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Forum News & Views

Does Leukotriene Affect Intracellular Glutathione Redox State in Cultured Human Airway Epithelial Cells?

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ABSTRACT

Leukotrienes (LTs) are one of the most important mediators in the pathophysiology of asthma. We measured the intracellular amounts of reduced glutathione (GSH) and oxidized glutathione (GSSG) in cultured human airway epithelial cells. LTC_4 affects the GSH/GSSG ratio by activating signals to increase interleukin-8 (IL-8) production. Pretreatment with a reducing agent, glutathione monochrome ester (GSH-OEt), and with a leukotriene receptor antagonist, montelukast, significantly suppressed LTC_4-induced time-dependent changes in the intracellular redox state, and also suppressed upregulation of IL-8 production by suppressing NF-κB activation. Our observations led to the hypothesis that LTC_4-induced oxidative stress is likely to contribute to amplification of airway inflammation. *Antioxid. Redox Signal.* 10, 821–828.

AIRWAY INFLAMMATION AND REDOX

ASTHMA IS A CHRONIC INFLAMMATORY AIRWAY DISEASE that affects children and adults of all ages (4). Although the pathogenesis of asthma remains incompletely defined, there is ample evidence that asthma is mediated by oxidative stress. The imbalance between reactive oxygen species (ROS) and antioxidants is termed oxidative stress. Most environmental factors, including oxidants, ultraviolet light, radioactivity, infections, and allergic responses to allergens, act as oxidative stress upon cells (6, 12). The cells obtained from the peripheral blood and lungs of patients with asthma generate increased amounts of ROS, such as superoxide radicals and hydrogen peroxide (H_2O_2), and the increase correlates with disease severity (16). Cell functions are activated and inactivated by the balance between intracellular oxidation and reduction (redox state), which in turn closely correspond to the surrounding environment. Epithelial cells are the first cells to encounter inhaled allergens, and asthma is a disorder involving the airway epithelium that is more vulnerable to environmental injury and responds to this by impaired healing, in addition to inflammation (9). As the first barrier of the airway, to maintain a steady state, airway epithelial cells possess mechanisms that eliminate oxygen radicals, tending to counteract intracellular shifts toward the oxidized state (20). Understanding how allergic respiratory diseases are exacerbated requires consideration of the effect of inflammation as an oxidative stress on airway epithelial cells, the initial site of injury from inflammatory cells, and/or pro-inflammatory mediators.

Glutathione, the most abundant nonprotein tripeptide containing a sulfhydryl group, plays a prominent role in antioxidant protection of the lung. In humans, glutathione is 100-fold more concentrated in the airway epithelial lining fluid than in plasma (29). Oxidants or oxidative stress have been reported to activate transcription factors, including NF-κB and phosphorylation of MAP kinase (19, 22). Inflammation-related changes in intracellular redox state in lung macrophages and monocytes have a potent effect on cytokine production (11, 28). Furthermore, the intracellular redox system is affected by oxidative stress induced by inflammatory cells during allergic reactions in the airway mucosa. However, changes of intracellular redox state and redox-related reactions in airway epithelial cells are largely uncharacterized.

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LEUKOTRIENE AND INFLAMMATION

Leukotrienes (LTs), one of the most important mediators of the pathophysiology of asthma, are known to induce bronchoconstriction, airway inflammation, edema, and mucus hypersecretion (18). There are two families of leukotrienes: LTB_4 and a second group (LTC_4, LTD_4, LTE_4), called cysteinyl-leukotrienes (Cys-LTs), bind to highly selective receptors to induce bronchoconstriction and inflammation associated with asthma (14). Analysis by enzyme immunoassay of exhaled breath condensate from patients with asthma has proved that asthma patients have higher levels of Cys-LTs and 8-isoprotane (a marker of oxidative stress) than normal subjects (1). In a B cell line (BL41-E95-A), oxidative stress (H_2O_2, diamide) enhanced activity of the cellular 5-lipoxygenase (5-LO) , which catalyzes the first two steps in leukotriene biosynthesis (30). Depletion of thiol antioxidants induced the production of Cys-LTs and selective phosphorylation of MAP kinase in lung fibroblasts (2). Previous studies have demonstrated an association between production of Cys-LTs and oxidative stress. In this study, we investigated whether Cys-LTs can induce oxidative stress in cultured human airway epithelial cells, and evaluated the direct effect of Cys-LTs as an oxidative stressor through measurement of reduced glutathione (GSH) and oxidative glutathione (GSSG). We also studied cytokine regulation induced by Cys-LTs with a leukotriene receptor antagonist, montelukast, which is widely used as an anti-asthmatic drug.

