Free Radical Research, March 2006; 40(3): 241–250

Airway inflammation in cadmium-exposed rats is associated with pulmonary oxidative stress and emphysema

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Accepted by Professor J. Vina

(Received 18 September 2005; in revised form 22 November 2005)

Abstract

The aim of this study was to test the hypothesis that pulmonary inflammation and emphysema induced by cadmium (Cd) inhalation are associated with pulmonary oxidative stress. Two groups of Sprague Dawley rats were used: one vehicle-exposed group undergoing inhalation of NaCl (0.9%, $n = 24$) and one Cd-exposed group undergoing inhalation of $CdCl_2$ (0.1%, $n = 24$). The animals in the vehicle-and Cd-exposed groups were divided into 4 subgroups ($n = 6$ per group), which underwent either a single exposure (D2) of 1H or repeated exposures 3 times/week for 1H for a period of 3 weeks (3W), 5 weeks (5W) or 5 weeks followed by 2 weeks without exposure (5W + 2). At sacrifice, the left lung was fixed for histomorphometric analysis (median inter-wall distance, MIWD), whilst bronchoalveolar lavage fluid (BALF) was collected from the right lung. Cytological analysis of BALF was performed and BALF was analysed for oxidant markers 8-iso-$PGF_{2\alpha}$, uric acid (UA), reduced (AA) and oxidised ascorbic acid (DHA) and reduced (GSH) and oxidised glutathione (GSSG). Cd-exposure induced a significant increase of BALF macrophages and neutrophils. 8-iso-$PGF_{2\alpha}$, UA, GSH and GSSG were significantly increased at D2. At 5W and 5W + 2, AA and GSH were significantly lower in Cd-exposed rats, indicating antioxidant depletion. MIWD significantly increased in all repeatedly Cd-exposed groups, suggesting development of pulmonary emphysema. 8-iso-$PGF_{2\alpha}$ and UA were positively correlated with macrophage and neutrophil counts. GSH, GSSG and 8-iso-$PGF_{2\alpha}$ were negatively correlated with MIWD, indicating that Cd-induced emphysema could be associated with pulmonary oxidative stress.

Keywords: *Cadmium inhalation, inflammation, bronchoalveolar lavage, 8-iso-$PGF_{2\alpha}$, glutathione, ascorbic acid*

Abbreviations: *AA, reduced ascorbic acid (μM); ARR, ascorbic acid redox ratio (= DHA/[AA + DHA]) (%); BALF, bronchoalveolar lavage fluid; DHA, dehydro-ascorbic acid (μM); oxidised ascorbic acid; GRR, glutathione redox ratio (= GSSG/[GSH + GSSG]) (%); GSH, reduced glutathione (μM); GSSG, oxidised glutathione (μM); MMP, matrix matelloprotease; ip, intra-peritoneal; MIWD, median inter-wall distance (μM); UA, uric acid (μM)*

Introduction

Chronic airway inflammation and oxidative stress are important features of chronic obstructive pulmonary disease (COPD), a condition that is characterised by a progressive and irreversible airflow limitation [1–3]. More than 90% of COPD-affected patients are smokers; cigarette smoking and inhalation of airborne pollutants including oxidant gases such as ozone, nitrogen dioxide or particulate air pollution lead to lung damage and activation of pulmonary inflammatory processes [3]. Cadmium (Cd) is an important component of tobacco smoke [4] and has been shown to accumulate during exposure to tobacco in man [5,6]. In animal models of COPD or emphysema, intra-tracheal administration or inhalation of Cd has been shown to induce a pulmonary inflammatory response as well as the subsequent development of emphysema [7–12], which mirrors the conditions observed in COPD-affected patients.

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760500494657

The pathogenesis of COPD and in particular the development of emphysema is under intensive investigation [1–3]. Oxidative stress, i.e. the disequilibrium between endogenous antioxidants and exogenous or endogenous oxidants in favour of the latter [13], appears to play a predominant role in the development of COPD [3,14]. Indeed, reactive oxygen species (ROS), such as superoxide anion or hydroxyl radical, might induce tissue damage by oxidising proteins, lipids and DNA [15,16]. When the endogenous antioxidant defence system is overwhelmed by the exogenous oxidative burden, cellular damage occurs and ROS generation might further be enhanced and even maintained by the respiratory burst of macrophages and neutrophils [17]. Moreover, the effects of ROS in airways may be mediated by the secondary release of inflammatory mediators, e.g. 4-hydroxy-2-nonenal; the latter can affect cell proliferation, apoptosis and activation of signalling pathways [18]. Another lipid peroxidation product, 8-iso-$PGF_{2\alpha}$, a member of the F_2-isoprostanes, is currently considered as one of the most reliable markers of lipid peroxidation in patients with asthma or COPD [19,20]. Beside its application as an oxidative "fingerprint" or marker, 8-iso-$PGF_{2\alpha}$ could also display some biological activity due to its structural similarity with its stereoisomer $PGF_{2\alpha}$ [19–21].

