CAT-2 Amplifies The Agonist-Evoked Force Of Airway Smooth Muscle By Enhancing Spermine-Mediated Phosphatidylinositol(4)-Phosphate 5-Kinase-γ Activity

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ABSTRACT

We investigated the effect the loss of the CAT-2 gene (CAT-2-/-) has on lung resistance ($R_L$) and tracheal isometric tension. The $R_L$ of CAT-2-/- mice at a maximal dose of acetylcholine (ACh) was decreased by 33.66% ($p = 0.05$, $n = 8$) as compared with that of C57BL/6 (B6) mice. The isometric tension of tracheal rings from CAT-2-/- mice showed a significant decrease in carbachol (CCh)-induced force generation (33.01%, $p < 0.05$, $n = 8$) as compared to controls. The isoproterenol (ISO)- or the sodium nitroprusside (SNP)-induced relaxation was not affected in tracheal rings from CAT-2-/- mice. The activity of iNOS and arginase in lung tissue lysates of CAT-2-/- mice were indistinguishable from that of B6 mice. Further, the expression of phospholipase-C-β (PLC-β) and phosphatidylinositol-(4)-phosphate-5-kinase-γ (PIP-5K-γ) was examined in the lung tissue of CAT-2-/- and B6 mice. The expression of PIP-5K-γ but not PLC-β was significantly reduced in CAT-2-/- compared to B6 mice. The reduced airway smooth muscle (ASM) contractility to CCh seen in the CAT-2-/- tracheal rings was completely reversed by pre-treating the rings with 100 µM spermine. This increase in the CAT-2-/- tracheal ring contraction upon spermine pretreatment correlated with a recovery of the expression of PIP-5K-γ. Our data indicates that CAT-2 exerts control over ASM force development through a spermine-dependent pathway which directly correlates with the expression level of PIP-5K-γ in the lung.
INTRODUCTION

CAT-2 is a membrane-bound transporter exhibiting comparably high affinities for cationic amino acids (11,8,40). Early studies demonstrated that CAT-2, could mediate the uptake of L-arginine by liver cells, and in subsequent studies, CAT-2 mRNA was found to be expressed in a variety of mammalian cells and tissues, including the lung (25,19). Since L-arginine is the key substrate for NO production by inducible nitric oxide synthase (iNOS), CAT-2 activity may play an important regulatory role in the production of NO. Likewise, CAT-2 activity may also regulate the production of polyamines (i.e. putrescine, spermine and spermidine) and proline which are formed as by-products of the metabolism of L-arginine to L-ornithine by arginase. Since polyamines and proline control cell proliferation and collagen production, respectively, it is conceivable that CAT-2 activity may influence many events involved in tissue remodeling.

In terms of airway responsiveness, the two arms of L-arginine metabolism may represent opposing regulatory forces. Many publications have demonstrated the importance of nitric oxide in mediating airway smooth muscle relaxation and bronchodilation. Extrahepatic arginase activity has been implicated in smooth muscle contraction, and airway hyperresponsiveness (AHR), by either limiting the availability of intracellular L-arginine for iNOS and/or by driving airway remodeling through the production of polyamines and proline (34,28,41). Chronic asthma is associated with a higher level of arginase expression and AHR, suggesting the two may be causally linked. Additionally, increased arginase activity underlies allergen-induced deficiency of NO and AHR in a guinea pig model of asthma (16). Perhaps even more importantly, the expression of type I arginase is increased by key inducers of allergic asthma, such as, the Th2 cytokines IL-4 and IL-13. Since L-arginine transport can also be inhibited by the arginase product, L-ornithine, and iNOS activity seems to be more sensitive to shifts in substrate availability than arginase, the balance may be further tipped in favor of arginase-driven L-arginine catabolism rather than iNOS-driven catabolism(27, 3).

Although one may assume that asthmatic airway responsiveness develops as a result of reduced NO production through iNOS substrate competition with arginase, the abnormal responsiveness of ASM could also arise as result of the downstream effects of additional proline and polyamine production from increased arginase activity. In this study we address
whether the reduction in L-arginine transport through the absence of CAT-2 is sufficient to promote or inhibit ASM responsiveness in tracheal ring sections. We demonstrate that absence of CAT-2 can result in a reduced contractile response to the muscarinic agonist, CCh, suggesting that the absence of NO production, at least through the CAT-2 pathway, is not sufficient to drive an increase in the contractile function of ASM under physiological conditions. Then, to understand the fundamental relationship between CAT-2 activity and ASM tone we examined the effects of CAT-2 absence on ISO (agonist of β2-adrenergic receptor (β2-AR))- and SNP (agonist of guanylate cyclase)-mediated relaxation responses. In additional experiments, we utilized specific pharmacological agents, L-NAME (inhibitor of iNOS), GTPγ-S (activator of PLC-β) and spermine (activator of PIP-5K-γ), to specifically address the underlying mechanism for reduced force response to muscarinic receptor activation upon CAT-2 absence.
MATERIALS and METHODS

Animals

Breeding pairs of CAT-2 deficient mice were kindly provided by Dr. Carol MacLeod (30). All animals were bred at Charles River Laboratories (Wilmington, MA). After weaning, male CAT-2-/- and B6 mice were housed at Wyeth Research Corporation under specific-pathogen-free conditions. Food and water were provided ad libitum. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as the Institutional Animal Care and Use Committee guidelines of Wyeth Research Corporation.