LEUKOTRIENE AND INTRACELLULAR REDOX STATE

LTC_4 exposure and intracellular redox state

To determine an informative concentration and duration of Cys-LTs exposure, we compared six various stimulated concentration of LTC_4: from 10^{-7} to 10^{-12} *M* at 2 and 6 h. LTC_4 exposure dose-dependently induced an oxidized state within Calu-3 cells at 2 h after stimulation (Fig. 1). We chose a final LTC_4 concentration of 10^{-8} *M* as suitable for subsequent study because it induced a reproducible oxidized state at 2 h and a rebound reaction at 6 h.

We measured the intracellular redox state in Calu-3 cells immediately and at 2, 4, 6, 12, 24, and 48 h after LTC_4 exposure. In the 10^{-8} *M* group after exposure for 2 h, the GSH/GSSG ratio decreased immediately to a mildly oxidized state, whereas the GSH/GSSG ratio fell below 100% compared with the control state. At 4 h after LTC_4 exposure, the ratio gradually declined to a severely oxidized state where the GSH/GSSG ratio fell to the minimal value, then the GSH/GSSG ratio increased dramatically to reach a maximum reduction at 6 h after LTC_4 exposure (Fig. 2A). Figure 2B shows the time course of the absolute values of GSH and GSSG. At 4 h after LTC_4 exposure, the GSH slightly decreased, and thereafter the GSH increased significantly to reach a maximum reduction at 6 h after LTC_4 exposure (Fig. 2B). The GSSG increased at 24 h after LTC_4 exposure. The control group, exposed to PBS as vehicle (10 μl/well), did not show any significant changes in the intracellular redox state at any time point (data not shown).

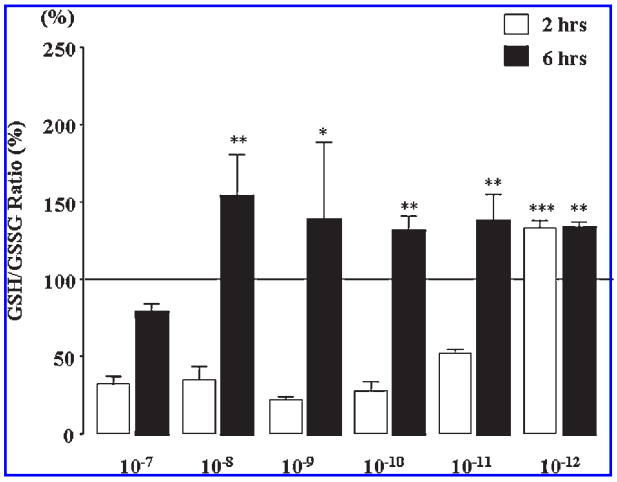

FIG. 1. Dose-dependent effect of LTC_4 on intracellular redox. Results following LTC_4 (10^{-7}–10^{-12} *M*) exposure for 2 h show the intracellular redox state declining to indicate an oxidized state, with the GSH/GSSG ratio showing <100% of control-state values ($n = 6$). At 6 h after exposure, the GSH/GSSG ratio increased. In 10^{-8} *M* concentration, LTC_4 induced a clear series of oxidation and reduction. $*p < 0.05$, $**p < 0.02$, $***p < 0.001$, compared with 10^{-7} *M* LTC_4.

LTC_4 exposure and pretreatment with glutathione modulators

Calu-3 cells were pretreated with the reducing reagent, glutathione monochrome ester (GSH-OEt), at concentrations of 10^{-4}, 10^{-5}, and 10^{-6} *M* for 4 h, and then exposed to LTC_4 at a concentration of 10^{-8} *M*. In the LTC_4 group (exposed to LTC_4 without pretreatment), the GSH/GSSG ratio at 2 h after LTC_4 exposure showed a decreased of >25%, indicating that cells were in an oxidized state, peaking at 6 h after LCT_4 exposure (Figs. 3A and B). On the other hand, in the OEt+LTC_4 group (GSH-OEt pretreatment and exposed to LTC_4), the GSH/GSSG ratio exceeded 100%, and a reduced intracellular state was maintained for 6 h after LTC_4 exposure. LTC_4-induced intracellular oxidative state at 2 h was inhibited by pretreatment with GSH-OEt, thus resulting in the continuous reductive state in human epithelial cells.

LTC_4 exposure and pretreatment with Cys-LTs receptor antagonists

Calu-3 cells were pretreated with Cys-LTs receptor antagonist, montelukast, at concentrations of 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} *M* for 2 h before and during exposure to LTC_4 at a concentration of 10^{-8} *M*. In the LTC_4 group (exposed to LTC_4 without pretreatment), the GSH/GSSG ratio at 4 h after LTC_4 exposure decreased to nearly 50%, indicating that cells were in a severely oxidized state (Fig. 4), while addition of montelukast at concentrations of 10^{-6} and 10^{-7} *M* attenuated the significant decreases (Fig. 4). There was no significantly difference in the GSH/GSSG ratio between montelukast pretreatment (10^{-6}, 10^{-7} *M*) group and control group at 4 h after LTC_4 exposure.

