

Choline supplementation reduces oxidative stress in mouse model of allergic airway disease

A. K. Mehta^{*,†}, N. Arora^{*}, S. N. Gaur[‡] and B. P. Singh^{*}

^{*}Allergy and Immunology Section, Institute of Genomics and Integrative Biology, Delhi, [†]Department of Biotechnology, University of Pune, Ganeshkhind, Pune, [‡]Department of Pulmonary Medicine, V. P. Chest. Institute, University of Delhi, Delhi, India

ABSTRACT

Background Asthma is a multi-factorial inflammatory disease associated with increased oxidative stress and altered antioxidant defences. We have evaluated the effect of choline on oxidative stress in a mouse model of airway disease.

Materials and methods Balb/c mice were sensitised with 100 µg of ovalbumin on days 0 and 14, and challenged with aerosolized ovalbumin on days 25–27. Mice were administered 1 mg kg⁻¹ of choline via oral gavage or intranasal route on days 14–27. Mice were also administered 100 mg kg⁻¹ of α -lipoic acid as standard antioxidant. Total cell counts, eosinophils and eosinophil peroxidase (EPO) activity were determined in bronchoalveolar lavage (BAL) fluid. Reactive oxygen species (ROS), lipid peroxidation and isoprostanes levels were measured in BAL fluid. IL-13 and tumour necrosis factor- α (TNF- α) levels were also measured in BAL fluid and spleen cell culture supernatant. Nuclear factor κ B (NF κ B) p65 protein expression was measured after last ovalbumin challenge in nuclear and cytosolic extracts of lungs.

Results Compared with ovalbumin-challenged mice, choline and α -lipoic acid treated mice had significantly reduced eosinophilic infiltration and EPO activity in BAL fluid. Choline and α -lipoic acid treatment reduced ROS production and isoprostanes level significantly in BAL fluid and thus suppressed oxidative stress. Choline and α -lipoic acid administration by either route decreased lipid peroxidation levels and down regulated NF κ B activity. Further, choline and/or α -lipoic acid treatment suppressed TNF- α level significantly as compared with that of ovalbumin-challenged mice.

Conclusions Choline administration reduces oxidative stress possibly by modulating the redox status of the cell and inhibits inflammatory response in a mouse model.

Keywords Asthma, choline, isoprostanes, lipid peroxidation, nuclear factor- κ B, reactive oxygen species.

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Introduction

Asthma is a chronic inflammatory disease of the airways characterised by eosinophilic infiltration, airway hyperresponsiveness (AHR) and intermittent pulmonary obstruction [1]. Oxidative stress plays an important role in allergic disorders and increased levels of oxidants are considered as markers of the inflammatory process [2]. Oxidative stress is caused by a variety of free oxygen radicals such as superoxide, hydrogen peroxide, hypochlorous acid and hydroxyl radicals, collectively known as reactive oxygen species (ROS) [2]. ROS generated are 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 [3–5].

Excess production of oxidants is supposed to be kept less by a well-coordinated and efficient endogenous antioxidants defence system. However, individuals with asthma demonstrate diminished levels of ascorbate and α -tocopherol, suggesting both increased ROS/reactive nitrogen species and decreased oxidative capacity [6]. There has been considerable interest in the possible modulation of asthma by supplementation of antioxidants e.g., hydrophilic and lipophilic antioxidants such as vitamins C and E respectively [7]. However, these oral supplements have failed to demonstrate overall convincing beneficial effects in asthma [8,9]. Interestingly, a recent study has indicated that higher levels of serum antioxidants such as vitamin C and β -carotene were associated with low risk of asthma [10].

Choline, a precursor to acetylcholine and cytidine 5-diphosphocholine formation, showed anti-inflammatory activity in animal model of arthritis [11]. Its deficiency results in loss of membrane phosphatidyl choline (PC) and induction of apoptosis *in vitro* [12]. Choline administration has shown lysophosphatidyl choline lowering effect and symptomatic improvement in asthmatics [13,14]. Further, mice fed with choline deficient diet (CDD) showed increase in oxidative stress and altered antioxidants [15–17]. To test the hypothesis that altered antioxidant defences in allergic asthma processes can be modulated by supplementation of choline, we have evaluated the antioxidant effects of nutritional choline to modulate the ensuing allergen-mediated airway immunological responses.