Preparation of Trachea and Lungs

CAT-2-/- and control B6 mice were sacrificed by CO2 asphyxiation. Trachea were rapidly excised and cleaned of adherent connective tissue. Each trachea was sectioned into 3 - 4 mm wide rings and cultured overnight in DMEM (0.1 mM nonessential amino acids, 2.0 mM L-glutamine, 0.05 mM β-mercaptoethanol, 100 µg/ml penicillin/streptomycin, and 5% heat-inactivated FBS). To observe any changes in airway structure resulting from ex vivo culturing, whole and sectional fresh trachea and trachea cultured in medium overnight were examined histologically after staining with a H & E solution (CAT hematoxylin, Edgar Degas Eosin Working Solution, Biocare Medical, Concord, Ca) and photographed under a light microscope at a x 4 and x 20 magnification. Tracheal rings were selected over tracheal strips because the configuration of the ASM bundles is largely preserved in this arrangement and their contraction is directly related to in vivo airway narrowing (17). After collection of tracheae, left lungs from either B6 or CAT-2-/- mice were harvested for Western blot analysis. Lungs were submerged in Krebs-Henseleit (K-H) solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 11.1 mM Dextrose, 1.2 mM MgSO4, 2.8 mM CaCl2, and 25 mM NaHCO3, pH 7.4) containing 10 mM HEPES, cleaned of adherent tissues and incubated at 370C for 60 min. in an atmosphere of 5% CO2 and 95% atmosphere with or without 100 µM of spermine (Sigma, St. Louis, MO). Treated and untreated tissues were homogenized separately in cell lysis buffer (1% Triton, 0.5% Deoxycholic Acid, 0.1% SDS in PBS, pH 7.3) containing a protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL). Tissue supernatants were centrifuged (100,000 x g) for
60 min at 4°C and the protein concentration of the cleared supernatant was determined by BCA assay (Pierce Biotechnology, Rockford, IL). Cleared supernatants were stored at -70°C until used.

**In vivo Responsiveness**

R_1_ from B6 and CAT-2-/- mice was examined by cannulation of the jugular vein as previously described (15, 14). Briefly, mice were anesthetized by an intraperitoneal injection (IP) of a mixture of ketamine and xylazine (100 mg/kg and 20 mg/kg, respectively) and maintained with a light plane of the anesthesia. Small saline-filled polyethylene tubing was inserted into a jugular vein for an intravenous administration of ACh (muscarinic receptor agonist). The trachea was opened in the position between the 2nd and the 3rd cartilage ring, rapidly intubated, and attached to a mouse ventilator (Harvard Model 687, USA) at a respiratory rate of 130 beats/min with 0.2 ml of the tidal volume. R_1_ derived from alveolar pressure and airflow rate was assessed using data automatically obtained with a pulmonary mechanics analyzer (Buxco Electronics Inc. NY, USA) and visualized on a recorder tracing. [ACh]_s ranging from 80 to 1280 (µg/kg) were intravenously administrated with a microinfusion syringe approximately 3-minutes apart until the R_1_ returned to a baseline level. The airway responsiveness of the mice to ACh challenges was performed within the same 25 minute period of each experiment at a total volume of 150 µl per mouse.

**Ex vivo ASM Studies**

The isometric tensions of fresh or cultured trachea from B6 and CAT-2-/- mice were examined as previously described (6,37). Briefly, the tracheal rings were supported longitudinally by a plexiglas rod with a stainless steel pin inserted into the base of a double-jacketed, glass organ bath filled with 15 ml of K-H solution. The solution was continuously gassed with a mixture of 5% CO₂ and 95% atmosphere at 37°C for the duration of each experiment. The upper support was attached by a loop of silk thread to an isometric force transducer (TSD125C, BIOPAC Systems, Goleta, CA) by which changes in tension were measured and fed into a preamplifier (MP150WS, BIOPAC Systems, Goleta, CA). The dose-response curves for agonists were synchronously recorded and displayed on a computer. All of the initial ASM tensions were set at approximately 0.5 g and maintained at a steady state.
for 1 hour before agonists were given. The tracheal rings were washed 4 times with K-H solution at 10 minute intervals. The contractile response curves to CCh (10^{-7} - 10^{-5} M) were obtained with tracheae from CAT-2/-/- and B6 animals that had been cultured overnight in DMEM. Concentrations of the agonist were increased only when the contractile response to the previous concentration had stabilized. To examine the relaxant responses to ISO and SNP, tracheal rings were first pre-contracted with CCh (1.0 µM). After reaching a stable contraction, ISO or SNP were introduced into each organ bath in increasing concentrations (3 x 10^{-8} - 10^{-5} M). 200 µM papaverine, a phosphodiesterase inhibitor, which has previously been shown to produce complete relaxation of the tracheal rings (5), was added to each organ bath at the end of the experiment to evaluate whether a maximum relaxation was achieved with the highest concentrations of ISO or SNP. The % relaxation was calculated as (relaxation to ISO or SNP/relaxation to papaverine) x 100.