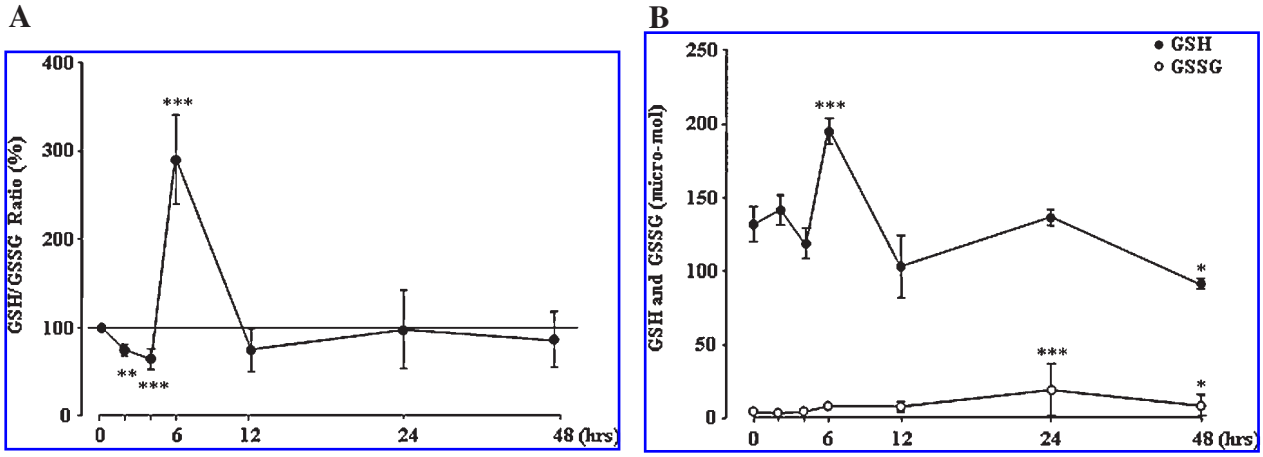

FIG. 2. (**A**) **Change of GSH/GSSG ratio after LTC_4 exposure.** Results following LTC_4 (10^{-8} *M*) exposure for 2 h show the intracellular redox state declining to indicate a mildly oxidized state, with the GSH/GSSG ratio showing <100% of control-state values ($n = 6$). At 4 h after exposure, the ratio gradually declined to severely oxidized state where the GSH/GSSG ratio fell to the minimal value. Then the GSH/GSSG ratio increased dramatically, and the maximum reduction was reached at 6 h after LTC_4 exposure. $^{**}p < 0.02$, $^{***}p < 0.001$, compared with 0 h. (**B**) Change of GSH and GSSG after LTC_4 exposure. At 4 h after LTC_4 exposure, the GSH slightly decreased, thereafter the GSH increased significantly to reach a maximum reduction at 6 h after LTC_4 exposure. The GSSG increased at 24 h after LTC_4 exposure ($n = 6$). $^{*}p < 0.05$, $^{***}p < 0.001$, compared with 0 h.

LEUKOTRIENE AND CYTOKINE/CHEMOKINE UPREGULATION

Cys-LTs -induced upregulation of cytokine production

The concentration of interleukin-8 (IL-8) in control cells unexposed to LTC_4 was 185.75 ± 23.92 ng/ml. After exposing Calu-3 cells to LTC_4, we measured the changes in production of IL-8. At 24 h after exposure, IL-8 values (282.49 ± 16.14 ng/ml) were significantly higher than in the control group ($p < 0.001$, Fig. 5). We also investigated the effects of the LTC_4-induced changes in IL-8 production following pretreatment with GSH-OEt (10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} *M*) or montelukast (10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} *M*). Pretreatment with GSH-OEt (10^{-3}, 10^{-4}, and 10^{-5} *M*) dose-dependently suppressed LTC_4-induced upregulation of epithelial cell-derived IL-8 production (Fig. 5). Also, pretreatment with 10^{-6} or 10^{-7} *M* montelukast

