INTRODUCTION

Studies have shown that continuous exposure to air pollution is associated with a range of respiratory and cardiovascular health effects and increased mortality (Brunekreef and Holgate, 2003). Oxidative stress has been identified as one potential feature underlying the toxic effect of air pollutants, which trigger a number of redox sensitive signalling pathways, inflammatory response and associated cytokine production (Mudway and Kelly, 2000; Kelly, 2003; Nel, 2005; Schlesinger et al., 2006). Toxicity may arise due to an imbalance of biological pro-oxidant and antioxidant processes linked to increased exposure to oxidants or the presence of impaired antioxidant defences (Bowler and Crapo, 2002; Cross et al., 2002; Misso and Thompson, 2005). The respiratory system is one of the primary interfaces with the external environment and has unique demand to handle and detoxify inhaled bio-particles (antigens) and pollutants. It is supposed to control and express inflammatory pathways in ways that preserve the primary functions of the respiratory system while protecting it from invasion by foreign infective agents or antigens. Inhaled air, even under relatively pristine conditions, contains large numbers of particles of diverse origin such as animal and plant proteins, inorganic dusts, and chemical materials from industry, transportation and suspended particulate matter from smoke and tobacco smoking.

Asthma is an immune inflammatory disease associated with infiltration of mast cells, eosinophils, neutrophils and lymphocytes in lung (Elias et al., 2003). Inflammation is a central feature of asthma and atopy. As a part of inflammation, reactive oxygen species (ROS) are formed, causing oxidation of DNA and lipids, leading to a variety of cytotoxic products (Barnes, 1990). The oxidative stress plays an important role in the pathogenesis of asthma including AHR, mucus hyper secretion, and bronchoconstriction (Talati et al., 2006). T-helper cell type 2 (Th2) response is important component for the development and perpetuation of asthma by release of Th2 cytokines such a IL-4, IL-5 and IL-13. The oxidative stress can enhance the production of these cytokines exacerbating the asthmatic symptoms (Crapo, 2003).
Airways are unique in exposure to high level of environmental oxidants and their unusually high concentration of extracellular antioxidants. In the resting state, the balance between antioxidants and oxidants is sufficient to prevent the disruption of normal physiologic function, but increase in oxidants or decrease in antioxidants can disrupt this balance (Halliwell and Gutteridge, 1999). The imbalance between oxidants and anti-oxidants leads to an increase in ongoing cycle of inflammation in the asthmatic airways that ultimately contributes to tissue injury (Comhair et al., 2005b). Reactive oxygen species (ROS) plays major role in asthma by activating AHR, mucus hyper secretion and tissue damage in lungs. Although it is difficult to get direct measurements of reactive oxygen species, studies of exhaled air from asthmatic patients had shown increased hydrogen peroxide and nitric oxide levels (Kharitonov et al., 1995; Ichinose et al., 2000; Emelyanov et al., 2001; Ganas et al., 2001). Furthermore, an increase in reactive oxygen species production is inversely correlated with forced expiratory volume in 1 second (FEV1) (Romieu et al., 2008).

Airway inflammatory cells e.g. macrophages from asthmatic patients produce more superoxide (oxidant) than control subjects (Calhoun et al., 1992). Antigen challenge is known to induce spontaneous release of reactive oxygen species from airway eosinophils in patients with asthma (Sanders et al., 1995). Here, circulating inflammatory cells might be a source for ROS. Peripheral blood monocytes are activated to secrete superoxide when IgE binds to membrane receptors (Demoly et al., 1994). The eosinophils isolated from asthmatic patients (Woschnagg et al., 1996) hours after antigen challenge produce more hydrogen peroxide when challenged with antigen (Evans et al., 1996). Peripheral blood eosinophils and monocytes also produce more reactive oxygen species in asthmatic patients compared to control subjects (Calhoun et al., 1992; Vachier et al., 1994). Thus both airway and intravascular inflammatory cells contribute to elevated oxidative stress in asthma. Increase in airway oxidative stress markers such as isoprostanes and ethane, as well as urinary isoprostanes, suggests that oxidative stress occur both at epithelial cell membrane and endothelial cell membrane (Montuschi et al., 1999; Paredi et al., 2000; Dworski et al., 2001).

