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Oxidative stress is one of the hallmarks of asthma. Inflammatory cells associated with asthma are considered to be the main source of oxidants. In addition, oxidants are continuously produced by resident pulmonary cells, including epithelial cells, during normal cellular respiration and aerobic metabolism as well as through non-phagocytic-NADPH oxidases (Duox 1, 2). Duox1 and -2 are found at the apical surface of tracheal and airway epithelial cells, and recently Duox1 was found to be inducible in response to the Th2 cytokines IL4 and IL13 (1, 3). Lastly, asthma exacerbations are often caused by environmental agents that increase the oxidative burden in the lung, like tobacco smoke (4), air pollution (5), ozone (6), and pollen (7).

Oxidative stress has been reported in patients with exacerbations. Elevated levels of the oxidants, hydrogen peroxide (H$_2$O$_2$) (8), and NO (9) are detected in exhaled breath, and increases in levels of 8-isoprostane (10), malondialdehyde (11), nitrotyrosine (12), and protein carbonyls (13) which reflect lipid or protein oxidation, are also present in these patients. In stable asthmatics in contrast, variable data have been reported with regard to oxidative stress. Because levels of oxidants correlate directly with disease severity, oxidants, or markers of oxidation currently serve as parameters for the assessment of disease severity (15), and as measures of the therapeutic effect of inhaled corticosteroids (15, 16).

Oxidants are believed to play a causal role in pathophysiology of asthma. Several studies have indicated that oxidants may contribute to the development of some of the hallmarks of asthma. For instance, oxidants are known to cause epithelial cell death through DNA damage (17) and the consequent loss of epithelial barrier function can increase airway hyperreactivity (18, 19). Furthermore, oxidants have been demonstrated to cause mucus hypersecretion and impair mucociliary clearance (20–22) which can lead to airflow limitation. Lower levels of CuZnSOD (23), MnSOD (24), and catalase activity (25) are also well known to occur in airway epithelial cells of asthmatics when compared with cells from nonasthmatics, contributing to the prooxidative environment.

A number of strategies to increase the antioxidant capacity of the lung have been evaluated in patients with asthma. These studies mainly investigated dietary supplementation of antioxidants like vitamins C (26) and E (27, 28), as well as cysteine precursors (29, 30), and reported variable success rates in alleviating asthma symptoms.

Additional approaches, aimed at restoring normal levels of antioxidant enzymes encompassed SOD mimetic compounds, which have been shown to improve inflammation and other features of allergic airway disease in animal models (31, 32). Catalase represents an important component of the endogenous antioxidant defense system of the lung. Catalase is responsible for detoxifying H$_2$O$_2$ produced under physiological conditions. Catalase transgenic mice have been shown to exhibit attenuated disease parameters in various models associated with oxidative stress, like hypoxia-reoxygenation (33) and doxorubicin toxicity in the heart (34) as well as oxidant injury to pancreatic $\beta$-cells (35). Most recently, mice that overexpress catalase in mitochondria were found to display an extended life span (36).
Because most studies that address the role of oxidants in the pathophysiology of allergic airways disease have relied on the use of antioxidant compounds with diverse reactivities, the causal role of endogenously generated H$_2$O$_2$ in the disease process remains unraveled. The goal of the present study therefore was to elucidate the contribution of H$_2$O$_2$ in the pathophysiology of allergic airway disease. For this purpose we used mice that systematically overexpress catalase and strain matched controls, in the OVA model of allergic airway disease.