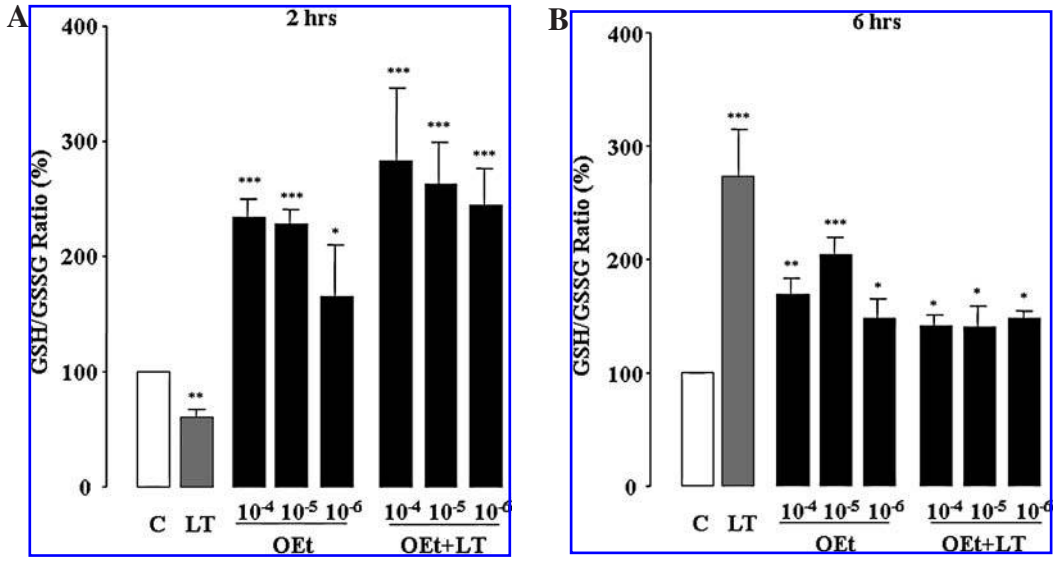

FIG. 3. (**A**) **Effect of antioxidant on LTC_4-induced redox change at 2 h.** GSH-OEt increased GSH/GSSG ratio after LTC_4 exposure in Calu-3 cells at 2 h ($n = 6$). Pretreatment of GSH-OEt was for 4 h, and incubation time of LTC_4 was 2 h. $^{*}p < 0.05$, $^{***}p < 0.001$, compared with control. (**B**) Effect of antioxidant on LTC_4-induced redox change at 6 h. GSH-OEt suppressed after LTC_4 -induced increase of GSH/GSSG ratio in Calu-3 cells at 6 h ($n = 6$). Pretreatment of GSH-OEt was for 4 h, and incubation time of LTC_4 was 2 h. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with control.

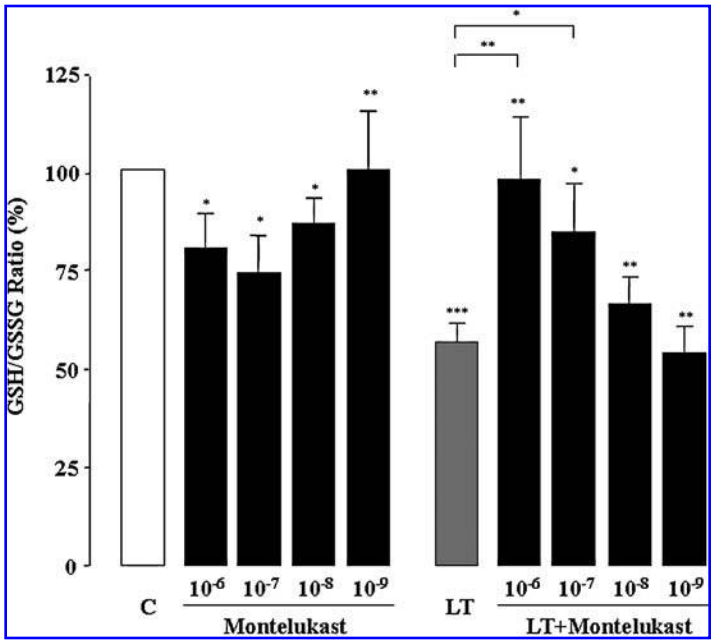

FIG. 4. Effect of LT antagonist on LTC_4-induced redox change. Montelukast had a protective effect on GSH/GSSG ratio after LTC_4 exposure in Calu-3 cells ($n = 6$). Pretreatment of montelukast was for 2 h, and incubation time of LTC_4 was 2 h. $^*p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.001$, compared with control.

significantly inhibited LTC_4-induced upregulation of epithelial cell-derived IL-8 production (Fig. 6).

Effect of Cys-LTs exposure upon NF-κB p65 binding activity

Compared with the control sample, the concentration of NF-κB p65 showed a significant increase after LTC_4 exposure ($p < 0.001$, Fig. 7). Pretreated with 10^{-4} or 10^{-5} *M*, GSH-OEt suppressed LTC_4-induced activation of NF-κB p65 in Calu-3 cells, and the inhibitory effect was dose dependent. This was also the case for montelukast at concentrations of 10^{-6} and 10^{-7} *M* (Fig. 7).