Antioxidant depletion is another characteristic feature in COPD. Non enzymatic antioxidants, which are of major importance in the pulmonary epithelial lining fluid [22], such as glutathione (GSH: reduced form, GSSG: oxidised form), vitamin E, ascorbic acid (AA: reduced form, DHA: dehydroascorbic acid, oxidised form) and uric acid (UA) have been reported to be decreased. There is also evidence that the activity of the antioxidant enzyme system (e.g. catalase, superoxide dismutase and glutathione peroxidase) may be disturbed [3].

Among the consequences of an impaired oxidant/antioxidant balance, inflammatory gene expression, mucus hypersecretion, apoptosis and a protease/antiprotease imbalance must also be considered. These factors are believed to play a significant role in development of emphysema and in airway remodelling [3,23]. Recently, an increased activity of metalloprotease 2 and 9 (MMP-2 and MMP-9) in bronchoalveolar lavage fluid (BALF) has been demonstrated in a rat model of Cd-induced emphysema, indicating that protease activity is up-regulated in this animal model [12]. In the present study, it was hypothesised that Cd-induced airway inflammation and pulmonary emphysema would be associated with pulmonary oxidative stress. In a vehicle-controlled study, pulmonary markers of oxidative stress (8-iso-$PGF_{2\alpha}$, UA, AA and GSH) and indices of pulmonary inflammation and emphysema were assessed after single and repeated vehicle or Cd inhalations in rats.

Materials and methods

Animals

Male Sprague Dawley rats ($n = 48$) weighing between 440 and 520 g at the start of the study were used. It was aimed by using relatively mature animals to minimise growth-related effects on the lung which could have an impact on Cd-induced emphysema. 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 two 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.

Study design

The animals were randomly assigned to one of two groups: one vehicle-exposed group undergoing saline (NaCl 0.9%) inhalation ($n = 24$) and one Cd-exposed group undergoing Cd ($CdCl_2$ 0.1% in 0.9% NaCl) inhalation ($n = 24$). The animals of the vehicle-and Cd-exposed groups were further divided in four subgroups of six animals each. Each Cd-exposed group was matched with a vehicle-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) or 5 weeks followed by 2 weeks without exposure (5W + 2). The animals were sacrificed the day after their last exposure. Bronchoalveolar lavage and subsequent BALF analyses were performed on the right lung, whereas the left lung was fixed for histomorphometric analysis.

Repeated Cd or vehicle nebulisation

Cd 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 an airflow of 30 l/min into a glass chamber (dimensions [length × width × height]: 58 × 38 × 34 cm^3) 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 exposed to nebulised Cd in the chamber for a period of 1 h, three times a week. The vehicle-exposed groups were exposed to nebulised sterile saline using the same chamber, which was carefully cleaned in order to avoid cross contamination.

Bronchoalveolar lavage and lung fixation

The rats were sacrificed by a lethal ip injection of 200 mg/kg pentobarbital (Dolethal, Vetoquinol, France). The chest wall was opened and the cardio-pulmonary 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 groups of animals. The right lung was discarded after lavage.

A second catheter was introduced into the left main bronchus and also ligatured. Only the left lung was immediately formalin-fixed (4%) by connection of the bronchial catheter to a formalin circuit kept at 4°C and allowing a constant pulmonary pressure of 25 cmH_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.

Bronchoalveolar lavage fluid processing

Six millilitre of BALF for total protein determination, 8-iso-$PGF_{2\alpha}$, ascorbic acid and UA analysis were centrifuged for 5 min at 2500*g* at 4°C. Supernatant was stored at − 80° C until analysis. For glutathione analysis, methanol was added to BALF (0.7 ml ml^{-1} BALF), which was centrifuged for 2 min at 13,000*g* at 4°C. Supernatant was withdrawn and stored in liquid nitrogen.

Cytological analysis of bronchoalveolar lavage fluid

One hundred microlitre of fresh BALF were mixed with 400 µl of Türck solution and a total cell count using a Thomas cell was performed within hours after collection. Four counts were performed for each BALF sample. Differential cell counts were performed on cytospin preparations (Shandon, Pittsburgh, PA, USA) made from 150 µl of BALF and stained with Giemsa. One hundred cells were systematically counted; epithelial cells were very rare and were not included in differential counts.

Determination of protein content in bronchoalveolar lavage fluid

Protein concentration was determined using a spectrophotometric assay (Microprotein, Elitech Diagnostics, France). A standard curve was run and each sample was analysed in duplicate. Working reagent (1 ml) was mixed with 20 µl of BALF and absorbance was read at 598 nm after 5 min of incubation. Coefficients of variation were lower than 5%.

