Glutathione ethyl ester supplementation prevents airway hyper-responsiveness in mice

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Background: Oxidative stress plays an important role in the pathogenesis of asthma. Glutathione (GSH) is considered to be one of the most important antioxidants. Our study systematically investigated the effect of the GSH alternative, glutathione ethyl ester (GSH-EE), on airway hyper-responsiveness (AHR) in mice.

Methods: Sixty-three male specific pathogen-free mice were used. Asthma was induced using a single dose of ovalbumin (OVA). The normal group (n=15) received vehicle only [Al(OH)₃ in saline]. Then, 48 mice were divided into two groups, including a control group who received sodium phosphate buffer (pH =7.4), and the GSH-EE group who received 0.1% GSH-EE. AHR was measured 2, 6, and 12 hours after exposure to nebulized OVA (0.01%). The animals were then sacrificed, and lung tissue and the bronchi-alveolar lavage fluid (BALF) were harvested. Factors involved in the antioxidant response to asthma were then measured in these tissues, including thiol content (from GSH and protein), γ-glutamylcysteine synthetase (γ-GCS) activity and expression, and nuclear factor-erythroid-2-related factor (Nrf2) expression.

Results: The GSH-EE group showed a significant attenuation of AHR (P<0.01) 2 hours after OVA challenge, and significantly enhanced thiol contents by approximately 45% (P<0.05) at 2 and 6 hours after the last OVA challenge, compared to the control group. γ-GCS activity was also higher in the GSH-EE group compared to the control group at different time points (P<0.01). γ-GCS and Nrf2 protein expression increased in the GSH-EE group and the control group compared with the normal group, but there was no statistically significant difference (P>0.05) between the GSH-EE group and the control group.

Conclusions: GSH-EE supplementation can prevent AHR in asthmatic mice during the early stages. It may function by serving as a precursor for GSH biosynthesis and by protecting sulfhydryl groups from oxidation.

Keywords: Glutathione ethyl ester (GSH-EE); glutathione (GSH); ovalbumin (OVA); γ-glutamylcysteine synthetase (γ-GCS); nuclear-factor-erythroid-2-related factor (Nrf-2); airway hyper-responsiveness (AHR)

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**Introduction**

Understanding the mechanisms underlying the development of asthma and finding effective therapeutic strategies are problems of great scientific and social interest. It has been reported that over 300 million people worldwide suffer from asthma across both adults and children (1,2). Asthma is a chronic inflammatory disorder of the airways associated with airway hyper-responsiveness (AHR) and airflow limitation in response to specific triggers. Oxidative stress plays an important role in the pathogenesis of asthma (1,3-5), promoting inflammation and shifting redox balance towards oxidation reactions (6-8).

Glutathione (GSH) is an antioxidant that was first recognized in the 1970s, and is considered to be one of the most important antioxidants (1). Early studies reported that it was significantly decreased in asthma patients compared to healthy controls (9). In early asthma and AHR animal models, the tissue contents of GSH were shown to decrease (10). It was also found that GSH can decrease bronchial smooth muscle contraction, which was induced by oxidant free radicals in response to different stimuli (11).

Since GSH has a short half-life, resulting in limited cellular uptake, several alternatives to increase intracellular levels of GSH have been developed (12-15). Glutathione ethyl ester (GSH-EE) is one of these GSH alternatives, and has been shown to boost thiol content in cells through hydrolysis to release GSH (16,17). GSH-EE injected intraperitoneally in mice was able to effectively decrease ovalbumin (OVA)-induced inflammatory cell infiltration and cytokine synthesis (10). GSH and GSH-EE atomization and intravenous injection have also been reported to relieve AHR and airflow limitation in response to early asthma (8,18). Furthermore, it has been reported that GSH and GSH-EE can decrease airway responsiveness (AR) by directly relaxing bronchial smooth muscle contractions (8,11). However, these previous studies did not perform a systematic investigation from *in vitro* to *in vivo*, and did not examine the antioxidant regulatory enzymes that are important in asthmatic pathologic processes.

To follow up on these findings, we systematically investigated how early asthmatic reactions induced by an allergen (OVA-induced asthma model) modulated antioxidant levels *in vivo* and *in vitro*, and tested whether supplementation with GSH-EE can prevent AHR and increase GSH content. We also investigated the response of GSH regulatory enzymes, including γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme of GSH, and nuclear-factor-erythroid-2-related factor (Nrf-2), which plays an important role in regulating the expression of intracellular γ-GCS (19,20).

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-7114).