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 five groups of 10 mice each and inhabited in an animal house at 25 °C and 60–70% humidity on a 12-h light/dark schedule, and with *ad libitum* access to water and standard diet. The mice were sensitised intraperitoneally (*i.p.*) with 100 µg of 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 min on days 25–27. Mice were treated once daily just after immunisation till last challenge (on days 14–27) with 100 µl saline (group 2), 1 mg kg⁻¹ of choline in 100 µl saline by oral gavage (group 3), 1 mg kg⁻¹ of choline in 50 µl saline intranasal (*i.n.*) (group 4) and 100 mg kg⁻¹ of α-lipoic acid by oral gavage (group 5). The control (group 1) mice were sensitised, challenged and treated with 100 µl of saline. After the last treatment/challenge, mice were killed on day 28. Mice receiving *i.n.* dose were lightly anaesthetized (3% isoflurane; *i.n.*) before each dose of treatment. Choline chloride and ovalbumin were procured from Sigma, St. Louis, MO, USA. Alpha-lipoic acid (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 the last challenge, mice were killed using sodium pentobarbital (100 mg kg⁻¹ *i.p.*). Blood was collected and sera separated for analysis of serum immunoglobulins. The trachea was cannulated and the 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 400g for 10 min at 4 °C. The BAL cell pellets were re-suspended in

phosphate-buffered saline (PBS) and total numbers of cells were determined. BAL cell smears were stained with Leishman stain to identify (%) eosinophils by counting minimum of 200 cells. Absolute eosinophils were calculated by multiplying total cell counts by percentage of eosinophils in BAL cell pellets.

Measurement of intracellular ROS

Reactive oxygen species were measured by a method described previously elsewhere [18]. BAL cell pellets were washed with PBS and cells were incubated with 3.3 µM of 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) in PBS for 10 min at room temperature in dark to label intracellular ROS. DCFH is membrane impermeable and rapidly oxidises to the highly fluorescent 2',7'-dichlorofluorescein in the presence of intracellular hydrogen peroxide and peroxidases [19]. The cells after incubation were examined under fluorescence activated cell sorter (Guava Technologies, CA, USA) and the mean fluorescence intensity of 5000 cells was analysed in each sample and corrected for auto-fluorescence of unlabelled cells.

Determination of eosinophil peroxidase (EPO) activity

Eosinophil peroxidase levels in BAL fluid were determined as described previously elsewhere [20].

Cytosolic or nuclear protein extractions for analysis of NFκB

Cytosolic or nuclear proteins were extracted using CellLytic™ NUCLEAR™ extraction kit (Sigma, St. Louis, MO, USA) by following the manufacturer's instructions. Briefly, 100 mg of lung tissue from each sample was rinsed two times with PBS and homogenised in 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 000g for 20 min at 4 °C. The nuclei pellet obtained was re-suspended in extraction buffer containing DTT and protease inhibitor cocktails with gentle stirring for 30 min and centrifuged at 21 000g for 5 min at 4 °C. The resulting supernatant was used as soluble nuclear proteins for the determination of NFκB levels.

Western blot

Cytosolic and nuclear extracts (30 µg of protein per lane) were loaded onto 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. After electrophoresis, separated proteins were transferred to polyvinylidene difluoride membranes using the wet transfer method at 250 mA for 120 min. Nonspecific sites were blocked with 3% defatted milk in PBS for 2 h at 37 °C, and the blots were then incubated with antibody against rabbit polyclonal NFκB p65 (Santa Cruz, CA, USA) after washing with

PBS for 2 h at room temperature. Anti-rabbit horseradish peroxidase-conjugated IgG was used to detect binding of antibody. Anti- β -actin was used to normalise for loading variations.

