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Allergen-induced formation of F₂-isoprostanes in a murine asthma model identifies oxidative stress in acute airway inflammation *in vivo* ☆

Sofia Jonasson^a, Josephine Hjoberg^a, Göran Hedenstierna^a, Samar Basu^{b,c,*}^a Department of Medical Sciences, Clinical Physiology, Uppsala University, Uppsala, Sweden^b Department of Public Health and Caring Sciences, Oxidative Stress and Inflammation, Faculty of Medicine, Uppsala University, Uppsala Science Park, SE-751 85 Uppsala, Sweden^c Center of Excellence-Inflammation, Uppsala University Hospital, Uppsala, Sweden

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ABSTRACT

F₂-isoprostanes have been associated with various forms of oxidant stress. The levels of F₂-isoprostanes in a murine asthma model were studied both *in situ* and *in vivo* and further investigated whether the formation of F₂-isoprostanes was associated with increased ovalbumin (OVA)-induced airway inflammation after a 17-day (OVA-17) or a 24-day (OVA-24) protocol. Bronchial reactivity was assessed by using a ventilator (FlexiVent). OVA-treated animals had higher lung resistance and lung compliance compared to control groups ($P < 0.001$). 8-Iso-PGF_{2α} levels in bronchoalveolar lavage (BAL) and 8-iso-PGF_{2α} immunoreactivity in lung tissue were analyzed. OVA-17 mice showed a 2.5-fold increased level of 8-iso-PGF_{2α} in BAL compared to PBS-17 mice ($P = 0.023$). Lung tissue from OVA-24 mice had more intense 8-iso-PGF_{2α} staining compared to OVA-17 mice. This study showed an accumulation of F₂-isoprostanes in acute airway inflammation and a markedly increased tissue damage caused by oxidative stress in an ongoing inflammation.

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1. Introduction

Asthma is a common chronic inflammatory disease of the airways characterized by reversible airflow obstruction, hyperresponsiveness to bronchoconstriction and also remodelling of the airways [1]. Infiltration and activation of inflammatory cells, such as mast cells, eosinophils and lymphocytes, into the airways is an inflammatory response in allergic asthma and may be important in the pathophysiology of the disease. Activation of inflammatory cells lead to increased formation and secretion of inflammatory mediators that promote inflammation and generate oxidative modification of tissues [2–5]. The number of eosinophils are increased in the asthmatic lung [6,7] and may be involved in the formation of the reactive oxygen species and reactive nitrogen species (ROS/RNS) [4,8,9]. This formation could also lead to tissue damage indirectly by the activation of other harmful mechanisms such as acute or chronic inflammation. The free radical nitric oxide (NO) is elevated in asthmatic patients and may be

bioconverted into more potent RNS at the site of inflammation [10,11]. During eosinophil activation, a respiratory burst occurs which may subsequently contribute to tissue damage in the airways by producing ROS/RNS [12,13]. ROS and RNS are also produced by epithelial cells as confirmed by the detection of nitrotyrosine [8]. Oxidative stress and inflammation are linked so that inflammatory processes lead to increases in ROS formation and vice versa [14].

The prostaglandin-like F₂-isoprostanes are formed *in vivo* through free radical catalyzed peroxidation of arachidonic acid, and are considered to be reliable indicators of oxidative stress [15–19]. F₂-isoprostanes, specifically 8-iso-prostaglandin F_{2α} (8-iso-PGF_{2α}, a major F₂-isoprostane) is the most abundant and well-studied bioactive isoprostane formed by ROS *in vivo* through peroxidation of arachidonic acid, independent of cyclooxygenases [20–22]. Isoprostanes are increased in various lung diseases, such as cystic fibrosis, asthma, acute respiratory distress syndrome and chronic obstructive pulmonary disease [3,21–23]. 8-Iso-PGF_{2α} is related to ongoing oxidative/nitrosative stress in the airways [3,13,14,20,24]. F₂-isoprostanes are assumed to be a biochemical link between airway inflammation and oxidative stress, and is associated with various forms of *in vivo* oxidant injury [3,21,22,25,26]. Allergen challenge in human atopic asthmatics causes oxidative stress, and measurement of F₂-isoprostanes appears a reliable approach for quantitative detection of oxidant stress in this acute condition [3,13,20,25].

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* Corresponding author at: Department of Public Health and Caring Sciences, Oxidative Stress and Inflammation, Faculty of Medicine, Uppsala University, Uppsala Science Park, SE-751 85 Uppsala, Sweden. Tel.: +46 18 611 79 58; fax: +46 18 611 79 76.

