Chapter 4

To evaluate the anti-oxidant activity of choline in mouse model of allergic airway inflammation
INTRODUCTION

Asthma is a complex disorder characterized by AHR, pulmonary obstruction and persistent airway inflammation (Elias et al., 2003). Although the inflammatory response is important for the initiation of tissue repair, the exaggerated response associated with asthma results in excessive oxidant formation and tissue destruction (Comhair et al., 2005a). Airways are unique in exposure to high levels of environmental oxidants and their unusually high concentration of extracellular antioxidants. In the resting state, the balance between anti-oxidants and oxidants is sufficient to prevent the disruption of normal physiologic functions; however, either increase in oxidants or decrease in antioxidants can disrupt this balance (Halliwell and Gutteridge, 1999). This imbalance between oxidants and anti-oxidant forces leads to an increase in ongoing cycle of inflammation in the asthmatic airways that ultimately contributes to airway injury (Comhair et al., 2005b). The state of imbalance is collectively referred to as oxidative stress and is associated with diverse airway pathologies (Halliwell and Gutteridge, 1999). 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). The major oxidants in airways are reactive oxygen and nitrogen species (ROS/RNS). ROS includes superoxide, hydrogen peroxide, hypochlorous acid and hydroxyl radicals (Reynaert et al., 2007). RNS include nitric oxide and its derivatives such as nitrogen dioxide and peroxynitrite (Ricciardolo et al., 2006). Other molecules that can contribute to oxidative stress include protein radicals and lipid peroxide radicals. Although these molecules collectively cause non-specific damage to cells and extracellular matrix when produced in excess, for e.g., superoxide is a crucial component of phagocytosis (Otonello et al., 1995) and nitric oxide mediates smooth muscle relaxation in blood vessels (Palmer et al., 1987) as well as airways (Dupuy et al., 1992).

ROS are diffused throughout the lung and are a byproduct of normal metabolism. Mitochondria are the largest producer of ROS because electrons leaked from the electron transport chain onto oxygen to form superoxide. It is estimated that 1-3% of O$_2$ reduced in cells may form superoxide in this manner (Halliwell and Gutteridge, 1999). Other sources of superoxide include cytosolic xanthine oxidase (Quinlan et al., 1997), mitochondrial respiration, and membrane nicotinamide adenine dinucleotide phosphate oxidases such as the cytochrome P450 system of the endoplasmic reticulum (Goeptar et al., 1995). Hydrogen peroxide is formed during the
dismutation of superoxide and also by glycolate oxidase in peroxisomes. Hydroxyl radical classically formed in the presence of metals and hydrogen peroxide and decomposition from other molecules like peroxynitrite may play a small role (Merenyi et al., 1998). Unlike nitric oxide, there are no well-described independent signaling roles for superoxide in the lung.

RNS are primarily derived from nitric oxide. In the resting state, nitric oxide is considered a signaling molecule. The sources of nitric oxide are three nitric oxide synthases (NOSs): (Halliwell and Gutteridge, 1999) constitutive NOS, found in respiratory epithelium, blood vessels, and nerve endings; (Ottonello et al., 1995) inducible NOS, found in respiratory epithelium and activated macrophages (Palmer et al., 1987); and neuronal NOS, found in the nerve plexus of the trachea (Guembe and villaro, 1999). When nitric oxide is produced in high concentrations, such as with inducible NOS, it can react with oxygen or superoxide to form the highly reactive compounds namely nitrogen dioxide and peroxynitrite. Only inducible NOS are highly upregulated by cytokines such as TNF-α and interleukin-1β (Radomski et al., 1993). Location determines the function of nitric oxide in the lung. In pulmonary vessels, nitric oxide is a vasodilator. In airway muscles, nitric oxide functions as a bronchodilator, and in airway epithelium, nitric oxide modulates the immune response.

Antioxidants are the primary defense against ROS/RNS. The antioxidant effect can be either enzymatic or non-enzymatic. Antioxidant enzymes include the families of superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione S-transferase, and thioredoxin. The non-enzymatic antioxidants include low molecular weight compounds such as glutathione, ascorbate, urate, α-tocopherol, bilirubin and lipoic acid. Concentrations of these antioxidants vary depending on both the subcellular and anatomic location. For instance, 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.
In many airway diseases, the balance between ROS/RNS and antioxidants is disrupted because of excess production of ROS/RNS or depletion of antioxidants. For instance, in the airways of patients with asthma, ROS/RNS react with proteins to form amino acid adducts such as nitrotyrosine or chlorotyrosine; with lipids to generate ethane and isoprostanones and with DNA to form base pair adducts such as 8-oxo-2-deoxyguanosine. In diseases such as asthma and cystic fibrosis, inflammation may be the most significant contributor to oxidative stress. Studies have shown that the footprints of oxidative stress correlate with disease activity (Horvath et al., 1998, Montuschi et al., 2000a). ROS generation is associated with the pathogenesis of asthma and increased levels correlate with the disease severity, AHR, lipid peroxidation, production of chemoattractants and enhance vascular permeability and initiate Th2 cytokine release (Barnes, 1990; Crapo, 2003; Nadeem et al., 2003;).