Determination of BALF oxidative stress markers

8-Iso-$PGF_{2\alpha}$ in BALF was purified and concentrated as described elsewhere and was analysed by an enzyme immuno-assay kit (Cayman, Ann Arbor, MI, USA) [24]. BALF samples were defrosted and pH adjusted to 2–2.5 with 2 normal hydrochloric acid (HCl 2 N). BALF was centrifuged for 10 min at 200*g* at 4°C. One microlitre of supernatant was passed through a C18 1 ml·100 mg^{-1} column (Bond Elut,Varian, Harbor City, CA, USA). The column was rinsed with 1 ml water and 1 ml hexane. Elution was then performed with 2 ml (4 × 500 µl) ethyl-acetate/methanol (95:5 V/V). Eluate was vacuum-dried and reconstituted with buffer provided by the kit manufacturer. The kit was consequently used according to manufacturer's instructions. Each sample was analysed in triplicate.

UA was analysed by high performance liquid chromatography (HPLC) according to the method of Grootveld and Halliwell [25].

Ascorbic acid (AA) and dehydroascorbic acid (DHA) were analysed by HPLC with UV detection [26]. Ascorbic acid redox ratio was expressed in % and was calculated as follows: ARR = DHA/[AA + DHA].

Reduced glutathione (GSH) and oxidised glutathione disulfide (GSSG) in BALF were measured by use of HPLC with electrochemical detection according to the method described by Smith et al. [27]. Glutathione redox ratio was expressed in % and was calculated as follows: GRR = GSSG/[GSH + GSSG].

Lung histomorphometry

After fixation, lungs were embedded in paraffin and two 3-µm sagittal slices were cut from the medial lung portion and stained with haematoxylin–eosin. Histomorphometry was performed on haematoxylin–eosin stained slices. 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 digitalised using a numeric camera (3 CCD Sony XP007P, Japan) and Image-Pro Plus software (MediaCybernetics, Silver Spring, Maryland, 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

MIWD for each animal, used as an index of the diameter of the alveoli and the alveolar ducts [28]. Shrinkage was not taken into consideration.

Statistical analysis

All data except MIWD data were normally distributed and are presented as mean ± SD. Differences over time and between treatment groups were investigated using a 2-way analysis of variance (ANOVA), followed by PLSD-Fisher tests when appropriate. MIWD data are shown as median and percentiles and were analysed by Kruskall–Wallis and Mann–Whitney tests. Correlation analyses were performed using Pearson's correlation or Spearman rank correlation. A p-value lower than 0.05 was considered as statistically significant.

Results

BALF cytology

There was a significant increase in total number of cells in BALF of all Cd-exposed groups, whereas saline-exposed rats showed no change over time. This increase was due to a significant increase of BALF macrophages (Figure 1a) and BALF neutrophils (Figure 1b), which both persisted up to 2 weeks after the end of the last Cd-exposure. Lymphocyte counts did not change significantly in any group at any time point (data not shown).

BALF total proteins

BALF protein concentration was significantly increased 24H after a single Cd exposure (1003 ± 702 mg/l) in comparison to vehicle-exposed rats (103 ± 18 mg/l, $p < 0.0001$). Time-related differences in vehicle-exposed rats were not observed (data not shown). Cd-exposed rats of group 3W, 5W, 5W + 2 did not differ from time-matched controls (data not shown).

Pulmonary oxidative stress markers

Cd exposure induced a significant increase of BALF 8-iso-$PGF_{2\alpha}$ in group D2, whereas Cd-exposed rats of groups 3W, 5W and 5W + 2 did not differ from respective controls (Figure 2).

BALF UA concentration followed a very similar pattern to that of 8-iso-$PGF_{2\alpha}$: UA concentration was significantly increased after Cd exposure in group D2, but was not significantly different from vehicle-exposed rats at 3W, 5W and 5W + 2 (Figure 3).

BALF AA and DHA were similar in vehicle-and Cd-exposed groups at D2 and 3W. At 5W and 5W + 2, AA in Cd-exposed rats was significantly lower than that in their respective controls (Figure 4). There were no

