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Glutathione Peroxidase-2 Protects from Allergen-Induced Airway Inflammation in mice

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Abstract

Objectives: To identify and validate the biological significance of new genes/ proteins involved in the development of allergic airway disease in a murine asthma model.

Methods: Gene microarrays were used to identify genes with at least a 2-fold increase in gene expression in lungs of two separate mouse strains with high and low allergic susceptibility, respectively. Validation of mRNA data was obtained by western blotting and immunohistochemistry, followed by functional analysis of one of the identified genes in mice with targeted disruption of specific gene expression.

Results: Expression of two antioxidant enzymes, glutathione peroxidase-2 (*Gpx-2*) and glutathione-S-transferase Omega (GSTO) 1-1 was increased in both mouse strains after induction of allergic airway disease and localized in lung epithelial cells. Mice with targeted disruption of the *Gpx-2* gene showed significantly enhanced airway inflammation compared to sensitized and challenged wild-type mice.

Conclusion: Our data indicate that genes encoding the antioxidants *Gpx-2* and GSTO *1-1* are common inflammatory genes expressed upon induction of allergic airway inflammation, independently of allergic susceptibility. Furthermore, we provide evidence to illustrate the importance of a single antioxidant enzyme, *Gpx-2*, in protection from allergen-induced disease.

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Airway hyperreactivity, asthma, Glutathione peroxidase, glutathione S-transferase

Introduction

Allergic asthma is a polygenetic disease that unfolds through the interplay of various genes with environmental factors. Despite the identification of several proteins and pathways involved in this inflammatory process, clinical trials in which key mediators were inhibited revealed that other and yet unknown factors might be causally involved in the allergic cascade (1). In search for these putative candidates, it is intriguing to speculate that antioxidant defense systems might be involved in the regulation of airway inflammation, since airways are naturally exposed to higher oxygen concentrations than most other tissues. Recent studies have identified increased levels of oxidative stress and alterations of antioxidant enzymes in the lungs of allergic individuals and in allergic animal models resulting in the hypothesis that an increase in oxidative stress may contribute to the characteristic features of asthma (2).
In search of new factors, different technologies may be applied. Several recent studies benefitted from microarray analysis of gene expression profiles in order to identify genes involved in the development of allergic airway inflammation, as reviewed in (3). This approach has significant advantages over conventional experimental approaches. Conventional approaches only permit the study of known mediators of inflammation, where previous studies usually already suggested a possible association with allergic airway disease. Deductive gene expression profiling via microarrays, on the other hand, might identify mediators without any known link to inflammation or airway disease, thereby introducing truly “novel” targets into the field of allergic airway research. To identify novel factors commonly involved in pulmonary inflammation, we therefore employed RNA microarray technology. Comparisons of naïve and treated mice, on the one hand side and two mouse strains with known different genetic suscepbility to the induction of allergic airway disease (4), on the other hand side, allowed us to identify common genes involved in pulmonary inflammation, indepently of genetic susceptibility to disease development. Among the identified genes were several genes involved in the regulation of oxidative stress, among these

the antioxidative enzymes, glutathione peroxidase-2 (*Gpx-2)* and glutathione-S-transferase Omega 1-1 (*GSTO 1-1)*. These two enzymes had not previously been recognized to be part of the allergen-mediated inflammation cascade. Our data indicate that *GSTO 1-1* and *Gpx-2* are upregulated in allergic airway inflammation. Furthermore, the absence of *Gpx-2* leads to an increase in the allergic airway inflammation. Manipulating this pathway in future studies will test the hypotheses that oxidative stress is involved in the pathogenesis of asthma.

Methods

Animals

Specific-pathogen-free female BALB/c and C57BL/6 mice (Harlan-Winkelmann, Borchen, Germany), and C57BL/6 mice with a targeted disruption of *Gpx-2 (Gpx-2*-KO) (5), 6–8 weeks old at starting point of experiments, were used. Five animals/ group were analyzed and three independent experiments were conducted. All experimental procedures were approved by the animal care facility (Berlin Office for Occupational Safety, Protection of Health and Technical Safety-LAGeSo).