A production of oxidants is regulated by a well coordinated and efficient endogenous antioxidants defense system. But individuals with asthma demonstrate diminished levels of ascorbate and α-tocopherol, suggesting both increased ROS/reactive nitrogen species and decreased oxidative capacity (Kelly et al., 1999). There has been considerable interest in the possible modulation of asthma by supplementation of anti-oxidants e.g. hydrophilic and lipophilic antioxidants like vitamin C and E, respectively (Bowler and Crapo, 2002). However, these oral supplements, have failed to demonstrate overall convincing beneficial effects in asthma (Caramori and Papi, 2004; Hubbard and Fogarty, 2004). Previously, a study indicated that higher levels of serum antioxidants such as vitamin C and β-carotene were associated with low risk of asthma (Rubin et al., 2004). Diet increasingly deficient in anti-oxidants has increased population susceptibility with consequent large increases in disease prevalence (Seaton, et al., 1994). Along with this, there is an increase in prevalence of allergic rhinitis, despite of no increase in pollen levels (Seaton et al., 1996).

Epidemiologic studies have reported association between antioxidants and lipid intake and asthma or atopic disease (Devereux and Seaton, 2005). Dietary saturated fat intake has been reported positively associated with bronchial hyperresponsiveness in asthma and in women with atopic sensitization and hay fever (Soutar et al., 1997; Huang and Pan, 2001; Trak-Fellermeier et al., 2004). Several cross-sectional studies have reported beneficial associations between dietary fish
intake, asthma and atopic disease (Hodge et al., 1996; Dunder et al., 2001). Investigations in asthma and dietary lipids suggest that asthma and atopy are a consequence of increasing n-6 polyunsaturated fatty acids and decreasing n-3 polyunsaturated fatty acids consumption that effect on inflammatory mediators and Th cell differentiation (Devereux and Seaton, 2005). Choline, a lipophilic agent, precursor to acetylcholine and cytidine 5-diphosphocholine showed anti-inflammatory activity in animal model of arthritis (Ganley et al., 1958). Its deficiency results in loss of membrane phosphatidyl choline (PC) and induction of apoptosis in vitro (Yen et al., 1999). Choline administration has shown lysophosphatidyl choline (LPC) lowering effect and symptomatic improvement in asthmatics (Szczeklik et al., 1990; Gaur et al., 1997). Choline is crucial during fetal development, when it influences stem cell proliferation and apoptosis, thereby altering brain structure and function (Loy et al., 1991; Albright et al., 1999; Craciunescu et al., 2003; Meck et al., 2003; Mellott et al., 2004). Diet deficient in choline leads to development of fatty liver (hepatosteatosis) and elevated serum amino-transferases (Albright et al., 1996; Buchman et al., 1995; Albright et al., 2005). Further, mice fed with choline deficient diet (CDD) showed increase in oxidative stress and altered antioxidant level (Grattagliano et al., 2000; Oliveira et al., 2003; Yoshida et al., 2006). The production of ROS was accentuated during severe non-alcoholic liver steatosis induced by CDD (Oliveira et al., 2002). In the present study, antioxidant effects of choline were investigated in mouse model of allergic airway disease.

MATERIALS AND METHODS

Sensitization and Treatment: Female Balb/c mice of 6-8 weeks were obtained from National Institute of Nutrition, Hyderabad (India). Mice were randomly divided into 5 groups of 10 mice each and inhabited in animal house at 25°C and 40-70% humidity on a 12 h light/dark schedule and with ad libitum access to water and standard diet. The mice were sensitized intraperitoneally (i.p.) with 100 µg ovalbumin (OVA) adsorbed on 2 mg of Al (OH)₃ in 100 µl of 0.9% NaCl (saline) on days 0 and 14. They were challenged with 2.5% aerosolized OVA (w/v in saline) in a plexiglass chamber using nebulizer (Omron, Tokyo, Japan) for 30 minutes on days 25-27. Mice were treated once daily just after immunization till last challenge on days 14-27 with 100 µl saline (group 2), 1 mg/kg of choline in 100 µl saline by oral gavage (group 3), 1 mg/kg of choline in 50 µl saline intranasal (i.n.; group 4) and 100 mg/kg of α-lipoic
acid by oral gavage (group 5). The control (group 1) mice were sensitized, challenged and treated with 100 µl of saline. After the last treatment/challenge, mice were sacrificed on day 28. Mice receiving i.n. dose were lightly anesthetized (3% isoflurane; i.n.) before each dose of treatment. α-lipoic acid (SRL Pvt. Ltd, Mumbai, India) was taken as standard antioxidant. The animal ethics committee of Institute of Genomics and Integrative Biology, Delhi approved the study protocol.

