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RESEARCH ARTICLE

Application of glutathione to antagonize H_2O_2-induced oxidative stress in rat tracheal epithelial cells

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Abstract **With increasing industrialization, numerous air pollutants are generated. This research aimed to investigate the effects of inhalation of oxidative pollutants. H_2O_2 was used to simulate oxidative air pollutants, and glutathione, a reducing agent that is widely distributed in organisms, was used as an antagonist, to protect cells from oxidative stress. H_2O_2 was diluted using two gradients (0.05 mM, 0.20 mM, 0.80 mM, 3.20 mM and 0.05 mM, 0.10 mM, 0.15 mM, 0.20 mM) and GSH was dissolved at 20 μM. MTT, MDA, ROS, GSH, and TSLP were used as biomarkers to evaluate oxidative stress and possible resulting molecular events. A dose–response relationship was observed between H_2O_2 concentrations and the above-mentioned biomarkers. Glutathione significantly reduced levels of oxidative stress.**

Keywords H_2O_2, glutathione, oxidative stress, TSLP

Introduction

The air contains many pollutants, such as formaldehyde (Lu et al., 2008), sulfur dioxide (Meng, 2003), and ozone (Schlagnhaufer et al., 1997), that are oxidative or that contain oxidative factors that can cause oxidative damage to the respiratory system.

Reactive oxidative species (ROS), including O_2^-, H_2O_2, $HO_2\cdot$, and $HO\cdot$, are important for maintaining the balance between oxidants and anti-oxidants in living organisms. However, some exogenous pollutants may disrupt this balance and cause oxidative stress levels to increase, leading to a series of subsequent molecular events (Nakamura et al., 2008; Li et al., 2014). In this research, H_2O_2 was used to simulate oxidative air pollutants to investigate correlated effects. Glutathione (GSH), a reducing agent that can decrease intracellular oxidative stress, was used to protect cells.

In the present study, rat tracheal epithelial cells (RTE) were exposed to various concentrations of H_2O_2 to simulate inhalation of different doses of air pollutants by humans. RTE cells were protected by GSH. The effects were evaluated by recording levels of biomarkers, such as cell viability (MTT), malondialdehyde (MDA), reactive oxygen species (ROS), and glutathione (GSH). Of the above biomarkers, MDA, ROS, and GSH were used to evaluate oxidative stress levels. We also hypothesized that associated allergic diseases would accompany rising oxidative stress levels. Thymic stromal lymphopoietin (TSLP), which is an important biomarker of potential inflammation-modulating factors, was chosen as an indicator of subsequent molecular events.

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Material and methods

Reagents and apparatus

MEM medium was purchased from Procell (Wuhan, China). Fetal calf serum was purchased from Gibco (Life Technologies, Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-thiobarbituric acid (TBA), and 2,7-dichlorodihydro-fluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The TSLP ELISAkit was purchased from BlueGene (Shanghai, China). The GSH kit was purchased from Jiancheng (Nanjing, China).

A CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA), a super clean bench (Suzhou, China), and a low-temperature refrigerated centrifuge (Eppendorf-5417R) were used in this study.

RTE cell lines were purchased from Procell.

Preparation of H_2O_2 and GSH

Based on previous tests, H_2O_2 was diluted in MEM medium at two sets of concentrations (0.05 mM, 0.20 mM, 0.80 mM, 3.20 mM and 0.05mM, 0.10 mM, 0.15 mM, 0.20 mM). GSH was first dissolved in stock solution at 40 μM and then diluted with MEM medium to 20 μM.

Cell culture and exposure to H_2O_2 and GSH

RTE cells, which are fusiform and adherent, were cultured in MEM medium supplemented with 15% fetal calf serum. Cells were divided into two groups. One group was incubated in GSH solution, and the other in MEM medium, for 3 h. Then the culture solution was replaced with different concentrations of $H_2O_2$and incubated for 2 h. For the MTT, intracellular ROS generation, and GSH depletion assays, cells were seeded into 96-well plates at a density of 1×10^5 cells/well; cells were allowed to reach 70% confluence before experiments were conducted. For the MDA and TSLP assays, cells were seeded into 6-cell plates at a density of 1×10^6 cells/well and cells were allowed to reach 70% confluence before experiments were conducted.

MTT assays

MTT assays were used to determine cell viability, with or without protection by GSH for 3 h and at different concentrations of H_2O_2 for 2 h. After the above procedure, wells were washed once with PBS, and a mixture of 180 μL of MEM medium and 20 μL of 5 mg/mL MTT was added after PBS was removed. After culturing for 4 h, the mixture was removed and cells were exposed to 150 μL of DMSO for another 10 min before the final test. The method of Li et al. (2010) was followed.

