Chapter 2

To evaluate the anti-inflammatory effect of choline in animal model
INTRODUCTION

Prevalence of asthma and allergic disorders has increased 2-fold in the past two decades (Umetsu et al., 2002). Asthma was viewed primarily as a disease of bronchoconstriction but now it is considered as a chronic inflammatory disease characterized by eosinophilic infiltration, airway hyperresponsiveness (AHR) and intermittent pulmonary obstruction. It is partly mediated by CD4+ helper T lymphocytes that activate numerous airway inflammatory cells such as mast cells, neutrophils and eosinophils leading to morphological changes in airway tissue and increased AHR (Elias et al., 2003). The activated mast cells and lymphocytes, particularly the Th2 subpopulation of cells, which release a family of pro-inflammatory cytokines including IL-4, IL-5 and IL-13. These cytokines assist in the recruitment and activation of eosinophils. Lymphocytes, along with epithelial cells, generate chemokines, including RANTES and eotaxin, which appear to be essential for the recruitment of eosinophils (Galli et al., 2008). Further, release of cysteinyl leukotrienes (LTC4, LTD4 and LTE4) induces AHR in asthmatics followed by bronchoconstriction, enhanced mucus secretion and eosinophil chemotaxis (Smith et al., 1991; Henderson, 1994).

Inflammation is a reaction of the immune system of the host under normal conditions that leads to swelling, pain, and redness in the affected tissue. Once the stimulus is cleared from the affected area, endogenous stop signals or anti-inflammatory mediators usually take over and the inflammation resolves (Van Hove et al., 2008). In a number of circumstances like the presence of stimulus or dysregulation of anti-inflammatory mechanism, the status of inflammation persists for an extended period and becomes chronic. Other factors, like eosinophils, mast cells, CD4+ T cells, epithelial cells and inflammatory mediators, which contribute in acute inflammation, are also evident in chronic inflammation (Van Hove et al., 2008).

Airway inflammation, a characteristic feature of asthma contribute significantly to bronchial obstruction, AHR and initiation of the injury-repair process (remodeling) in asthmatics (Tattersfield et al., 2002; Elias et al., 2003). The pattern of inflammation varies considerably and depends on the stage of the disease: acute, chronic or remodeling. The degree of airway inflammation varies with severity and chronicity of the disease and may determine the responsiveness of the patient to treatment (Tattersfield et al., 2002). Tissues from patients who died of asthma showed a distinct airway inflammation that includes denudation of airway epithelium, mucus
plugging of segmental bronchi and bronchioles, collagen deposition beneath the basement membrane, edema of the sub-mucosa, infiltration of inflammatory cells, and smooth muscle hypertrophy/hyperplasia (Jeffery, 2000).

Airway smooth muscle contraction occurs due to several stimuli that lead to excessive airway narrowing. There are many factors or mediators (histamine, leukotrienes, prostaglandins etc) in the airways that contribute to airway smooth muscle tone. Many of the mediators can also increase membrane permeability to cause mucosal edema that leads to variable airway obstruction (Tattersfield et al., 2002).

Some patients have an injury-repair process of airways with irreversible airway obstruction termed as remodeling. A number of components are present in airway remodeling like smooth muscle hypertrophy, mucus glands, goblet cell hyperplasia etc. Lipid mediators are one of the potent regulators of airway tone and remodeling like slow reacting substance of anaphylaxis (SRS-A), is a mixture of the leukotrienes LTC4, LTD4 and LTE4 collectively called cysteinyl leukotrienes (CystLTs) etc. CysLTs have a key regulatory role in tissue damage and make structural changes in the airways. Remodeling promotes collagen synthesis by smooth muscle cells, develops pulmonary fibrosis and induces cell proliferation in human tracheal smooth muscle cells (Ogawa and Colhoun, 2006; Van Hove et al., 2008).