Analysis of lipid peroxidation

The amount of lipid peroxidation was assessed by measuring the thiobarbituric acid reactive substances (TBARS). Briefly, BAL fluid was mixed with 10% trichloroacetic acid to precipitate the protein. The precipitate was separated by centrifugation at 10 000g for 15 min at 4 °C and the supernatant collected was treated with 0.67% TBA at 100 °C for 15 min. Absorbance was measured at 532 nm after cooling and compared with standard malanodialdehyde [21].

Measurement of 8-isoprostanes (8-iso PGF₂ α)

BAL fluid samples were assayed in duplicates for 8-isoprostanes using enzyme immunoassay kits (Cayman Chemical Co., MI, USA) by following the manufacturer's instructions. The detection limit for 8-isoprostanes was 2.7 pg mL⁻¹.

Splenocyte culture

Splenocytes (5 × 10⁶ cells mL⁻¹) were cultured in RPMI-1640 medium (supplemented with 10% heat-inactivated foetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin; Sigma) for 72 h at 37 °C in a CO₂ incubator. The cells were stimulated with 100 µg mL⁻¹ OVA. Culture supernatant was collected to measure cytokines and eicosanoids.

Determination of cytokine by Enzyme-linked immunosorbent assay (ELISA)

IL-13 and tumour necrosis factor- α (TNF- α) (R & D System, Minneapolis, MN, USA and BD Pharmingen, San Diego, CA, USA respectively) levels were determined in BAL fluid and culture supernatants by ELISA using paired antibodies according to manufacturer's instruction. The detection limit for IL-13 and TNF- α was 15.6 pg mL⁻¹.

Statistical analysis

Groups were analysed using one-way analysis of variance 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 are presented as mean $\pm$ SD for each group.

Results

Choline suppresses intracellular Reactive oxygen species production

There was enhanced production of ROS in BAL cells of ovalbumin-sensitised and challenged mice after last challenge

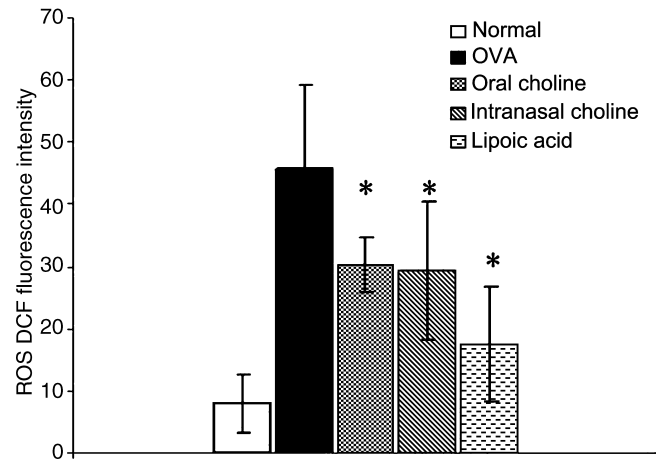


Figure 1 Effect of choline treatment on relative ratio of reactive oxygen species levels in bronchoalveolar lavage (BAL) fluid of ovalbumin-sensitised and challenged mice. □, Normal control; ■, ovalbumin-sensitised, challenged and saline treated mice; ▨, ovalbumin-sensitised, challenged and oral choline treated mice; ▩, ovalbumin-sensitised, challenged and *i.n.* choline treated mice; ▤, ovalbumin-sensitised, challenged and α -lipoic acid treated mice. Bars present $\pm$ SD from six mice per group. * $P < 0.05$ vs. ovalbumin-sensitised and challenged mice.

compared with that of saline control mice (Fig. 1). The ROS production reduced markedly by choline treatment in both oral and *i.n.* groups ($P < 0.05$). The treatment with α -lipoic acid also decreased intracellular ROS levels in BAL fluid ($P < 0.05$).

Choline suppresses allergen-induced eosinophilic airway inflammation

A significant reduction in BAL cell count and eosinophils was observed in orally ($P < 0.05$) or *i.n.* ($P < 0.05$) choline treated mice compared to OVA-challenged saline treated mice (Fig. 2). Treatment with α -lipoic acid 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 compared with that in OVA-challenged group by staining with H & E. In OVA-challenged saline treated group, numerous eosinophils were observed in the lung interstitium around airways and blood vessels along with narrowing of airway lumen (data not shown). Choline administration orally or *i.n.* significantly reduced the inflammatory infiltrates ($P < 0.05$). Mice treated with α -lipoic acid also showed reduced inflammation and eosinophil infiltration in the lungs.