Collection of bronchoalveolar lavage (BAL) fluid and blood: After last airway challenge, mice were sacrificed using sodium pentobarbital (100 mg/kg i.p.). Blood was collected after cardiac puncture for total and differential cell counts. Sera were separated and used for analysis of serum immunoglobulins. The trachea was cannulated after the collection of blood. A 19 gauge needle was inserted into the trachea and lungs were lavaged three times with 0.5 ml of chilled saline and volume recovered was approximately 1.2 ml from each mouse. BAL fluid was centrifuged at 400 × g for 10 min at 4°C and supernatant was collected for analysis of cytokine and leukotriene levels and eosinophil peroxidase (EPO) activity. The cell pellets were resuspended in 1 ml of PBS and the total cell counts were determined using hemocytometer under light microscope and cell viability was determined by adding trypan blue (0.4 %). BAL cell smears were stained with Leishman’s stain and eosinophils were identified (%) by counting minimum of 200 cells in high magnification (× 400). Absolute eosinophils were calculated by multiplying total cell counts by percentage of eosinophil in BAL cell pellets.

Measurement of Intracellular ROS: Reactive oxygen species were measured by a method described previously (Lee et al., 2006). BAL cell pellets were washed with PBS and cells were incubated with 3.3 µM 2’’,7’’-dichlorofluorescein diacetate (DCFH-DA) in PBS for 10 min at 25°C in dark to label intracellular ROS. DCFH is a membrane impermeable and rapidly oxidized to the highly fluorescent 2’’,7’’-dichlorofluorescein in the presence of intracellular hydrogen peroxide and peroxidases (Szejda et al., 1984). The cells after incubation were washed with PBS and examined under fluorescence activated cell sorter (Guava Technologies, CA, USA) and the mean fluorescence intensity of 5,000 cells was analyzed in each sample and corrected for auto-fluorescence of unlabeled cells.

Determination of eosinophil peroxidase (EPO) activity: EPO levels in BAL fluid supernatant were determined as described previously (Tomkinson et al., 2001).
Briefly, 100 µl of substrate solution containing 0.1 mM o-phenylene-diamine-dihydrochloride (OPD); (Sigma), 0.1% Triton X-100, 1 mM hydrogen peroxide in 0.05 M Tris-HCl was added to 100 µl of BAL fluid supernatant in microtiter plates and incubated for 30 minutes at 37°C. Reaction was stopped by adding 50 µl of 4 M sulphuric acid and absorbance read at 492 nm.

**Cytosolic or nuclear protein extraction for analysis of NFκB:** Cytosolic or nuclear proteins were extracted using Celllytic™ NUCLEAR™ extraction kit (Sigma, St. Louis, MO, USA) following manufacturer’s instructions. Briefly, 100 mg of lung tissue from each sample was rinsed two times with PBS and homogenized in 1 ml of lysis buffer (50 mM Tris HCl, pH 7.5, 10 mM MgCl₂, 15 mM CaCl₂ and 1.5 M sucrose) containing 0.1 M of dithiothreitol (DTT) and protease inhibitor cocktails. The homogenates were centrifuged to obtain cytosolic fraction at 11,000 × g for 20 min at 4°C. The nuclear pellet obtained was resuspended and homogenized again in extraction buffer (20 mM HEPES, pH 7.9; 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, and 25% Glycerol) containing 0.1 M DTT and protease inhibitor cocktails with gentle stirring for 30 min and centrifuged at 21,000 × g for 5 min at 4°C. The resulting supernatant was used as soluble nuclear proteins for the determination of NFκB levels.

**Protein estimation:** Protein content of the cytosolic or nuclear proteins was determined by bicinchoninic acid assay (BCA) (Stoscheck, 1990). Bovine serum albumin (BSA) was used as reference standard for calibration [Appendix 4].

**Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE):** Cytosolic or nuclear proteins were subjected to SDS-PAGE on 12% resolving gel using discontinuous buffer (0.025 M Tris, 0.2 M glycine and 0.1% SDS) system (Laemmli, 1970). Thirty microgram of the extract was boiled for 5 min at 100°C in sample buffer containing β-mercaptoethanol and electrophoresed at a constant voltage of 120 V. The protein bands were detected by staining the gel with 0.1% coomassie brilliant blue R-250 [Appendix 5].