**Methods**

**Materials**

OVA and GSH-EE were purchased from Sigma Chemical Company (Beijing, China). BALB/c mice were provided by Animal Laboratories of Chinese PLA general hospital (Beijing, China). Protein and enzyme quantification kits were obtained from Jiancheng Biology Company (Nanjing, China). All other chemicals and instruments were obtained from local companies.

**Animals**

The experiments lasting over 3 weeks were performed using 2-month-old male specific pathogen-free mice (n=63), which were housed in individual polypropylene breeding cages under a day/night cycle of 12 hours at 20–25 °C room temperature, and provided with food and water ad libitum. Animal care and protocols were in accordance with the guidelines for animal care and approved by the Institutional Animals Ethics Committee at the Chinese People’s Liberation Army General Hospital.

**OVA sensitization and challenge (8)**

All animals weighing 20–25 g were sensitized by injecting a mixture of 20 µg OVA and 2 mg of adjuvant Al(OH)₃ in 1.0 mL saline. All animals were randomly assigned to each experimental group. Al(OH)₃ in saline was administered to the normal controls (n=15). Each animal was injected intraperitoneally on days 0 and 14, and repeated airway challenges with aerosolized 1% OVA in PBS (30 min) on days 28, 29, and 30 were performed as described previously (21,22).

**GSH-EE supplementation**

Five mg of GSH-EE was dissolved in 5 mL of sodium phosphate buffer solution (pH 7.4). The pH value of the GSH-EE solution was adjusted to 7.2 before use. The GSH-EE group (n=24) was treated with 0.1 mL GSH-EE injected intraperitoneally once daily during the
3 days before OVA challenge. Sodium phosphate buffer solution was used as the drug vehicle control (n=24). GSH-EE was tested for drug safety and utility by preliminary experiments. There was no statistically significant difference between drug-treated mice and untreated mice when observed for irritation or abdominal symptoms. The GSH-EE solutions were made fresh daily and preserved in a 4 °C icebox. During injection, the rats were held by assistants, and continuously held for a 2–3 minutes light massage of the injection site.

Measurement of airway responsiveness

Two, six, and twelve hours after mice were exposed to nebulized OVA (0.01% in saline), AHR was measured in unrestrained mice by using barometric whole-body plethysmography (WBP), as previously described (18,21,23). Each mouse was placed in a main plexiglas Buxco chamber of the body plethysmograph, exposed to increasing concentrations of nebulized methacholine (MCh) from 0–50 mg/mL to establish a dose-response relationship for 2 minutes, followed by a 3-minute period during which airway responses (enhanced pause, Penh values) were recorded. Penh values that were twice the baseline levels were recorded as PC100, log2(10PC100) and was statistically analyzed for airway responses. As an index of in vivo airway obstruction, AHR was calculated from 3 separate experiments (21-24).

Bronchi-alveolar lavage fluid (BALF) analysis

Following euthanasia of mice (pentobarbionate sodium, 0.6 g/kg body weight, intraperitoneally), the lungs were lavaged with 500 µL of sterile saline through a cannula in the trachea at 32 °C, and withdrawn after 10 seconds. This procedure was repeated twice. The BALF was centrifuged at 1,500 rpm for 5 minutes at 4 °C, and the supernatant was retained for measurements of GSH/GSSG levels and protein levels. Once the supernatant was removed, the cell pellets were pooled for differential cell counts using 100 µL of the re-suspended cells.

Thiol determination in lung tissue and BALF (18,24,25)

Thiol levels from GSH and protein were determined using the dithiobis-nitrobenzoic acid (DTNB) recycling assay measured by absorption at 412 nm. Values were expressed as nmol per mg tissue protein (wet weight) for lungs, and µM for BALF.

γ-GCS activity assay in lung tissue

Using the classic dual enzymatic method (26), absorbance was measured at 340 nm in 1 mL total volume of reaction solution, and γ-GCS activity was defined as (U) =1 nmol/min/mg protein.

Reverse transcription and real-time PCR

To measure γ-GCS mRNA and Nrf2 mRNA levels in lung tissue, total RNA was isolated from the right lung, and reverse-transcribed into cDNA. Real-time PCR was performed using the SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan). GAPDH mRNA was used as an internal standard. The primers used in this study were as follows: γ-GCS mRNA, the forward primer was 5’-AAGCTCTTGGCCGAAAACCCA-3’, and the reverse primer was 5’-GGAGCACGTCACATCCAGTT-3’. Nrf2 mRNA, the forward primer was 5’-GGACATGGAGCAAGTTTGGC-3’, and the reverse primer was 5’-CCAGCGAGGAGATCGATGAG-3’.

