in rats is dependent of pulmonary inflammation as well as of MMP production. These findings would even be more relevant if the correlation analyses were run separately for each time point, but given the small number of animals per group, significance could not be reached. Further studies using anti-inflammatory therapeutics and/or MMP inhibitors should provide further insight on the role of MMPs in development of emphysema in this animal model. Moreover, a potentially existing disequilibriumbetweenMMPs and theirs inhibitors (TIMPs) warrants further investigation.

Beside assessment of the inflammatory and morphological changes induced by cadmium or elastase, the present study also investigated respiratory function using single chamber B WBP for unrestrained animals. Although this technique has been recently very severely criticised (Adler et al., 2004; Bates et al., 2004) and should be used with caution, it might be considered as a non-specific test, detecting airflow limitation in spontaneously breathing animals (Hamelmann et al., 1997; Halloy et al., 2004; Kirschvink et al., 2005). In the present study, significantly increased Penh-values and a modified plethysmograph box pressure trace were observed 24 h after the first cadmium-exposure, and corresponded to a clinically apparent inspiratory and expiratory dyspnoea. Cytology and protein concentration of BALF were suggestive of an acute inflammatory response and decreased MIWD values even suggested the presence of alveolar oedema. Earlier studies performed in cadmium-exposed rats effectively describe the presence of alveolar oedema after acute cadmium exposure (Paterson, 1947; Thurlbeck and Foley, 1963; Snider et al., 1973; Dervan and Hayes, 1979), but respiratory function has, to our knowledge, not yet been investigated in cadmium-exposed rats. Penh was still significantly different from time-matched control rats at 3W, but no more detectable modification of the plethysmograph box pressure change or the presence of airflow limitation could be evidenced at later time points. Elastase-treated rats did not show signs of airflow changes, although earlier studies using ventilatory mechanics evidenced lung function impairment in elastase-induced emphysema (Damon et al., 1982; Johanson and Pierce, 1972). Given that BWBP is not a very sensitive technique, detection of moderate modifications of pulmonary resistance or dynamic compliance in these animals was unlikely (Kirschvink et al., 2005). Nevertheless, these results suggest that repeated cadmium inhalation did not induce important changes of respiratory function that could be related to bronchial or interstitial remodelling.

With regard to human COPD, the rat model used in our study bears some interesting comparative aspects, such as the presence of persistent lower airway inflammation, MMP production and development of emphysema. However, as many animal models of COPD, the major limitations of this model are the initial acute irritant response and the lack of progressive inflammatory, functional and histomorphological changes once the cadmium exposure is stopped. In the light of the potential role of reactive oxygen species in COPD, investigations addressing cadmium-induced pulmonary oxidative stress in this rat model, such as described by Hart et al. (1996, 2001), appear as future challenges.

In conclusion, this study describes a model of cadmium-induced emphysema associated with increased MMP-9 and MMP-2 levels measured in BALF, and correlating with pulmonary inflammation. This model might by of interest for testing MMP inhibitors and anti-inflammatory agents aiming at prevention of emphysema.

Acknowledgements

This study was supported by the Walloon Region (DGTRE) and UCB Pharma, Belgium. G. Vincke was supported by a grant from FRIA, Belgium. Didier Cataldo is a postdoctoral researcher from the FNRS, Belgium.

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