Therapeutic interventions available for asthma are focused on attenuating the airway inflammation. The current anti-inflammatory therapies such as glucocorticoids, non-steroidal anti-inflammatory drugs and anti-histamines are largely based on inhibiting the synthesis or action of inflammatory mediators. Administration of such drugs relieves symptoms but does not reverse the progression of the disease and has potential side effects that limit their usefulness (Barnes and Pederson, 1993). Inhaled glucocorticosteroids are the most effective treatment available for long-term management of persistent asthma. However, controlled clinical trials have demonstrated that long-term treatment with high doses of inhaled glucocorticosteroids may be associated with side effects, including skin thinning and easy bruising, adrenal suppression and decreased bone mineral density (Lipworth et al., 1999). Nasal corticosteroids are considered as the most potent medication for treatment of allergic rhinitis. They control itching, sneezing, rhinorrhea, and stiffness in most patients, but do not alleviate ocular symptoms. They have a relatively good safety profile, but long-term perennial use or prolonged use in children, may be problematic (Juniper et
Among the other drugs available, β2-adrenergic suppressors are the most potent and rapid acting drugs for treatment of asthma. Apart from relaxing the smooth muscle, they also enhance mucociliary clearance, decrease vascular permeability and modulate mediator release from mast cells (Lemanske and Busse, 2003). Inhaled short acting β agonist (SABA) such as salbutamol and terbutaline are effective for rapid relief in acute attacks of asthma. They can be given as and when required during emergencies, but long-term use of these bronchodilators does not provide benefit to the patients (Tattersfield et al., 2002). By contrast, inhaled LABA (salmeterol and formoterol) induces bronchodilation for 12 hours, improves asthma symptoms and reduces acute attacks (Barnes et al., 1996a). Monotherapy with LABA is not recommended as it could mask worsening airway inflammation due to side effects. A combination of inhaled corticosteroids and LABA are currently the most effective treatment for asthma and may improve patient compliance (Holgate and Polosa, 2008).

Theophylline, a methylxanthine, is a bronchodilator medication that may have mild anti-inflammatory effects as well with activity as both a cAMP phosphodiesterase (PDE) inhibitor and an adenosine-receptor antagonist (Holgate and Polosa, 2008). At higher doses (10 mg/kg body weight/day or more), theophylline intoxication can occur involving multiple organs of the body. Gastrointestinal discomforts, nausea and vomiting are the most common symptoms. Further, theophylline intoxication in children and adults can result in seizures, arrhythmias and even death.

Leukotriene modifiers, the new class of anti-asthma drugs likes lipoxygenase inhibitors namely zileuton and the leukotriene antagonist such as montelukast and zafirlukast are used with some inhibitory effects on patients of mild to moderate asthma and rhinitis. (Drazen et al., 1999; Scow et al., 2007). Leukotriene modifier therapy has been associated with liver toxicity, and there are reports of Churg-Strauss syndrome (Health advisory for new asthma drug, 1998). Allergic disorders including asthma impair quality of life and cause loss of several working hours. Despite the progress made in pharmacotherapy, new drugs are required to control immune inflammation with minimal or no side effects.

Several murine models of atopic asthma have been described in earlier studies (Temelkovski et al., 1998; Southam et al., 2008). Multiple sensitization and repeated
transbronchial challenge are the standard procedures to induce a Th2 dominant response, with high specific IgE production and pulmonary eosinophilic infiltration. Studies demonstrated epithelial abnormalities including hypertrophy, goblet cell hyperplasia/metaplasia, and sub-epithelial fibrosis along with marked airway reactivity to methacholine in chronic mice model (Temelkovski et al., 1998; Southam et al., 2008). The experimental murine models simulate morphological and functional changes of human asthma and can offer valuable information in several domains of asthma pathogenesis and treatment.

Choline, a lipotrophic factor, plays a role in mobilization of fats in liver, is essential for acetylcholine (ACh) formation (Blusztajn, 1998) and used is for phosphatidyl choline (PC) synthesis by de novo pathway (Pelech and Vance, 1984). It has shown anti-inflammatory activity in arthritis animal model (Ganley et al., 1958). Choline magnesium trisalicylate improved symptoms in aspirin induced asthma patients (Szczeklik et al., 1990). Further, tricholine citrate reduced symptoms and drug requirement in asthma (Gupta and Gaur, 1997; Gaur et al., 1997). These reports suggest the benefit of choline administration in asthma but how it improves the airway inflammation is still unknown. The present study investigates the anti-inflammatory effect of choline in ovalbumin induced mouse model of allergic airway inflammation.

