Catalase Overexpression Fails to Attenuate Allergic Airways Disease in the Mouse


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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 (H2O2) (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 (11, 14). 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 apoptosis (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 H2O2 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 ß-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 systemically 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' and 3' 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) via 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 confluence on 10 cm Collagen I coated culture dishes in DMEM/F12 medium containing 20 ng/ml cholera toxin, 4 µg/ml insulin, 5 µg/ml bovine pituitary extract, 10 ng/ml EGF, 100 nM dexamethasone, 2 mM t-glutamine, 50 µM penicillin, and 50 µg/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 x 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 to the mice. Pressures and volumes were recorded and volume to the constant phase model of the lung: Z($\omega$) = R$_e$ + Jol + (G$_\infty$ + Jh$_2$)/ω$^2$ (43). We determined the following physiological parameters: R$_e$ (a measure of central airways resistance), H$_e$ (elastance) and G$_\infty$ (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.
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 was used for 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 cata-
alase 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 H₂O₂. 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. 3A), and BAL (Fig. 3, B and C). As expected, sensitization and challenge with OVA caused prominent perivascular and peribronchial cell infiltration in C57BL/6 mice, and marked increases in eosinophils in BAL. Surprisingly, catalase transgenic mice displayed inflammatory responses to Ag (Fig. 3) that were indistinguishable from C57BL/6 mice. Evaluation of BAL cytokines revealed no detectable levels of IL12 (p70), IL13, IL10, IFN-γ, TNF-α, or eotaxin in mock immunized mice, nor detectable expression of GM-CSF, IL2, or IL3 in response to OVA sensitization plus challenge in C57BL/6 or catalase transgenic mice (data not shown). Similar OVA-dependent increases in IL4, IL5, IL6, MCP1, G-CSF, and MIP1β occurred in both C57BL/6 and catalase transgenic mice (Table I). Although KC and IL12 (p40) increased in an OVA-dependent manner in both mouse strains, these increases were enhanced in C57BL/6 mice compared with catalase transgenic mice. In contrast, IL1α, RANTES, and IL17 were found only to be significantly elevated after OVA immunization and challenge in catalase transgenic mice (Table I).

Catalase transgenic mice display enhanced mucin gene expression in response to OVA, compared with C57BL/6 controls

Because oxidants have been reported to stimulate mucus production (22), we next evaluated mucus metaplasia. PAS positive epithelial cells were detected both in C57BL/6 and catalase transgenic mice in response to OVA (Fig. 4A). Although scoring of

![Image](image.png)

**FIGURE 4.** Mucus metaplasia and mucin gene expression following sensitization and challenge with OVA in catalase transgenic and C57BL/6 mice. A, Representative sections from paraffin-embedded lungs, stained using PAS reagent to visualize mucus producing airway cells. B, Airways with a length to diameter ratio of <2:1 were evaluated for PAS positivity and the percentage of PAS positive airway epithelial cells was recorded. Data are expressed as mean of four mice per group, using multiple airways per mouse (±SEM). RNA was collected from lungs, reverse-transcribed, and analyzed for CLCA3 (C) and MUC5AC (D) expression relative to HPRT by semiquantitative TaqMan PCR. Data are expressed as mean RQ from six mice per group (±SEM). * p < 0.05 between Alu/OVA and OVA/OVA. Cat tg, Catalase transgenic mice.

<table>
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<tr>
<th>cytokine</th>
<th>C57Bl/6 Alu/OVA</th>
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<tr>
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<td>IL5</td>
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<td>12.76 ± 4.76</td>
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<td>MCP1</td>
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<td>141.10 ± 51.43</td>
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<td>KC</td>
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<td>2.18 ± 1.64</td>
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<td>IL12 (p40)</td>
<td>372.07 ± 148.03*</td>
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<td>132.34 ± 40.38*</td>
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*p < 0.05 between Alu/OVA and OVA/OVA. Cat tg, Catalase transgenic mice.
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 (Fig. 5A) 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 in response to sensitization and challenged with OVA. How-ever mRNA increases CLCA3 and MUC5AC were more pronounced in catalase transgenic mice compared with C57BL/6 strain matched controls.

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**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 (42). OVA-induced increases in central airway resistance (Rc) after challenge with methacholine occurred to similar extents in C57BL/6 and catalase transgenic mouse strains (Fig. 6A). Intriguingly, tissue visco-elastic properties and/or airflow heterogeneity (Gp) were elevated in naive catalase transgenic mice after metha-choline challenge compared with naive C57BL/6 controls, masking increases that were observed in response to OVA challenge in C57BL/6 mice (Fig. 6B). Lastly, tissue elastance (Ht) responses to methacholine were similar in naive animals of the two mouse strains, although OVA-induced increases in elastance were enhanced in mice that overexpress catalase (Fig. 6C).

**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 hyperresponsive-ness, and enhanced expression of genes important to mucus pro-duction, 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, cyclo-oxygenase (COX)-2 dependent formation of PGD₂ was shown to be a mediator of H₂O₂-induced bronchoconstriction, whereas...
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 H2O2 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 H2O2. Glutathione peroxidase and the more recently discovered members of the peroxiredoxin family of enzymes are also capable of eliminating H2O2 (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 H2O2 in the extracellular space, like the extracellular lining fluid of the lung where eGPx would be more likely to account for H2O2 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 H2O2 added to cell culture medium more efficiently than 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 H2O2 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 PGE2 has been demonstrated to be involved in H2O2-induced repression of airway smooth muscle contractility (49–51). H2O2 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 H2O2.

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 H2O2 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

FIGURE 6. Assessment of airway hyperresponsiveness following OVA sensitization and challenge in catalase transgenic and C57BL/6 mice. Pulmonary hyperresponsiveness to increasing doses of nebulized methacholine was assessed from forced oscillations and expressed as Rh (central airways resistance), Gm (tissue visco-elastic properties and/or heterogeneity), and Hm (elastance). Solid lines: Alu/OVA; dotted lines: OVA/OVA; • C57BL/6; ▲ Cat tg. Data are derived from the constant phase model and are expressed as percentage changes from baseline measurements (±SEM) and comprised of 7–8 mice per group. *, p < 0.05 between C57BL/6 Alu/OVA and C57BL/6 OVA/OVA, †, p < 0.05 between Cat tg Alu/OVA and Cat tg OVA/OVA, ‡, p < 0.05 between C57BL/6 Alu/OVA and Cat tg Alu/OVA, §, p < 0.05 between C57BL/6 OVA/OVA and Cat tg OVA/OVA.
would suggest that the enhanced hyperresponsiveness and muci-
gin 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$_x$) 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$_x$ and inflammation. Regard-
less of its effects on inflammation, NO is a well-known broncho-
dilator. 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 detoxi-
fication of a single oxidant will therefore have consequences for
the formation of multiple oxidant species and will complicate de-
fining the mechanism by which 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 mucus metaplasia and
illustrates the importance of H$_2$O$_2$ in regulating a diverse spectrum
of physiological functions. These findings appear to warrant re-
consideration of the damaging role of H$_2$O$_2$ in the pathophysiology
of allergic airway disease.

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