MDA assays

MDA assays indicate the level of oxidative stress in cells. The method of Shuai et al. (2015) was followed and the concentrations of MDA were calculated using the formula: $C(\mu mM) = 6.45(A_{532} - A_{600}) - 0.56A_{450}$.

Intracellular ROS generation assays

Intracellular ROS is a known direct biomarker of oxidative stress. The signal at 428 nm(excitation)/525 nm (emission) was recorded to determine the content of ROS. The method described by Crow (1997) was followed.

TSLP and GSH assays

The manufacturer's instructions were used to perform that TSLP and GSH assays.

Statistical analyses

Data are presented as mean±standard error and plotted using GraphPad Prism 6. Dose–response (p_{trend}) analyses for multiple groups, shown in part A of the figures, were performed using linear regression trend testing. Differences between the two groups, shown in part B of the figures, were determined using the *t*-test in Origin 6.1. $p < 0.05$ and 0.01 indicated statistical significance.

Results

MTT assays (C_{max} = 3.20 mM)

MTT assays were used to determine cell vitality. A significant dose–response relationship was observed ($p < 0.0001$) (Fig. 1A). However, the group treated with the lowest dose showed hormesis. GSH significantly protected the cells ($p < 0.01$) (Fig. 1B).

MDA assays (C_{max} = 3.20 mM)

MDA contents reflect the level of oxidative stress. A decrease

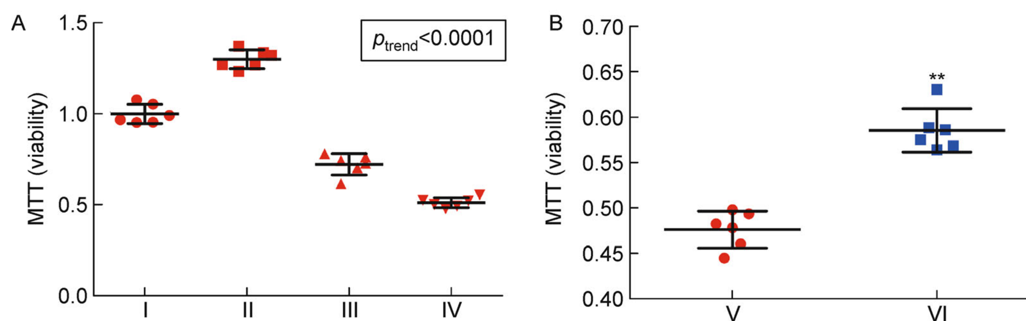

Figure 1 Results of the MTT assay. I, $C_{peroxide}$ = 0 mM; II, $C_{peroxide}$ = 0.05 mM; III, $C_{peroxide}$ = 0.20 mM; IV, $C_{peroxide}$ = 0.80 mM; V, $C_{peroxide}$ = 3.20 mM; VI, $C_{peroxide}$ = 3.20 mM + GSH. Cell viability decreased as $C_{peroxide}$ increased, except when $C_{peroxide}$ = 0.05 mM. GSH significantly reduced toxicity when $C_{peroxide}$ = 3.20 mM.

was observed as the H_2O_2 concentration increased ($p < 0.0001$) (Fig. 2A). The GSH and GSH-free groups differed significantly ($p < 0.05$) (Fig. 2B).

GSH assays (C_{max} = 3.20 mM)

Glutathione concentrations can also demonstrate the level of oxidative stress. No trend was observed as the concentration of H_2O_2 increased (p = 0.3854) (Fig. 3A). No significant difference was observed between the GSH and GSH-free protected groups (Fig. 3B).

TSLP assays (C_{max} = 3.20 mM)

The TSLP content can indicate possible subsequent molecular events. No increase in TSLP content was observed as the H_2O_2 concentration increased (p = 0.0782) (Fig. 4A). However, a significant difference was found between the GSH and GSH-free protected groups ($p < 0.05$) (Fig. 4B), indicating that GSH prevented a rise in TSLP.

MDA and ROS assays (C_{max} = 0.20 mM)

From previous MTT data, the effect of H_2O_2 was not observed below a concentration of 0.20 mM. In the effect of treatment with 0.05 mM to 0.20 mM H_2O_2, MDA and ROS were used as indicators of oxidative stress.

The MDA and ROS contents illustrated that the dose–response relationship persisted ($p < 0.0001$). GSH also protected cells from oxidative stress ($p < 0.05$, $p < 0.01$ respectively).

Discussion

Numerous air pollutants have been found to be oxidative or to contain oxidative factors. In this study, H_2O_2, a well-known oxidant, was used to simulate such pollutants and to investigate the effects of oxidative pollutants, primarily oxidative stress (Oda et al., 2000). Further experiments were conducted to determine levels of TSLP, which can reveal possible downstream molecular events, which can ultimately lead to allergic diseases (Soumelis et al., 2002). GSH, known as a reducing agent, was used to protect cells from oxidation (Wu et al., 2004) and was found to be effective. RTE cells were used to simulate the effects of inhalation of oxidizing pollutants by humans.