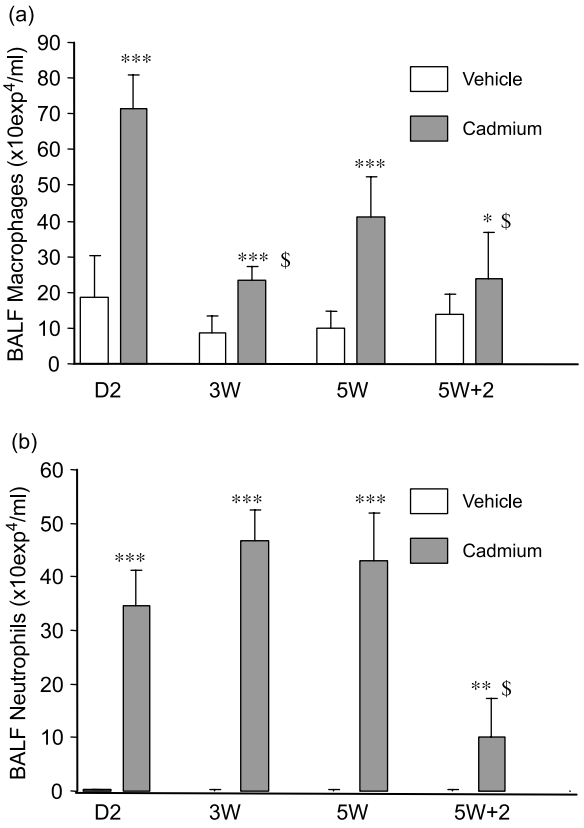

Figure 1. BALF macrophage (a) and neutrophil (b) counts in vehicle-and cadmium-exposed rats ($n = 6$ rats per group) 24 h after the last exposure with either vehicle (NaCl 0.9%) or cadmium. D2: single exposure, W3 and W5: 3 exposures per week during 3 and 5 weeks, respectively, W5 + 2: 3 exposures per week during 5 weeks with 2 weeks of recovery. ⋆significantly different from respective vehicle-group (⋆$p < 0.05$, ⋆⋆$p < 0.01$, ⋆⋆⋆$p < 0.001$), $^{\$}$significantly different from respective D2-group, $p < 0.05$.

significant treatment or time-related changes in ARR (data not shown).

BALF GSH and GSSG were both significantly increased after Cd exposure in group D2. A progressive decrease of GSH occurred in all repeatedly

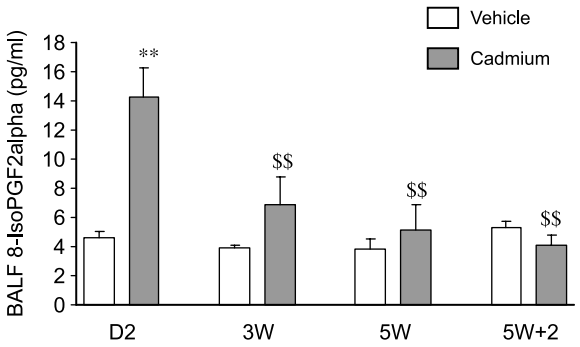

Figure 2. BALF 8-iso-$PGF_{2\alpha}$ concentration in vehicle-and cadmium-exposed rats ($n = 6$ rats per group). Refer to legend of Figure 1 for X-axis abbreviations. ⋆significantly different from respective vehicle-group (⋆$p < 0.05$, ⋆⋆$p < 0.01$), $^{\$}$significantly different from respective D2group ($^{\$}p < 0.05$, $^{\$\$}p < 0.01$).

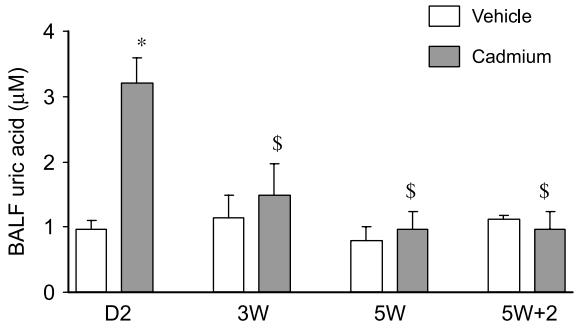

Figure 3. BALF uric acid concentration in vehicle-and cadmium-exposed rats ($n = 6$ rats per group). Refer to legend of Figure 1 for *X*-axis abbreviations. ⋆significantly different from respective vehicle-group ($^{\star}p < 0.05$), $^{\$}$significantly different from respective D2-group ($^{\$}p < 0.05$).

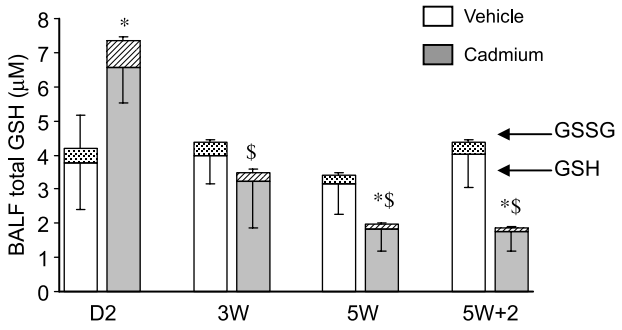

Figure 5. BALF GSH and GSSG concentrations in vehicle-and cadmium-exposed rats (6 rats per group). Refer to legend of Figure 1 for *X*-axis abbreviations. ⋆total glutathione (GSH + GSSG) significantly different from respective vehicle-group ($^{\star}p < 0.05$), $^{\$}$significantly different from respective D2-group ($^{\$}p < 0.05$), significantly different from all other GSSG concentrations, $p < 0.05$.