Sensitization and challenge protocol

Mice were sensitized by intraperitoneal (i.p.) injection of 20 µg ovalbumin (OVA) grade VI (Sigma-Aldrich, München, Germany) in 2 mg of aluminum hydroxide on days 1 and 14. Airway inflammation was induced by intranasal instillation of OVA grade V (Sigma-Aldrich,) (50 µg in 50 µl phosphate-buffered saline) on day 28 (for microarray analyses) and 29 (for quantitative real-time polymerase-chain reaction (RT-PCR)). For studies with *Gpx-2*-null animals, mice were systemically sensitized by i.p. injection of OVA and aluminum hydroxide. Non-sensitized mice received aluminum hydroxide without OVA. On days 28, 29 and 30, all mice were challenged with OVA, and killed at day 32. For microarray analyses, animals were sacrificed 16 h after the single intranasal challenge on day 28. For RT-PCR,

Western blotting and for studies with *Gpx-2*-null animals, animals were sacrificed 48 h after last challenge, i.e. either 48h after two challenges on day 28 and 29 or 48h after challenges on day 28, 29 and 30.

Detection of the allergic phenotype

Immunoglobulins (Igs): 48 h after thelast challenge, blood was drawn from the tail vein, and serum levels of total IgE and OVA-specific IgE were measured by ELISA, as previously described (6).

Bronchoalveolar lavage: Sixteen hours after a single allergen challenge and forty-eight hours after multiple challenges (day 28 and 29 or days 28, 29 and 30, see above), lungs were lavaged and cytospin slides were prepared, stained with Diff-Quik (Dade Behring AG, Liederbach, Switzerland) and 200 cells were characterized according to morphologic criteria via light microscopy.

Airway reactivity*:* Airway reactivity was measured by whole body barometric plethysmography (WBP, corresponding to the Buxco-system provided by EMKA Technologies, Paris, France), as previously described (6).

Invasive lung function measurement in isolated perfused mouse lungs after three challenges (days 28, 29 and 30) of WT and KO mice*:* Mouse lungs were prepared, ventilated and analyzed as described (7). After a steady state period of 30 min, MCh was administered to the perfusate for 30 sec at 12-min intervals. Airway resistance values were determined at the end of the steady state period, as well as at the maximum level of resistance increase. The change in airway resistance was expressed as "relative fold airway resistance", representing the increase in responsiveness due to OVA-sensitization by normalizing fold airway resistance values of OVA lungs to corresponding mean values of control groups.

Preparation of RNA

Total RNA was extracted from mouse lungs using Qiagen RNeasy Total RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Microarray analysis

CRNAs were hybridized individually to mouse genome MG-U74Av2 chips (Affymetrix, High Wycombe, UK). In total, 8 lungs were analyzed, two treated mice vs. two controls in two different mouse strains (BALB/c and C57BL/6) respectively. The gene chips were scanned with an Affymetrix Gene Chip Instrument and scaled using Affymetrix's Microarray Suite software 5.0 (MAS5). We made four-way comparison of the arrays; between two different mouse strains and between treated and controls (2x2 matrix). Only those genes which were found to be similarly regulated in all 4 comparisons were classified as differentially expressed genes. The signal log ratio (SLR) was converted to a standard on a logarithmic scale and the mean fold change of all 4 comparisons was calculated. We consecutively focused on those genes, which were similarly regulated in both strains of mice and with more than 2-fold changes between treated and untreated mice. A more detailed description of the microarray analysis is attached as supplementary material.

Real-time PCR

PCR amplification and analysis were performed using an ABI PRISM 7700 (Perkin Elmer, Rodgau, Germany) and SDS software version 1.7. (A more detailed description of the real time RT-PCR can be found in the supplementary materials)

Primer design and sequences

Complementary DNA PCR primers for amplification were designed using Primer3 Input software (primer3_www.cgi version 2.0) for DNA and RNA sequences obtained from GenBank.. The list of primers, and sequences were archived as supplementary material.

Protein preparation, SDS-PAGE and Western blotting

One gram of mouse lung tissue (snap-frozen, stored at -80°C) was homogenized in digestion buffer. Aliquots of the lung homogenates were analyzed via SDS-PAGE and western blotting using anti *Gpx-2* or anti *α-GSTO* antibodies (8;9) (A detailed description is provided in the supplementary material).