MTT assays indicated that cell vitality decreases as the H_2O_2 concentration increases; the group treated with the lowest dose showed hormesis, indicating that cells will respond positively to low concentrations of the toxicant (Stebbing, 1982; Sagan, 1987). The MDA and ROS assays revealed a dose–response relationship between the H_2O_2 concentration and cellular oxidative stress. Furthermore, GSH

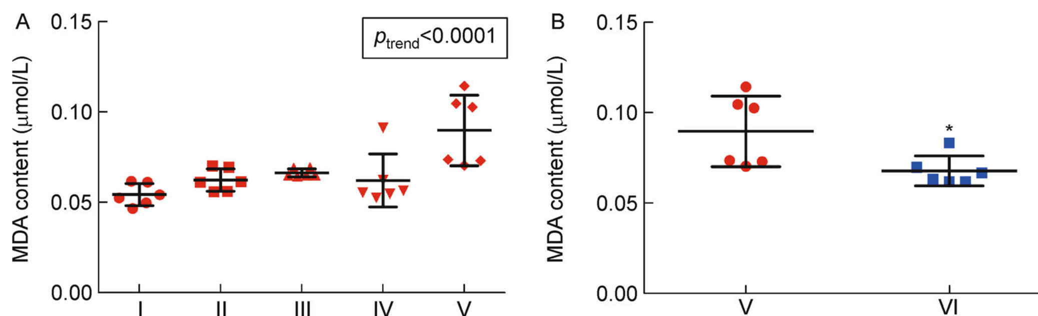

Figure 2 Results of the MDA assay. I, $C_{peroxide}$ = 0 mM; II, $C_{peroxide}$ = 0.05 mM; III, $C_{peroxide}$ = 0.20 mM; IV, $C_{peroxide}$ = 0.80 mM; V, $C_{peroxide}$ = 3.20 mM; VI, $C_{peroxide}$ = 3.20 mM + GSH. Oxidative stress levels increased as $C_{peroxide}$ increased, and GSH reduced the effect.

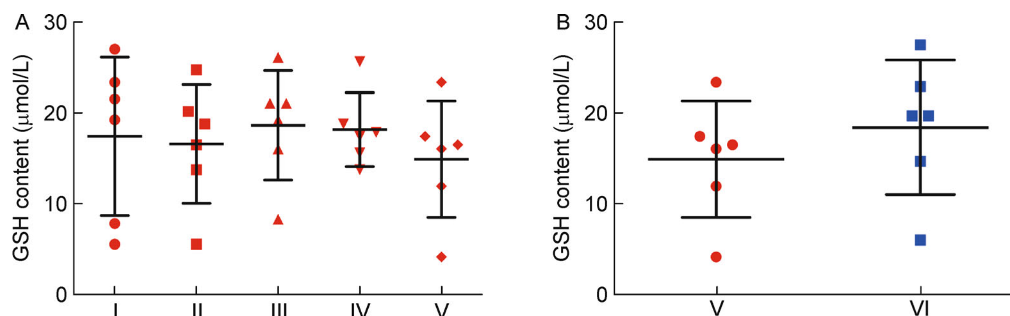

Figure 3 Results of the GSH assay. I, $C_{peroxide}$ = 0 mM; II, $C_{peroxide}$ = 0.05 mM; III, $C_{peroxide}$ = 0.20 mM; IV, $C_{peroxide}$ = 0.80 mM; V, $C_{peroxide}$ = 3.20 mM; VI, $C_{peroxide}$ = 3.20 mM + GSH. No changes in oxidative stress levels or protective effects were observed in the results of the GSH test.

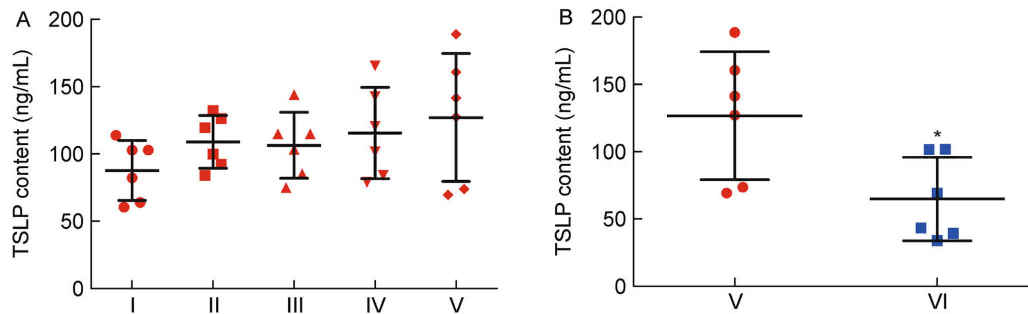

Figure 4 Results of the TSLP assay. I, $C_{peroxide}$ = 0 mM; II, $C_{peroxide}$ = 0.05 mM; III, $C_{peroxide}$ = 0.20 mM; IV, $C_{peroxide}$ = 0.80 mM; V, $C_{peroxide}$ = 3.20 mM; VI, $C_{peroxide}$ = 3.20 mM + GSH. A dose–response relationship was not observed. However, a significant protective effect of GSH was observed when $C_{peroxide}$ = 3.20 mM.