E-mail address: samar.basu@pubcare.uu.se (S. Basu).

In this study, mice were sensitized and subsequently challenged with the antigen ovalbumin (OVA) in a well-established model of experimental asthma. This subsequently induces airway hyperresponsiveness to bronchoconstrictors and an eosinophilic inflammation in the airways [27]. We measured the levels of 8-iso-PGF_{2α} in bronchoalveolar lavage (BAL) fluid, determined the localization of 8-iso-PGF_{2α} *in situ* in lung tissue by immunohistochemistry, and investigated whether this formation was associated with increased acute airway inflammation. We also assessed the effect of different OVA protocols for challenge of the airways and determined the time-course of the formation of F₂-isoprostanes. Studies on the localization of F₂-isoprostanes in lung tissues in different pulmonary diseases or experimental model of such have been lacking. This study demonstrates the profound bioactivity of these compounds *in vivo*.

2. Materials and methods

2.1. Animals

Female BALB/c mice (Taconic (M&B), Denmark) were used in this study. They were housed in plastic cages with absorbent bedding material and were maintained on a 12 h daylight cycle. Food and water were provided *ad libitum*. Their care and the experimental protocols were approved by the Regional Ethics Committee on Animal Experiments in Uppsala, Sweden (C86/5).

2.2. Preparation of animals and analysis of lung mechanics

Mice were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital sodium (90 mg · kg⁻¹, from local suppliers). They were tracheostomized with an 18-gauge cannula and mechanically ventilated in a quasi-sinusoidal fashion with a small animal ventilator (FlexiVent®, Scireq, Montreal, PQ, Canada) at a frequency of 2.5 Hz and a tidal volume (V_T) of 12 ml · kg⁻¹ body weight. Once ventilation was established bilateral holes were cut in the chest wall so that pleural pressure would equal body surface pressure and so that the rib cage would not interfere with lung movement. This enabled strict lung mechanics measurements. Positive end-expiratory pressure (PEEP) of 3 cmH₂O was applied by submerging the expiratory line in water. Four sigh maneuvers at three times the tidal volume were performed when beginning the experiment to establish stable baseline lung mechanics and ensure a similar volume history before the experiments. The lateral tail vein was cannulated for intravenous (i.v.) injections. The mice were then allowed a 5 min resting period before the experiment began. Dynamic lung mechanics were measured by applying a sinusoidal standardized breath and analyzed using the single compartment model and multiple linear regression, giving us lung resistance (R_L) and lung compliance (C_L) [28]. In this study thorough evaluations of lung mechanics were made using forced oscillation technique (FOT) and the constant phase model during 4 s (prime 4), at the mouse's airway opening. The constant phase model has the capacity to partition the respiratory properties into central and peripheral airways and to distinguish between different tissue properties [29–31]. The parameter obtained was tissue damping (G), closely related to tissue resistance and reflecting energy dissipation in the lung tissues [32].

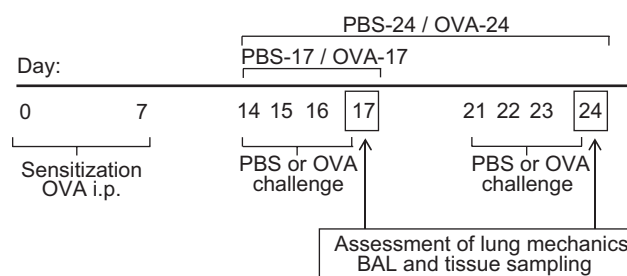


Fig. 1. Schematic presentation of the mouse model of asthma. Assessment of lung physiology, bronchoalveolar lavage (BAL), and tissue sampling were performed on day 17 or day 24. OVA; ovalbumin, PBS; phosphate buffered saline.

2.3. Experimental protocols

Airway inflammation was induced by i.p. injections of 10 µg ovalbumin (OVA, Sigma-Aldrich) emulsified in Al(OH)₃ (Sigma-Aldrich) on days 0 and 7. Mice were then challenged with 1% OVA diluted in phosphate-buffered saline (PBS, Sigma-Aldrich). Animals in the shorter acute protocol (OVA-17, Fig. 1) were exposed to aerosolized OVA for 30 min, on days 14, 15 and 16. Animals in the extended OVA-protocol (OVA-24, Fig. 1) were exposed to aerosolized OVA for 30 min, on days 14, 15, 16, 21, 22 and 23. Aerosol exposure was performed in a chamber coupled to a nebulizer (DeVilbiss UltraNeb®, Sunrise Medical Ltd., UK). The chamber was divided into pie-shaped compartments with individual boxes for each animal, providing equal and simultaneous exposure to allergen. The experiment ended with assessment of lung mechanics on day 17 or on day 24, 24 h after last allergen exposure. Control mice were sensitized with OVA i.p. and challenged with aerosolized PBS using the same protocol as for OVA-17 and OVA-24 described above. To assess airway responsiveness, mice were given incremental doses of MCh (MCh, acetyl-β-methylcholine chloride, Sigma-Aldrich) i.v. (0 = PBS, 0.03, 0.1, 0.3, 1 and 3 mg · kg⁻¹) at 5 min intervals. MCh was diluted in PBS with 10 U · ml⁻¹ of heparin, and a volume of 2000 µl · kg⁻¹ was given at each injection.