Western blot

To measure γ-GCS and Nrf2 protein expression, lung lysates were mixed with NuPAGE LDS sample buffer (4x), boiled, and loaded onto NuPAGE Bis-Tris Mini Gels. Proteins were transferred to nitrocellulose, and immunodetection for γ-GCS and Nrf2 protein was performed using anti-γ-GCS antibody (S-16, Santa Cruz) and anti-Nrf2 primary antibodies (C-20, Santa Cruz).

Statistical analysis

Data are expressed as mean ± SEM. One-way ANOVA was used for testing statistical significance between groups. P<0.05 was considered statistically significant. All statistical analysis was performed using the SPSS statistical package version 21.0 (SPSS Inc., Chicago, USA).

Results

Effect of GSH-EE on AHR

The GSH-EE group (n=24) was treated with GSH-EE
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**Figure 1** Comparison between airway hyper-responsiveness (AHR) in mice at different time points post-ovalbumin (OVA) challenge. Dots on figures represent the dose of methacholine (Mch) causing 100% increase in airway resistance from baseline levels (PC100). (A) 2 hours (h), (B) 6 hours, and (C) 12 hours after the last challenge with OVA, the AR was recorded. OVA-induced asthmatic mice [control group n=24; glutathione ethyl ester (GSH-EE) group, n=24] showed significantly higher AR (#, P<0.01) compared to normal control mice (normal group, n=15). (A) The GSH-EE group showed significantly decreased AHR (#, P<0.01) compared to the control group 2 hours after OVA challenge. (B,C) However, there were no obvious differences between the two groups 6 and 12 hours after OVA-challenge. # represents a significant difference compared to controls (#, P<0.01).

**Figure 2** Inflammatory cells counts in bronchi-alveolar lavage fluid (BALF). Bars above represent cells counts at different time points after the last challenge with ovalbumin (OVA), * represents a significant difference compared to controls (*, P<0.01). (A) 2 hours and (B) 12 hours after the last challenge, OVA-induced asthmatic mice (grey bars, control group; black bars, glutathione ethyl ester (GSH-EE) group) showed significantly increased numbers of inflammatory cells in BALF compared to the normal group (white bars; P<0.01). No significant difference was found between the control group and the GSH-EE group. Total, total number of cells; E, eosinophils; M, monocytes; L, lymphocytes; N, neutrophils (the bar indicates mean ± SEM, n=8 for control group and GSH-EE group, n=5 for normal group).

daily before the first OVA challenge, while the control group was treated with sodium phosphate buffer (n=24) daily. AHR was measured at 3 different time points (2, 6, 12 hours) after the last OVA challenge. OVA-induced asthmatic mice (control group and GSH-EE group) showed significantly higher AHR (P<0.01) compared to normal control mice (normal group n=15) across the entire observation period (Figure 1A,B,C). GSH-EE treated mice showed a significantly greater ability to attenuate AHR (P<0.01) compared to vehicle treated mice 2 hours after OVA-challenge (Figure 1A), while no obvious differences were found between the two groups at 6 and 12 hours after OVA-challenge.

**Analysis of inflammatory cells in BALF**

OVA-induced asthmatic mice had significantly increased inflammatory cell counts (P<0.01, Figure 2A,B), especially
eosinophil (E), monocyte (M), and neutrophil (N) cell counts, compared to normal mice. The GSH-EE group showed no significant change in total and differential cell counts compared with the control groups at different time points (Figure 2A,B).

Thiol levels (from GSH and protein)

After the last challenge, OVA-induced asthmatic lungs demonstrated a 25–55% decrease in the level of thiol contents in lung tissue (P<0.01) and a 20–30% decrease in the level of thiol contents in BALF (P<0.01) compared to control mice at each time point (Figure 3A,B). However, these differences were not time-dependent. Thiol levels were lower in BALF fluid compared to lung tissue. Surprisingly, GSH-EE significantly enhanced thiol levels by approximately 45% (P<0.05) compared to the control group at 2 and 6 hours after the last OVA challenge (Figure 3A), however, there was no significant difference at 12 hours. Furthermore, no significant differences were found between the GSH-EE group and the control group in terms of thiol levels in BALF.

γ-GCS activity, γ-GCSmRNA, and γ-GCS protein expression in lungs: γ-GCS activity in the lungs of OVA-induced asthmatic mice (control group and GSH-EE group) was significantly higher than the normal mice (P<0.01, Figure 4A), and was higher in the GSH-EE group compared to the control group at different time points (P<0.01, Figure 4A). γ-GCSmRNA and γ-GCS protein expression in lung tissue were consistent with the results of γ-GCS activity at different time points (Figure 4B,C). Relative γ-GCSmRNA and γ-GCS protein expression in the lung tissue of the control group and the GSH-EE group were significantly higher than normal mice (P<0.01, Figure 4B,C).