Materials and Methods

Animals

Homozygous Tg (CAT)$^{+/+}$ mice were a gift from Dr. H. Van Remmen (University of Texas at San Antonio, San Antonio, TX (37)). Briefly, catalase transgenic mice were generated using a 80-kb genomic DNA fragment containing the human CAT gene and $5^\prime$ and $3^\prime$ flanking regions (38), that led to the integration of a 65-kb fragment. Thus the human CAT gene is controlled by endogenous regulatory elements which leads to systemic gene expression. Mice were backcrossed 12 times onto the C57BL/6 background. Age- and sex-matched C57BL/6 mice (The Jackson Laboratory) were used as controls. Mice were administered 20 μg of OVA with 2.25 mg of Imject Alum (OVA-sensitized, OVA/OVA) or 2.25 mg of Imject Alum alone (mock-sensitized, Alu/OVA) i.p. injection on days 0 and 7. All mice were challenged for 30 min with aerosolized 1% OVA in PBS on day 14, 15, and 16, as previously described (39). Grade V OVA was purchased from Sigma-Aldrich and Imject Alum from Pierce. Mice were euthanized by a lethal dose of pentobarbital via intraperitoneal injection, 48 h after the last challenge. The Institutional Animal Care and Use Committee granted approval for all studies.

Cell culture and flow cytometry

Primary tracheal epithelial cells were isolated from C57BL/6 or catalase transgenic mice according to Wu and Smith (40) with minor modifications (41). For experiments, cells were grown to confluency on 10 cm Collagen I coated culture dishes in DMEM/F12 medium containing 20 mg/ml cholera toxin, 4 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml bovine pituitary extract, 10 mg/ml EGF, 100 μM dexamethasone, 2 mM t-glutamine, 50 μM penicillin, and 50 mg/ml streptomycin (PS). Forty-eight hours before analysis, cells were switched to phenol red free DMEM containing P/S and t-glutamine. To assess baseline levels of H$_2$O$_2$, DCF was added at a final concentration of 10 μM for 30 min, or was omitted as a control. The medium was then aspirated, cells were washed twice with PBS, harvested by trypsinization, spun at 500 × g, resuspended in HBSS and analyzed by flow cytometry.

Pulmonary function assessment

Anesthetized mice were tracheotomized and mechanically ventilated for the assessment of pulmonary function using the forced oscillation technique as described previously (42) (flexiVent; SCIREQ). Briefly, mice were ventilated at a rate of 2.5 Hz with a tidal volume of 0.2 ml and 3 cmH$_2$O positive end-expiratory pressure. Data from before methacholine challenge were collected to establish the baseline for each animal. Next, inhaled doses of aerosolized methacholine (Sigma-Aldrich) in saline were administered in successive increasing concentrations (0, 3.125, 12.5, and 50 mg/ml). Multiple linear regression was used to fit impedance spectra derived from measured pressure and volume to the constant phase model of the lung: $Z(f) = R_x + jωf + [G_y + jωf]^n$ (43). We determined the following physiological parameters: $R_x$ (a measure of central airways resistance), $H_y$ (elastance) and $G_y$ (a measure of visco-elastic properties and/or airflow heterogeneity, (44)). The peak response for each variable was determined, and the percentage change from baseline, as measured at the beginning of the protocol, was calculated.

Bronchoalveolar lavage (BAL)$^3$

BAL fluid was collected from euthanized mice, using 1 ml of PBS for the assessment of total and differential cell counts.

Plasma collection and Ig analysis

Following euthanasia, blood was collected by heart puncture, transferred to plasma separator tubes, centrifuged, and plasma was kept frozen at −80°C.

$^3$Abbreviations used in this paper: BAL, bronchoalveolar lavage; RT, room temperature.

For Ig ELISAs, 96-well plates were coated with 1 μg/ml OVA in PBS overnight at 4°C and washed with PBS containing 0.05% Tween 20 (PBS-T). After blocking with 1% BSA in PBS for 1 h, plates were washed with PBS-T, plasma was applied at dilutions of 1/2–1/250 and incubated for 2 h. Plates were washed with PBS-T and incubated with biotinylated secondary Abs (BD Biosciences Pharmingen), followed by incubation with streptavidin/peroxidase (R&D Systems) for 1 h and detection using reagents from R&D Systems. ODs were read at 405 nm with a wavelength correction at 540 nm (Bio-Tek Instruments). Data is reported as δ OD values ($\pm$SEM) from identical dilutions within the linear range of the readings.