2.4. Bronchoalveolar lavage

After completion of the lung mechanics experiment on day 17 or 24, the animals were exsanguinated and subjected to BAL. The lungs were lavaged three times via the tracheal tube with a total volume of 1 ml PBS containing 0.6 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich). The BAL fluid was then immediately centrifuged and the supernatant was removed for 8-iso-PGF_{2α} analysis. Leukocytes were counted manually in a hemacytometer so that 50,000 cells could be loaded and centrifuged using a cytospin centrifuge. The total cell counts of pulmonary inflammatory cells were calculated.

2.5. Assay of 8-iso-PGF_{2α} (indicator of oxidative stress)

The BAL samples from this study were analyzed for free 8-iso-PGF_{2α} by a radioimmunoassay developed and characterized by Basu [16]. In brief, an antibody was raised in rabbits by immunization with 8-iso-PGF_{2α} coupled to BSA at the carboxylic acid by 1,1'-carbonyldiimidazole method. The cross-reactivity of the antibody with 8-iso-15-keto-13,14-dihydro-PGF_{2α}, 8-iso-PGF_{2β}, PGF_{2α}, 15-keto-PGF_{2α}, 15-keto-13,14-dihydro-PGF_{2α}, TXB₂, 11β-PGF_{2α}, 9β-PGF_{2α} and 8-iso-PGF_{3α} was 1.7%, 9.8%, 1.1%, 0.01%, 0.01%, 0.1%, 0.03%, 1.8% and 0.6%, respectively. The detection limit of the assay was 23 pmol · l⁻¹. This method has further been applied to detect oxidative stress in an independent multi-center

network study, designated as Biomarkers of Oxidative Stress Study (BOSS), organized by the National Institute of Health, USA, it was found that outcome of the results from this method is similar to that of other GC–MS methods [33].

2.6. Histological evaluation of airway inflammation

Following BAL, the lungs were inflated with 4% paraformaldehyde solution to a pressure of 20 cmH₂O without removing the lungs from the chest. The trachea was tied off, the lungs were then removed and stored overnight in 4% paraformaldehyde, then stored in 70% ethanol at room temperature (RT) until time for embedding. After embedding in paraffin, the tissue was cut into 4 µm sections and mounted on positively charged slides. To evaluate the airway inflammation, the sections were deparaffinized, dehydrated, and stained with hematoxylin and eosin (H&E). H&E stained sections were examined by bright field microscopy (Axioskop 40 microscope, Carl Zeiss, Göttingen, Germany) and images were captured with a high-resolution digital camera system (AxioCam, Axiovision 2007, Carl Zeiss Imaging Systems, Göttingen, Germany).

2.7. Immunohistochemistry protocols

After embedding in paraffin, the tissue was cut into 4 µm sections and mounted on positively charged slides. Tissue sections were deparaffinized in xylene and alcohol. Slides were rinsed in running H₂O for 5 min. All slides were then heated in a microwave (750 W) in citrate buffer (citric acid and dH₂O, pH 6.0) for 2 × 5 min. After cooling, tissue sections were rinsed in PBS and thereafter incubated in 3% H₂O₂ for 30 min to block endogenous peroxidase activity. Slides were rinsed in PBS before following protocols for each primary antibody. After the specific procedure for each primary antibody, the staining was visualized using DAB (3,3'-diaminobenzidine tetrahydrochloride, Vector SK4200) for 5 min. Sections were lightly stained in haematoxylin for 40 s and rinsed in running H₂O for 10 min. Slides were then dehydrated with ethanol and xylene. Stained sections were examined using the same equipment as for histology evaluation.