Immunohistochemistry

Localization of the *Gpx-2* and *GSTO 1-1* proteins was detected via immunohistochemistry (IHC) using 4 µm paraffin sections of lung tissue. Antigen retrieval was performed by heating the tissue sections for 6 minutes in pre-heated Dako target retrieval solution (TRS, Dako,Hamburg,Germany), using a pressure cooker. For detection of *GSTO* 1-1, the rabbit antiserum was diluted 10,000 fold (9)and detection of *Gpx-2* was performed with a rabbit polyclonal anti-*Gpx-2* antibody (8). Biotinylated secondary anti-rabbit antibodies were used at a dilution of 1:10,000 (Amersham Pharmacia Biotech, Freiburg, Germany). For signal amplification and visualization of anti-*GSTO 1-1* and anti-*Gpx-2*, a tyramine amplification system (CSA kit, Dako, Hamburg, Germany) was used. As chromogen for the peroxidase-reaction, 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dako, Hamburg,Germany) was used.

Statistical analysis

Data pertaining to the allergic phenotype was analyzed statistically with the Mann-Whitney-U-Test. Micro-Array analysis was done with Affymetrix's Microarray Suite software 5.0 (MAS5) using a non-parametric statistical test (Wilcoxon signed rank test).

Results

Analysis of the allergic phenotype

Systemic sensitization with the allergen ovalbumin (OVA) in BALB/c mice leads to a significant increase in both total and OVA-specific IgE as compared to animals that received only PBS (total IgE 1639 ng/ml versus 924 ng/ml; OVA-specific IgE 423 LU/ml versus < 6.2 LU/ml). The inflammatory reaction in the airways showed a specific and time-dependent pattern for the different cell types in the bronchoalveolar lavage (BAL) fluid (supplementary Table 1). 16 h after the single airway allergen challenge, mostly neutrophils, but virtually no eosinophils were detected. At this time point, airway hyperreactivity, measured via whole body plethysmography (6) had not yet developed (data not shown). At a later time point (48h), the BAL contained a robust eosinophilic and lymphocytic infiltration, corresponding to development of in vivo AHR.

Identification of upregulated inflammatory genes in lung tissues of sensitized and challenged animals.

RNA isolated from whole lung tissue was used to generate Affymetrix based gene expression profiles. Lung tissue was obtained at 16h after a single allergen airway challenge to analyze genes at an early time point of AI in order to identify genes involved in the development of the characteristic Th2 phenotype of this model. Gene expression was compared between BALB/c mice and C57BL/6 mice because of their known differences in the development of AI and AHR (4). While both strains develop significant AI, AHR and systemic sensitization parameters such as allergen-specific IgE are much more pronounced in the BALB/c strain. We postulated that the comparison of these two strains would strengthen our aim (“two identify of signature genes of AI”) considerably compared to an approach utilizing only one mouse strain, increasing the probability of identifying genes truly relevant in the development of allergic AI. OVA-sensitized and -challenged C57BL/6 mice had an altered expression of

370 probe sets compared to the naive control mice, whereas OVA-challenged BALB/c mice had 2128 probe sets changed in their expression levels. Between these two sets, 95 probe sets were consistently up-regulated in both sensitized and challenged BALB/c and C57BL/6 mice, but only 31 probe sets coding for 27 different genes were up-regulated at least two-fold in both mouse strains (the list of up regulated genes after OVA challenge is provided in the supplementary material as Table II). Gene ontogeny analysis revealed that among these common inflammatory genes, several were involved in response to oxidative stress. Two of them, *Gpx2* and *GSTO1*, had not yet been reported in the context of allergic airway reaction, and were thus analyzed further.

Upregulation of Gpx-2 and GSTO 1-1 in allergen-induced airway inflammation is confirmed by quantitative RT-PCR.

Quantitative RT-PCR was used to confirm the gene chip result. Sensitized and challenged mice (OVA/OVA) showed about two- and five-fold higher levels of *Gpx2* and *GSTO 1-1* mRNA in lung tissue as compared to animals in which airway inflammation was not induced (PBS/PBS) (Figure 1). Although the upregulation of *Gpx*-2 (Figure 1A) and GSTO *1-1* (Figure 1B)was found in both mouse strains after induction of allergic airway inflammation, in BALB/c mice the increase was even higher, as determined by the relative difference in fluorescence intensity between the target mRNAs and β-actin mRNA, a housekeeping gene.