Histopathology and morphometry

Following euthanasia and BAL, the left lung lobe was instilled with 4% paraformaldehyde in PBS and placed into 4% paraformaldehyde at 4°C overnight, before embedding in paraffin. Next, 7-μm sections were cut, affixed to glass microscope slides, deparaffinized with xylene, and rehydrated through a series of ethanol and stained with H&E periodic acid Schiff (PAS) stains or Pico Sirius red, coveredslipped, and examined by light microscopy (20× objective). Sections with a length: diameter ratio of <2:1 were evaluated for PAS positivity and collagen deposition. The percentage of PAS positive airway epithelial cells was recorded. For the assessment of pulmonary fibrosis, Pico Sirius red stained sections were visualized by differential interference contrast microscopy (45) and scored using a scale of 1 to 3 for airway as well as parenchyma associated collagen deposition by two independent, blinded observers. The cumulative score from each mouse was then averaged according to treatment group.

Catalase activity

Lung tissue was pulverized in liquid nitrogen using a mortar and pestle and dissolved in nine volumes of sodium phosphate buffer, and different dilutions were reacted with 30 mM H$_2$O$_2$. Decomposition of H$_2$O$_2$ was followed spectrophotometrically at 240 nm (46). Recombinant catalase was

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Assessment of catalase activity and oxidative stress in lungs or tracheal epithelial cells derived from catalase transgenic or C57BL/6 mice. A, Pulverized lung tissue was homogenized in nine volumes of Na-phosphate buffer, and reacted with 30 mM H$_2$O$_2$. Decomposition of H$_2$O$_2$ was recorded spectrophotometrically at 240 nm, and compared with a standard curve generated with catalase of known catalytic activity. Data were normalized to protein, and are expressed as units. *, $p < 0.05$ between Alu/OVA and OVA/OVA. Cat tg, Catalase transgenic mice. B, Tracheal epithelial cells were isolated from catalase transgenic or C57BL/6 mice, grown to confluence and trypsinized for the evaluation of DCF oxidation via flow cytometry, as described in Materials and Methods. Data on y-axis represent log DCF oxidation whereas the x-axis represents cell counts.
used to generate a standard curve. Data are expressed in units, where 1 unit equals the amount of enzyme that will decompose 1 μM H₂O₂ per minute at 25°C, and results are normalized to protein content.

**Semiquantitative PCR**

Total RNA was DNase treated and reverse transcribed into cDNA. Semiquantitative TaqMan PCR was performed using TaqMan Gene Expression Assays for MUC5AC, CLCA3, and COL1A1 (Applied Biosystems). Values were normalized to the expression levels of HPRT.

**Bio-Plex analysis**

The Bio-Plex (Bio-Rad) kit used allowed analysis of twenty three different cytokines, and was used according to the manufacturer’s instructions. Standard curves were established using a stock of lyophilized multiplex cytokine. The anti-cytokine beads were vortexed and a 25-fold working dilution was prepared in a stock solution of Assay Buffer A. The bead solution was added to the plate and washed twice with Bio-Plex wash buffer A. Standards and samples were added to the plate and incubated for 30 min at room temperature (RT) with shaking. Following this incubation, the plate was washed three times with Bio-Plex wash buffer A. Detection Ab A was incubated for 30 min at RT with shaking, washed three times and incubated with streptavidin-PE for 10 min at RT with shaking. After three washes, the beads were resuspended in Bio-Plex wash buffer A and the plate was read on the Bio-Plex suspension array reader.

**Statistical analysis**

All data were expressed as mean ± SEM and compared by ANOVA. Differences were considered significant when p < 0.05. Pulmonary function assessment was evaluated by staining representative sections from paraffin-embedded lungs with H&E. BAL fluid was collected and total (B) and differential cell counts were performed (C). Values are means (±SEM) from 10 to 13 mice/group. *, p < 0.05 between Alu/OVA and OVA/OVA. Cat tg, Catalase transgenic mice.