Eosinophil peroxidase activity in choline treated mice: EPO levels were significantly high with increased infiltration of eosinophils in BAL fluid of OVA-challenged and saline treated mice (Fig. 3). The choline treatment by both the routes significantly

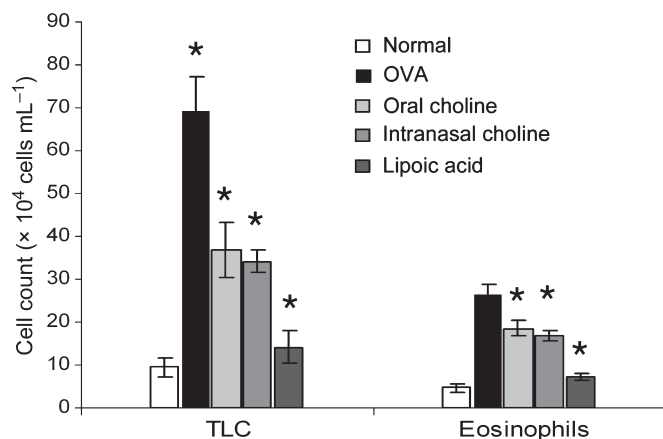


Figure 2 Effect of choline and α -lipoic acid treatment on total leucocyte count (TLC) and eosinophil number in BAL. Bars present $\pm$ SD from six mice per group. * $P < 0.05$ vs. ovalbumin-sensitised and challenged mice.

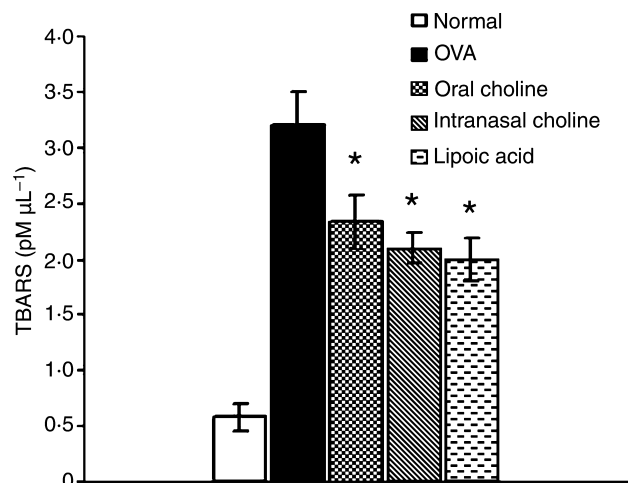


Figure 4 Oxidative stress marker in BAL fluid. Effect of choline and α -lipoic acid treatment on TBARS level in BAL fluid. Bars present $\pm$ SD from six mice per group. * $P < 0.05$ vs. ovalbumin-sensitised and challenged mice.

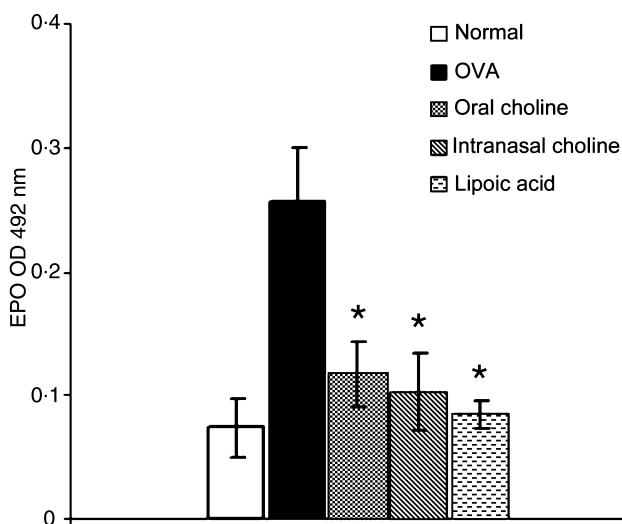


Figure 3 Eosinophil peroxidase activity in BAL fluid supernatant after choline and α -lipoic acid treatment. Bars present $\pm$ SD from five mice per group. * $P < 0.05$ vs. ovalbumin-sensitised and challenged mice.

reduced EPO activity ($P < 0.05$) and inflammation in the airways. Treatment with α -lipoic acid also reduced the EPO activity significantly ($P < 0.05$) than that in OVA-challenged saline treated group.