**Western blot:** SDS-PAGE separated cytosolic or nuclear proteins were electrophoretically transferred onto nitrocellulose membrane in Tris-glycine buffer (25mM Tris, 192 mM glycine, 20% methanol, pH 8.3) using a BIO-RAD transfer apparatus at 200 mA for 2.5 hours (Towbin et al., 1979) [Appendix 6]. The transferred proteins were checked by reversible staining with Ponceau S (0.1% in 1%
acetic acid). After washing with PBS, non-specific sites were blocked with 3% defatted milk in PBS for 2 hours at 37°C. The blots were washed with two to three changes of PBS and then incubated with 1: 250 v/v diluted rabbit polyclonal NFκB p65 (Santa Cruz, CA, USA) antibody for 2 hours at 25°C. The blots were then washed with two to three changes of PBS and probed with 1: 500 v/v diluted anti-rabbit-IgG conjugated with horse radish peroxidase (HRP) (Sigma) for 2 h at 37°C. The unbound antibodies were removed by washing three times with PBS. The NFκB p65 reactive bands were visualized with 3'-3'-diaminobenzidine (DAB) and hydrogen peroxide in 0.05 M sodium acetate buffer, pH 5.0. The reaction was stopped by washing the blots with distilled water.

**Analysis of lipid peroxidation:** The amount of lipid peroxidation was assessed by measuring the thiobarbituric acid reactive substances (TBARS). Malanodialdehyde (MDA) and thiobarbituric acid (TBA) react to form a product with maximum absorption at 532 nm. Briefly, BAL fluid was mixed with 10% trichloroacetic acid to precipitate the protein. The precipitate was separated by centrifugation at 10,000 × g for 15 min at 4°C and the supernatant collected was treated with 0.67% TBA in 100°C for 15 min. Absorbance was measured at 532 nm after cooling and compared with standard malanodialdehyde (Koca et al., 2005).

**Measurement of 8-isoprostanes (8-iso PGF$_2$α):** BAL fluid samples were assayed in duplicates for 8-isoprostanes using enzyme immunoassay kits (Cayman Chemical) following manufacturer’s instructions. Briefly, 50 µl of standards (8 serial dilutions) and undiluted BAL fluid were added to the wells in duplicates, followed by adding 50 µl of 8-isoprostanes tracer and 8-isoprostanes antiserum into each well and incubated at 4°C for 18 h. After washing with wash buffer, substrate was added and plate was incubated in dark at 25°C for 90-120 min with gentle shaking. The absorbance was read periodically at 420 nm until the maximum binding wells reached a minimum of 0.3 A.U. The detection limit for 8-isoprostanes was 2.7 pg/ml.

**Histopathology:** Lungs were inflated and fixed with 10% neutral-buffered formalin. After fixation, lung sections of 4 µm were cut and stained with hematoxylin and eosin (H & E) for histological evaluation, monitoring of inflammatory cells and mucus production. Sections were scanned using light microscope for antigen-induced peribronchial and perivascular inflammation. The eosinophils or lymphocytes infiltration around the airways in the bronchial epithelium were quantified and graded
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as, 0: not present; 1: very slight, 2: slight, 3: moderate, 4: moderate to marked and 5: marked. An inflammatory reaction affecting <20% of the airways was defined as ‘1’, ‘2’ was defined as 20-40% of the airways affected, ‘3’ 40-60%, ‘4’ 60-80% and ‘5’: >80% of the airways affected (Inoue et al., 2007).

**Splenocyte culture:** For *in-vitro* experiments, spleen from the sacrificed mice were collected aseptically and single-cell suspensions were prepared in complete Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Sigma, St. Louis, MO, USA), supplemented with 10 % fetal bovine serum (FBS; Invitrogen, NY, USA), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin and 100 µg/ml streptomycin [Appendix 1]. Cell viability was determined using the trypan blue exclusion test [Appendix 2]. Splenocytes (5 ×10⁶ cells/ml) from mice immunized with OVA were cultured and stimulated with 100 µg/ml OVA. Splenocytes stimulated with phytohaemagglutinin (PHA; 5 µg/ml) were taken as positive control while unstimulated cells were used for basal proliferation. Cells were cultured in triplicates for 72 hours at 37°C in a CO₂ incubator and the culture supernatant was collected and stored at -70°C for further analysis.