In order to dissect out the underlying mechanism responsible for the reduced contractile response to CCh in tracheae from CAT-2/-/- mice, CAT-2/-/- tracheal rings were pretreated overnight at 37°C with 10 µM GTP\gamma-S, 100 µM spermine or 100 µM L-NAME in DMEM containing 10 mM HEPES in an atmosphere of 5% CO₂. The following morning, tissues were washed with K-H solution and isometric force contraction was measured, as described above.

In each study described, doses of the agonists refer to the final concentration. At the end of each experiment, tracheae were blotted on gauze pads and weighed. Isometric tensions of the tracheal rings were calculated as milligram of tension per milligram of weight (mg/mg) and expressed as an individual percentage (%) of 10^{-5} M CCh-evoked force normalized to mean values from B6 mice. The concentration of CCh required to produce 50% effective contraction (EC_{50}) was determined and this EC_{50} value converted to a log scale. All values were expressed as mean ± SEM. The student’s unpaired t-test (two tailed) was used to determine significance between tracheal responses from B6 and CAT-2/-/- mice, and responses from the B6 tracheal rings that were incubated overnight in the absence and presence of L-NAME. One-way analysis of variance (ANOVA) was used to compare differences among groups that had been treated with or without GTP\gamma-S or spermine. A p-value of less than 0.05 was considered significant.
Nitrite Assay

Nitrite concentrations in either peritoneal macrophages, airway macrophages or lung tissues were examined by measuring the concentrations of an end product, nitrite, using a method based on the Griess reaction (42). Briefly, the peritoneal cavities of CAT-2/- and B6 mice were lavaged with 7.0 ml of PBS and the peritoneal lavage fluid collected. 80% of the cells from this primary lavage were determined to be macrophages by FACS and were cultured in DMEM supplemented with 12.5 ng/ml lipopolysaccharide (LPS) and 12.5 ng/ml Interferon-γ (IFNγ) for approximately 24 hours. Cells were then centrifuged and supernatants collected for analysis of nitrite concentration (see below). Lung tissue was also removed, placed in lysis buffer and incubated at 37°C for 24 hr. The lysates were then centrifuged and supernatants collected for analysis of nitrite concentrations. To assess nitrite levels, aliquots of supernatants (50 µl) from each sample were mixed with 50 µl Griess reagent (Bio-Rad, Hercules, CA) at room temperature for 10 min. The absorbance was measured at 540nm in an automated microplate reader. Sample nitrite concentrations were calibrated using a standard curve of sodium nitrite (µM) prepared as 200, 100, 50, 25, 12.5, 3.125 and 0. (The lower limit of detection for this method was 2.5 µM)

Urea Assay

Analysis of the urea content within lung tissue from CAT-2/- and B6 mice was carried out as previously described (29). Briefly, supernatants from lung tissue lysates were prepared as described above for the measurement of nitrite. Supernatants (12.5 µl) were added to an activation solution (10 mM MnCl2, 50 mM Tris-HCl at pH 7.5) and incubated at 55°C for 10 min. The solutions were then mixed with L-arginine (0.5 M) and incubated at 37°C for 60 minutes. The reaction was stopped by the addition of 100µl stop solution (H2SO4 : H3PO4 : H2O = 1 : 3 : 7). 9% 1-phenyl-1, 2-propanedione (v/v) was added to each solution, the mixtures vortexed and boiled at 100°C for 5 minutes. The solutions were cooled to room temperature prior to reading the absorbance values at 540 nm in an automated microplate reader. The quantity of urea produced in each lung sample was determined by comparing the sample OD to that obtained from a standard curve of urea (µg/ml) prepared as 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 and 3.9 (The lower limit of urea detection for this assay was 3.0 µg/ml).
Western Blotting

Protein expression of phosphatidylinositol-4-phosphate 5-kinase (PIP-5K-γ) and phospholipase C (PLC-β) was determined using Western blotting analysis. Briefly, aliquots of lung tissue lysate (100 µg/well) were loaded onto a 4-20% SDS polyacrylamide gel. The electrophoresed proteins were transferred to nitrocellulose membranes and blocked with 5% nonfat dried milk in TBS at 4° C overnight. The membranes were incubated with the primary antibody (1:250), anti-mouse PIP-5K-γ, PLC-β (BD Biosciences, San Diego, CA) and β-actin (Sigma, USA) for 60 min and then washed with a wash buffer. The membrane was incubated with peroxidase-conjugated anti-mouse IgG (1:2000) (Jackson ImmunoResearch, Laboratory Inc. East Grove, PA) for 60 min and washed again. A mixture of western blotting detection reagent I and II (GE Healthcare Life Sciences, Piscataway, NJ) was poured on the membrane and incubated at room temperature for 1 minute. The bands for PIP-5K-γ and PLC-β were visualized by autoradiography along with the band for β-actin in the same tissue sample for protein loading normalization.
RESULTS

The effect of the CAT-2 gene on induced NO in macrophages.