Effect of choline in lipid peroxidation

To determine whether increased ROS levels led to increased membrane peroxidation, we determined the malondialdehyde

levels, an important biomarker released from the oxidation and decomposition of polyunsaturated fatty acids. Choline treatment by either route reduced the lipid peroxidation levels significantly compared with that of OVA-challenged saline treated mice (Fig. 4; $P < 0.05$). Treatment with α -lipoic acid administration also reduced TBARS level substantially.

Effect of choline on 8-isoprostanes level

The isoprostanes (8-isoprostanes) levels were measured in BAL fluid of different groups of mice as a biomarker of lipid peroxidation. After the last challenge, 8-isoprostanes level was significantly elevated in OVA-challenged saline treated group than that in saline control mice (Fig. 5). Choline treatment by either route significantly reduced the level of isoprostanes ($P < 0.05$).

Effect of choline on NF κ B p65 protein level in lung tissues

NF κ B p65 protein expression was measured after last OVA challenge in nuclear and cytosolic extracts from lung tissues. Western blot analysis revealed that the levels of NF κ B p65 in nuclear protein extracts from lung tissues were increased in saline treated OVA-challenged mice (Fig. 6a; lane 2). The increased NF κ B p65 protein expression was inhibited after choline treatment by either route (Fig. 6a; lanes 3 and 4). In contrast, level of cytosolic NF κ B p65 from lung tissues decreased after last ovalbumin challenge compared with that in normal control (Fig. 6b; lane 2). The decreased NF κ B p65 levels in cytosolic protein extracts were increased by administration of choline (Fig. 6b; lanes 3 and 4). These results indicate that

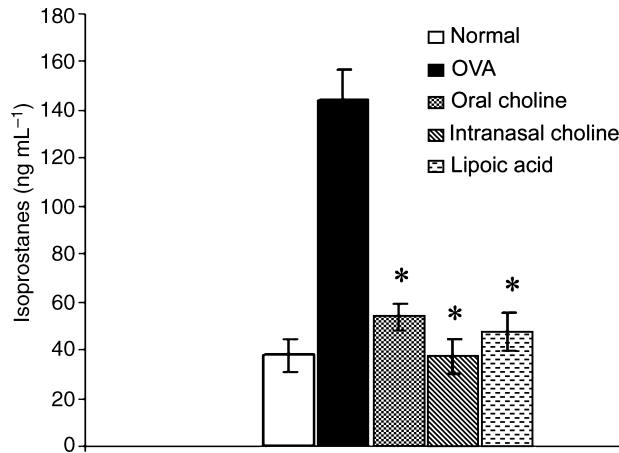


Figure 5 Levels of isoprostanes in BAL fluid of mice after choline and α -lipoic acid treatment. Bars present $\pm$ SD from six mice per group. * $P < 0.05$ vs. ovalbumin-sensitised and challenged mice.

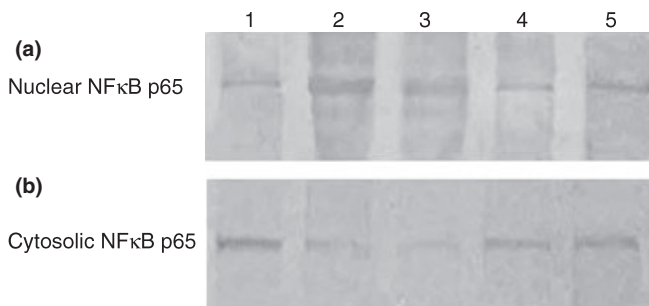


Figure 6 Effect of choline on NF κ B p65 protein level in lung tissues. Representative of six mice per group. (a) Nuclear extract (b) Cytoplasmic extract. Lane 1, normal mice sensitised, challenged and treated with saline; lane 2, OVA-challenged saline treated mice; lane 3, OVA-challenged mice treated with oral choline; lane 4, OVA-challenged mice treated with *i.n.* choline and lane 5, OVA-challenged mice treated with α -lipoic acid.

choline and α -lipoic acid inhibit NF κ B p65 activity by preventing translocation of NF κ B p65 from cytoplasm to nucleus.