**Determination of specific antibody levels by ELISA:** OVA-specific serum IgE, IgG1 and IgG2a titres were measured in duplicates by ELISA [Appendix 3]. Briefly, microtiter plates (Nunc-Immuno™ modules, Roskilde, Denmark) were coated with 5 µg/ml of OVA in 0.1 M carbonate buffer (pH 9.6) in separate wells and incubated overnight at 4°C. The plates were washed with PBS and blocked with 3% defatted milk for 3 hours at 37°C. The plates were washed with PBS and mice sera diluted to 1:10 (for IgE) and 1:50 (for IgG1 and IgG2a) was added 100 µl/well and incubated overnight at 4°C. Pre-immune sera was also added and processed simultaneously. After washing with PBST (0.05% Tween-20) and PBS, the plates were incubated for 3 hours at 37°C 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 incubated at 25°C for 90 minutes. Following washing, it was incubated with streptavidin-peroxidase (1:1000; BD Pharmingen) for 30 minutes. The plates were washed with PBST and PBS and developed using OPD and absorbance read at 492 nm.

**Determination of cytokine levels in BAL fluid and culture supernatant by ELISA:** Cytokines IL-4, IL-5, IL-13, TNF-α and IFN-γ were determined in BAL
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fluid and spleen cell culture supernatants by ELISA following manufacturer’s instructions (BD Pharmingen, USA and R & D, MN, USA). Briefly, capture antibody (100 µl; 1:250 v/v) for each cytokine was coated separately in the microtiter plates in carbonate buffer, pH 9.6 and incubated overnight at 4°C. After washing, the wells were blocked with PBS containing 10% FBS at 25°C for 1 h. After washing, 100 µl of standards (7 serial dilutions) and undiluted BAL fluid and culture supernatants were added to the wells in duplicates and incubated at 25°C for 3 h. The plates were washed again and incubated with biotinylated detector antibody labeled with avidin-HRP at 25°C for 1 h. After washing with PBST and PBS, substrate was added and plates were incubated in dark at 37°C for 30 min. The reaction was stopped with 2 N H₂SO₄ and absorbance was read at 450 nm. The cytokine concentrations were calibrated from the standards.

**Determination of leukotriene levels in BAL fluid and culture supernatant by ELISA:** Leukotriene (LT) B4 and cysteinyl leukotriene (Cys-LT) were determined in BAL fluid and spleen cell culture supernatants using enzyme immunoassay kit (Cayman Chemical Co. MI, USA) according to manufacturer’s instructions. Briefly, 50 µl of standards (8 serial dilutions) and undiluted BAL fluid and culture supernatants were added to the wells in duplicates, followed by 50 µl of tracer and antiserum of respective leukotrienes into each well and incubated at 25°C for 18 h. After washing with wash buffer, substrate was added and plates were incubated in dark at 25°C for 90-120 mins with gentle shaking. The absorbance was read periodically at 420 nm until the maximum binding wells have reached a minimum of 0.3 A.U. The detection limit for LTB4 and Cys-LT was 13 pg/ml.

**Statistical analysis:** Groups were analyzed using one-way analysis of variance followed by Dunnett’s multiple comparison tests to examine difference between OVA challenged saline treated and choline treated groups. Difference was considered significant at p < 0.05. Data are presented as mean ± SD for each group. The software package GraphPad Instat was used for data analysis.
RESULTS

Out of 10 mice in each group, 6 mice were taken from each group to sacrifice and data expressed as means of 6 mice per group.

**Choline suppresses intracellular ROS production:** There was enhanced production of ROS in BAL cells of OVA sensitized and challenged mice after last challenge compared to saline control mice (Figure 4.1). The ROS production reduced significantly by choline treatment in oral gavage group (p < 0.05). Intranasal choline treatment also suppressed ROS production significantly as compared to OVA challenged saline treated mice (p < 0.05). The treatment with \(\alpha\)-lipoic acid decreased intracellular ROS levels significantly in BAL cells (p < 0.05).

**EPO activity in choline treated mice:** The OVA-induced increase in eosinophil count was accompanied with a significant rise in EPO activity in the cell-free BAL fluid. There was approximately fourfold increase in EPO activity in OVA-challenged group as compared to saline control group (Figure 4.2). The choline treatment by both the routes significantly inhibited OVA-induced EPO activity (p < 0.05) and inflammation in the airways. Treatment with \(\alpha\)-lipoic acid also reduced the EPO activity significantly (p < 0.05) than OVA challenged saline treated group.

**Effect of choline in lipid peroxidation:** Antioxidant levels in BAL fluid were measured in terms of lipid peroxidation. MDA is an end product of the oxidation and decomposition of polyunsaturated fatty acids containing three or more double bonds. MDA is derived from TBA to form the MDA-TBA adduct. This adduct is most commonly quantified using a spectrophotometric assay (Pincemail et al., 1996). Choline treatment by either route reduced the lipid peroxidation levels significantly as compared to OVA challenge saline treated mice (Figure 4.3; p < 0.05). Treatment with \(\alpha\)-lipoic acid reduced TBARS level substantially.