Nrf2 mRNA and Nrf2 protein expression in lung tissue:

There were no significant differences in Nrf2 mRNA levels in the normal group and the OVA-induced asthmatic mice (control group and GSH-EE group, Figure 5A,B). However, a higher level of Nrf2 protein expression in the lung tissue of the control group and GSH-EE group was observed compared with the normal group (P<0.01, Figure 5B), though no significant difference was found between the GSH-EE group and the control group at different time points.

Discussion

GSH is the abundant intracellular antioxidant thiol and plays an important role to redox defense during oxidative stress. This study showed that thiol content (GSH and protein) was markedly depleted in an experimental asthma model. Our data showed that thiol levels in lung tissue decreased during the early stages of experimental AHR with supplementation of an GSH donor, GSH-EE. The acute thiol depletion and bronchoconstriction that accompanied the allergic reaction are probably causally related, since supplementation with GSH-EE prevented AHR during the early stages of experimental AHR.

The occurrence of oxidative stress during primary AHR is interesting, since inflammatory cells have not yet been recruited to the lungs as a result of prior respiratory allergen challenge (27,28). Repeated OVA challenge consistently
Figure 4 γ-GCS activity, γ-GCSh mRNA, and γ-GCSh protein expression in lung tissue. (A) The curve represents the level of γ-GCS activity at different time points after the last challenge with ovalbumin (OVA). # represent significant differences compared to controls (P<0.01). The control group and the glutathione ethyl ester (GSH-EE) group showed higher levels of activity compared to the normal group at different time points (P<0.01). The GSH-EE group showed higher levels of γ-GCS activity compared with the control group at 2, 6, and 12 hours after the last OVA challenge (P<0.01). (B) Results of RT-PCR showed higher levels of relative γ-GCSh mRNA expression in the control group and the GSH-EE group compared to the normal group at different time points (P<0.01), though there was no significant difference between the GSH-EE group and the control group. (C) Western blotting demonstrated consistent results, with a higher level of γGCSh protein expression in the GSH-EE group and the control group, though there was no significant difference between the GSH-EE group and the control group (the bar and dots indicate mean ± SEM, n=8 for control group and GSH-EE group, n=5 for normal group).

Figure 5 Nrf2 mRNA and Nrf2 protein expression in lung tissue. (A) Results of RT-PCR showed no significant differences in relative expression levels of Nrf2 mRNA between the three groups at different time points. (B) Western blot demonstrated a higher level of Nrf2 protein expression in the glutathione ethyl ester (GSH-EE) group and the control group compared to the normal group at different time points, though there was no significant difference between the GSH-EE group and the control group at 2, 6, and 12 hours after the last ovalbumin (OVA) challenge (the bar indicated mean ± SEM, n=8 for control group and GSH-EE group, n=5 for normal group).
Figure 6 Schematic diagram illustrating the proposed mechanisms of glutathione (GSH) synthesis by glutathione ethyl ester (GSH-EE) supplementation. GSH-EE is one of the GSH alternatives, and has easier cellular uptake. It undergoes hydrolysis to form GSH, and partly boosts cysteine (Cys) levels. GSH-EE supplementation had no obvious effect on alleviating inflammatory cell infiltration, though they are in accordance with previous research demonstrating that GSH and GSH-EE decrease AHR by directly relaxing bronchial smooth muscle contraction (8,11).

Although the source of the oxidative burst is unknown, changes in the contents of antioxidant thiols can affect signaling pathways that participate in a broad range of physiological responses (1,29). The early loss of thiol levels in asthma may result from the inactivation of various enzymes including γ-GCS, which plays a key role in maintaining GSH levels (29,30). Our data suggests that the beneficial effects of GSH-EE supplementation were associated with γ-GCS activity improvement, while no effects accompanied γ-GCS mRNA and protein expression.

Nrf2 has been demonstrated to regulate both the basal and inducible expression of many antioxidant response element (ARE) genes and the induction of the γ-GCS gene (30-34). The data showed Nrf2 protein expression increased in asthmatic mice, although GSH-EE supplementation had no effect on altering Nrf2 mRNA levels and protein expression in our experiments. In previous studies, Nrf2 has been shown to play a central role in controlling the redox equilibrium of dendritic cells, thereby influencing antigen presentation activity (35). These studies warrant investigation of Nrf2 activity in patients with asthma, which may aid in providing a novel therapeutic target.
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asthma.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal care and protocols were in accordance with the guidelines for animal care and approved by the Institutional Animals Ethics Committee at the Chinese People’s Liberation Army General Hospital.

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