The primary defense against reactive oxygen species is endogenous antioxidants, which can be subdivided into enzymatic and nonenzymatic categories.
The enzymatic antioxidants include the families of superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione-S-transferase, and thioredoxin (Bowler and Crapo, 2002). The nonenzymatic category of antioxidant defenses includes low molecular weight compounds, such as glutathione, ascorbate, urate, \(\alpha\)-tocopherol, bilirubin, and lipoic acid (Bowler and Crapo, 2002). Concentrations of these antioxidants vary depending on both the sub-cellular and anatomic location e.g. glutathione is 100-fold more concentrated in the airway epithelial lining fluid compared to plasma (Van der Vliet et al., 1999). Other high molecular weight molecules that might be considered as antioxidants include proteins with oxidizable thiol groups e.g. albumin or proteins that can bind free metal like transferrin. Albumin and transferrin are found in high concentration in serum but are present at a much lower concentration in airway lining fluid (Reynolds and Newball, 1974). Thus, both the lung parenchyma and airways have several antioxidant systems. The increase in reactive oxygen species during an asthma exacerbation might overwhelm these endogenous antioxidant defenses. Since ROS participate in the inflammatory response in airway disease, radical scavengers or antioxidants could play a useful role in therapy because antioxidants can mobilize and upregulate the antioxidative capacity of cells to annihilate excessive ROS formation. This can be achieved through two approaches, either by increasing the endogenous antioxidant defenses or by enhancing the defenses through dietary or pharmacological supplementation.

Oxidative stress plays an important role in allergic disorders and increased levels of oxidants are considered markers of the inflammatory process (Reynaert et al., 2007). ROS perpetuates the inflammatory process in lung, activates bronchoconstriction mechanism, and precipitates asthmatic symptoms (Tamer et al., 2004). This imbalance between oxidants and antioxidants favors skewing of the Th2 immune response while suppressing Th1 differentiation (Kim et al., 2007). Dietary intake of antioxidants, including vitamins C and E resulted in therapeutic benefit in asthma patients (Cho et al., 2004; Silva Bezerra et al., 2006). However, \(\alpha\)-lipoic acid, N-acetylcysteine, superoxide dismutase, ambroxol, and a mutated GST produced limited suppression of allergic airway inflammation and AHR in mouse model of asthma (Cho et al., 2004; Silva Bezerra et al., 2006).
Genetic variations in enzymes that detoxify ROS and other products generated by several inflammatory cells may contribute to the wide variation seen in the atopic reaction after exposure to an environmental trigger. The members of the glutathione-S-transferase (GST) superfamily of genes are important in the protection of cells from ROS due to their ability to utilize substrates of a wide range of products of oxidative stress (Hayes and Strange, 1995). Because of their role in protecting cells from oxidative stress, several GSTs have been suggested as candidate genes for asthma and atopy. There are conflicting reports regarding association between GSTM1 and GSTT1 and asthma. Ivaschenko et al. (2002) observed that the null genotypes of GSTM1 and GSTT1 were associated with an increased risk of atopic asthma in a case control study, however Fryer et al. (2000) found no association between GSTM1 and GSTT1 and asthma or atopy.

Glutathione-S-transferase (GST) superfamily protects cells from ROS by conjugating reactive intermediates with reduced glutathione (GSH) to produce water soluble compounds (Mannervik and Danielson, 1988; Rahman et al., 1999). The oxidant scavenging and anti-inflammatory properties of GST may be exploited to modulate the innate immune response to achieve therapeutic benefit. Previously, a study (Chapter 2) with mutated GST (mGST) has reduced oxidative stress and Th2 response to a limited extent in mouse model. Studies suggest that combination of antioxidants may give optimal effect (Silva Bezerra et al., 2006; Okamoto et al., 2006). Moreover, one of the major detoxification pathways involves the conjugation of xenobiotics with GSH and these reactions are catalyzed by GST (Rahman et al., 1999). The present study was aimed to enhance the effect of mGST by addition of reduced glutathione (GSH) in mouse model of allergen induced airway inflammation.