**Effect of choline on 8-isoprostane levels:** The 8-isoprostanes levels were measured in BAL fluid of different group of mice as a biomarker of lipid peroxidation. Isoprostanes can be found in all normal animal and human biological fluids and esterified in normal animal tissues. But during oxidant injury, isoprostanes levels are increased remarkably. After last OVA challenge, isoprostanes level was significantly elevated in OVA challenged saline treated group than in saline control mice (Figure 4.4). However, choline treatment by either route significantly reduced the level of isoprostanes (p < 0.05). Treatment with \(\alpha\)-lipoic acid also reduced isoprostanes significantly.
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Figure 4.1: Effect of choline treatment on relative ratio of reactive oxygen species levels in bronchoalveolar lavage (BAL) fluid in different groups of mice. Normal Saline control; ovalbumin sensitized, challenged and saline treated mice; ovalbumin sensitized, challenged and oral choline treated mice; ovalbumin sensitized, challenged and i.n. choline treated mice; ovalbumin sensitized, challenged and α-lipoic acid treated mice. Bars present ± SD from 6 mice per group. *, p < 0.05 (versus ovalbumin sensitized and challenged mice).

Figure 4.2: Eosinophil peroxidase activity in BAL fluid after choline treatment. Bars present ± SD from 6 mice per group. *, p < 0.05 (versus ovalbumin sensitized and challenged mice).
Figure 4.3: Oxidative stress marker in BAL fluid in terms of TBARS level. Effect of choline treatment on TBARS level in BAL fluid. Bars present ± SD from 6 mice per group. *, p < 0.05 (versus ovalbumin sensitized and challenged mice).

Figure 4.4: Level of isoprostanes in BAL fluid of mice after choline treatment. Bars present ± SD from 6 mice per group. *, p < 0.05 (versus ovalbumin sensitized and challenged mice).
Effect of choline on NFκB p65 protein level in lung tissues: NFκB p65 protein expression was measured after last OVA challenge in nuclear and cytosolic extracts from lung tissues. Western blot analysis revealed increased levels of NFκB p65 in nuclear protein extracts of lung tissues from saline treated OVA challenged mice (Figure 4.5A; lane 2). The increased NFκB p65 protein expression was inhibited after choline treatment by either routes (Figure 4.5A; lane 3 and 4). In contrast, cytosolic NFκB p65 from lung tissues were decreased after last ovalbumin challenge as compared to saline control (Figure 4.5B; lane 2). The decreased NFκB p65 levels in cytosolic protein extracts were increased by administration of choline (Figure 4.5B; lane 3 and 4). These results indicate that choline inhibits NFκB p65 activity by preventing translocation of NFκB p65 from cytoplasm to nucleus. α-lipoic acid administration also showed similar effect.

Choline treatment reduces Th2 cytokine levels in BAL fluid and culture supernatant: Th2-dominant response produces oxidative stress in the airways, and it is thought to be one of the crucial components of asthma pathogenesis. After last OVA challenge, Th2 cytokine levels were markedly elevated in OVA challenged saline treated mice than in saline control mice (Figure 4.6A and 4.6B). IL-4, IL-5, and TNF-α levels reduced significantly in BAL fluid and in culture supernatants with choline treatment by oral and or i.n. route (p < 0.05). Intranasal choline treatment induced slight increase in IFN-γ level in spleen cells culture supernatant. IL-13 levels showed decrease by choline treatment but the reduction was not significant (Fig. 4.6B). The levels of IL-4 and IL-5 also reduced by α-lipoic acid treatment (p < 0.05). α-lipoic acid administration decreased IL-13 levels in spleen cell culture supernatant (p < 0.05).

Choline suppresses allergen induced eosinophilic airway inflammation: Bronchoalveolar lavage fluid was collected 24 hours after OVA aerosol challenge, and total and eosinophil cell counts in BAL fluid were analyzed. OVA challenge significantly increased total cell numbers in BAL as compared to saline control group (p < 0.05). Eosinophils counts in BAL were also significantly high as compared to saline control group (p < 0.05). A significant reduction in BAL total cell counts and eosinophils was observed on choline treatment orally (p < 0.05) or i.n (p < 0.05) as compared to OVA challenged saline treated group (Figure 4.7). Treatment with α-lipoic acid also led to significant reduction in eosinophils numbers (p < 0.05) in BAL.
Figure 4.5: Effect of choline on NFκB p65 protein level in lung tissues. Data representative of 6 mice per group. A) Nuclear extract and B) Cytoplasmic extract. Lane 1, normal mice sensitized, challenged and treated with saline; Lane 2, OVA-challenged saline treated mice; Lane 3, OVA-challenged mice treated with oral choline; Lane 4, OVA-challenged mice treated with i.n. choline and Lane 5, OVA-challenged mice treated with α-lipoic acid.