GPX-2 and GSTO 1-1 proteins are expressed at higher levels in mice with allergic airway inflammation.

Western blotting with specific anti-*Gpx-2* and anti-*GSTO 1-1* antibodies was used to verify that an upregulation in mRNA levels leads to an increase in tissue protein levels of these enzymes in mice with allergic airway inflammation. Elevated expression levels of both proteins were detected in the lung tissue of mice challenged with OVA (OVA/OVA) as

compared to PBS-treated control animals (PBS/PBS) (Figure 2). While these values attained statistical significance for *Gpx-2* protein expression levels (Figure 2A), comparison of OVA-challenged mice to PBS-controls revealed only trends towards higher expression levels for *GSTO 1-1*.

Expression pattern of Gpx-2 and GSTO1 in mouse lung.

Immunohistochemistry for *Gpx-2* and *GSTO 1-1* revealed distinct expression patterns for these proteins in mouse bronchial epithelium (Figure 3). Expression patterns of both proteins were similar in the lungs of untreated animals as well as in sensitized and challenged animals with regards to localization. *Gpx-2* expression, which so far had not been detected in the lung on a protein level, was found in basal cells (arrows in Figure 3A), revealing a pattern compatible with expression in the cells responsible for epithelial regeneration. *GSTO 1-1* was found mainly in the apical parts of epithelial cells, sometimes appearing to be "budding" from the surface of the cells (arrows in Figure 3B), but secreted proteins were never detected by immunohistology inside the airway lumen.

Gpx-2 protects against airway inflammation.

In order to evaluate the biological significance of our findings, we evaluated the consequences of *Gpx-2* absence in the context of acute allergen-induced AI by utilizing mice genetically deficient for *Gpx-2* expression. As shown in Figure 4, direct comparison of Gpx-2 knock-out (KO) mice with wild type (WT) littermates revealed significantly higher levels of AI in Gpx-2 knock-out mice, mainly due to significant increased number of lymphocytes and eosinophils (Figure 4A). OVA-specific total IgE and IgG1 levels were also increased in Gpx-2 knock-out mice but on a non significant level (Figure 4B and 4C). In order to evaluate functional consequences of Gpx-2 deficiency, we analyzed airway resistance after methacholine

provocation in isolated and perfused lungs from WT and KO. Here, we observed a 32% increase in relative fold airway resistance in KO mice in comparison to WT mice (Figure 4D).

Discussion

In the present study, we utilized gene expression profiling in lung tissues of two different mouse strains to identify novel and common inflammatory genes involved in allergic airway disease. We detected two antioxidants, GSTO 1-1 and *Gpx-2,* which had yet not been recognized in this context and which were significantly upregulated, both on the transcriptional and translational levels. Our data support recent evidence that chronic allergic airway inflammation is, in part, a result of and mediated by reactive oxygen species (ROS) (2). Furthermore, increased levels of inflammation and airway reactivity in *Gpx-2*-null mice support the notion that *Gpx-2* plays a protective role in airway inflammation, similar to its anti-inflammatory role in the GI tract (5).

The family of *Gpx* consist of four selenoproteins, *Gpx1-4*, which are key enzymes in the redox cycle. Their differential expression patterns and additional enzymatic capacities indicate that they play an important role in exerting cell- and tissue-specific roles in metabolic regulation (10). *Gpx-1-4* have all been reported to be expressed in human lungs (11), yet functional studies revealing their contributions to health and disease in this organ remain sparse. Hoffmann et al. have recently shown that *Gpx-1*, but not *Gpx-4* protein was elevated (2.8-fold) in lung tissues of challenged C57BL/6J mice analyzed on day 29 (12). In our analysis, we were not able to reproduce this increase. However, induction of *Gpx-1* gene expression might occur later in the time course of allergic inflammation than the time point analyzed in our study.

Most studies of *GPX-2* so far were confined to the gastrointestinal tract ((5;13;14). Although mRNA *Gpx-2* expression was found in mouse lungs, localization in this organ has

not been elucidated (15). In humans, *Gpx-2* protein expression was not detected in the lung (13) and *Gpx* activity in the lung was mainly attributed to *Gpx-1* activity (16).