**MATERIALS AND METHODS**

*Animal study protocol:* Female Balb/c mice of (6-8 weeks), weighing 18-20 grams were obtained from National Institute of Nutrition, Pune, India. Mice were quarantined for 10 days to get acclimatized in experimental conditions. They were placed in cages at 22-25 °C, with 40-70% relative humidity and controlled 12-hour light: dark cycle. Water and standard chow diet were given. All animal experiments
were carried out in the morning to minimize the effects of circadian rhythm. Mice were randomly divided in 11 groups of six mice each. Group 1 mice were sensitized, challenged and treated with PBS as control. Group 2-11 mice were given i.p. injection of 10 µg OVA with 1 mg alum in 100 µl of PBS on days 1 and 14. The mice were subjected to airway challenge with OVA (4 µg/mice) on days 28, 29, 30. After 1h of the challenge, group 2 mice were treated with PBS (positive control) and group 3-11 were treated intranasally with different antioxidant(s) individually or in combinations (Figure3.1). The study protocol was approved by animal ethics committee of the Institute of Genomics and Integrative Biology, Delhi following guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals, Chennai, India.

**Collection of bronchoalveolar lavage fluid (BALF) and blood:** After inhalation treatment, the mice were sacrificed with an overdose of sodium pentobarbital on day 31 and tracheotomy was performed. Blood BALF and lungs were collected and processed as explained in chapter 2. Briefly, ice-cold PBS (0.5 ml) was instilled into the lungs and bronchoalveolar lavage fluid (BALF) was obtained by aspiration three times (total 1.5 ml) via tracheal cannulation. BALF was centrifuged, supernatant collected and stored at −70°C before use. The total inflammatory cell number was assessed by haemocytometer after excluding dead cells by staining with trypan blue. One hundred microlitre of BALF was spread on the slide, fixed and stained with Leishman for differential cell counts. Blood was collected, sera separated and used for analysis of serum immunoglobulins.

**Determination of immunoglobulins and cytokines by ELISA:** OVA-specific IgE, IgG1 and IgG2a were measured in serum by ELISA as described elsewhere (Chapter 2). Briefly, microtiter plates (Nunc, Denmark) were coated with 5 µg/ml of OVA. The plates were washed with PBS, blocked with 3% defatted milk and incubated with mice sera for IgE (1:10), IgG1 (1:50) and IgG2a (1:50), individually. The plates were washed with PBST followed by PBS and incubated with IgG1-peroxidase and IgG2a-peroxidase (1:1000 PBS; BD Pharmingen, San Diego, CA, USA). For IgE, biotinylated anti-mouse IgE (2 µg/ml, BD Pharmingen) was used, plate was washed
**Figure 3.1: Immunization protocol:** Mice were immunized with OVA on day 0 and 14 (i.p.). The mice were challenged intranasally on day 28, 29 and 30 with OVA and treated with antioxidant(s) / combinations 1h after each challenge. Mice were sacrificed on day 31 and blood, BALF and lungs were collected.
and incubated with streptavidin-peroxidase (1:1000; BD Pharmingen). Color was
developed using orthophenylene diamine (OPD) and absorbance read at 492 nm.

IL-4, IL-10 and IFN-γ levels were determined in BALF by ELISA using paired
antibodies according to manufacturer’s instruction (BD Pharmingen, San Diego). Briefly
capture antibody (1:250 v/v) for each cytokine was coated separately on microtitre plates,
incubated overnight and blocked with 10% fetal calf serum (FCS). BALF samples (1;2
v/v) were added to wells and incubated for 2h at 30°C. Biotinylated anti IL-4 or anti IL-
10 or anti IFN-γ were used to detect specific cytokine levels. The detection limit for IL-4
was 7.8 pg/ml and for IL-10 and IFN-γ, it was 31.3 pg/ml.

**Histopathology:** Lungs were fixed with 10% neutral-buffered formalin (pH-7.0) and
embedded in blocks containing paraffin. The sections of 4 μm were cut and stained with
hematoxylin and eosin (H & E) or periodic acid schiff (PAS). Twelve slides were made for
each type of staining (2 slides per mice) from every group and these were analyzed
using light microscope, for antigen-induced peribronchial and perivascular
inflammation. Coded (blinded) samples of lung tissues were made and given to a
pathologist. Semi-quantification of inflammation score was made on the scale of 0–4 for
perivascular accumulation of inflammatory cells followed by statistical analysis (Singh et
al., 2005). Lung inflammation score was calculated in terms of eosinophil infiltration and
mucus secretion. Score 0 was assigned to no or occasional inflammatory cells, score 1
with a few inflammatory cells, score 2 with scattered aggregates of inflammatory cells,
score 3 with thin layer of inflammatory cells surrounding the airways and vessels and
score 4 with a thick layer of inflammatory cells surrounding the airways and vessels.