Cd-exposed groups and reached significance at W5 and W5 + 2 (Figure 5). There were no significant treatment or time-related changes in GRR (data not shown).

Histomorphometry

At lung dissection, no macroscopic lesions were seen in any of the Cd-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 Cd group D2. Bronchial walls were thickened, whilst no mucus was seen within the airways. After 3 and 5 weeks of exposure, distal airspace enlargement was apparent and was most homogenous in the dorsal part of the lung. Alveolar oedema was no longer 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 the whole lung section. In group 5W + 2, alveolar accumulation of inflammatory cells further decreased, whereas peri-bronchial and multifocal inflammatory nodules persisted. Airspace enlargement was further detectable, but appeared to be more heterogeneous. Representative slides for saline- and Cd-exposed rats are shown in Figure 6. As described in an earlier study using Masson's trichrome stains [12], very mild collagen deposition was observed in rats undergoing at least 3 weeks of Cd exposure.

The histomorphometric analysis indicated that rats undergoing repeated Cd-exposure had a significantly increased MIWD in comparison to their respective controls (Figure 7). Except for the Cd group D2, where MIWD was significantly decreased compared with the vehicle-exposed group, no significant differences in MIWD between Cd groups were observed. The MIWD of saline-exposed groups remained unchanged throughout exposures, suggesting that no growth-related changes of alveolar diameter had occurred.

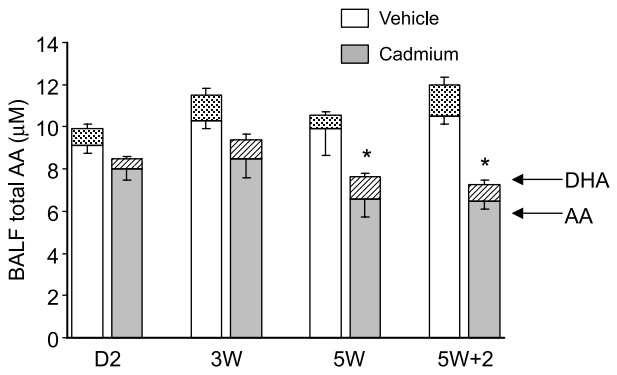

Figure 4. BALF AA and DHA concentrations in vehicle-and cadmium-exposed rats (6 rats per group). Refer to legend of Figure 1 for *X*-axis abbreviations. ⋆total ascorbic acid (AA + DHA) significantly different from respective vehicle-group ($^{\star}p < 0.05$).

Relationship between selected variables

Among the pulmonary oxidant markers, 8-iso-$PGF_{2\alpha}$ and UA were positively and significantly correlated with indices of pulmonary inflammation, whereas an opposite trend was observed for AA. In addition, 8-Iso-$PGF_{2\alpha}$, GSH and GSSG were negatively correlated with MIWD, whereas ARR correlated positively with MIWD (Table I). Macrophage count was also negatively correlated with MIWD ($r = -0.32$, $p < 0.05$). There were significant positive correlations between 8-iso-$PGF_{2\alpha}$ and UA ($r = 0.59$, $p < 0.0001$), 8-iso-$PGF_{2\alpha}$ and GSH ($r = 0.39$, $p < 0.005$) and 8-iso-$PGF_{2\alpha}$ and GSSG ($r = 0.46$, $p < 0.001$). GSH was also positively correlated with AA (Figure 8) and UA ($r = 0.31$, $p < 0.05$).