**MATERIALS AND METHODS**

**Sensitization and Treatment:** Female Balb/c mice (n = 100) of 6-8 weeks were obtained from National Institute of Virology, Pune (India). Choline chloride (Sigma, St. Louis, MO, USA) was administered via oral gavage or intranasal (i.n.) route before and after OVA challenge in sensitized mice using two different protocols. In protocol A, mice were randomly divided into 5 groups of 10 mice each. Control group was sensitized, challenged and treated with 100 μl of 0.9% NaCl (Saline; group 1). The remaining four groups were sensitized intraperitoneally (i.p.) with 100 μg OVA adsorbed on 2 mg of Al (OH)₃ in 100 μl of saline on days 0, 7 and 14. They were challenged with 2.5% aerosolized OVA in saline in a plexiglass chamber using nebulizer (Omron, Tokyo, Japan) for 30 minutes on days 25-27. OVA sensitized and challenged mice were treated with 100 μl saline (group 2), 1 mg/kg body weight of choline in 100 μl saline by oral gavage 1 hour before each OVA challenge on days
25-27 (group 3), 1 mg/kg body weight of choline (i.n.) in 50 µl saline 30 minutes before each OVA challenge (group 4). Dexamethasone phosphate (i.p.) was given (1 mg/kg body weight) in 100 µl saline 30 minutes before each OVA challenge (group 5). After the last treatment / challenge, AHR was measured on day 28 and mice sacrificed on day 29.

In protocol B, mice were randomly divided into 5 groups of 10 mice each and sensitized as mentioned in protocol A. Further, they were challenged with 2.5% aerosolized OVA in saline for 30 minutes on days 25-30. Twenty-four hours after the last challenge, mice were treated with 100 µl of saline (group 2), choline (1 mg/kg body weight) by oral gavage for 10 days from day 31-40 in saline (group 3). Same dose (1 mg/kg body weight) of choline (i.n.; in 50 µl saline) was given in group 4 and dexamethasone in group 5 (i.p.; in 100 µl saline) on alternate days from day 31-40. Finally, a booster challenge with OVA was done on day 38. AHR was measured on day 41 after the challenge / treatment and mice sacrificed on day 42. Control group was sensitized, challenged and treated with 100 µl of saline (group 1). Mice receiving i.n. dose were lightly anesthetized (3% isoflurane) before each treatment. The dose of choline selected here was derived from an anti-inflammatory activity study of choline in mice model of collagen-induced arthritis (Ganley et al., 1958). In addition, dose response analysis was made with different doses of choline chloride and the dose of 1 mg/kg body weight was selected for the present study. Dose of dexamethasone, was similar to those used in previous studies on anti-inflammatory effects of dexamethasone and other drugs (Blyth et al., 1998; Kumar et al., 2003). The study protocol was approved by animal ethics committee of Institute of Genomics and Integrative Biology, Delhi, India.

**Determination of AHR:** The assessment of AHR allows the measurement of physiologically relevant parameters that seem representative of the clinical manifestations of allergic inflammation in airways. Airway reactivity in response to methacholine (Mch; Sigma, St. Louis, MO, USA) challenge was measured in conscious, unrestrained mice in a preconditioned whole body plethysmograph (Buxco Electronics Inc., Troy, NY, USA). Mice from each group were nebulized with phosphate buffered saline (PBS), followed by increasing concentration of Mch (4-50 mg/ml) to induce bronchoconstriction. Lung function was recorded and calculated as enhanced pause (Penh) (Hamelmann et al., 1997).
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 immunoglobulins. The trachea was cannulated after the collection of blood. A 19-gauge needle was inserted into the trachea, lungs were lavaged three times with 0.5 ml of chilled saline and approximately 1.2 ml of BAL fluid was recovered from each mouse. BAL fluid was centrifuged at 400 × g for 10 min, 4°C and supernatant was collected for analysis of cytokine, 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 using 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 eosinophil counts were calculated by multiplying total cell counts with percentage of eosinophil in BALF cell pellets.

Determination of eosinophil peroxidase (EPO) activity: EPO levels in BAL fluid were determined as described (Tomkinson et al., 2001). Briefly, 100 µl of substrate solution containing 0.1 mM o-phenylene-diamine-dihydrochloride (OPD; Sigma, St. Louis, MO, USA), 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.