**Results**

Enhanced catalase activity in lungs from catalase transgenic mice and dampened DCF oxidation in tracheal epithelial cells

We first determined the basal level of catalase activity in whole lung homogenates of control or catalase transgenic mice. Catalase transgenic mice demonstrated ~8-fold higher levels of lung catalase activity compared with C57BL/6 control mice (Fig. 1A), which was slightly increased in mock sensitized mice receiving OVA. To corroborate that the enhanced catalase activity decreased steady state levels of H₂O₂ in catalase transgenic mice, we isolated...
tracheal epithelial cells from transgenic and strain matched controls for evaluation of oxidation of DCF, which is sensitive to H$_2$O$_2$. Results in Fig. 1B demonstrates that baseline oxidation of DCF was attenuated in tracheal epithelial cells isolated from catalase transgenic mice, compared with controls, suggesting that as expected, baseline cellular oxidation is dampened by the enhanced expression of catalase.

**Catalase overexpression does not alter Ig production following Ag sensitization and challenge**

To ensure that overexpression of catalase did not affect the immunization process, plasma levels of OVA-specific immunoglobulins were measured. OVA sensitization and challenge increased plasma levels of OVA-specific IgE, IgG1, and IgG2a in C57BL/6 mice (Fig. 2). Catalase transgenic mice demonstrated similar increases in levels of these OVA-specific immunoglobulins (Fig. 2), indicating that catalase transgenic mice mounted an immune response to OVA that is similar to C57BL/6 mice.

**Catalase overexpression does not confer protection against cellular infiltration following Ag sensitization and challenge**

We next evaluated the impact of catalase overexpression on OVA-induced pulmonary inflammation in tissue (Fig. 3, A and B). Although scoring of inflammation by guest on April 17, 2017 http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/
percent PAS-positive cells in the airways indicated a trend toward enhanced goblet cell metaplasia in catalase transgenic mice (Fig. 4B), this failed to reach statistical significance. Evaluation of mRNA expression of Calcium activated Chloride Channel 3 (CLCA3) (Fig. 4C) and Mucin 5 subtype A & C (MUC5AC) (Fig. 4D) in lung tissues revealed marked increases in both strains of mice in response to sensitization and challenged with OVA. However mRNA increases CLCA3 and MUC5AC were more pronounced in catalase transgenic mice compared with C57BL/6 strain matched controls.

Catalase overexpression does not alter collagen expression or deposition

To address whether H₂O₂ could affect the development of subepithelial fibrosis, lung sections were stained with Pico Sirius red and collagen deposition scored in airways as well as parenchymal regions (Fig. 5B). Although subepithelial fibrosis can be detected in OVA sensitized and challenged mice (47) and C57BL/6 mice are prone to the development of fibrosis (48), the acute exposure regimen used here was not sufficient to enhance collagen deposition (Fig. 5, A and B), and no differences were observed between C57BL/6 and catalase transgenic mice. However, increases in mRNA expression of collagen type I α 1 (COL1A1) were detected in this acute study, and comparable increases in COL1A1 mRNA occurred in both mouse strains (Fig. 5C).

Enhanced airway hyperresponsiveness following OVA sensitization and challenge in catalase transgenic mice compared with C57BL/6 controls

To assess whether catalase overexpression affected respiratory physiology, pulmonary function was determined using the forced oscillation technique and the constant phase model of the lung physiology, pulmonary function was determined using the forced oscillation technique and the constant phase model of the lung