2.7.1. 8-Iso-PGF_{2α} immunoreactivity

The tissue slides were carefully rinsed with PBS after blocking endogenous peroxidase activity and incubated (in a humidified box, at RT) with 10% normal blocking goat serum (NGS, X0907 DAKO) in PBS/1% bovine serum albumin (PBA) for approximately 30 min. The excess PBA was shaken off and the primary antibody 8-iso-PGF_{2α} (1:1000, [34]) diluted in PBA+5% NGS was added to the slides and stored overnight at 4 °C in a humidified box. Next day, slides were rinsed in PBS, followed by incubation for 60 min with biontinylated secondary antibody, goat anti-rabbit in PBA, (1:200, DAKO). Slides were rinsed in PBS, followed by incubation with strept avidin–biotin complex (ABC technique) for 30 min, using standard procedure (VECTASTAIN Elite ABC Kit). Slides were rinsed in PBS before staining was visualized with the protocol above. All these procedures were also carried out in sections of lung tissue that had been incubated in the absence of primary antibody (negative control).

2.7.2. Anti-nitrotyrosine immunoreactivity

After blocking the endogenous peroxidase activity and rinsing, sections were blocked with PBA for 60 min at RT, followed by incubation with 2% normal blocking goat serum in PBA (X0907 DAKO) for approximately 30 min at RT to reduce immunologic binding of antibody. The excess PBA was shaken off and the primary antibody anti-nitrotyrosine was diluted in PBA (1:142,

no. 06-284, Upstate cell signaling solutions) and added to the sections and stored overnight at 4 °C in a sealed humidity chamber to prevent air drying of the tissue sections. Next day the slides were rinsed twice in PBS, followed by incubation for 45 min at RT with secondary antibody, goat anti-rabbit in PBA (1:100, DAKO). Slides were rinsed twice in PBS and excess PBS was removed. Slides were incubating for 1 h with strept avidin-biotin complex (ABC technique) using standard procedure (VECTASTAIN Elite ABC Kit). Slides were rinsed twice in PBS. All these procedures were also carried out in sections of lung tissue that had been incubated in the absence of primary antibody (negative control). For positive control, slides were incubated after having been deparaffinized for 20 min in the following solution; 1 mM sodium nitrite, 1 mM hydrogen peroxide in 100 nM sodium acetate pH 5.0. Slides were then processed using the protocol above.

2.8. Statistical analysis

Results are presented as mean ± standard error of mean (SEM). Statistical significance was assessed by parametric methods using two-way analysis of variance (ANOVA) to analyze differences between groups, followed by Bonferroni *post hoc* test. When appropriate, one-way ANOVA, Student's unpaired *t*-test or linear regression analysis was used. A statistical result with *P* < 0.05 was considered significant. Statistical analysis and preparations of graphs were performed with GraphPad Prism (version 4.0 GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Bronchoalveolar lavage cell count and histology

Mice undergoing the 17-day (OVA-17) or 24-day (OVA-24) ovalbumin challenge protocol (Fig. 1) had clear signs of airway inflammation compared to control animals (PBS-17 and PBS-24). OVA-24 group had approximately a 47-fold increase in total BAL cell count and OVA-17 had a 6-fold increase compared to control groups (both *P* < 0.001). Animals in the OVA-24 had a significant higher total cell count in BAL than OVA-17 (*P* < 0.0001). Light microscopic examination of hematoxylin and eosin sections from OVA-17 and OVA-24 animals revealed an eosinophilic inflammation with a patchy distribution of eosinophils surrounding the airways and within the alveolar spaces. A more pronounced inflammation was seen in tissue from animals having undergone the longer OVA-protocol (OVA-24) than OVA-17 animals. OVA-24 animals had a significantly increased perivascular inflammation (Fig. 2, Table 1).

3.2. Lung mechanics

3.2.1. Lung resistance (*R_L*)

Animals in the OVA-24 group had a significantly higher MCh-induced maximum *R_L* than OVA-17 animals (8.8 ± 0.9 and 7.2 ± 0.7 cmH₂O · s · ml^{−1} respectively, *P* = 0.0017, Fig. 3). Both OVA groups had significant higher maximum *R_L* than PBS-treated mice (*P* < 0.0001, Fig. 3). There were no differences between PBS-treated controls.