**Oxidative stress markers in BALF:** Oxidative stress was determined by measuring
thiobarbituric acid reactive substance (TBARS) concentration spectrophotometrically
(Koca et al., 2005). Malanodialdehyde (MDA) and thiobarbituric acid (TBA) react to form
a product with maximum absorption at 532 nm. BALF (200 μl) was mixed with 500 μl of
10% w/v trichloroacetic acid to precipitate the protein. The precipitated protein was
pelleted by centrifugation at 10,000xg at 4°C for 15min. The collected supernatant was
collected and treated with 0.67%TBA in boiling water for 15 min. The different
concentrations of malanodialdehyde (Sigma, USA) diluted in saline is taken as standard and incubated with 0.67% TBA in boiling water. The mixture was cooled and centrifuged at 1500xg at 25°C for 10 minutes. The supernatant was collected and absorbance was recorded at 532 nm. The malanodialdehyde equivalents in the samples were calculated from the malanodialdehyde standards.

The concentration of 8-isoprostanes was measured using 8-Isoprostane EIA kit (Cayman chemical Company, Ann Arbor, MI) as per manufacturer’s instruction. Briefly, plates were pre-coated with mouse monoclonal antibody and blocked with defatted milk (Bio-Rad, USA). After washing, 50µl of standard/ BALF sample was added to the corresponding wells and incubated for 18h at 4ºC. The wells were washed five times with wash buffer. Eliman’s reagent (200 µl) was added to each well and kept in dark for 90-120 minutes for color development. The plates were read at 420 nm and the blank was subtracted. The concentration of 8-isoprostanes was calculated from standard curve plotted with standard samples.

**Nuclear extract preparation and NF- kB (p65) estimation:** Nuclear extract of lung tissues was prepared using NuCLEAR Extraction Kit (SIGMA, USA) following manufacturer’s instruction. PBS rinsed tissue (100mg) was suspended in 1 ml of lysis buffer containing dithiothreitol (DTT) and protease inhibitors. The tissue was homogenized and disrupted cell suspension was centrifuged at 10,000xg for 20 minutes. The pellet was resuspended in 150 µl of extraction buffer with DTT, protease inhibitor for 30 minutes and centrifuged for 5 minutes at 20,000xg. The supernatant was stored at -70°C as nuclear extract.

NF-kB (p65) transcription factor was determined in above fraction using an immunoassay kit (Cayman Chemical Company, USA). Complete transcription factor binding buffer (CTFB) was added 90 µl to each well of microtiter plate pre-coated with dsDNA consensus sequence containing the NF-kB response sequence. Ten microlitre of each sample was added to wells and incubated overnight at 4ºC without shaking. Wells were washed 5 times with wash buffer followed by addition of 100 µl of diluted NF-kB
(p65) antibody and incubated for 1h at 25°C. After 5 times washing with wash buffer, 100 µl of secondary antibody was added per well and incubated for 1h at 25°C. One hundred microlitre of developing solution was added to each well, reaction was stopped after 30min and absorbance was read at 450nm. The blank and positive controls were taken as supplied with the kit.

**Statistical analysis:** Data was expressed as the mean±standard deviation. The data was analyzed using GraphPad Prism 5.00 (Graphpad software Inc. USA). The sample size for each parameter tested was six mice. Statistical significance was calculated by paired t-test to determine intra-group differences of means between untreated and treated mice. Probability of significance was assigned by non-parametric ANOVA using Kruskal-Wallis test. Each parameter was compared in all the groups using Dunn’s multiple comparison post test. P<0.05 was considered significant in all cases.