Gpx-2 is upregulated by nuclear factor erythroid 2-related factor 2, and increased levels in hyperoxia-induced lung injury in *Nrf2*-null mice points towards a role of *Gpx-2* in protection against oxidative stress (15;17). The present study extends these findings to another adverse lung event that is known to generate ROS as well as inflammatory mediators: allergic airway inflammation. The finding of increased airway inflammation in the absence of *Gpx-2* strongly supported a protective role in allergic airway inflammation for this protein.

Considering that Ho *et al.* have shown that 95% of the glutathione peroxidases activity in the lung is attributable to *Gpx-1*(16), it might be possible that the protective role of *Gpx-2* in this organ is due to different enzymatic activities. In fact, glutathione peroxidases have been reported to inhibit prostaglandin synthesis (18), thus reducing the expression of pro-inflammatory mediators known to play an important role in the pathogenesis of allergic asthma,. Also, *Gpx- 2* knock-out studies suggested an involvement in anti-inflammatory mechanisms (5). The exact mechanism by which *Gpx-2* decreases airway inflammation will be the subject of further studies as our preliminary studies concerning changes in classical inflammatory mediators (IL-4, IL-5, IL-10, IFN-gamma) upon allergen restimulation did not show significant differences (data not shown).

The other gene identified in our present study belongs to the supergene family of glutathione S-transferases, of which we identified the mouse homologue of glutathione S-transferase Omega 1-1 (*GSTO 1-1*). In the human lung, the enzyme *GSTO 1-1* is reported to be exclusively expressed in alveolar macrophages (19) while the mouse homologue has been shown to be expressed ubiquitously, with expression levels highest in the lung and liver (9).

Up to now, this enzyme has not been implicated in the pathology of bronchial asthma. The GST family was initially described to provide an important detoxification step for various ROS (20) but many of the six distinct subclasses perform additional reactions. (21). Human

GSTO 1-1 acts as a glutathione-dependent-thioltransferase, which might serve to restore enzymatic function after exposure to oxidative stress (22). Human *GSTO 1-1* has also been shown to inhibit IL-1β-dependent apoptosis via cytokine release inhibitory drugs (23), suggesting a new type of regulatory operation performed by this enzyme. The expression of the mouse homologue of *GSTO 1-1*, p28, was initially discovered in a radiation-resistant lymphoma line, pointing towards a possible role in conferring resistance to radiation-induced cell death (9). This role is supported by studies showing that GSTs inhibit certain stress kinases such as JNK (24), which in turn inhibits apoptosis and allows cell repair (25).

Integrating the functional results pertaining to *GSTO 1-1* into our mouse model, inhibition of apoptosis by upregulation of *GSTO 1-1* may lead to adverse effects in cells which under normal circumstances would be deleted. One hypothesis concerning the effects of ROS proposes three levels of response to oxidative stress. (i) Low amounts of oxidative stress induce protective responses via the induction of cytoprotective and anti-inflammatory mediators. (ii) An intermediate level of oxidative stress causes the induction of cytokines, chemokines, and adhesion molecules, leading to an inflammatory response. And finally, (iii) a high amount of oxidative stress causes apoptosis and necrosis, which leads to the induction of inflammation and remodeling in which induction of *GSTO 1-1* might play a role, as reviewed in (25).

Other findings pointing towards a possible role of *GSTO 1-1* and *Gpx-2* in allergic airway diseases arise from studies on genetic heterogeneity. The individual's ability to deal with an oxidant burden may depend in part on the genetic background. Polymorphisms of different subclasses of GSTs have been shown to be associated with asthma, lung function and susceptibility to xenobiotic enhancement of allergic symptoms (21;26;27). Recently, functional data have been added, suggesting that *GSTs* are able to modify the adjuvant effect of diesel exhaust particles and thereby attenuate local and systemic allergic inflammation (26). Such an association has not yet been reported for the *GSTO 1-1* or the *Gpx-2* iso-

enzyme. Yet two independent studies were able to link the development of asthma to chromosome 14q24, which is the chromosomal location of *Gpx-2* (28;29). Taken together, our data suggest that different activity levels of *GSTO 1-1* and *Gpx-2* due to genetic polymorphisms might contribute to the relative risk of disease development, a hypothesis which should be tested in association studies in disease cohorts.