Nitrite concentrations were examined in both resting and activated peritoneal and airway macrophages derived from B6 and CAT-2-/- mice. Levels of nitrite were undetectable in untreated macrophages derived from either B6 or CAT-2-/- mice. However, when peritoneal and airway macrophages were activated with LPS/IFNγ, only the cells from the B6 mice produced a high concentration of nitrite. In contrast, nitrite levels remained undetectable in the peritoneal and airway macrophages from the CAT-2-/- mice treated with LPS/IFNγ (Fig. 1A, B). These results showed that the release of NO was significantly reduced in either peritoneal or airway macrophages in the absence of the CAT-2 gene, as is consistent with what has been previously reported (26).

The basal nitric oxide synthase and arginase levels in CAT-2-/- mice.

In order to confirm that the level of activity of enzymes immediately downstream of the CAT-2 transporter was comparable in B6 or CAT-2-/- mice, we examined the activity of nitric oxide synthase and arginase. As a measure of tissue nitric oxide synthase activity we measured nitrite levels and as a measure of arginase activity, urea levels in lung tissue lysates. Our results showed that nitrite (µM) levels in the lung tissues from B6 and CAT-2-/- mice were 35.57 ± 16.85 and 21.68 ± 7.69 and urea (µg/ml) levels were 151.74 ± 46.21 and 187.43 ± 62.17, respectively. We did not detect a significant difference as measured by the Student’s unpaired T-test in the levels of nitrite produced in lung tissue lysates from either B6 or CAT-2-/- mice (Fig. 2A) nor did we detect a significant difference in the levels of urea produced in the lung lysates from knockout or control animals (Fig. 2B) suggesting that, at least in the lung, under physiological conditions the level of neither iNOS nor arginase is significantly changed by a deficiency of the CAT-2 gene.

ACh-induced R_L in CAT-2-/- mice.

ACh has previously been shown to be a potent agonist of ASM contraction (14, 15). To assess, under physiological conditions, the possible changes in airway function resulting from a loss of the CAT-2 gene, the in vivo responsiveness of these mice was examined by measuring the R_L after intravenous ACh administration. Throughout a range of 80 to 1280µg/kg,
the ACh was able to cause a dose-dependent increase in the RL. The RL was found to have a slight decrease only at 1280 µg/kg in the response of the CAT-2-/- mice compared to B6 mice (Fig 3). At this dose a 33.66% decrease was observed in the CAT-2-/- mice compared to B6 mice. The maximal values (cmH₂O/ml/sec) of RL were shown to be 1.09 ± 0.14 and 0.72 ± 0.09 for the B6 and CAT-2-/- mice, respectively. This difference in the RL at the maximal dose of ACh between these two groups was found to be statistically significant (p = 0.05, n = 8) using the Student’s unpaired T-test.

**Airway structure and CCh-evoked force generation in tracheal rings from CAT-2-/- mice.**

Airway structure was examined histologically under a light microscope at a x 4 and x 20 magnification in whole and sectional fresh rings and rings cultured overnight from B6 and CAT-2-/- mice (Fig, 4A). The epithelium and smooth muscle were histologically identical in the fresh and cultured tracheas. There was no evidence of tissue edema, epithelial denudation or patchy shedding of epithelial cells observed in the cultured trachea compared to freshly isolated trachea. Original tracks for the CCh-induced force generation are shown for the cultured trachea from B6 and CAT-2-/- mice (Fig 4B).

To address whether the absence of the CAT-2 gene would be sufficient to promote or inhibit airway responsiveness in tracheal ring sections, trachea from B6 and CAT-2-/- mice were cultured overnight in DMEM. Our results indicate that the force response from fresh trachea reaches only 65% of force developed from cultured trachea (data not shown). Maximal tensions (%) were 100.00% ± 6.27% and 66.91% ± 10.20% in the cultured rings from B6 and CAT-2-/- mice, respectively (Fig. 4C). The maximal contractile response of trachea from CAT-2-/- mice was approximately 30% less than that observed in trachea from B6 mice. Table 1 shows LogEC₅₀ values for CCh-induced contraction in trachea from B6 and CAT-2-/- mice. There were no significant differences in LogEC₅₀ values between the responses observed in trachea from B6 and CAT-2-/- mice as determined by the Student’s unpaired T-test.

**L-NAME mediated effects on CCh-evoked contraction.**

In order to investigate the physiological response of ASM to a decrease in the production of NO, the CCh-evoked contractile response was examined in tracheal rings from B6 mice that were incubated overnight in the presence or absence of L-NAME (100 µM). In contrast
to untreated tracheal rings, rings treated with L-NAME exhibited a slight increase in their contractile response over the entire CCh-dose response curve (Fig. 5). Maximal tensions (%) were 117.76% ± 6.94% in rings that had been treated with L-NAME compared to 100.00% ± 10.09% (n = 8) in the untreated rings; however, the differences did not reach significance as determined by the Student’s unpaired T-test. In addition, fresh trachea from B6 mice were incubated with L-NAME for one hour and showed a similar result to that of the cultured trachea with the exception of an overall 35% decrease in the force generated by fresh trachea compared to the cultured trachea in response to CCh (data not shown).