Figure 4.6A: Effect of choline administration on cytokine concentrations (IL-4, IL-5 & IFN-γ) in BAL fluid (BALF) and splenocyte culture supernatant of OVA-sensitized and challenged mice. Bars present ± SD from 6 mice per group. *, p < 0.05 (versus ovalbumin sensitized and challenged mice).
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Figure 4.6B: Effect of choline administration on cytokine concentrations (TNF-α & IL-13) in BAL fluid (BALF) and splenocyte culture supernatant of OVA-sensitized and challenged mice. Bars present ± SD from 6 mice per group. *, p < 0.05 (versus ovalbumin sensitized and challenged mice).

Figure 4.7: Effect of choline treatment on total leukocyte counts (TLC) and eosinophil counts in BAL. Bars present ± SD from 6 mice per group. *, p < 0.05 (versus ovalbumin sensitized and challenged mice).
The recruitment of inflammatory cells in the airways was monitored in choline treated groups as compared to OVA challenged group (Figure 4.8). Lung tissue was collected 24 h after the last OVA challenge. In OVA challenged saline treated group, numerous eosinophils were observed into the lung interstitium around airways and blood vessels along with narrowing of airway lumen. Choline administration orally or \(i.n.\) significantly reduced the inflammatory infiltrates (\(p < 0.05\)). Mice treated with \(\alpha\)-lipoic acid also showed reduced inflammation and eosinophil infiltration in the lungs. These results were further confirmed by a reduction in total inflammation score in choline treated groups compared to OVA challenged group (\(p < 0.05\)).
Figure 4.8: Hematoxylin and eosin stained sections of mouse lungs. (A) Normal mice, saline sensitized and challenged, (B) OVA-challenged saline treated mice, (C) OVA-challenged mice treated with oral choline, (D) OVA-challenged mice treated with i.n. choline and (E) OVA-challenged mice treated with alpha-lipoic acid. (F) Total inflammation score graded on a scale from 0-4. Bars present ± SD from 6 mice per group. *, p < 0.05 versus ovalbumin sensitized and challenged mice. Scale = 100 µm.
DISCUSSION
Oxidative stress is involved in the pathogenesis of a variety of diseases such as asthma, COPD, cystic fibrosis etc. The strategies that counterbalance oxidative process may have a chemoprotective role in clinical or restorative medicine (Castro and Freeman, 2001). Epidemiological studies suggest that changes in diet, in particular reduced antioxidant intake contributes to increase in asthma prevalence and severity and raises the possibility that dietary interventions may improve asthma (Fogarty and Britton, 2000). While oxidant damage is an established consequence of inflammation, the role of antioxidants and ROS in asthmatic airway inflammation is vital (Wood et al., 2003). Choline, a dietary component is important for the structural integrity of cell membranes, methyl metabolism, cholinergic neuro-transmission, transmembrane signaling, lipid and cholesterol transport and metabolism. Its deficiency activates production of ROS as one of the pathogenic mechanism of mitochondrial and oxidative damage (Ossani et al., 2007). In the present study, we have investigated the protective effect of choline against oxidative stress in mouse model of allergic airway disease. In addition, α-lipoic acid has been tested as a standard antioxidant. It is a naturally occurring antioxidant used clinically for the treatment of oxidant induced diseases, such as ischemia-reperfusion injury and diabetic neuropathy (Cao and Phillis, 1995; Van Dam, 2002; Ametov et al., 2003). Dosages of α-lipoic acid taken in our study are similar to those used in previous studies (Lee et al., 2005; Lee et al., 2006).