**RESULTS**

*Intranasal treatment with antioxidant(s) suppresses allergen induced inflammatory cells:* Analysis of BALF showed large number of total cells \((40.67±2.52) \times 10^4\) and eosinophils \((15.50±2.50) \times 10^4\) in OVA-challenged mouse than PBS control \((2.87±0.31) \times 10^4\) and \((1.31±0.34) \times 10^4\) (Figure 3.2). Intranasal administration of antioxidants or their combinations significantly reduced the cellular infiltration \((p<0.01)\). The combination of mGST+GSH treatment showed higher reduction in total cells \((6.31±0.91) \times 10^4\) and eosinophils \((5.41±1.90) \times 10^4\) infiltration as compared to mGST \((18.84±2.05) \times 10^4\) and \((7.41±1.00) \times 10^4\) and GSH group \((15.10±1.76) \times 10^4\) and \((5.41±1.90) \times 10^4\) group. The reduction in cellular infiltration was more with mGST+GSH treatment than α-lipoic acid \((6.31±0.91) \times 10^4\) and \((8.28±0.86) \times 10^4\), respectively, taken as standard antioxidant and other antioxidant or their combinations.

*Administration of antioxidants reduces OVA-specific IgE:* Specific IgE levels were elevated significantly in OVA immunized and challenged mice \((0.380±0.032 \text{ OD}_{492\text{nm}})\) than PBS control \((0.033±0.010 \text{ OD}_{492\text{nm}})\) (Figure 3.3). IgE levels decreased substantially
Figure 3.2: Total cell and eosinophil counts in BALF post treatment in OVA sensitized and challenged mice and PBS control: Total cell count was determined using haemocytometer. One hundred microlitre of BALF was spread on the slide, fixed and stained with Leishman for eosinophil count. Data are presented as mean±SD. n=6 mice per group.
Figure 3.3: Serum immunoglobulin levels after intranasal treatment with different antioxidants in OVA sensitized and challenged mice. OVA-specific IgE, IgG1 and IgG2a were measured in serum by ELISA. Microtiter plates were coated with OVA, incubated with mice sera for IgE (1:10), IgG1 (1:50) and detected using respective secondary antibodies separately. Data is presented as mean±SD. n=6 mice per group.
(0.262±0.010 to 0.213±0.023 OD492nm) in different treatment groups (individual antioxidants or in combinations) as compared to OVA immunized PBS treated mice. The reduction in IgE levels was significant (0.213±0.023 OD492nm) post treatment with mGST+GSH since it has reached to nearly half the value of OVA challenged-PBS treated mice. IgG1 levels however did not change on treatment with different antioxidant(s) or combinations. Specific IgG2a levels were below detectable level in all treatment groups.

**Antioxidant(s) treatment reduces Th2 cytokines in BALF**: IL-4 levels in BALF of OVA immunized and challenged mice was 112.00±6.00 pg/ml whereas in PBS control it was below detection limit (7.8 pg/ml). Individual antioxidant treatment with mGST or GSH reduced IL-4 level to 52.77±4.90 pg/ml and 37.30±3.34 pg/ml, respectively whereas mGST+GSH in combination decreased IL-4 level to one sixth (18.67±1.52 pg/ml) to that of the OVA challenged-PBS treated mice. Treatment with mGST+GSH combination showed maximum reduction in IL-4 level than individual antioxidant(s) and their combinations (Figure 3.4). The reduction in IL-4 level by treatment with mGST+GSH was comparable with α-lipoic acid (21.33±2.08 pg/ml). IL-10 and IFN-γ level did not change post treatment in all the groups (Figure 3.4).

**Individual or combination of antioxidant(s) reduces airway inflammation**: OVA challenged mice showed numerous eosinophils into the lung interstitium around airways and blood vessels along with narrowing of airway lumen (Figure 3.5a). Mice treated with individual antioxidant(s) or their combinations showed substantial reduction in cellular infiltration in airways as compared to OVA challenged PBS treated mice, score-4 (Figure 3.5a). mGST+GSH treatment showed maximum reduction in cellular infiltration as compared to the individual antioxidant(s) or other combinations. Reduction in cellular infiltration in mGST+GSH and α-lipoic acid treated mice was similar with inflammation score-1, whereas it ranged from score 1.5 to 3.5 in other treatment groups.
In PBS control group, a few PAS stained epithelial cells were observed (Score-0). Contrary to this, epithelial cells were enlarged in OVA challenged mice, along with airway narrowing, mucus plugs and increased goblet cell hyperplasia (Figure 3.5b). The treatment with mGST+GSH has decreased mucus production and goblet cell hyperplasia better than other antioxidant(s) or their combination.