**ISO- and SNP-induced relaxation**

To examine the relaxant properties of ASM from CAT-2/-/- and B6 mice, tracheal rings were first contracted with 1.0 µM CCh and then increasing concentrations of either ISO or SNP were added to the organ baths. ISO- and SNP-induced relaxation showed a dose-dependent response that was not significantly different in tracheal rings from CAT-2/-/- mice compared to responses observed in rings from B6 mice (Fig. 6A,B).

**ASM contractile responses in the presence of GTPγ-S and spermine**

The original tracings for CCh-evoked force generation in cultured trachea from B6 and CAT-2/-/- mice in the presence and absence of either 10 µM GTPγ-S or 100 µM spermine are shown (Fig 7 A). The level of expression of PLC-β and PIP-5K-γ in the lung tissue of B6 and CAT-2/-/- mice was examined using Western blotting analysis. A specific band for PLC-β was determined to have a similar level of expression in lung tissue from both B6 and CAT-2/-/- mice in reference to the β-actin expression level in the same tissues (Fig. 7 B). In association with this finding, CCh-induced contraction of tracheal rings from B6 and CAT-2/-/- mice was determined in the absence or presence of GTPγ-S (10 µM) (Fig. 7 B). No statistically significant difference was detected in the force generation observed in the tracheal rings of CAT-2/-/- mice treated with GTPγ-S as compared to the tracheal rings from CAT-2/-/- mice that were not treated with GTPγ-S as determined by the One-way ANOVA test.

The expression of PIP-5K-γ in lung tissue from B6 and CAT-2/-/- mice was also examined by Western blot analysis in reference to the β-actin expression level in same tissues. Although a specific band for PIP-5K-γ was clearly observed in lung tissue from B6 mice,
the expression levels were significantly reduced in lung tissue from CAT-2/- mice (Fig. 7 C). Pretreatment of lung tissue from CAT-2/- mice with 100 µM spermine increased the expression of PIP-5K-γ to a level similar to that seen in the tissue from B6 mice. Furthermore, the reduced contractile response to CCh, observed in the tracheal rings from CAT-2/- mice was completely restored to the response observed in the trachea from B6 mice when the rings from CAT-2/- mice were incubated with 100 µM spermine overnight prior to the measurement of CCh-induced contraction (Fig. 7 C). Maximal tensions (%) were shown to be 100% ± 12.63% in B6, 69.85% ± 9.68% in CAT-2/- and 92.62% ± 16.89% in CAT-2/- treated with spermine. There was no statistically significant difference detected in the contractility of ASM between B6 and CAT-2/- mice treated with spermine (n = 7) as determined by the Student’s T test.
DISCUSSION

Deletion of the CAT-2 gene was initially confirmed with the observation that peritoneal and airway macrophages from CAT-2-/- mice are significantly defective in inducible NO production. Under resting conditions, levels of NO were minimal in the macrophages from both B6 and CAT-2-/- mice. However, after stimulation with LPS/IFNγ only peritoneal and airway macrophages from B6 mice produced a high level of NO. The NO levels remained below the level of detection in activated macrophages from CAT-2-/- mice. This result is completely consistent with previous reports indicating that macrophage NO produced by iNOS is significantly impaired in the absence of CAT-2 (21). This not only indicates that a CAT-2-dependent deficiency in arginine transport significantly reduces iNOS-mediated NO production in these cells but also confirms the effect of the deletion of the CAT-2 gene in these mice.

We next investigated the inherent enzymatic activity of iNOS and arginase in the CAT-2-/- mice because the loss of this transporter would cause a reduction in the cellular uptake of L-arginine and the subsequent change in substrate availability could affect the function of these enzymes. The activities of iNOS and arginase in lung tissue lysates were examined by measuring the products of these enzymes, nitrite and urea. Our results showed that there were no significant differences in these products produced by either B6 or CAT-2-/- mouse tissue, suggesting that the functional activities of the enzymes were normal when supplied with an excess of substrate in the homogenized tissue.

CAT-2 may play an important role in regulating ASM tone since it can transport L-arginine into the cell for metabolism by either iNOS or arginase. L-arginine metabolism by iNOS leads to the generation of NO, a major neurotransmitter of the inhibitory nonadrenergic (iNANC) nervous system, the most effective bronchodilating neural pathway of the airways (24). Furthermore, there is a general agreement among in vitro studies that exogenously applied NO can relax ASM with a potency intermediate between the β-adrenoceptor agonist, isoprenaline, and the PDE inhibitor, theophylline. In vivo studies in several species, including humans, have also demonstrated a bronchodilator effect for inhaled NO (1). S-nitrosoglutathione (GSNO), another product of iNOS activation, which is formed in the presence of oxygen and glutathione, has also received attention as a potent endogenous bronchodilator. Mice rendered
genetically incapable of breaking down GSNO are protected from allergen-induced AHR (32), whereas children who are intubated intratracheally for asthmatic respiratory failure have accelerated GSNO catabolism (13). Although the iNOS arm of L-arginine metabolism appears to mediate bronchodilation and inhibition of AHR, L-arginine metabolism through the arginase pathway may contribute to AHR through the production of polyamines (i.e. putrescine, spermidine and spermine). Coburn, et al., have shown that spermine increases the PI(4,5)P$_2$ content in HL60 cells (10) and polyamines have been demonstrated to activate the kinase, PIP-5K, producing PI(4,5)P$_2$ in vitro (4). Recently, Zimmermann, et al., demonstrated that CAT-2, arginase I and arginase II were particularly prominent among asthma signature genes in expression profiling studies of lung tissue from mice with experimental asthma (43). They also demonstrated increased enzymatic activity for arginase in lung tissue and elevated levels of lung polyamine after allergen challenge in mice. In humans, they showed that arginase I protein expression was elevated in bronchoalveolar lavage cells and that arginase I mRNA positive cells were strongly detected in the asthmatic lung but were almost completely undetectable in normal individuals. While these results are correlative in nature, the data is strongly suggestive that increased arginase activity has pathophysiological consequences in asthma.