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) or alcian blue/periodic acid-schiff (AB/PAS) 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 and the proliferation of goblet cells in the bronchial epithelium were quantified and graded 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’ (< 20% goblet cells stained with AB/PAS), ‘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; Bangalore Genei, Bangalore, India; 5 µg/ml) were taken as positive control while unstimulated cells were considered for basal proliferation. Cells were cultured in triplicates at 37°C in CO₂ incubator and the culture supernatant was collected after 72 hours 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-Immundo™ 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 plate was washed with PBS and mouse sera diluted to 1:10 (for IgE) and 1:50 (for IgG1 and IgG2a) were added 100 µl/well and incubated overnight at 4°C. Pre-immune sera was also processed simultaneously in separate wells. After washing with PBST (PBS + 0.05% Tween-20) and PBS, the plates were incubated for 3 hours at 37°C with IgG1-peroxidase and IgG2a-peroxidase antibody (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, the plates were 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 and IFN-γ were determined in BAL fluid and spleen cell culture supernatants by ELISA following manufacturer’s instructions (BD
Pharmingen, 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 10% fetal bovine serum (FBS; v/v in PBS) at 25°C for 1 h. After washing, 100 µl of standards (7 serial dilutions) and undiluted BALF 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-horseradish peroxidase (HRP) at 25°C for 1 h. After washing with PBST and PBS, plate was developed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution and incubated in dark at 37°C for 30 min. The reaction was stopped with 2 N H$_2$SO$_4$ and absorbance was read at 450 nm. The cytokine concentrations were interpolated from the standards. The detection limit for IL-4, IL-5 and IFN-γ was 7.8, 15.6 and 31.3 pg/ml, respectively.

**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 (PBST), Ellman substrate solution was added and plates were incubated in dark at 25°C for 90-120 mins. 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 LTB$_4$ and Cys-LT was 13 pg/ml.

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

Choline suppresses allergen induced eosinophilic airway inflammation: The immunization protocols used in this study is presented in Figure 2.1. Out of 10 mice in each group, 5 mice were taken from each group to sacrifice and data expressed as means of 5 mice per group. Two different schedules of choline administration, before (preventive) and after (therapeutic) OVA challenge were used to demonstrate the changes occurred after choline treatment in allergen induced mouse model of airway disease. A significant reduction in BAL fluid total cell counts and eosinophils was observed after choline treatment by oral gavage (p < 0.05) or i.n. (p < 0.05) as compared to OVA challenged saline treated mice in both the study protocol A and B (Figure. 2.2 and 2.3). Treatment with dexamethasone also led to significant reduction in eosinophils numbers (p < 0.05).

The recruitment of inflammatory cells in the airways was monitored in choline treated groups as compared to OVA challenged saline treated group by H & E staining in both the protocols (Figure 2.4B and 2.5B). In OVA challenged saline treated group, numerous eosinophils were observed in the lung interstitium around airways and blood vessels alongwith narrowing of airway lumen (Figure 2.4 and 2.5). The choline treatment administered orally or i.n. significantly reduced the inflammatory infiltrates in both the protocols (Figure 2.4C, 2.4D, 2.5C and 2.5D). Mice treated with dexamethasone also showed reduced inflammation and eosinophil infiltration in lungs (Figure 2.4E and 2.5E). These results were further confirmed by a significant reduction in total inflammation score in choline treated groups compared to saline treated groups (p < 0.05) (Figure. 2.4F and 2.5F).

In control groups, either no or very few AB/PAS positive epithelial cells were observed in protocol A and B (Figure. 2.6A and 2.7A). Contrary to this, many of the epithelial cells were enlarged in OVA challenged mice, alongwith airway narrowing and mucus plugging with increased goblet cell hyperplasia visualized by AB/PAS staining (Figure. 2.6B and 2.7B).
Figure 2.1: Schematic presentation of immunization protocol A and B.
Figure 2.2: Total cell count and eosinophil number in bronchoalveolar lavage (BAL) from different groups of mice in protocol A. Data are expressed as means ± SD of 5 mice per group; *p < 0.05 compared with OVA mice.