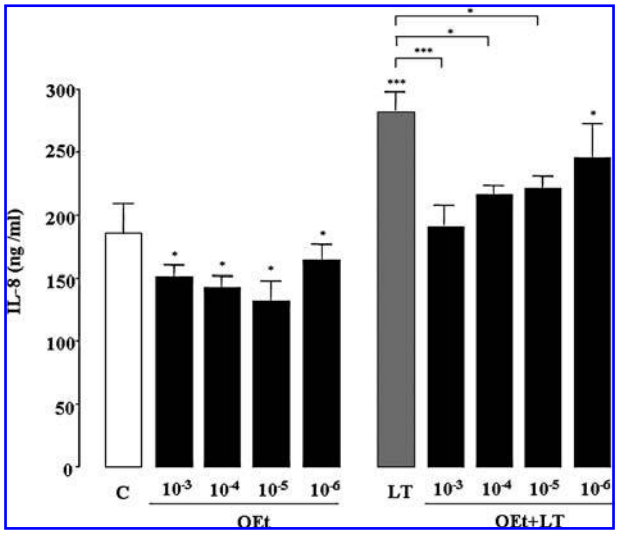

FIG. 5. Effect of LTC_4 and antioxidant on IL-8 production in Calu-3 cells. GSH-OEt suppressed LTC_4-induced IL-8 production in Calu-3 cells ($n = 6$). Pretreatment of GSH-OEt was for 4 h, and incubation time of LTC_4 was 2 h. $^*p < 0.05$, $^{***}p < 0.001$, compared with control.

CONCLUSION AND OPEN QUESTIONS

A vast body of evidence suggests that the intracellular redox state regulates various aspects of the cellular function (12), while that in various cell types of glutathione constitutes the first line of cellular defense against oxidative injury, acting as the major intracellular redox buffer (20). However, in the studies published so far, little has been reported regarding whether some important inflammatory molecules, such as Cys-LTs, can induce oxidative stress on the glutathione redox system in human airway epithelial cells or how changes in the intracellular redox state affect cytokine regulation. In this study, we examined whether Cys-LTs can induce oxidative stress in cultured human airway epithelial cells (Calu-3) and evaluated the direct effect of Cys-LTs as an oxidative stressor through measurement the intracellular redox state in Calu-3 cell. We monitored the intracellular glutathione redox balance, as indicated by the GSH/GSSG ratio. We also studied cytokine regulation induced by this oxidative stress.

One key finding of the present study is that the glutathione redox balance in airway epithelial cells was affected by LTC_4-induced oxidative stress. At 4 h after Calu-3 cells were exposed to 10^{-8} *M* LTC_4, the intracellular redox state gradually changed from a reduced to a severely oxidized state, followed by a prolonged reduced state. Airway epithelial cells have effective mechanisms to prevent a prolonged oxidized state, particularly the glutathione redox system, which can rapidly return cells from an oxidized to a reduced state (27). Although previous studies have demonstrated an association between the production of Cys-LTs and oxidative stress (2, 30), this is the first study of the effect of Cys-LTs on the intracellular redox state,

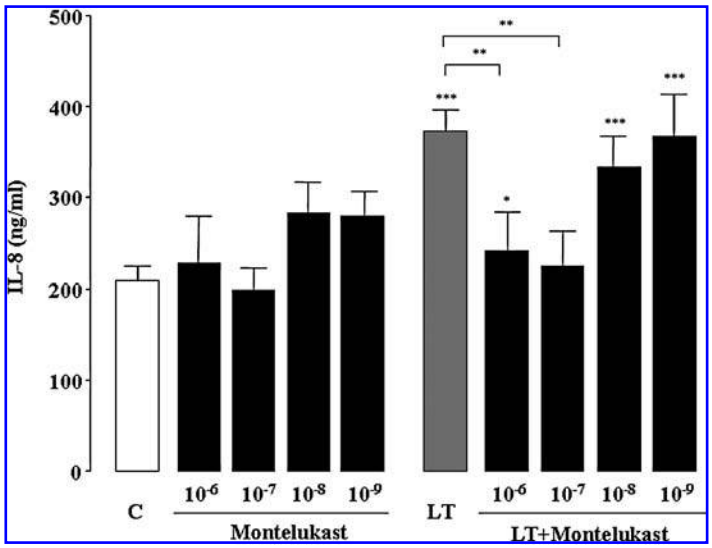

FIG. 6. Effect of LTC_4 and montelukast on IL-8 production in Calu-3 cells. Montelukast had a significant effect on LTC_4-induced IL-8 production in Calu-3 cells ($n = 6$). Pretreatment of monterukast was for 2 h, and incubation time of LTC_4 was 2 h. $*p < 0.05$, $**p < 0.02$; $***p < 0.001$, compared with control.

and it proved that Cys-LTs can induce oxidative stress in cultured human airway epithelial cells. The decreased intracellular GSG/GSSG ratio in Calu-3 cells may be induced by the increased ROS formation after exposure to LTC_4. Electron spin resonance is the only method that can be used to measure free radicals directly, but the evanescent nature of many ROS makes them difficult to measure. Therefore, the most common technique is to quantify ROS indirectly by measuring the products damage caused by ROS, including the shift in the balance of reduced and oxidized glutathione (6). GSH is capable of reducing a wide variety of disulfides by transhydrogenation and acts as a major reductant of cellular protein disulfides (8).