In order to investigate the physiological activity of ASM in the absence of CAT-2, we used the combined techniques of in vivo $R_L$ measurement and the ex vivo measurement of tracheal ring force generation in response to muscarinic agonists. Our previous study has demonstrated that ACh prepared in fresh solution may be used to measure $R_L$ with an effective window to observe its induced changes in ASM responses (15). Another reason for using ACh in this study is that this is only mediator found naturally in the human body so that the ACh-induced changes in $R_L$ are more closely linked to a normal physiological response. In initial experiments, the $R_L$ to a maximal dose of ACh in CAT-2/-/- mice showed a significant decrease of 33.66% as compared to that of the B6 mice, suggesting that this transporter can act on the airway to modify the underlying ASM function to the agonist. Since the data obtained showed a significant decrease in $R_L$ only at the highest dose of ACh, it leads to us to speculate that $R_L$ could be insensitive to observed airway hypo-responsiveness under physiological conditions. In a support of mechanical ventilation with tidal breathing, the change in $R_L$ is smaller because the
mechanically assisted tidal force may overcome a basic resistance in normal airways, which could lead to a decreased magnitude in the $R_L$ response at low doses of the agonist.

A comparison was made of the physiological responses of fresh and cultured trachea. Based on histological findings, no tissue edema, epithelial denudation or patchy shedding of epithelial cells was observed in the trachea incubated in medium as compared with fresh trachea. Since cultured trachea may generate 35% more force compared to fresh trachea, original tracings of the CCh-induced force generation were recorded in cultured trachea from B6 and CAT-2/- mice. To further investigate the functional consequences of the absence of the CAT-2 gene within the airway, the in vitro force response of tracheal rings cultured overnight was measured in B6 and CAT-2/- mice. In association with a low nitrite level in macrophages from the BAL from CAT-2/- mice, we established that trachea from CAT-2/- mice were severely refractory to CCh-induced contraction. Our results indicate that the reduction of both iNOS and arginase activity does not lead to an enhancement of ASM tone, as one would expect from a lack of iNOS activity, but rather results in reduced ASM contractility.

To investigate the effect iNOS inhibition has on ASM tension in our system, we examined the tracheal ring contractile response to CCh after pre-incubation with the iNOS competitive inhibitor, L-NAME. Although not statistically significant, cultured trachea which have been treated with L-NAME were slightly more reactive to CCh than untreated trachea. A similar result was observed in fresh trachea (data not shown), indicating that NO production can potentially be involved in controlling the intrinsic tone of the airways. This is in agreement with the observation that naturally released NO apparently modulates the function of acetylcholine to balance the intrinsic tone in the airways (39). Since a decrease in NO production by an inhibition of iNOS results in no effect, to possibly a slight increase, in the response of ASM to agonists, we eliminate the possibility that the inhibition of the L-arginine/NO pathway resulting from the absence of CAT-2 contributes to a decrease in the CCh-evoked force generation seen in CAT-2/- mice.

Having demonstrated a significant role for CAT-2 in driving muscarinic receptor-mediated airway smooth muscle contraction, we were curious as to the relationship of CAT-2 to $\beta_2$-AR/cAMP-mediated relaxation of airway smooth muscle. To investigate this
interaction, tracheal rings were pre-contracted with CCh and then incubated in the presence of increasing doses of ISO, an agonist for the β₂-AR. For comparison, pre-contracted trachea was also incubated in the presence of increasing doses of SNP, a NO donor that mediates relaxation through cGMP. Given that we observed no significant difference in the relaxation in tracheal rings from B6 or CAT-2-/− mice, we conclude that the reduction of the CCh-induced contractile response in trachea from CAT-2-/− mice is not due to an inhibition of the Gs-coupled β₂-AR response.

Since the reduced contractile response associated with the CAT-2 deficiency appears not to be due to alterations in NO metabolism, defective β₂-AR signaling or changes in the functional activities of iNOS or arginase, we postulated that changes in polyamine production were involved in the refractory phenotype. Despite the enormous interest being focused on the changes in NO associated with AHR in asthmatics (2,35,12), this present study is the first to document a predominantly physiological role for CAT-2 and the associated L-arginine/polyamine pathway in the maintenance of ASM tone. It is well established that CCh contracts ASM by binding to muscarinic receptors, resulting in an increased turnover of phosphatidylinositol (4,5)-bisphosphate (PIP₂), a precursor of inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), leading to Ca²⁺ release from the sarcoplasmic reticulum (7,9). A variety of enzymes, such as PLC and PIP-5K, are involved in the synthesis and turnover of IP₃ and DAG (20, 21). Although presently, it is not known whether CAT-2 acts directly on these enzymes, it is more likely that CAT-2 acts indirectly through intermediate factor(s) to regulate their activity.