Discussion

The implementation of catalase transgenic mice in various disease models has suggested a damaging role of H₂O₂ in their pathophysiology. In addition, a recent study demonstrated the oxidative inactivation of catalase in a murine model of allergic airway disease as well as decreased catalase activity in lungs of patients with asthma (25). In contrast, the present study demonstrating that catalase transgenic mice exhibit worsened airways hyperresponsiveness, and enhanced expression of genes important to mucus production, points to a protective role of H₂O₂ in these manifestations of Ag-induced allergic airway disease. In line with our findings, the same catalase transgenic mice were shown to have increased sensitivity to γ-irradiation induced death (37). Although the results from the present study might be surprising, conflicting reports exist on the role of H₂O₂ in airways hyperresponsiveness. For example, studies using isolated organ preparations have demonstrated that H₂O₂ could increase airway contractility by decreasing epithelial barrier function through damage to epithelia, thereby increasing epithelial permeability to methacholine (18, 19). Furthermore, cyclooxygenase (COX)-2 dependent formation of PGD₂ was shown to be a mediator of H₂O₂-induced bronchoconstriction, whereas

Figure 5. Collagen deposition and COL1A1 mRNA expression in catalase transgenic and C57BL/6 mice after OVA sensitization and challenge. A. Representative sections from paraffin-embedded lungs that were stained using Pico Sirius red, which stains collagen red in bright-field microscopy (upper panels) and bright green when visualized by differential interference contrast microscopy (lower panels). B. Scoring by two blinded observers of collagen deposition in airways (■) or parenchyma (□) using a scale from 1 to 3. The cumulative score for each mouse was averaged according to treatment group. C, RNA was collected from lungs, reverse-transcribed, and analyzed for COL1A1 mRNA expression by TaqMan PCR. Data are expressed as mean RQ from six mice per group (±SEM), following normalization to the housekeeping gene, HPRT. *, p < 0.05 between Alu/OVA and OVA/OVA.
levels of IL-17 in the BAL fluid of catalase transgenic mice compared with C57BL/6 controls (Table I) could therefore be responsible for the observed mucus metaplasia. This scenario, which remains to be formally tested, would suggest an indirect role for \( \text{H}_2\text{O}_2 \) in the alterations of mucin gene expression seen in catalase transgenic mice compared with C57BL/6 controls in response to Ag challenge.

Catalase transgenic mice appear to be capable of mounting a normal immune response against OVA, based upon comparable increases in levels of immunoglobulins seen in both strains of mice subjected to Ag sensitization and challenge (Fig. 2). Yet recent evidence supports a role for oxidant production, and a second messenger function of oxidants, in cells of the adaptive immune response after receptor activation (for review (57)). In particular, oxidants have been shown to regulate Ag processing and presentation by dendritic cells (58). Some evidence also points to a role of ROS in skewing Th-2 responses (for review (59)). However, levels of the Th-2 cytokines, IL-4 and IL-5, and lung inflammation were similar in catalase transgenic and C57BL/6 mice, suggesting that these observed effects in catalase transgenic mice may not be due to altered regulation of immune cell function. Catalase transgenic mice showed no alterations in the recruitment of inflammatory cells, despite elevated levels of RANTES and KC in the BALF of Catalase transgenic mice, which are known to be involved in the recruitment of eosinophils (60) and chemotraction of neutrophils (61), respectively.