**mGST and GSH combination reduces oxidative stress significantly:** Oxidative stress level in BALF was measured in terms of lipid peroxidation (TBARS) and 8-isoprostanes level. OVA challenged mice showed elevated TBARS level of 3.25±0.34 pM/µl. mGST and GSH administration could reduce this level to 2.07±0.33 pM/µl and 2.13±0.23 pM/µl, respectively. mGST+GSH treatment showed significantly higher reduction in TBARS level (1.33±0.27 pM/µl) in BALF (p < 0.01) than individual or combinations of antioxidant(s). Reduction in TBARS level with mGST+GSH treatment was comparable to α-lipoic acid (1.39±0.07 pM/µl) taken as standard antioxidant (Figure 3.6a).

The level of 8-isoprostanes, a potent pulmonary vasoconstrictor was significantly raised in OVA immunized and challenged mice (82.79±4.58 pg/ml) compared to PBS control (22.55±2.40 pg/ml) (Figure 3.6b). The level of 8-isoprostanes reduced substantially after treatment with mGST or GSH, individually (61.82±3.59 pg/ml, 56.65±5.33 pg/ml, respectively). Its level reduced to almost half in mGST+GSH treated group (44.53±6.78 pg/ml) than OVA immunized and challenged mice, whereas other combinations or individual antioxidant(s) have showed higher levels of 8-isoprostanes. The reduction in 8-isoprostanes level with mGST+GSH treatment was comparable to α-lipoic acid (48.80±4.28 pg/ml).

**mGST with GSH seems most effective in reducing NF-κB (p65) level in lung’s nuclear extract:** OVA immunized and challenged mice showed significantly raised NF-κB (p65) level (0.404± 0.037 OD_{490nm}) in lung’s nuclear extract than PBS control (0.161± 0.02 OD_{490nm}). Intranasal administration of different antioxidant(s) or combinations
significantly reduced the NF-κB (p65) level in nuclear extract of lung tissues (Figure 3.7). Here mGST+GSH treatment showed two fold reduction in NF-κB level (0.196±0.04 OD490nm) to that of ovalbumin challenged PBS treated mice. Mice treated with mGST and GSH alone showed comparatively less reduction in NF-κB level (0.315±0.038 and 0.255±0.052, OD490nm, respectively) than treatment with mGST+GSH in combination. NF-κB (p65) level was nearly half in post treatment with mGST+GSH or α-lipoic acid (0.188± 0.02 OD490nm).
Figure 3.4: Cytokine profile in BALF of different antioxidant treatment group mice. IL-4, IL-10 and IFN-γ were determined by ELISA. Capture antibody (1:250 v/v) for each cytokine was coated on microtiter plate, blocked, incubated with BALF samples (1:2 v/v) and detected using biotinylated anti IL-4 or anti IL-10 or anti IFN-γ. Data is presented as mean±SD. n=6 mice per group.
Figure. 3.5a: Hematoxylin and Eosin (H&E) stained sections of the lungs showing cellular infiltration in different treatment groups. OVA and PBS control. Lungs were fixed with formalin, embedded in paraffin, sections cut and stained with H&E. (a) PBS control; (b) OVA challenged-PBS treated; (c) mGST treated; (d) GSH treated; (e) α-tocopherol; (f) mGST+α-lipoic acid; (g) mGST+α-tocopherol; (h) GSH+α-lipoic acid; (i) GSH+α-tocopherol; (j) mGST+GSH treated; and (k) α-lipoic acid treated (l) Inflammation Score.
Figure 3.5b: PAS stained sections of the lungs showing mucus secretion and goblet cell hyperplasia in different treatment groups, OVA and PBS control. Lungs were fixed with formalin, embedded in paraffin, sections cut and stained with PAS. (a) PBS control; (b) OVA challenged-PBS treated; (c) mGST treated; (d) GSH treated; (e) α-tocopherol; (f) mGST+α-lipoic acid; (g) mGST+α-tocopherol; (h) GSH+α-lipoic acid; (i) GSH+α-tocopherol; (j) mGST+GSH treated; and (k) α-lipoic acid treated (l) Inflammation Score.
Figure 3.6a: Oxidative stress in BALF of different treatment groups, OVA and PBS control: Oxidative stress in BALF was determined by measuring thiobarbituric acid reactive substance (TBARS) concentration, spectrophotometrically. Data is presented as mean±SD. n=6 mice per group. (b): 8-isoprostanines in BALF of different treatment groups, OVA and PBS control: 8-Isoprostanines a potent pulmonary vasoconstrictor was determined using EIA kit (Cayman Chemical Company, USA). Data is presented as mean±SD. n=6 mice per group.
Figure 3.7: NF-kB level in lung nuclear extract of different treatment groups, OVA and PBS control: NF-κB (p65) levels in nuclear extract of lung tissues was determined by ELISA kit (Cayman Chemical Company, USA). Data are presented as mean±SD. N=6 mice per group.
DISCUSSION