This study clearly demonstrated that LTC_4 stimulated airway epithelial cells to produce IL-8. Cys-LTs are one of the most important mediators of the pathophysiology of asthma (18). They are produced mainly by eosinophils and mast cells. Studies have proved that Cys-LTs not only elicit bronchoconstriction as potent constrictors of smooth muscle, but also play an important role in airway remodeling. Recently, Perng *et al*. reported that LTC_4 induced transforming growth factor β_1 (TGF-β_1) in the airway epithelium through a P38 mitogen-activated protein (MAP) kinase activation mechanism (21). In our experiment, LTC_4 upregulated epithelial cell-derived IL-8 production. IL-8, a member of the α-chemokine family and one of the most abundant cytokines produced by airway epithelial cells, exhibits a variety of biological activities including neutrophils and T-lymphocyte chemotactic activity (3, 9). Recently, IL-8 has been implicated in the pathogenesis of the allergic inflammation of asthma, and neutrophils have been found to predominate over eosinophils as the major inflammatory cell type in bronchoalveolar lavage fluid and sputum samples of patients with acute exacerbated asthma (5).

Studies of inflammation-related changes in the intracellular redox state in pulmonary macrophages and monocytes show that these changes have a potent effect on cytokine production (11, 28). The IL-8 gene has been reported to be activated by

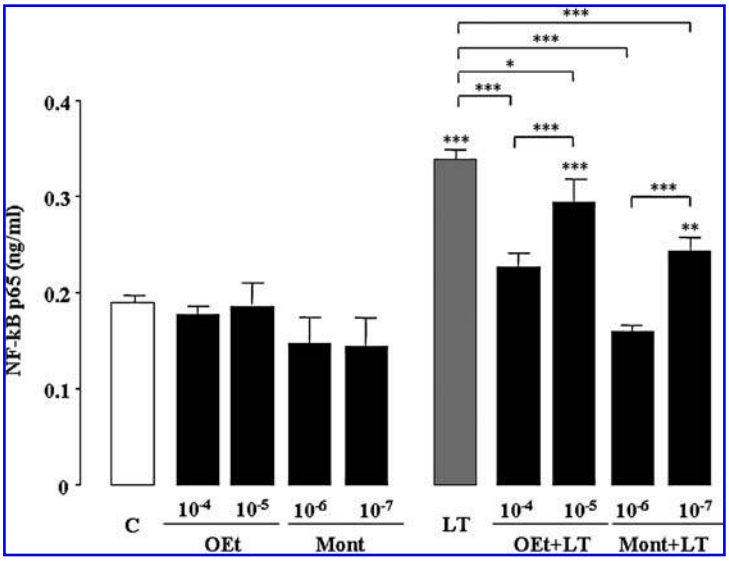

FIG. 7. Effect of LTC_4 on NF-kB p65 binding activity in Calu-3 cells. LTC_4 had an effect on NF-kB p65 binding activity in Calu-3 cells ($n = 5$). Pretreatment of GSH-OEt and montelukast was for 4 h and for 2 h. Incubation time of LTC_4 was 2 h. $*p < 0.05$, $**p < 0.02$, $***p < 0.001$, compared with control.

oxidative stress (10, 17). In our previous study, oxidative stress affected the signal transfer system in airway epithelial cells, resulting in upregulation of cytokine and chemokine production, especially of IL-8 (27). It may be surmised that LTC_4 upregulated IL-8 production by means of oxidative stress in Calu-3 cells.

Oxidants or oxidative stress have been reported to activate transcription factors, including NF-κB, and to promote phosphorylation of MAP kinase (19, 22). We also confirmed that oxidative stress influenced the NF-κB pathway. Like most inflammatory mediators, IL-8 expression can be induced by activation of specific transcription factors such as NF-κB, which then bind to DNA sites located in the promotor region of the relevant gene (17). NF-κB has been shown to be redox state responsive (19), with binding of NF-κB to IL-8 promotors being highly sensitive to changes in the intracellular redox state (29).