3.2.2. Lung compliance (*C_L*)

In both OVA-treated groups, MCh-challenge induced a greater reduction in lung compliance (*C_L*) as compared to control mice (PBS-treated). In the OVA-24 group, *C_L* decreased by 87% from baseline, the decline being significantly larger than the OVA-17

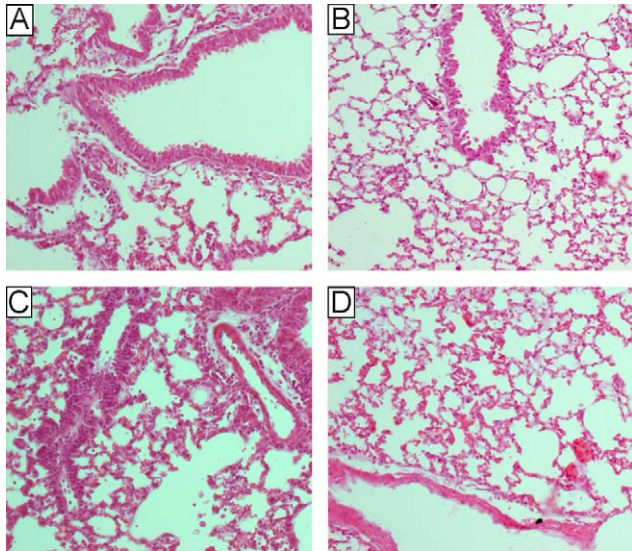


Fig. 2. Representative histological sections (hematoxylin and eosin (H&E) stained) from PBS-treated controls (PBS-17 and PBS-24) and from animals with airway inflammation (OVA-17 and OVA-24). (A) OVA-17, (B) PBS-17, (C) OVA-24 and (D) PBS-24.

Table 1

Histology and immunohistochemical staining for 8-iso-PGF_{2α} and nitrotyrosine in lung tissue from OVA-challenged mice (OVA-17 and OVA-24) and from PBS-challenged mice (PBS-17 and PBS-24).

Treatment	Cell infiltration	H&E staining	8-Iso-PGF _{2α}	Nitrotyrosine
PBS-17	+	+	+	+
OVA-17	++	+++	++	++(+)
PBS-24	+	+	+	+
OVA-24	+++	+++	+++	+++

The results of immunochemical staining are shown in five different grades, from + to +++++ according to positive staining.

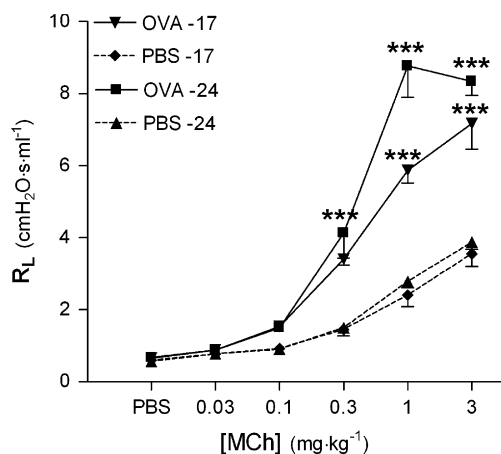


Fig. 3. The effects of incremental doses of methacholine (MCh) on lung resistance (R_L) in animals with OVA-induced airway inflammation (OVA-17 and OVA-24) and PBS-treated controls (PBS-17 and PBS-24). All values are mean $\pm$ SEM, *** P <0.001.

group (36%, P <0.001, Fig. 4). Both OVA groups had significantly greater maximum reduction in C_L than PBS-treated mice (PBS-24; 24%, P <0.001 and PBS-17; 21%, P <0.05, Fig. 4). There were no differences between PBS-treated controls.

3.2.3. Tissue resistance (G)

During bronchial reactivity assessment, the 4 s perturbation of forced oscillation (Prime 4) before each dose of PBS and MCh,

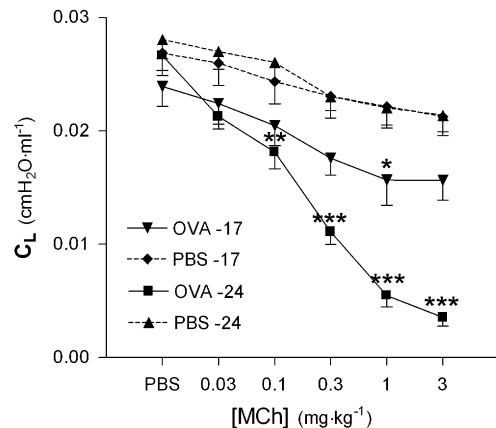


Fig. 4. Effects of incremental doses of methacholine (MCh) on lung compliance (C_L) in animals with OVA-induced airway inflammation (OVA-17 and OVA-24) and PBS-treated controls (PBS-17 and PBS-24). All values are mean $\pm$ SEM, * P <0.05, ** P <0.01 *** P <0.001.