Studies suggest that asthmatic patients have enhanced production of reactive oxygen species in blood and its level correlate with severity of the disease (Cluzel et al., 1987; Chanez et al., 1990; Vachier et al., 1992,1994). Furthermore, concentration of natural antioxidants including GST, glutathione peroxidase, superoxide dismutase, glutathione, vitamin C and E, are reduced in the blood cells, plasma or BALF in asthmatic patients (Nadeem et al., 2003). There are increased levels of oxidized glutathione in BALF and increased nitric oxide concentration in exhaled air (Nadeem et al., 2003). GSH is important antioxidant for the detoxification of xenobotics catalyzed by GST to reduce ROS. Previously, mGST had shown limited benefit compared to α-lipoic acid in improving oxidative stress and Th2 response (chapter 2). In the present study, combination of mGST and GSH has been investigated for reducing oxidative stress and airway inflammation in mouse model. Individual antioxidant(s) and their random combinations have also been evaluated for comparison.

Cellular infiltration is the hallmark of asthma and eosinophil is a central effector cell in inflamed asthmatic airways (Holgate and Roche, 1991; Duez et al., 2004). The inflammatory cells recruited to the asthmatic airways have an exceptional capacity for producing oxidants. Once recruited in the airspaces, inflammatory cells may become activated and generate reactive oxidants in response to various stimuli. Activated eosinophils, neutrophils, monocytes, and macrophages, and also resident cells such as bronchial epithelial cells, can generate oxidants (Barnes et al., 1990, 1998; Dworski, 2000; Henricks and Nijkamp,2001; Bowler and Crapo, 2002). Eosinophils possess several times greater capacity for generating oxidants than neutrophils and the EPO content of eosinophils is several times higher than that of MPO in neutrophils (Aldridge et al., 2002). Previous studies with different antioxidants supplemented through oral route have shown reduction in inflammatory cells and mucus secretion in lung tissues (Cho et al., 2004; Okamoto et al., 2006). In the present study, OVA challenged mice showed severe eosinophil infiltration in airways that reduced substantially on treatment with
antioxidant(s). Lung tissues of mGST+GSH treated mice exhibited maximum reduction in inflammatory cells than individual or other combination of antioxidants. The better therapeutic action of mGST+GSH combination may be attributed to synergistic effect of both the antioxidants, similar to natural system.

Inflammation and airway remodeling in allergic asthma are caused by the secretion of a series of Th2 or proinflammation cytokines. IL-4, a Th2 cytokine is required for differentiation of T cells and is a key factor for class switching to IgE in B cells (Finkelman et al., 1988). In the present study, repeated exposure of sensitized mice with OVA caused significant increase in IL-4 and IgE than PBS control. Intranasal administration of mGST+GSH could effectively reduce serum IgE and IL-4 levels in BALF than individual or combination of antioxidants. Zheng et al (1999) reported that higher doses of vitamin E supplementation suppress serum IgE in nasal allergy mice model. Both IL-4 and IL-5 reduced significantly in the BALF of α-tocopherol supplemented mice (Okamoto et al., 2006). IL-5 and serum IgE were also decreased in probucol-supplemented mice, but no change was there in IL-4 levels.