Figure 2.3: Total cell count and eosinophil number in bronchoalveolar lavage (BAL) from different groups of mice in protocol B. Data are expressed as means ± SD of 5 mice per group; *p < 0.05 compared with OVA mice.
Figure 2.4: Hematoxylin & Eosin stained sections of mouse lungs in protocol A showing preventive effect of choline before antigen challenge (A) normal control, saline sensitized and challenged, (B) OVA-challenged saline treated mouse, (C) OVA-challenged mouse treated with oral choline, (D) OVA-challenged mouse treated with *i.n.* choline and (E) OVA-challenged mouse treated with dexamethasone (Dex) at 20× magnification. (F) Total inflammation score graded on a scale from 0-4. Score was significantly reduced in choline treated group. Data are expressed as means ± SD of 5 mice per group; *p < 0.05 compared with OVA mice.
Figure 2.5: Hematoxylin & Eosin stained sections of mouse lungs in protocol B showing therapeutic effect of choline after antigen challenge (A) normal control, saline sensitized and challenged, (B) OVA-challenged saline treated mouse (C) OVA-challenged mouse treated with oral choline (D) OVA-challenged mouse treated with i.n. choline and (E) OVA-challenged mouse treated with dexamethasone (Dex) at 20× magnification. (F) Total inflammation score graded on a scale from 0-4. Score was significantly reduced in choline treated group. Data are expressed as means ± SD of 5 mice per group; * p < 0.05 compared with OVA mice.
Figure 2.6: Alcian blue/Periodic acid-Schiff staining of lung sections from mouse in protocol A. Lung histology of (A) normal control, saline sensitized and challenged, (B) OVA-challenged saline treated mouse (C) OVA-challenged mouse treated with oral choline and (D) OVA-challenged mouse treated with i.n. choline at 20× magnification. (E) Degree of infiltration of goblet cells was estimated and score graded on a scale from 0-4. Score was significantly reduced in choline treated group. Data are expressed as means ± SD of 5 mice per group; * p < 0.05 compared with OVA mice.
Figure 2.7: Alcian blue/Periodic acid-Schiff staining of lung sections from mice in protocol B. Lung histology of (A) normal control, saline sensitized and challenged, (B) OVA-challenged saline treated mouse, (C) OVA-challenged mouse treated with oral choline and (D) OVA-challenged mouse treated with i.n. choline at 20× magnification. (E) Degree of infiltration of goblet cells was estimated and score graded on a scale from 0-4. Score was significantly reduced in choline treated group. Data are expressed as means ± SD of 5 mice per group; * p < 0.05 compared with OVA mice.
Choline treatment before OVA challenge (protocol A) by either route (oral/i.n.) inhibited the mucus production and goblet cell hyperplasia (Figure 2.6C and 2.6D). Also choline treatment down-regulated the accumulation of mucus in the airways and prevented airway obstruction after antigen challenge (protocol B; Figure 2.7C and 2.7D). There was a significant reduction in total inflammation score in choline treated mice compared to saline treated mice (p < 0.05) (Figure 2.6E and 2.7E).

**Choline reduces methacholine induced AHR:** Methacholine induced airway resistance was measured on day 28 and on day 41 for both the protocols (A and B). Saline sensitized and challenged control mice virtually had no increase in AHR. OVA challenged saline treated mice developed AHR as demonstrated by a dose dependent elevation in Penh in response to Mch (Figure 2.8 and 2.9). Treatment with oral choline before challenge exhibited a modest decrease in AHR, though it was not significant (protocol A). However, *i.n.* treatment demonstrated significant reduction (p < 0.05) in Penh at Mch concentrations of 12-50 mg/ml (Figure 2.8).

In protocol B, the Penh values significantly decreased in oral choline treatment group (20-50 mg/ml). However, *i.n.* treatment was more effective and showed statistically significant reduction (p < 0.05) in Penh at Mch concentrations of 4-50 mg/ml (Figure 2.9). Dexamethasone treatment also inhibited the development of AHR in both the study protocols (p < 0.05).

**Loss of EPO activity in choline treated mice:** EPO activity was measured as a marker for the activation of eosinophils. Significantly high levels of EPO with increased infiltration of eosinophils in BAL fluid were observed in OVA challenged saline treated mice. The choline treatment (Figure 2.10 and 2.11) by both the routes reduced EPO activity (p < 0.05) significantly and thus reduced inflammation in the airways. Treatment with dexamethasone also reduced the EPO activity in both the protocols (p < 0.05).