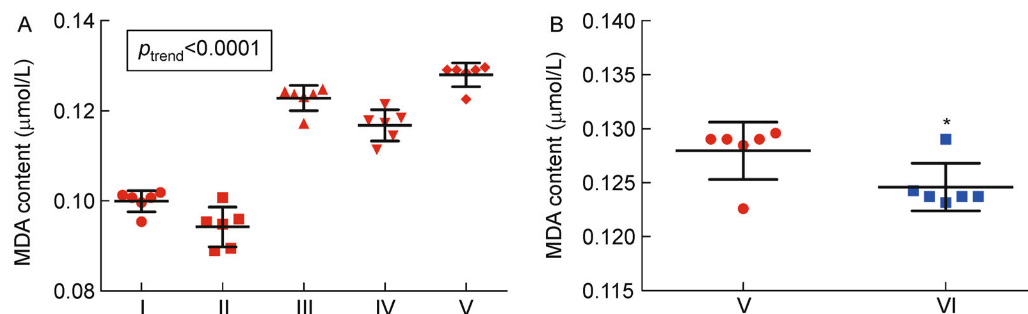

Figure 5 Results of the MDA assay. I, $C_{peroxide}$ = 0 mM; II, $C_{peroxide}$ = 0.05 mM; III, $C_{peroxide}$ = 0.10 mM; IV, $C_{peroxide}$ = 0.15 mM; V, $C_{peroxide}$ = 0.20 mM; VI, $C_{peroxide}$ = 0.20 mM + GSH. A dose–response relationship was observed and protection by GSH was significant.

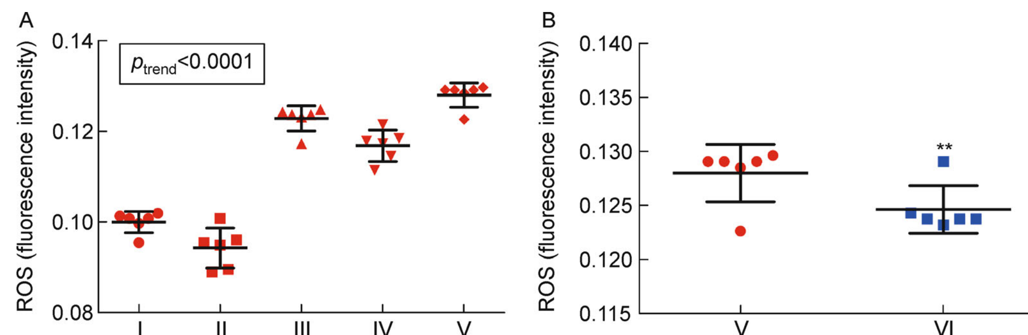

Figure 6 Results of the ROS assay. I, $C_{peroxide}$ = 0 mM; II, $C_{peroxide}$ = 0.05 mM; III, $C_{peroxide}$ = 0.10 mM; IV, $C_{peroxide}$ = 0.15 mM; V, $C_{peroxide}$ = 0.20 mM; VI, $C_{peroxide}$ = 0.20 mM + GSH. The oxidative stress levels rose significantly as $C_{peroxide}$increased, and GSH prevented toxicity when $C_{peroxide}$ = 0.20 mM.

significantly reduced oxidative stress and prevented the increase in TSLP.

TSLP is a potential inflammation-modulating factor. It has been shown that TSLP can induce the proliferation of CD4 + T cells and dendritic cells (Al-Shami et al., 2005). It has also been reported that activation and maturation of dendritic cells can help to induce CD4 + T cells to produce Th2 inflammatory cytokines, leading to the generation of IL-4 and IgE, which are indicators of possible allergic diseases (Pandey et al., 2000; Nakamura et al., 2008). The results of this experiment suggested that the increase in TSLP levels could be prevented by adding GSH to reduce oxidative stress, which indicated a potential positive relationship between oxidative stress and TSLP levels. We also found that GSH can effectively prevent step 2, and also reduce production of correlated biomarkers.

Conclusion

Thus, we can deduce a series of molecular events in cells: 1) Inhalation of an oxidative air pollutant; 2) ROS content of

cells increases and oxidative stress occurs; 3) TSLP contents rise; 4) Related inflammation may occur and cause allergic diseases.

Compliance with ethics guidelines

The authers declare that they have no competing interets.

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