It is of importance to consider that catalase is not the only enzyme responsible for the detoxification of \( \text{H}_2\text{O}_2 \). Glutathione peroxidase and the more recently discovered members of the peroxiredoxin family of enzymes are also capable of eliminating \( \text{H}_2\text{O}_2 \) (for review see (62)). One of the potential pitfalls of using catalase transgenic mice is that catalase in most cells is primarily localized to peroxisomes (63) and that this approach therefore would limit investigating the role of \( \text{H}_2\text{O}_2 \) in the extracellular space, like the extracellular lining fluid of the lung where eGPx would be more likely to account for \( \text{H}_2\text{O}_2 \) detoxifying activity. eGPx has furthermore been demonstrated to be elevated in asthmatic patients compared with control individuals (64). However, primary hepatocytes derived from catalase transgenic mice have been shown to detoxify \( \text{H}_2\text{O}_2 \) added to cell culture medium more efficiently then cells derived from control animals (37). Results in Fig. 1B also indicate that steady state levels of DCF-oxidizing species are lower in primary tracheal epithelial cells from catalase transgenic mice compared with cells from C57BL/6 mice. Additionally, catalase transgenic mice used in the present study overexpress catalase in all tissues, and all compartments of the lung making it impossible to precisely assess the local environments and cell types in which \( \text{H}_2\text{O}_2 \) affects hyperresponsiveness or mucin gene expression. Tissue specific or inducible transgenic approaches would be required to unravel these questions. It is furthermore critical to consider that most antioxidant systems are interrelated and interconnected. Compensation in response to changes in the steady state activities of a particular antioxidant module by other functionally related enzyme systems has been recognized (64 – 67). However, previous studies that addressed antioxidant compensation in catalase transgenic mice failed to detect changes in levels of MnSOD, CuZnSOD, GPx in various tissues, including the lungs, compared with wild-type controls (37). We have in addition not found altered concentrations of GSH or GSSG in lung homogenates of catalase transgenic mice compared with C57BL/6 controls (GSH C57BL/6 3.13 ± 0.32, Cat tg 3.13 ± 0.23 nmol/mg protein; GSSG C57BL/6 0.010 ± 0.003, Cat tg 0.019 ± 0.009 nmol/mg protein; mean ± SEM, values were corrected for protein content). Based upon these considerations, the present data

conversely, COX-2 dependent formation of PGE\(_2\) has been demonstrated to be involved in \( \text{H}_2\text{O}_2 \)-induced repression of airway smooth muscle contractility (49 – 51). \( \text{H}_2\text{O}_2 \) has furthermore been shown to up-regulate the expression of eNOS (52, 53), and the consequent rise in NO production could also account for the bronchoprotective effects of \( \text{H}_2\text{O}_2 \).

Our present findings indicate that catalase overexpression may enhance mucus production, in association with increased expression of MUC5AC and CLCA3 in allergic airway disease. No other studies to date have indicated a direct role for \( \text{H}_2\text{O}_2 \) in the repression of mucus hyperproduction or mucin gene expression. In contrast, oxidants generated through the activity of neutrophil elastase (54), Duox 1 (22) as well as xanthine/xanthine oxidase (20) have been demonstrated to increase the expression of MUC5AC, consequently leading to increased mucus secretion. However, studies have shown that IL-17 (IL-17) is a potent inducer of mucus metaplasia through the induction of MUC5AC (55, 56). The higher
would suggest that the enhanced hyperresponsiveness and mucin gene expression are due to changes in steady state levels of H$_2$O$_2$, and not to changes in other antioxidant defenses.

It is also important to consider that H$_2$O$_2$ is not the sole oxidant produced during allergic airway inflammation. For instance, it is well established that levels of exhaled NO (NO$_2$) are elevated in patients with asthma, due to the enhanced expression of iNOS, and correlate with FEV1. However, conflicting data exist on the role of NO in the development of allergic airway disease. Some reports point to an anti-inflammatory role for NO whereas other studies find negative correlations between NO and inflammation. Regarding its effects on inflammation, NO is a well-known bronchodilator. It is furthermore important to keep in mind that multiple reactive oxygen and nitrogen species are produced at the same time and will interact, forming more reactive species with different properties. For review of these complex events and their role in allergic airway disease see (68). Altering the production or detoxification of a single oxidant will therefore have consequences for the formation of multiple oxidant species and will complicate the explanation of how a single oxidant contributes to disease pathology.

Taken together, the present study indicates a protective role for H$_2$O$_2$ in airway hyperresponsiveness and mucous metaplasia and illustrates the importance of H$_2$O$_2$ in regulating a diverse spectrum of physiological functions. These findings appear to warrant reconsideration of the damaging role of H$_2$O$_2$ in the pathophysiology of allergic airway disease.

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