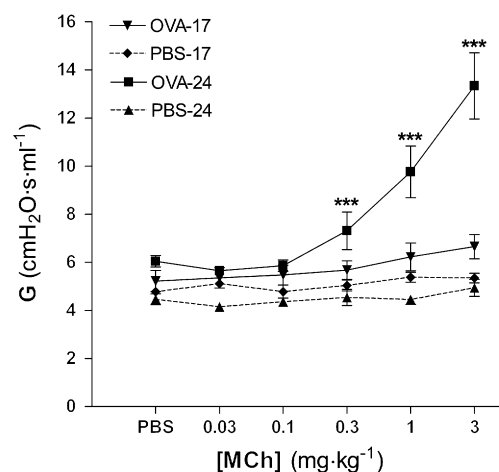


Fig. 5. Measurements of tissue damping (G) were performed with forced oscillation technique (Prime 4 perturbation, Zrs measurements) before each i.v. injection of phosphate buffered saline or methacholine (MCh) in control animals (PBS-17 and PBS-24) and from animals with airway inflammation (OVA-17 and OVA-24). Values are mean $\pm$ SEM, *** P <0.001.

OVA-24 mice had significantly higher maximum tissue resistance (G) than OVA-17 animals (13.3 ± 1.4 and 6.7 ± 0.5 cmH₂O $\cdot$ s $\cdot$ ml⁻¹, respectively, P <0.001, Fig. 5). OVA-24 animals had significantly higher maximum G than PBS-24 (4.9 ± 0.4 cmH₂O $\cdot$ s $\cdot$ ml⁻¹, P <0.001, Fig. 5). There were no differences in G between OVA-17 and PBS-17 animals (5.3 ± 0.2 cmH₂O $\cdot$ s $\cdot$ ml⁻¹, Fig. 5) or between PBS-treated controls. Regression analysis showed strong correlation between the maximal response in G and levels of 8-iso-PGF_{2α} in OVA-24 animals ($R^2 = 0.83$, $P = 0.033$). In the other groups, there were no correlations between G and 8-iso-PGF_{2α}.

3.3. 8-Iso-PGF_{2α} levels in bronchoalveolar lavage (BAL) fluid

OVA-17 mice showed a 2.5-fold increased level of 8-iso-PGF_{2α} in BAL fluid compared to PBS-17 animals ($P = 0.023$; Fig. 6). There were no detectable differences between animals having undergone the 24-day long protocol (OVA-24 and PBS-24) or between OVA-17 and OVA-24, respectively, (P >0.05, Fig. 6). Basal levels of 8-iso-PGF_{2α} could also be detected in naïve mice under normal physiological conditions.

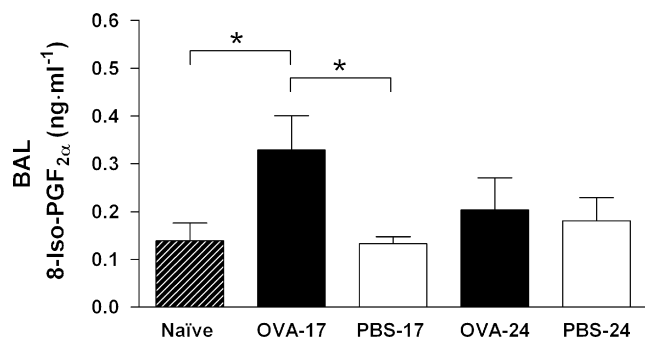


Fig. 6. The levels of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$), in bronchoalveolar lavage (BAL) from control animals (Naïve, PBS-17 and PBS-24) and from animals with airway inflammation (OVA-17 and OVA-24). Values are mean $\pm$ SEM, * $P < 0.05$.

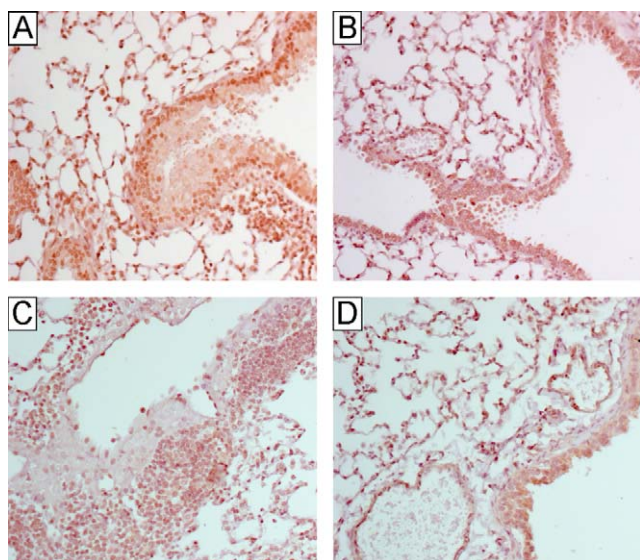


Fig. 7. 8-Iso-prostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$) staining. Representative histological sections from control animals (PBS-17 and PBS-24) and from animals with airway inflammation (OVA-17 and OVA-24). (A) OVA-17, (B) PBS-17, (C) OVA-24 and (D) PBS-24.