**Effect of choline on serum OVA-specific IgG1, IgE and IgG2a:** Oral and *i.n.* choline treatment resulted in significant decrease of IgG1 and IgE (p < 0.05) levels in both the study protocols (Figure 2.12 and 2.13). Dexamethasone treatment also reduced IgG1 and IgE levels in mice. However, no significant difference was observed in IgG2a antibody levels after choline treatment.
Figure 2.8: AHR to increasing concentrations of aerosolized methacholine was determined by Penh values in Protocol A. Penh is expressed as means ± SD of 5 mice per group; * p < 0.05 compared with OVA mice.

Figure 2.9: AHR to increasing concentrations of aerosolized methacholine was determined by Penh values in Protocol B. Penh is expressed as means ± SD of 5 mice per group; * p < 0.05 compared with OVA mice.
Figure 2.10: EPO activity in BAL fluid from different groups of mice in protocol A. Data are expressed as means ± SD of 5 mice per group; * p < 0.05 compared with OVA mice.

Figure 2.11: EPO activity in BAL fluid from different groups of mice in protocol B. Data are expressed as means ± SD of 5 mice per group; * p < 0.05 compared with OVA mice.
Figure 2.12: OVA-specific serum antibody levels as determined by ELISA in protocol A. Normal controls and OVA-challenged mice treated with saline, choline (oral/i.n.) or dexamethasone. Data are expressed as means ± SD of 5 mice per group; * p < 0.05 compared with OVA mice.

Figure 2.13: OVA-specific serum antibody levels as determined by ELISA in Protocol B. Normal controls and OVA-challenged mice treated with saline, choline (oral/i.n.) or dexamethasone. Data are expressed as means ± SD of 5 mice per group; * p < 0.05 compared with OVA mice.
Choline treatment reduces Th2 cytokine levels in BAL fluid and culture supernatant: IL-4 and IL-5 levels reduced significantly with choline treatment by oral and or i.n. route (p < 0.05) in BAL fluid and in culture supernatants (Figure 2.14 and 2.15). In protocol A (Figure 2.14), i.n. choline treatment showed increase in IFN-γ level in BAL fluid (p = 0.035) but not in oral treatment group (p = 0.149). But in contrast to i.n. (p = 0.76) choline treatment, oral choline administration induced IFN-γ levels significantly (p < 0.05) in spleen cells culture supernatant. In protocol B (Figure 2.15), choline administration by oral or i.n. routes showed increase in IFN-γ level in BAL fluid as compared to normal mice but this was not evident in culture supernatant.

Choline inhibits the release of leukotriene in BAL fluid and culture supernatant: In protocol A, LTB4 level was reduced in culture supernatant but not in BAL fluid in oral choline treatment group (Figure 2.16). However, LTB4 showed a significant decrease in BAL fluid as well as in culture supernatant (p < 0.05) with choline treatment by either route after challenge with OVA in protocol B (Figure 2.17).

Apart from LTB4, choline treatment significantly (p < 0.05) inhibited the release of Cys-LT not only in BAL fluid but also in culture supernatant (Fig. 2.16 and 2.17). This inhibition of Cys-LT was observed in both the protocols (A and B).
Figure 2.14: Cytokine levels in BAL fluid (BALF) and in spleen cell culture supernatant as determined by ELISA in Protocol A. Normal controls and OVA-challenged mice treated with saline, choline (oral/i.n.) or dexamethasone. Data are expressed as means ± SD of 5 mice per group; *p < 0.05 compared with OVA mice.

Figure 2.15: Cytokine levels in BAL fluid (BALF) and in spleen cell culture supernatant as determined by ELISA in Protocol B. Normal controls and OVA-challenged mice treated with saline, choline (oral/i.n.) or dexamethasone. Data are expressed as means ± SD of 5 mice per group; *p < 0.05 compared with OVA mice.
Figure 2.16: Leukotriene levels in BAL fluid (BALF) and in spleen cell culture supernatant as determined by ELISA in Protocol A. Normal controls and OVA-challenged mice treated with saline, choline (oral/i.n.). Data are expressed as means ± SD of 5 mice per group; *p < 0.05 compared with OVA mice.