On the other hand, Cys-LTs are recognized by at least two receptor types: Cys-LT_1 and Cys-LT_2. The Cys-LT_1 receptor mRNA is found in the spleen, lung tissue, and smooth muscle cells (1), and Cys-LT_2 receptor mRNA is confirmed in human and rat airway epithelial cells (25). It remains unclear which of these is involved in the mechanism of LTC_4-induced upregulation of IL-8 production. Also, we cannot ascertain whether LTC_4 is able to directly induce NF-κB activation via the Cys-LTs receptor in human airway epithelial cells. A previous study reported that LTC_4 did not directly induce NF-κB activation in U-937 cells (human monocytic leukemia cell line) (15). Further investigations are necessary to clarify the relationship between the LTC_4-induced intracellular redox state and IL-8 related signal transfer in airway epithelial cells.

Our results also suggested that pretreatment with montelukast, a Cys-LT_1 receptor antagonist, suppressed LTC_4-induced oxidation and significantly reduced the upregulation of IL-8 production in cultured human airway epithelial cells. At present, montelukast is widely used in the treatment of asthma. The anti-asthmatic effect may be rendered not only by the antileukotriene activity, but also by other types of pharmacological activity. Simeonova *et al*. demonstrated that montelukast inhibited TNF-α-induced NF-κB activation in THP-1 cells (human monocytic leukemia cell line) (24). Pranlukast, another Cys-LT_1 receptor antagonist, has been reported to inhibit TNF-α-induced NF-κB activation in U-937 cells, which have Cys-LT_1 receptors on their membranes, and T cells (Jurkat) which do not have Cys-LT_1 receptors on their membranes (15). The underlying mechanism of the inhibitory effect on NF-κB activation is unclear. Taking a previous study into consideration (15, 24), antagonism of Cys-LT_1 receptor may be partially related to the inhibition of NF-κB activation, and it is likely that Cys-LT_1 receptor antagonists have another antagonism that inhibits NF-κB activation. Cys-LT_1 receptor antagonists compose a new class of drugs, currently being investigated, and further research will expand our knowledge of their anti-inflammatory potential.

Our results demonstrated that, in cultured human airway epithelial cells, pretreatment with a reducing agent significantly suppressed LTC_4-induced time-dependent changes in the intracellular redox state and LTC_4-induced upregulation of epithelial cell-derived IL-8 production by suppressing NF-κB activation, which in turn confirmed that the LTC_4-induced upregulation of IL-8 production was mainly a consequence of oxidative stress. This finding suggests that pretreatment with antioxidative agents could protect against LTC_4-induced oxidative stress. Accordingly, pretreatment with reducing agents may protect against ozone-induced upregulation of IL-8 production in cultured human airway epithelial cells (27), so antioxidative drugs may have a prophylactic effect against inflammation-induced exacerbation of respiratory symptoms. The modulation of IL-8 production, in relation to oxidative stress, has an important role in inflammatory cell recruitment and activation (24), and treatment with reducing agents might benefit patients with airway diseases associated with inflammation, such as asthma. The protective effects of reducing reagents on Cys-LTs exposure *in vivo* require further preclinical investigation.

In conclusion, LTC_4 can affect the intracellular glutathione redox state in human airway epithelial cells, thereby activating signals and thus causing them to increase the production of cytokine. These findings may provide a basis for understanding the interrelationships between oxidative stress and airway inflammation. Therapeutic interventions that either augment endogenous antioxidant defenses or result in a decreased exposure to environmental oxidative stress might therefore be beneficial as adjunctive therapies for asthma and allergic respiratory disorders.

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ABBREVIATIONS

GSH, reduced glutathione; GSH-OEt, glutathione monochrome ester; GSSG, oxidized GSH; redox, reduction-oxidation' NF-κB, nuclear factor-κB.

APPENDIX

Culture of human airway epithelial cells

The Calu-3 cell line obtained frozen from the American Type Culture Collection (Rockville, MD), was grown in T75 tissue-culture flasks (Coster, CA), containing a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 10% fetal calf serum (FCS) at 37°C in an atmosphere including 5% CO_2 and 95% air. When the cells were 80–90% confluent, they were detached with 0.05% trypsin and 0.02% ethylenediamine tetraacetate (EDTA), and seeded into wells in six-well plastic tissue culture dishes at 10^5 cells/cm^2. They then were grown to confluence, which typically required 10–12 days (23).

LTC_4 stimulation

In experiments involving Cys-LTs stimulation, confluent Calu-3 cells were serum-starved for 24 h before the addition of LTC_4. Cells were stimulated with LTC_4 at various concentrations or exposed to ve-

hicle (phosphate buffer solution, PBS). Based on previous experiments of cultured airway epithelial cell, we finally exposed confluent Calu-3 cells to 10^{-8} *M* LTC_4.