Given that PLC-β is known to hydrolyze phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P₂) producing IP₃ and thus contributing to the release of Ca²⁺ (36), we hypothesized that reduced PLC-β activity may account for the reduced contractile phenotype in CAT-2-/− mice. To explore this hypothesis, we examined the expression of PLC-β in the lungs of both B6 and CAT-2-/− mice. We observed similar levels of expression of PLC-β in the lungs of both CAT-2-/− and B6 mice. We also measured the CCh-induced contractile response in trachea from CAT-2-/− mice that were incubated in the presence of GTPγ-S, a physiological activator of PLC-β (33). The reduced contractile phenotype in trachea from CAT-2-/− mice was not repaired when
tissues were incubated overnight in the presence of GTPγ-S, leading us to conclude that CAT-2/PLC-β interactions, if they exist, are not involved in the altered response to CCh. Also, since the muscarinic receptors are coupled preferentially to Gi-proteins directly activating PLC-β, it is unlikely that an effector(s) upstream of PLC-β contributes to the muscarinic receptor-induced contractile dysfunction in CAT-2-/- mice (22).

Since the absence of CAT-2 did not appear to affect the contractile response through alterations in PLC-β activity, we explored the possibility that CAT-2 may influence the activity of PIP-5K to regulate the contractile response. There are two types of PIP-5K (type I and II) found in mammalian cells and three isoforms (α, β and γ) of type I have been identified based on their size and sensitivity to certain compounds (18,38,31,23). Moreover, all of the isoforms are enzymatically active and undoubtedly contribute to the conversion of PIP to PIP2, which when hydrolyzed by PLC-β to IP3, stimulates the release of intracellular Ca2+ leading to contraction. We only examined the expression level of PIP-5K-γ protein in lung tissue samples since this is the only isoform for which an antibody is commercially available. Interestingly, protein expression of PIP-5K-γ was significantly reduced in the absence of CAT-2. In addition, the reduced muscarinic receptor-mediated contractile response in trachea from CAT-2-/- mice, as well as the reduced protein expression of PIP-5K-γ in the lung tissue of these animals, was fully reversible by the pretreatment of the tissues with 100 µM spermine. This implies that CAT-2, as a carrier of L-arginine, regulates spermine biosynthesis and through the action of spermine on PIP-5K-γ, it controls the contractility of the muscle to muscarinic agonists. Indeed, previous studies have shown spermine is required for the activity of PIP-5K-γ in ASM cells, although, the mechanism(s) by which this kinase is activated remains unknown (4,10).

Our working model for the mechanism of the effect of CAT-2 on the regulation of the ASM contractile response, based on the phosphoinositol cycle (23), is shown diagrammatically in figure 8. It is an indirect effect, by which CAT-2 transporting arginine into the cell is acting as a regulator of substrate availability for enzymes involved in polyamine biosynthesis. The increased production of polyamines, or possibly exclusively spermine, increases the expression and enzymatic activity of PIP-5K (10). The increased activity of PIP-5K results in a more effective replenishment of PIP2, the substrate for PLC-β. This maintains the available
substrate pool for PLC-β allowing it to sustain its signaling, leading to a prolongation of the intracellular Ca\(^{2+}\) flux and PKC activation.

In conclusion, our findings indicate that CAT-2 modulates the mechanisms underlying ASM function and predominantly serves to amplify ACh-induced \(R_L\) and CCh-generated force by spermine-mediated regulation of PIP-5K-γ activity in the contracted muscle. We describe a hypothetical pathway connecting the regulation of ASM force to CAT-2-controlled polyamine (spermine) metabolism and PIP-5K activity.
REFERENCES


FIGURE LEGENDS

Figure 1. CAT-2-/- macrophages are deficient in inducible nitrite production.

Peritoneal (A) or airway (B) macrophages from B6 and CAT-2-/- mice were stimulated for 24 hours with PBS or LPS/IFNγ. The amount of nitrite accumulated in the media was measured using the Griess reaction. The data are expressed as the Mean ± SE (n = 6 - 8).

Figure 2. The effect of the loss of the CAT-2 gene on the basal Nitrite and Urea levels in the lung.

Lung tissue from B6 or CAT-2-/- mice was isolated, lysed, and either the nitrite concentration was measured in the cleared lysate by the Griess reaction (A) or the urea concentration measured by the absorbance in the presence of ISPF in the cell lysate after the addition of the L-arginine substrate (B). The data are expressed as the Mean ± SE (n = 5).

Figure 3. The loss of the CAT-2 gene reduces ACh-induced R_L

ACh-induced R_L was measured in B6 or CAT-2-/- mice on a ventilator after administration i.v. of 80 to 1280 µg/kg ACh. All of the values of R_L were normalized to the value of B6 mice treated with a maximal dose of ACh. Results are expressed as the Mean ± SE (n = 8). *: p = 0.05 as compared with the vehicle.