Figure 2.17: Leukotriene levels in BAL fluid (BALF) and in spleen cell culture supernatant as determined by ELISA in Protocol B. Normal controls and OVA-challenged mice treated with saline, choline (oral/i.n.). Data are expressed as means ± SD of 5 mice per group; *p < 0.05 compared with OVA mice.
DISCUSSION

Asthma is one of the commonest chronic diseases in industrialized as well as in developing countries. There is every indication that its prevalence, severity and mortality may increase, despite better recognition and increasing therapy (Sears, 1991; Burney 1992; Bousquet et al., 2005). Asthma remains under-diagnosed and undertreated and treatment is often inappropriate or suboptimal even in hospitals (Barnes, 1991). Asthma is recognized as a chronic inflammatory condition of the airways. Recent research has emphasized that, even in mild asthma, the airways are chronically inflamed, with a special type of inflammatory response characterized by eosinophils and T-lymphocyte infiltration and mast cell activation (Djukanovic et al., 1990; Barnes, 1992). The uncontrolled airway inflammation may lead to structural changes, such as fibrosis and smooth muscle hypertrophy, which are likely to underlie the irreversible narrowing of the airways as evident in poorly controlled asthma (Knox, 1994). It is therefore logical to direct treatment for reducing and controlling the inflammation, rather than dealing only with symptomatic manifestations.

Identification of new drugs for asthma has been relegated in recent years especially for those who respond poorly to conventional therapy. In the past decade, treatment of asthma has emphasized long-term suppression of airway inflammation and relief in symptoms with rapid acting bronchodilators (Barnes, 2006). Inhaled corticosteroids are the most effective agents for symptomatic control of asthma and improvement in pulmonary function (Barnes, 1996a), but their potential side effects when used in escalating doses have led to use of adjunctive therapies (Allen, 1996; Lemanske and Allen, 1997). Nevertheless, whether used alone or in combination with other therapies, corticosteroids do not consistently abrogate airway inflammation in asthmatics (Jeffery et al., 1992; Booth et al., 1995; Godfrey et al., 1995). Furthermore, glucocorticosteroids are associated with systemic side effects (Lipworth, 1999). Consequently, there is need for the development of new agents to treat airway disease like asthma.

The nicotinic acetylcholine receptors (nAChR) agonist is known for protective effect on the development of airway inflammation through cholinergic anti-inflammatory pathway (Wang et al., 2003; Gallowitsch-Puerta and Tracey, 2005). The nAChRs are expressed on various inflammatory cells such as eosinophils, lymphocytes, alveolar macrophages and airway smooth cells. The nAChR agonist like 1, 1-dimtheyl-4-phenylpiperazinium (DMPP) induces a potential down-regulating
effect on eosinophils function by inhibiting LTC4 and eosinophils migration (Blanchet and Langlois, 2007). Choline, a selective agonist of alpha-7-nicotinic receptors suppressed passive joint anaphylaxis and showed anti-inflammatory effect in guinea pigs (Ganley et al., 1958; Papke et al., 1996; Wang et al., 2005). In the present study, choline was demonstrated as a potent inhibitor of airway inflammation suppressing the accumulation of eosinophils and release of EPO in BAL fluid. The effects may be mediated through the activation of alpha-7-nicotinic receptor via cholinergic anti-inflammatory pathway.

Eosinophils on activation release eosinophil cationic protein, major basic protein and EPO that correlates with the disease severity or AHR (Cheng et al., 1993; McFadden, 1994). In the present study, choline administration was effective in inhibiting AHR both before and after OVA challenge. Choline treatment prevented the development of AHR and its resolution may be associated with loss of eosinophil activation. Choline administered through i.n. route gives a localized effect whereas oral route gives systemic effect in reducing AHR. Aerosol therapy via i.n. route is the preferred asthma therapy for adults and children, since it offers a rapid onset of drug action directly into the target organ requires smaller doses and reduces systemic side effects (O'Hagan and Illum, 1990).