3.4. 8-Iso-PGF $_{2\alpha}$ expression in lung tissue

Immunohistochemistry with an unlabelled antibody to 8-iso-PGF $_{2\alpha}$ was performed on lung sections from OVA- and PBS-treated mice to characterize the tissue expression of 8-iso-PGF $_{2\alpha}$. Both OVA-treated groups (OVA-17 and OVA-24) had more detectable 8-iso-PGF $_{2\alpha}$ staining as compared to PBS-treated animals. The cells surrounding the airways showed the characteristic brown appearance of positive cells. OVA-24 mice showed more intense 8-iso-PGF $_{2\alpha}$ staining of large and small airways as compared to OVA-17 animals (Fig. 7, Table 1). Pulmonary arterioles in connection with the respiratory bronchioles displayed less 8-iso-PGF $_{2\alpha}$ immunoreactivity.

3.5. Nitrotyrosine expression in lung tissue

Immunohistochemistry was performed on lung sections from OVA- and PBS-treated mice to characterize the tissue expression of nitrotyrosine. Immunoreactivity of nitrotyrosine was detected around bronchioles and in tissue surrounding pulmonary arterioles and bronchioles. Stronger staining was observed in OVA-treated animals as compared to PBS-treated mice. Tissue from OVA-24 animals expressed more intense staining than

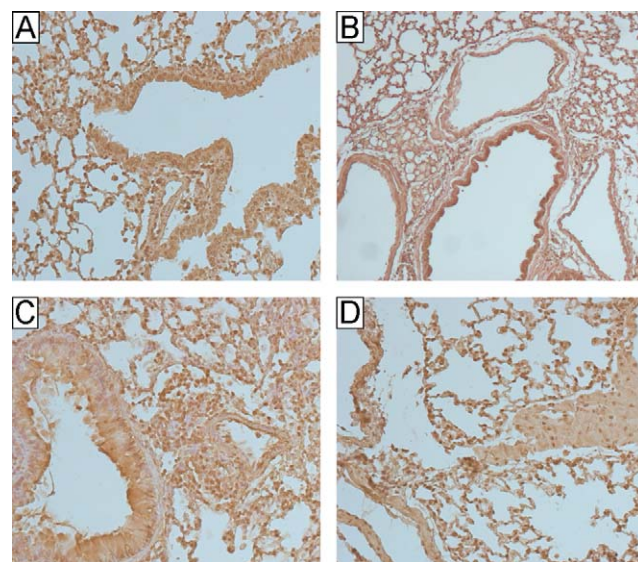


Fig. 8. Anti-nitrotyrosine staining. Representative histological sections from control animals (PBS-17 and PBS-24) and from animals with airway inflammation (OVA-17 and OVA-24). (A) OVA-17, (B) PBS-17, (C) OVA-24 and (D) PBS-24.

OVA-17. There were no differences between PBS-treated animals (Fig. 8, Table 1).

4. Discussion

The aim of this investigation was to study the levels of F_2 -isoprostanes in a murine asthma model and whether the increased formation of F_2 -isoprostanes and their appearance in lung tissues were associated with increased airway inflammation. In this well-established experimental murine model of allergen-induced asthma, sensitization and subsequent aerosol challenge of antigen, was found to cause an inflammatory reaction in the lung and increased airway reactivity. 8-Iso-PGF $_{2\alpha}$ is a non-cyclooxygenase catalyzed product of arachidonic acid formed on membrane phospholipids by the action of reactive oxygen species [35]. 8-Iso-PGF $_{2\alpha}$ was measured in bronchoalveolar lavage at different time-points and simultaneously the localization of 8-iso-PGF $_{2\alpha}$ *in situ* in the lung tissue was performed. Our most important findings in this study are that high levels 8-iso-PGF $_{2\alpha}$ could be detected in acute airway inflammation, both in bronchoalveolar lavage fluid and *in situ* in lung tissue following intervention.