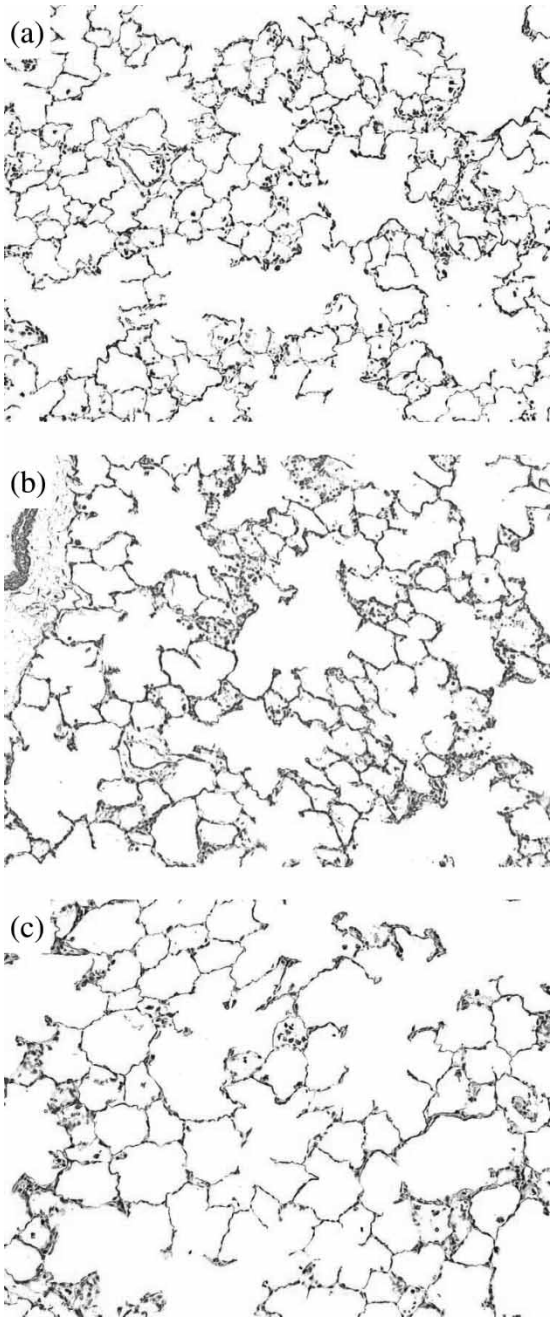

Figure 6. Histological appearance of medial-left lung sections of rats following saline exposure (a), single cadmium exposure (D2) (b) and 3-week cadmium exposure (3W) (c). Sections were stained with haematoxylin and eosin. Magnification 100×. Note the apparent alveolar oedema and inflammation after a single cadmium exposure (D2) (b) and airspace enlargement after 3-week cadmium exposure (3W) (c).

Discussion

The aim of the present study was to investigate the oxidant/antioxidant status in BALF of rats exposed to nebulised Cd and to test the hypothesis that Cd-induced airway inflammation and pulmonary emphysema would be associated with pulmonary oxidative stress.

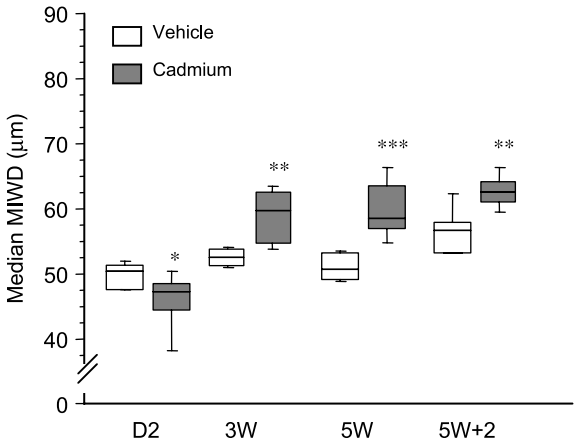

Figure 7. Median inter-wall distance (MIWD) in vehicle-(open boxes) and cadmium-exposed (filled boxes) rats ($n = 6$ rats per group). Refer to legend of Figure 1 for *X*-axis abbreviations. The horizontal line within each box represents the median value, the upper and lower edges of the box represent the 75th and 25th percentiles, respectively, and the upper and lower whiskers represent the 90th and 10th percentiles, respectively. *Significantly different from respective vehicle-group, $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

As described in earlier studies [7,8,12,29,30], a single inhalation of Cd induced a significant and acute pulmonary inflammation response as shown by increases in BALF total cell count and numbers or proportions of macrophages and neutrophils. Similarly, BALF protein concentration was significantly increased at D2 in Cd-exposed rats, reflecting the acute irritating effect of Cd. With repeated Cd exposure, airway inflammation progressively decreased but still persisted even after cessation of Cd nebulisation (5W + 2). However, BALF protein concentration at this time was not different from time-matched control rats.

With regard to MIWD, a significant decrease of this histomorphometric variable was noted at D2, suggestive of pulmonary oedema, which was also visible when the slides were examined by light microscopy. This finding was in line with the increased total protein concentration in BALF. After 3 weeks of exposure, the MIWD of Cd-exposed rats was significantly different from their time-matched controls and was suggestive of development of pulmonary emphysema that persisted at 5W and 5W + 2. Although the correlation between MIWD and BALF macrophages indicated a potential link between airway inflammation and development of emphysema, no further development of airspace enlargement despite persistent airway inflammation could be observed.

8-Iso-$PGF_{2\alpha}$, which is considered as one of the most reliable peroxidation markers in COPD patients [19,20], increased significantly in BALF after a single exposure but was not different from the vehicle-exposed values at or beyond 3 weeks. These results parallel observations made in ozone-exposed calves,

Table I. Correlation between BALF oxidative stress markers, BALF cytology and median inter-wall distance (MIWD) in vehicle ($n = 24$)- and cadmium-exposed rats ($n = 24$).