In summary, we have identified two common inflammatory genes that were not previously recognized as being involved in the development of allergen-mediated airway disease. Knowledge of the mechanism underlying oxidative stress in the lungs may allow the development of novel antioxidant interventions. These strategies will then have to test the hypothesis that oxidative stress is involved in the pathogenesis of asthma, not only by direct injury to cells, but also as a fundamental factor in airway inflammation.

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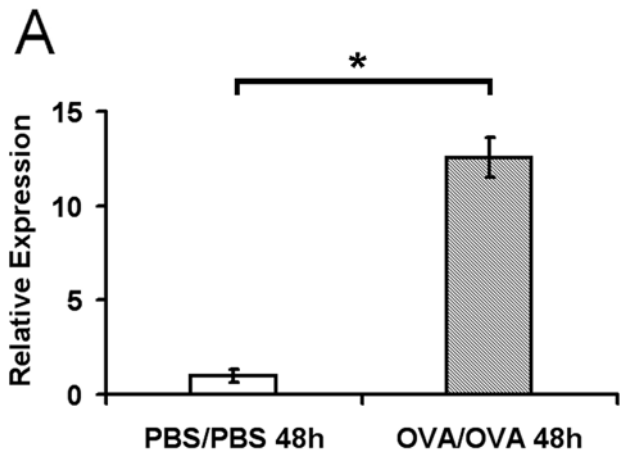
Figure Legends

Figure 1. *Gpx-2* and *GSTO 1-1* mRNA levels in the murine lungs.

mRNA levels of *Gpx-2* gene (A) and *GSTO-1* gene (B) were determined at 48 h after last challenge from mice subjected to sensitization and challenge (OVA/OVA) versus control mice (PBS/PBS) by real-time PCR. mRNA levels were initially normalized to □-actin mRNA levels. Comparisons were made by setting the value of control mice to one. Significance of mRNA-Expression (* $p \leq 0.05$., Mann-Whitney-test) was calculated via ΔΔCT-method.



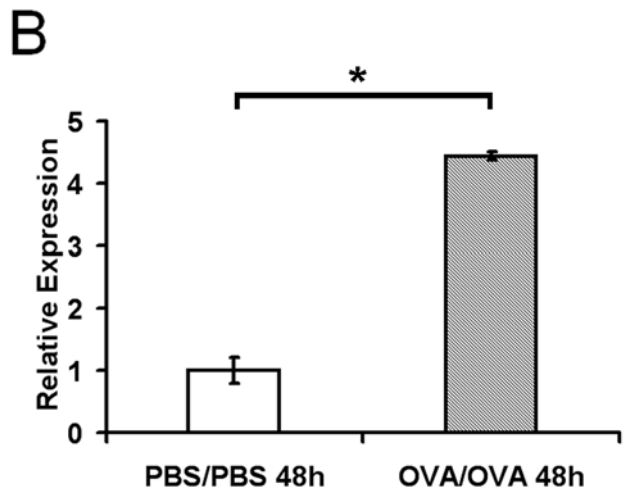


Figure 2. *Gpx-2 and GSTO 1-1 protein levels in murine lungs.*

Relative quantity of *Gpx-2* (A) or *GSTO 1-1* (B) protein levels were compared to protein levels of ß-actin using integrated density values from western blot analysis (right hand of the graph) 48h after last challenge. * $p \leq 0.05$, Mann-Whitney-test.