Clinical and experimental evidence implicates Th2 cytokines in orchestrating the inflammatory response and AHR in asthma (Elias et al., 2003). In the present study, the anti-inflammatory effects of choline were at least partly mediated by suppression of Th2 cytokines in BAL fluid and spleen cells culture supernatant with significant decrease in IgE and IgG1. IL-4/IFN-γ ratio in BAL fluid also decreased in choline treated mice (data not shown). The reduction of IL-5, a potential activation and survival factor for eosinophils, was concomitant with the loss of EPO activity in BAL supernatant.

Leukotrienes are the key mediators of allergen-induced airway eosinophils infiltration and mucus release (Henderson, 1994). High levels of LTB₄ and Cys-LT were demonstrated in BAL fluid of asthmatics (Wenzel et al., 1990; Wenzel et al., 1991). Cys-LT₁ receptors are expressed on eosinophils and their activation by Cys-LT induces eosinophil infiltration in airways and thus Cys-LT is considered potent eosinophil chemo-attractant (Figueroa et al., 2001). Choline (nAChR agonist) treatment also inhibited the release of Cys-LT and eosinophilic infiltration in the
allergen-induced airway inflammation. The eosinophils infiltration may be mediated through the activation of alpha-7-nicotinic receptor as evident by studies with nAChR agonist DMPP (Blanchet and Langlois, 2007).

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 results in loss of membrane PC and induction of apoptosis in PC12 cells in vitro (Yen et al., 1999). It is involved in eliciting a variety of pharmacological effects in many diseases including stroke, dementia, Alzheimer’s, Parkinson’s etc (Boyd et al., 1977; Eberhardt et al., 1990; Alvarez et al., 1999; Adibhatla et al., 2006). Previously, asthma patients have shown improvement in symptom-drug score on oral administration of choline (Gupta and Gaur, 1997; Gaur et al., 1997). Recently, choline has been shown to produce antinociceptive effects against inflammatory pain that is blocked by alpha-7 nAChRs antagonist methylcaconitine citrate, which strongly supports the involvement of alpha-7 nAChRs in the antinociception of choline (Wang et al., 2005). Phosphatidylcholine is synthesized by de novo and transmethylation pathway, the two being reciprocally compensatory (Pelech and Vance, 1984). The activity of transmethylation pathway is coupled to phospholipase A2 activation, calcium influx and arachidonic acid formation with release of leukotrienes, prostaglandins and lysophosphatidyl choline (Hirata and Axelrod, 1980). Choline supplementation increases membrane PC via de novo pathway and inhibits transmethylation pathway thereby decreases mediator release from human basophils (Morita et al., 1981).

Several of the functions that are governed by ACh are disturbed in allergic airway disease and there is evidence that dysfunctioning of the cholinergic system is involved in the pathogenesis of asthma (Lips et al., 2007). Acetylcholine synthesis requires uptake of choline that is mediated by high affinity choline transporter-1 (CHT1) present in airway epithelial cells. Choline supplementation might restore this damage, as choline uptake via the CHT1 is the rate-limiting step in the ACh synthesis by choline acetyltransferase (Tucek, 1988). However, it is difficult to explain this discrepancy based on the existing data.

The mechanism underlying the modulating effect of choline is yet not clear. Various inflammatory cells like lymphocytes express most components of the cholinergic system including ACh, muscarinic and nicotinic ACh receptors (mAChRs
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and nAChRs, respectively) etc which helps in eliciting intracellular Ca\(^{2+}\) response. The smooth muscle contraction induced by methacholine is reduced by choline treatment. One of the major steps of smooth muscle contraction is intracellular calcium mobilization (Kuo et al., 2003). In this study Ca\(^{2+}\) level was not measured but it was demonstrated that nicotinic agonist could deplete Ca\(^{2+}\) level in lymphocytes (Kalra et al., 2000). Similarly, in rat model of endotoxin shock, choline restrained the increase in calcium influx and release of TNF-\(\alpha\) by activated macrophages possibly by increasing membrane PC, thereby preventing lung injury and improving survival (Rivera et al., 1998). This down regulating effect of nicotinic agonist on Ca\(^{2+}\) mobilization could be responsible for both anti-inflammatory and broncho-protective effects of choline.

In conclusion, choline was effective in inhibiting antigen induced airway inflammation in mouse model of airway hyperresponsiveness.