	8-Iso-$PGF_{2\alpha}$ (pg/ml)	UA (μM)	AA (μM)	DHA (μM)	ARR(%)	GSH (μM)	GSSG (μM)	GRR (%)
BALF total cell count	$r = 0.61$ ***	$r = 0.53$ ***	$r = -0.45$ **	ns	ns	ns	ns	ns
BALF macrophages	$r = 0.68$ ***	$r = 0.54$ ***	$r = -0.4$ **	ns *	ns	ns	$r = 0.35$	ns
BALF neutrophils	$r = 0.41$ **	$r = 0.43$ **	$r = -0.42$ **	ns	ns	ns	ns	ns
MIWD (μm)	$r = -0.4$ **	ns	ns	ns	ns	$r = -0.3$ *	$r = -0.37$ **	ns

UA: uric acid, AA: ascorbic acid, DHA: dehydro-ascorbic acid, ARR: ascorbic acid redo ratio, GSH: reduced glutathione, GSSG: oxidised glutathione, GRR: glutathione redox ratio, r: coefficient of correlation, *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$, ns: non significant.

which also showed a significant increase of BALF 8-iso-$PGF_{2\alpha}$ after a single ozone exposure, but which rapidly adapted within 7 days of exposure [24]. In COPD patients, 8-iso-$PGF_{2\alpha}$ in BALF, sputum or exhaled breath condensate remains elevated even after smoking cessation and might be further increased during exacerbations [20]. Although, BALF neutrophil count of our rats remained significantly elevated after cessation of Cd inhalation, it might be possible that neutrophils were less activated and did not further maintain the respiratory burst generating [31]. In this context, it would have been interesting to assess the activity of myeloperoxidase (MPO) as indicator of neutrophil activation.

BALF UA profile was similar to that observed for 8-iso-$PGF_{2\alpha}$ and was therefore, also suggestive of either adaptation or a decreased neutrophilic respiratory burst. It might be worth noting that UA can be considered as hydrophilic antioxidant or as oxidant marker [32,33]. Indeed, if UA synthesis is catalysed by xanthine dehydrogenase (XDH) it is not associated with ROS generation and can be considered to be acting as an important hydrophilic antioxidant, especially in pulmonary epithelial lining fluid [22]. However, under circumstances of oxidative stress, XDH might be converted into xanthine oxidase (XO) that also generates UA, but by liberating superoxide anions [32]. It has been shown in exercising horses that the switch from XDH to XO does occur and that UA generation via XO activity might be blocked using allopurinol, a XO inhibitor [33]. Given the positive correlation between BALF UA and BALF neutrophils and macrophages in the present study, UA appears as an oxidant marker related to on the degree of airway inflammation. However, further investigations determining the XDH/XO ratio or using allopurinol in the present model would be necessary to answer this question.

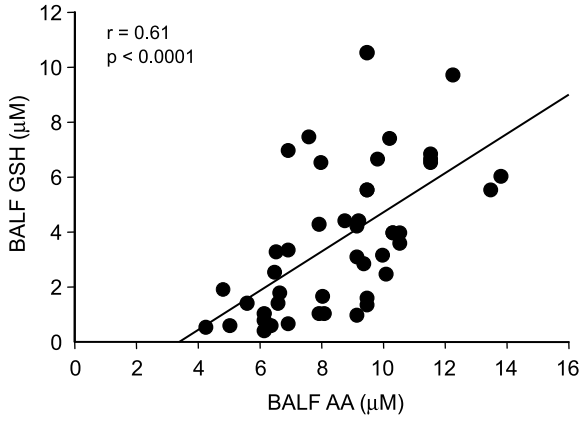

Figure 8. Scatter plot of BALF GSH concentration as a function of BALF AA concentration in vehicle-and cadmium-exposed rats ($n = 48$, all groups combined). Coefficient of correlation (R) and p-value are indicated.

Ascorbic acid in BALF did not undergo significant changes after the first Cd exposure and a significant decrease of its reduced form (AA) was only detectable at 5W and 5W + 2, indicating that depletion of this hydrophilic antioxidant occurred and persisted even after cessation of Cd inhalation. Interestingly, the decrease of AA did not parallel an increase of DHA and ARR and suggested that AA was potentially involved in detoxification or antioxidant processes that did not imply DHA accumulation [22]. Depletion of BALF and plasma AA has also been reported in man after ozone exposure as well as in COPD [3]. However, it should be born in mind that systemic and pulmonary AA concentrations in man depend on dietary ascorbic acid intake, whereas rats are able to synthesise AA [34]. Consequently, this comparison must be interpreted with care but nevertheless suggests that chronic Cd exposure increases the pulmonary turnover of this hydrophilic antioxidant. Increased lipid peroxidation in liver, kidney and serum has been described in guinea pigs that received oral Cd in conjunction with a diet low in ascorbic acid [35]. By increasing the dietary AA concentration, oxidative processes were decreased, indicating that AA played an important antioxidant action either by complexing Cd or by regenerating other antioxidants such as vitamin E or UA [36].