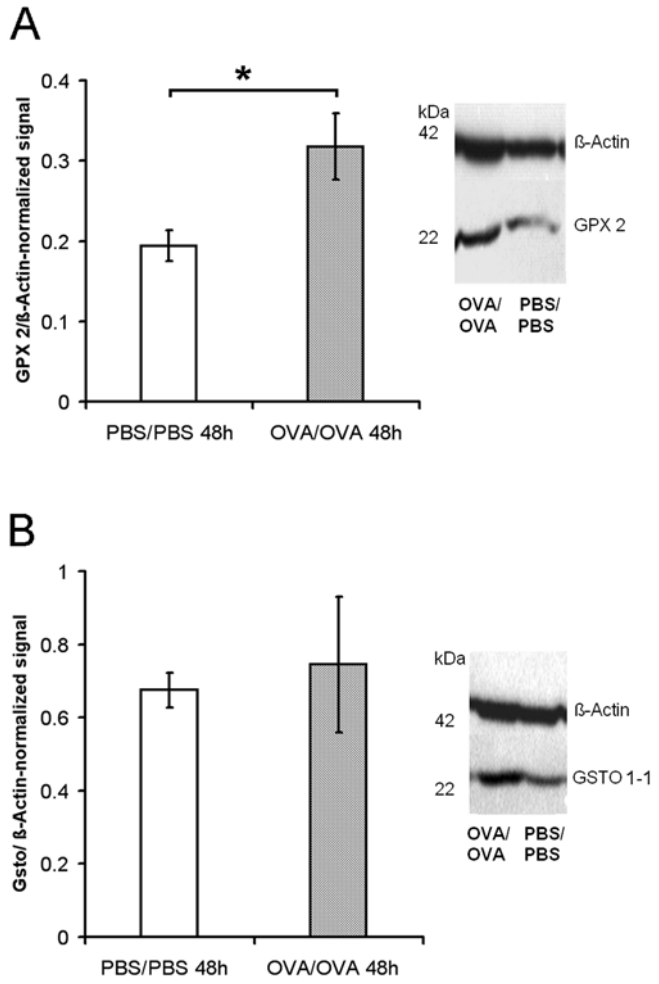


Figure 3. *Localization of Gpx-2 and GSTO 1-1 in murine lungs.*

Immunohistochemical detection of *Gpx-2* (A) and *GSTO 1-1* (B) protein expression in murine lungs. The *GSTO 1-1* protein was found mainly in apical parts of epithelial cells (arrows) while the *Gpx-2* protein was localized in basal epithelial cells (arrows). (BR = bronchus). Protein expression was revealed via immunohistochemistry of paraffin cuts (400 x magnification) in lungs harvested 48h post-challenge