We implemented both the constant phase model (low-frequency oscillation technique, FOT) and the single compartment model to measure the airway physiology. FOT has the capacity to partition the respiratory properties into central and peripheral airways and also into airway and tissue properties [29–31]. Inflammation of distal airways and lung parenchyma directly affects lung physiology by increasing lung resistance, as well as by decreasing pulmonary static and dynamic compliance [36]. In the current study, mice subjected to a shorter protocol of acute airway inflammation (OVA-17) had increased lung resistance and reduced lung compliance following methacholine provocation. Further increase in lung resistance and decrease in lung compliance was seen after longer period of aerosolized allergen challenge (OVA-24). The levels of 8-iso-PGF $_{2\alpha}$ were significantly elevated on day 17 and confirmed an ongoing process of oxidative stress. Further, it showed that the levels of 8-iso-PGF $_{2\alpha}$ in BAL were not as pronounced in OVA-24 as in OVA-17. The BAL levels of 8-iso-PGF $_{2\alpha}$ were reduced close to baseline levels. On the other

hand, the immunoreactivity of 8-iso-PGF_{2α} was higher in airway tissue after 24 days than after 17 days. This suggests that after 24 days, more tissue damage and airway inflammation was present. This is strengthened by our finding that levels of 8-iso-PGF_{2α} correlated to tissue resistance (G) in OVA-24 mice. Thus, it is conceivable that 8-iso-PGF_{2α} and oxidative stress may contribute to an increased airway heterogeneity. Tissue resistance reflects the parenchymal distortion that occurs when the airways constrict and G increases with an elevated degree of regional airflow heterogeneity throughout the lung [37].

Nitrotyrosine is an indicator of cell damage, inflammation and nitric oxide production. It is also a major product of the peroxynitrite reaction and evidence for a role of peroxynitrite *in vivo* [4,8]. OVA-24 mice demonstrated an increased pulmonary nitrotyrosine immunostaining as compared to OVA-17 and control animals, suggesting that nitrotyrosine and inflammation are linked, so that inflammatory processes lead to increases in nitrotyrosine formation and vice versa. It is believed that ROS and RNS participate in the airway inflammation that characterizes asthma and the evidence of their involvement is not clearly understood.

8-Iso-PGF_{2α} is only one specific and major product of arachidonic acid catalyzed through ROS among other 64 different isomers of PGF_{2α} formed *in vivo* [38]. Thus, it could be assumed that the total amount of the ROS catalyzed products of the F₂-isoprostane family will be much higher than the amount we have quantified biochemically *in vivo* or detected immunohistochemically *in situ*. This demonstrates that F₂-isoprostanes are produced in a significant amount in both *in situ* and biochemically on days 17 and 24 but with different kinetics of appearance and disappearance in BAL and *in situ* in lung tissue, respectively. It has earlier been shown that with 4 min of allergen challenge to humans no F₂-isoprostanes could be detected [39]. It is possibly due to the fact that there is a delay in the time-course of arachidonic acid oxidation on the cell membrane phospholipids and the appearance of free 8-iso-PGF_{2α} release through hydrolysis of esterified F₂-isoprostanes [40,41]. It is conceivable that there are a delay in activation of phospholipase and subsequent hydrolysis of the esterified F₂-isoprostanes. The reduction of 8-iso-PGF_{2α} levels from day-17 to day-24 in BAL and a concomitant increase of 8-iso-PGF_{2α} in lung tissue over that period might be unexpected. The decrease in 8-iso-PGF_{2α} in BAL on day-24 may possibly be due to more efficient activation of major enzymes (phospholipase, 15-PGDH (15-prostaglandin dehydrogenase) and Δ¹³-reductase) are responsible for the metabolism of 8-iso-PGF_{2α} [40,41].

In this study we found an increase of free 8-iso-PGF_{2α} in the BAL fluid on day 17, and this increase corroborates with the human allergen challenge study as described above [39]. Talati et al. [14] demonstrated that murine 8-iso-PGF_{2α} was generated during normal physiological conditions and that the levels increase during allergen-induced inflammation. They suggested that F₂-isoprostanes are IgE-dependent and rapidly generated by epithelial cells and macrophages in response to allergen [14].

In conclusion, this study shows that oxidative and nitrate stress through different reactive radical species can contribute to airway inflammation and hyperresponsiveness in asthma. We have shown where the effects of 8-iso-PGF_{2α} take place in a murine airway and that sensitization and subsequent challenge with the antigen ovalbumin leads to an increased reactivity to methacholine. Based on our current results, the formation and time-course of 8-iso-PGF_{2α}, nitrotyrosine and tissue damage in tissue are associated with increased inflammation, and more heterogeneous airway airflow. This study demonstrates a link between airway physiology and the involvement of oxidative stress pathways *in vivo*.

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