Glutathione is considered as one the most important antioxidants in pulmonary epithelial lining fluid and has been extensively studied in pulmonary antioxidant research [14]. The acute increase of BALF GSSG in Cd-exposed rats at D2 indicates that oxidative processes were increased, but balanced by a simultaneous increase of GSH. A similar pattern of response has been described in rat alveolar epithelial cells that were exposed *in vitro* to Cd chloride [37] and could be explained by a rapid increase of gamma-glutamyclcysteine synthetase and glutathione S-transferase expression, thereby offering protection against Cd toxicity [38]. With repeated Cd exposures in the present study, a significant and progressive decrease of BALF GSH was observed, whereas BALF GSSG concentrations were similar to time-matched vehicle-exposed rats. This pattern was similar but even more pronounced than that observed for AA and is in agreement with findings made in COPD patients. Indeed, reduced GSH concentrations in BALF and blood have been reported, particularly during exacerbations of disease [3,39,40]. Mechanisms similar to those inducing AA depletion might account for these changes [36]. It has further been demonstrated by Hart [41] that repeated Cd inhalation in rats induces a time-related increase of the Cd-binding protein metallothionein in the lung, as well as in the liver and the kidneys, showing that detoxification processes might undergo adaptation. Despite these adaptive responses, BALF GSH depletion occurs and might have considerable repercussions on GSH-dependent redox signalling [14,17].

Given that several other non enzymatic and enzymatic antioxidants play an important role in the pulmonary defence against ROS [22] and have been suggested to be involved in the development of emphysema [42], it would have been interesting to include other markers such as vitamin E, vitamin A, catalase or superoxide dismutase in this study. However, the volume of BALF recovered per rat was small and further analyses were not possible. As COPD is known to have systemic repercussions [43], it would also be interesting in future studies to assess the blood oxidant/antioxidant status of Cd-exposed rats.

Investigation of the relationship between pulmonary oxidative markers and BALF cytology suggested that 8-iso-$PGF_{2\alpha}$ and UA strongly parallel the inflammatory changes that occurred over time. Accordingly, 8-iso-$PGF_{2\alpha}$ and UA, whose concentrations are increased in presence of inflammatory cells [20,24,44], appear to be strongly dependent on the underlying inflammatory process. An opposite trend was found for AA as evidenced by the observation that AA depletion essentially occurred when the inflammatory process started to decrease. Given that the correlation analyses were run on data obtained at each time point and in both rat groups, these results might have been biased. However, due to the small number of rats in each group, separate correlations at each time point could not be performed.

Of particular interest are the significant negative correlations between MIWD and GSH and MIWD and GSSG, indicating that a down-regulation of the glutathione system or increased GSH turnover with impaired recycling coincides with airspace enlargement and development of pulmonary emphysema in this animal model. Again, the potential bias introduced by the analytical approach should be noted and might also account for the negative correlation that was found between MIWD and 8-iso-$PGF_{2\alpha}$.

Among the correlation analyses performed between pulmonary oxidative markers the most interesting correlation was probably that between BALF GSH and BALF AA. This observation further underlines the interaction between these important hydrophilic antioxidants and supports the notion of synergy existing between different antioxidant systems [45]. Similarly, UA was found to be positively correlated with GSH. Such data strongly support the recommendations that antioxidant trials should be performed using several antioxidants in order to benefit from synergistic antioxidant effects [46].

The rat model used in this study bears some interesting comparative aspects for COPD research, such as the presence of persistent lower airway inflammation, chronic and persisting AA and GSH depletion and development of emphysema. However, one limitation of this model is the initial acute irritant response and the lack of progressive histomorphological changes towards worsening emphysema during and after Cd exposure. In the light of the potential implication of oxidative stress in protease/antiprotease disturbances [3], it would be interesting to assess BALF oxidative stress markers and BALF MMP activities in this animal model of emphysema. The potential therapeutic effect of enhanced antioxidant defences on airway inflammation, MMP activity and the development of emphysema could be explored further in interventional studies using oral, injectable or inhaled antioxidants.

In conclusion, the present study has shown that pulmonary oxidative stress occurs in a rat model of Cd-induced emphysema and persistent airway inflammation. Significant correlations between airway inflammation, emphysema and pulmonary oxidative markers as well as between markers of pulmonary oxidative stress were found. The markers 8-iso-$PGF_{2\alpha}$ and UA were increased in BALF during the early response to Cd inhalation, whereas BALF GSH and BALF AA significantly decreased over time with repeated exposure. Further studies are indicated to determine if enhancement of pulmonary antioxidant defences can attenuate Cd-induced emphysema in this model.

Acknowledgements

The authors thank Mrs M. Belleflamme and Mrs G. Keesemaecker for valuable technical assistance and animal care. Mr F. Delvaux is acknowledged for realising the 8-iso-$PGF_{2\alpha}$ analyses. This study was supported by grants of RW DTRE and UCB, Belgium.

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