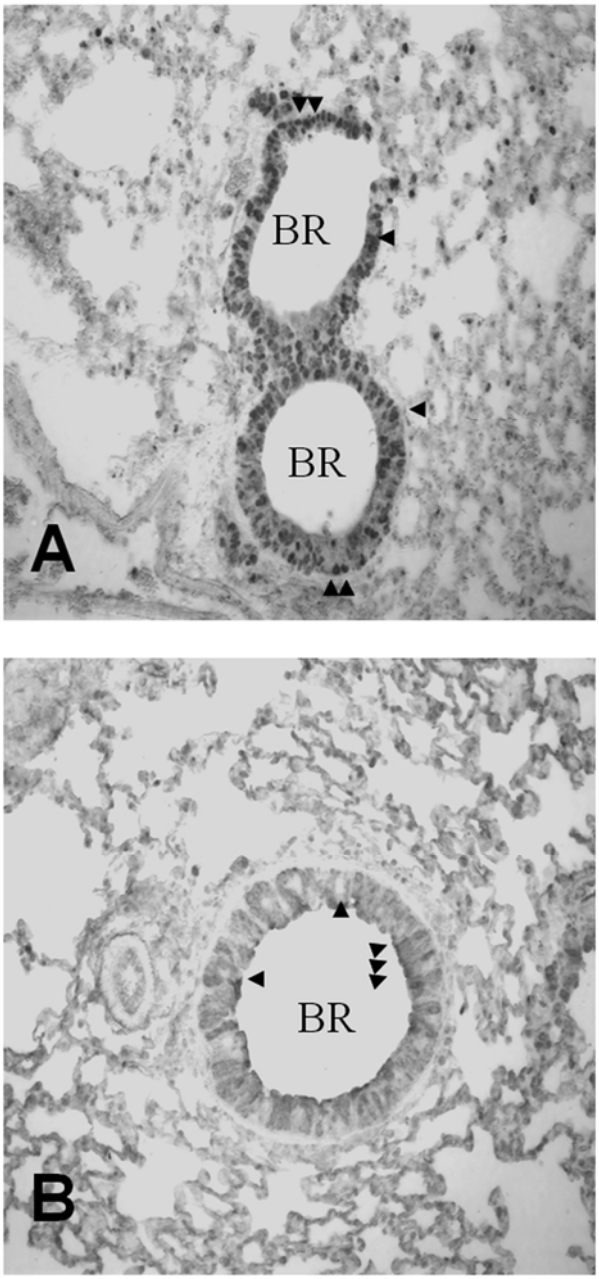
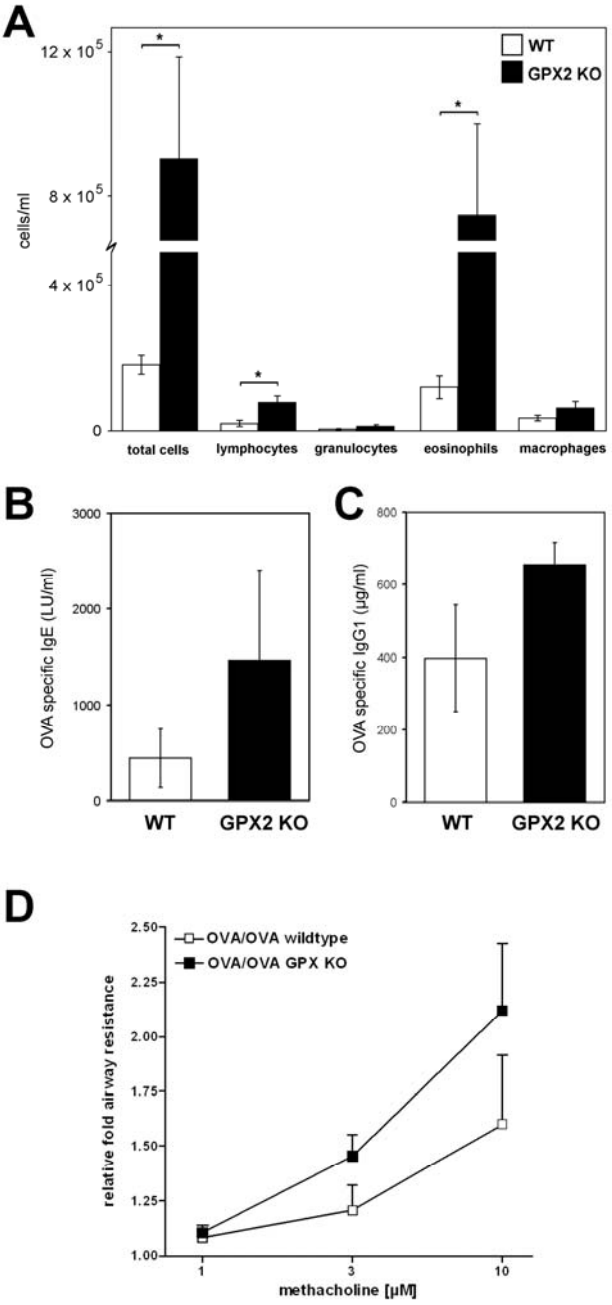


Figure 4. *Gpx2 deficiency enhances allergic airway inflammation and airway reactivity***.** Compared with WT littermates (wt, n=6), Gpx2 KO mice (n=6) showed increased airway inflammation, due to an increased influx of eosinophils and lymphocytes after sensitization and challenge with antigen. **(A)**. OVA-spec. IgE and IgG1 were increased in Gpx-2 KOmice but to a significant level **(B, C)**. Comparing airway resistance in isolated perfused lungs of Gpx2 KO and wild type mice (n= 6 each) after sensitization and challenge, we found a higher relative fold airway resistance in Gpx2 KO mice than in the corresponding WT controls **(D)**. $*P < 0.05$


A
12 x 10^5
8 x 10^5
4 x 10^5
0
cells/ml
WT
GPX2 KO
*
*
total cells
lymphocytes
granulocytes
eosinophils
macrophages
B
3000
2000
1000
0
OVA specific IgE (LU/ml)
WT
GPX2 KO
C
800
600
400
200
0
OVA specific IgG1 (µg/ml)
WT
GPX2 KO
D
2.50
2.25
2.00
1.75
1.50
1.25
1.00
relative fold air way resistance
OVA/OVA wildtype
OVA/OVA GPX KO
1
3
10
methacholine [µM]