Figure 4. Histologic observation and the loss of CAT-2 transport function reduces CCh-evoked force

Histologic examination at a magnification of x 4 and x 20 was performed on whole and sectional fresh tracheas and tracheas cultured overnight from B6 and CAT-2-/- mice (A). Original tracings for CCh-induced force generation are shown for cultured trachea (B). CCh-generated force was examined at the end of the culture period. Tracheal rings were stimulated with a range of CCh between 0.1 and 10 µM, and the CCh evoked force measured in B6 and CAT-2-/- mice (C). The measurements were expressed as the Mean ± SE (n = 8). *: p < 0.05 compared with the vehicle.
Figure 5. iNOS inhibition has no effect on ex vivo tracheal contraction.

The contraction of tracheal rings from B6 mice was examined after pretreatment with L-NAME (100 µM) or PBS. The rings were stimulated with a range of CCh between 0.1 and 10 µM. The results from each group are expressed as the Mean ± SE (n = 6 - 8).

Figure 6. A deficiency in the CAT-2 gene does not effect ISO- and SNP-induced relaxation.

Tracheal rings were isolated from B6 or CAT-2-/- mice and contracted with the addition of 1.0 µM CCh. After reaching a stable contraction, ISO (A) and SNP (B) were introduced in increasing concentrations into each bath and contractile force measured. Maximum relaxation was assessed by the addition of 200 µM papaverine. Measurements are expressed as the Mean ± SE (n = 8).

Figure 7. The expression of PIP-5K-γ, not PLC-β, correlates with the CCh-evoked force generated in tracheal rings.

The original tracings are shown for CCh-evoked force generation in cultured trachea from B6 and CAT-2-/- mice in the presence and absence of either 10µM GTPγ-S or 100µM Spermine (A). The expression of PLC-β and PIP-5K-γ was measured in lung tissue lysates in reference to the level of β-actin expression in same tissues by Western blot analysis. The contractile response to CCh of tracheal rings from B6 or CAT-2-/- mice was examined ex vivo in the presence and absence of either 10 µM GTPγ-S (B) or 100 µM Spermine (Sp) (C). The results of CCh-evoked force generation are expressed as the Mean ± SE (n = 8).

Figure 8. A schematic representation of the CAT-2-mediated signals controlling ASM force generation.

Enzymatic conversions are represented by solid arrows, signaling mechanisms are represented by bold, solid arrows, and regulation of activity by a dashed arrow. Abbreviations are given in the text except: PtdIns, phosphatidylinositol; Ins, inositol; PtdIns[4]P, phosphatidylinositol 4-phosphate; Ins[1,4,5]P3, inositol 1,4,5-trisphosphate; PtdA, phosphatidic acid.
Table 1  ASM sensitivities to CCh (Mean ± SE)

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<tr>
<th>Groups</th>
<th>LogEC50 (µM)</th>
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<tr>
<td>B6</td>
<td>-0.66 ± 0.06</td>
<td>8</td>
</tr>
<tr>
<td>CAT-2-/-</td>
<td>-0.48 ± 0.14</td>
<td>8</td>
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</tbody>
</table>
**Fig 1**

**A**
- B6
- CAT-2-/-
- B6/LPS/IFNγ
- CAT-2-/-/LPS/IFNγ

P < 0.001

**B**
- B6
- CAT-2-/-
- B6/LPS/IFNγ
- CAT-2-/-/LPS/IFNγ

P < 0.001
**Fig 2**

A) Nitrite in Lung (µM)

- B6
- CAT-2-/-

**P = 0.48**

B) Urea in Lung (µg/ml)

- B6
- CAT-2-/-

**P = 0.66**

![Bar chart for Nitrite and Urea in Lung with statistical significance levels](image-url)
Fig 3

Changes in RL (CmH2O/ml/Sec)

[B6] [CAT-2-/-

[ACh] µg/kg

80 160 320 640 1280

*
**Fig 4**

**A**

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<th>Cultured</th>
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<td><img src="image8.png" alt="Image" /></td>
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</table>

**B**

CCh (µM) 0.1 0.3 1.0 3.0 10

B6 CCh (µM) 0.1 0.3 1.0 3.0 10

CAT-2/-

---

**C**

% Contraction

![Graph](image9.png)

[CCh] µM

---

* * *
Fig 5
**Fig 6**

A. Graph showing the effect of different concentrations of ISO (µM) on the % Relaxation of B6 and CAT-2/- mice.

B. Graph showing the effect of different concentrations of SNP (µM) on the % Relaxation of B6 and CAT-2/- mice.
A

CCh (µM) 0.1 0.3 1.0 3.0 10
B6
CAT-2-/-

B

% Contraction

[CCh] µM

P < 0.05

B6 CAT-2/-
CAT-2/-GTP

% Contraction

[CCh] µM

P < 0.05

B6 CAT-2/-
CAT-2/-Sp

C

% Contraction

[CCh] µM

P < 0.05

B6 CAT-2/-
CAT-2/-Sp

Fig 7