Cell exposure to glutathione modulators or pretreated with Cys-LTs receptor antagonists

To increase the concentration of cellular GSH, some Calu-3 cells were either incubated with GSH-OEt at concentrations of 10^{-4}, 10^{-5}, or 10^{-6} *M* for 4 h before and during exposure to LTC_4 (7). Glutathione modulators were added in the presence of FCS-free medium to minimize the influence of FCS on cellular GSH. At the same time, FCS-free medium was added to the control samples. In selected experiments, Calu-3 cells were incubated with the Cys-LTs receptor antagonist, montelukast, at concentrations of 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} *M* for 2 h before and during exposure to LTC_4. Cell viability, determined by trypan blue dye exclusion, always exceeded 90%.

Measurements of intracellular GSH and GSSG concentration

Calu-3 cells were washed three times with cold wash buffer (0.1 *M* sodium phosphate and 5 m*M* EDTA; pH 7.5) and immediately thawed in 100 μl of lysis buffer (0.1% Triton-X, 0.1 *M* sodium phosphate, and 5 m*M* EDTA; pH 7.5) for 5 min. Lysates then were acidified with 15 μl of 0.1 *N* HCl, and protein was precipitated with 15 μl of 50% sulfosalicylic acid. After centrifugation, the supernatant was collected for GSH and GSSG assays. The total cellular glutathione concentration was assayed by a GSSG-reductase-DTNB recycling procedure by the method of Tietze (26), as modified by Buchmuller–Rouiller and coworkers (7). GSH was oxidized by DTNB and then reduced by β-NADPH in the presence of glutathione reductase.

Formation of 2-nitro-5-thiobenzoic acid was monitored by comparing absorbance at 405 nm with that of standard samples of GSH in lysis buffer. GSSG was assayed by Griffith's method (13). Briefly, standard solutions of GSSG or aliquots of samples were mixed with 2 μl of 2-vinylpyridine per 100 μl of sample volume. All solutions were adjusted to pH 7.5 with triethanolamine. After incubation for 60 min at room temperature, the assay was performed as described for total glutathione.

Quantitation of IL-8

Concentrations of IL-8 in culture supernatants were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems, Minneapolis, MN), in accordance with the manufacturer's instructions. Samples of each supernatant were collected 24 h after LTC_4 or PBS exposure. The assays could detect IL-8 concentrations exceeding 3 pg/ml.

Nuclear protein extraction and quantitation of NF-κB p65 binding activity

Nuclear protein extraction was carried out using a nuclear extract kit (Active Motif, Carlsbad, CA) as follows. Calu-3 Cells (10^5/cm^2) in six-well plastic tissue culture dishes were cultured for 10–12 days. After incubation with varied concentrations of GSH-OEt for a pretreatment time of 4 h or with montelukast for a pretreatment time of 2 h, the cells were exposed to LTC_4 or PBS, respectively. At 6 h following the LTC_4 exposure, nuclear proteins were extracted. All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were harvested after washing twice with 2 ml of ice-cold PBS, centrifuging at 500 *g* for 5 min after each wash. Cells were resuspended in lysis buffer (10 m*M* HEPES at pH 7.9, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 1 m*M* dithiothreitol (DTT), and protease inhibitor cocktail). A syringe with a 27-gauge needle was used to disrupt the cells by 10 repetitions of drawing the cell suspension and then ejecting it. The disrupted cell suspension then was centrifuged at 11,000 *g* for 20 min; the supernatant was removed, and the nuclear pellet was resuspended in extraction buffer [20 m*M* HEPES at pH 7.9, 1.5 m*M* $MgCl_2$, 0.42 *M* NaCl, 0.2 m*M* EDTA, 25% (vol/vol) glycerol, 1 m*M* DTT, and protease inhibitor cocktail]. Nuclei were disrupted with a syringe using the method described above, and centrifuged for 5 min at 21,000 *g*. Protein concentration of the nuclear extract was measured by the Lowry method.

NF-κB p65 expression was measured using the TransA NF-κB p65 kit (Active Motif).

Reagents

GSH-OEt, LTC_4, IL-4, and TNF-α were purchased from Sigma Chemical. (St. Louis, MO). montelukast sodium was purchased from Merck Co. (Whitehouse Station, NJ). Nicotinamide adenine dinucleotide phosphate (β-NADPH), 5-5,-dithiobis-2-nitrobenzoic acid (DTNB), and glutathione reductase were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Statistical analysis

All values are expressed as mean ± SE. Nonparametric analysis of variance (Kruskal–Wallis method) was used to determine significant overall differences between groups. We used the Mann–Whitney *U* test to determine significant differences between individual groups. A value of $p < 0.05$ was considered to indicate significance.

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