A previous study has demonstrated the generation of ROS in the airways of OVA-induced asthma model (Lee et al., 2006). In the present study, increased production of ROS was observed in BAL cells which mainly consist of recruited inflammatory cells, in ovalbumin sensitized mice. The ROS generation was substantially reduced by oral and i.n. administration of choline. Eosinophils are the most prominent effector cells in asthma and produce various mediators including ROS that contribute to inflammation (Barnes, 1990). Furthermore, it cannot be ruled out that influx and/or activation of eosinophils cause part of the increased production of ROS after antigen challenge (Henricks and Nijkamp, 2001). Eosinophil peroxidase, a specific marker for eosinophil is responsible for protein nitration that leads to oxidative injury in allergic inflammatory diseases (Duguet et al., 2001). Our results indicate that choline inhibits eosinophils influx thereby reducing EPO activity which might restrain protein nitration and prevent oxidative burst.
Choline supplementation alters plasma methionine-homocysteine cycle metabolites, which leads to increase plasma methionine and S-adenosylmethionine (SAM)/S-adenosylhomocysteine (SAH) ratio and an improved glutathione antioxidant status in children with cystic fibrosis (Innis et al., 2007). An elevation of SAM can prevent induction of inducible nitric oxide synthase (Majano et al., 2001), attenuate production of NFκB (Chawla et al., 1998), and increase the production of glutathione (Song et al., 2003), which in turn is involved in cytokine regulation (Grimble, 2006). Further, choline supplementation in diet supports PC biosynthesis, reduces oxidative stress in terms of lipid peroxidation and promotes conservation of retinol and α-tocopherol (Sachan et al., 2005; Detopoulou et al., 2008; Grattagliano et al., 2008). Also in the present study, choline administration by oral or i.n. routes reduced the lipid peroxidation significantly in terms of TBARS levels as compared to OVA challenged saline treated mice.

Elevated level of isoprostanes have been observed in a variety of respiratory diseases, including cystic fibrosis (Montuschi et al., 2000a; Wood et al., 2001), interstitial lung diseases (Montuschi et al., 1998), COPD (Montuschi et al., 2000b) and asthma (Montuschi et al., 1999; Wood et al., 2000). Isoprostanes are the most reliable biomarker to measure oxidative stress. They are structurally stable, produced in vivo and are present in relatively high concentrations (Awad et al., 1996). Levels of isoprostanes detected exceed those of cyclooxygenase-derived prostanoids by one to two orders of magnitude (Morrow et al., 1990). Indeed, as a marker of oxidative stress, 8-iso-PGF2 determination of carbon tetrachloride induced lipid peroxidation has been shown 20 times more sensitive than measurement of TBARS (Awad et al., 1996). The increase in oxidative stress markers namely hydroxyoctadecadienoic acid (HODE) and 8-iso-PGF\textsubscript{2α} has been demonstrated in liver and plasma induced by choline deficient diet (CDD) in mice (Yoshida et al., 2006). Studies suggest that fatty liver induced by CDD is associated with low level of antioxidants (Grattagliano et al., 2000; Oliveira et al., 2003). In the present study, 8-isoprostanes level was significantly high in OVA challenged mice, but choline treatment reduced 8-isoprostanes level significantly by both the routes. Previously, 8-isoprostanes have been reported as potent constrictor of smooth muscle as observed in vitro in human and guinea pig airways (Kawikova et al., 1996). Also 8-isoprostanes have been shown to elicit AHR in isolated perfused mouse lungs (Held and Uhlig, 2000) and cause airway obstruction and airway plasma exudation in guinea pigs in vivo (Okazawa et
In our study, choline treatment inhibited the development of AHR in mice as mentioned earlier (chapter 2).

The development of oxidant/antioxidant imbalance in the lungs of asthmatics may lead to activation of redox-sensitive transcription factor NFκB (Henderson et al., 2002). Furthermore, ROS can induce cytokine and chemokine production through induction of the oxidative stress sensitive transcription of NFκB in bronchial epithelial cells (Biagioli et al., 1999). NFκB is present in most cell types and regulates many of the cytokines such as IL-4, IL-5, IL-9, IL-15 and TNF-α when it gets activated under inflammatory conditions (Henderson et al., 2002). Administration of choline or α-lipoic acid inhibits translocation of NFκB p65 to nucleus and suppressed Th2 cytokines (IL-4 and IL-5). In addition, TNF-α levels showed reduction with choline administration. It has been reported that ROS are needed for TNF-α induced NFκB activation (Garg and Aggarwal, 2002), so the anti-inflammatory effects of choline may be partially mediated by the reduction of ROS production.

The increase in ROS during asthmatic attack might overwhelm endogenous antioxidant defenses. Airway glutathione is increased in asthmatics, but the ratio of oxidized to reduced glutathione is also increased. The increase in reduced glutathione is considered as an adaptive response; however, antioxidants namely ascorbate and α-tocopherol are decreased and superoxide dismutase activity is diminished in cells from lavage (Smith et al., 1997; Kelly et al., 1999). Oxidant/antioxidant balance in the airways is important for asthma and improvement in the antioxidant status is likely to improve the inflammatory process. Two strategies are suggested to treat oxidative stress in asthma, firstly, reducing exposure to oxidants and secondly, augmenting antioxidant defenses. In conclusion, the present study demonstrates that choline administration by oral or i.n. route limits oxidative stress and attenuates the Th2 immunological responses in mouse model of allergic airway disease.