Oxidative stress is an important consequence of the inflammatory response in asthma (Nadeem et al., 2003, 2008). Several markers of oxidative stress and a wide range of antioxidants are reported to be altered in asthma (Shanmugasundaram et al., 2001; Corradi et al., 2003a, 2003b; Nadeem et al., 2003, 2008). ROS-generation is enhanced in bronchoalveolar lavage cells of stable asthmatics and increases on antigen challenge (Sanders et al., 1995; Kelly et al., 1988). Biological specimens contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. TBARS return to normal levels over a period of time, depending upon the presence of antioxidants (Armstrong and Browne, 1994). 8-isoprostane (8-iso PGF2α) are potent pulmonary and renal vasoconstrictors and are implicated as a mediator of pulmonary oxygen toxicity (Banerjee et al., 1992; Vacchiano and Tempel, 1994). 8-isoprostanes have been proposed as a marker of antioxidant deficiency and oxidative stress and elevated levels have been found in heavy smokers (Morrow et al., 1995). In the present
study, OVA immunization and challenge increased oxidative stress markers namely TBARS and 8-isoprostanones that reduced significantly on intranasal treatment with mGST+GSH in combination than individual or combination of other antioxidants.

Oxidants, either inhaled or produced by inflammatory cells, are involved in the inflammatory responses in lung cells via signalling mechanisms. Transcription factors NF-kB is redox-sensitive and demonstrated to be activated in epithelial cells and inflammatory cells during oxidative stress/inflammation, leading to the upregulation of a number of pro-inflammatory genes (Li and Karin, 1999; Janssen-Heininger et al., 1999). Thus NF-kB is increased in asthmatic airways (Hart et al., 1998). It is activated by cytokines, radiation and oxidative stress (Van den Berg et al., 2001). Recent studies have provided some insight for NF-kB activation by cytokines (Van den Berg et al., 2001) but role of oxidative stress is still not established. In the present study, mGST+GSH reduced NF-kB levels most effectively in the lung tissues of mice than other antioxidants(s) individually or in combination. A previous study had also shown that α-lipoic acid effectively suppressed NF-kB activity in lung tissues of mice (Cho et al., 2004).

Lungs are directly exposed to the ROS and may reduce oxidant burden by increasing GSH or its dependent antioxidative enzymes (Rahman et al., 1999). Exogenous addition of catalytic and non-catalytic antioxidants may take care of oxidative burden. Earlier supplementation of single antioxidant has shown change in the ROS status (Cho et al., 2004; Chapter 2). In the present study, both substrate and enzyme that can modify the antioxidant capacity of the lungs have been used successfully to attenuate oxidative stress and airway inflammation. GSH alone could not work effectively may be because of its inefficient cellular uptake. In combination, mGST has enhanced the effect of GSH.

Antioxidant augmentation and/or dietary supplementation have been suggested as an adjunct therapy for management of asthma (Okamoto et al., 2006). In the present study, intranasal administration of mGST and GSH being complementary antioxidants in combination proved most effective in reducing oxidative stress and subsequent airway inflammation. The results suggest that the combination of these two antioxidants may
offer therapeutic benefit in asthma. Allergic disorders like asthma and rhinitis are multifactorial and blocking oxidative stress alone cannot lead towards the complete resolution of the disease. The proper knowledge of the mechanisms involved in regulation of endogenous and exogenous antioxidants may evolve new therapeutic strategies in the treatment of airway inflammatory diseases including asthma.

In conclusion, asthmatic inflammation is characterized by ongoing inflammation and accompanied by increased oxidative stress and subsequent lung injury. ROS generation through endogenous mechanisms or exogenous environmental exposure is critical to the inflammatory response through perpetuation and amplification of pro-inflammatory signaling pathways. At the same time, endogenous antioxidant mechanisms are present to counteract this ROS-mediated inflammatory response. It is when these two opposing mechanisms are out of balance that a chronic inflammatory state emerges. Modulation of these events by enhancing antioxidant levels offers unique opportunities for therapeutic prevention or inhibition of inflammation. Before this approach becomes reality, additional research is needed to better understand not only the molecular events involved in the pathogenesis of asthmatic inflammation, but also the complex actions and interplay between antioxidants and ROS in both the healthy and the asthmatic lung.

A better understanding of these issues would provide useful information in designing more appropriate antioxidant-based treatments and intervention trials in the future. These trials could be successful only if they show that supplemented antioxidant can: (i) be effectively delivered (bioavailable) to the sites of inflammation in the lung; (b) prevent deleterious oxidations; and (c) most importantly, be useful in the prevention and/or treatment of the clinical manifestations of asthma. In the same effort we found mGST in combination with GSH has synergistic effect in reducing airway inflammation as compared to other antioxidants and has potential for the treatment of asthma.