Choline treatment reduces cytokine level in BAL fluid and culture supernatant:

To confirm whether the enhanced expression of NF κ B leads to up regulation of inflammatory molecules, we measured the expression of TNF- α in BAL fluid and spleen cell culture supernatant. A significant increase in TNF- α level was observed in OVA-challenged mice. TNF- α levels reduced significantly in BAL fluid and in culture supernatants with choline treatment by oral or *i.n.* route ($P < 0.05$). In choline treated groups, the

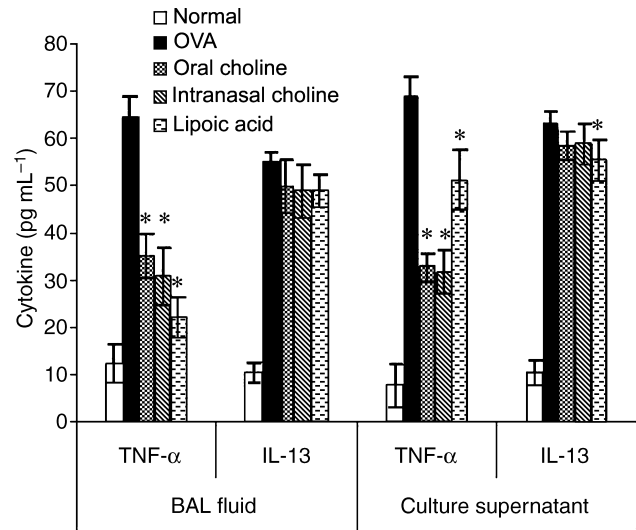


Figure 7 Effect of choline and α -lipoic acid administration on cytokine concentrations (TNF- α and IL-13) in BAL fluid of OVA-sensitised and challenged mice. Bars present $\pm$ SD from six mice per group. * $P < 0.05$ vs. ovalbumin-sensitised and challenged mice.

level of IL-13 also decreased although not significantly (Fig. 7). IL-13 levels showed reduction by α -lipoic acid administration in spleen cells culture supernatant ($P < 0.05$).

Discussion

Oxidative stress is involved in the pathogenesis of a variety of diseases including asthma, chronic obstructive pulmonary diseases (COPD), cystic fibrosis, etc. The strategies that counterbalance oxidative process could have a chemoprotective role in clinical or restorative medicine [22]. Choline, a dietary component, is important for the structural integrity of cell membranes, methyl metabolism, cholinergic neuro-transmission, transmembrane signalling and lipid and cholesterol transport and metabolism. Its deficiency activates the production of ROS as one of the pathogenic mechanisms of mitochondrial and oxidative damage [23]. In this study, we have investigated the protective effect of choline against oxidative stress in a 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 ischaemia-reperfusion injury and diabetic neuropathy [24,25].

A previous study has demonstrated that the generation of ROS in the airways of OVA-induced asthma model [18]. In this study, increased production of ROS was observed in BAL cells, which mainly consist of recruited inflammatory cells, in

ovalbumin-sensitised 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, which contribute to inflammation [3]. Eosinophil peroxidase, a specific marker for eosinophil, is responsible for protein nitration that leads to oxidative injury in allergic inflammatory diseases [26]. Our results indicate that choline inhibits eosinophil influx, thereby reducing EPO activity that might restrain protein nitration and thus prevent oxidative burst.

Choline supplementation alters plasma methionine-homocysteine cycle metabolites, which lead to increase in plasma methionine and S-adenosylmethionine (SAM)/S-adenosylhomocysteine ratio and an improved glutathione antioxidant status in children with cystic fibrosis [27]. An elevation of SAM can prevent the induction of inducible nitric oxide synthase [28], attenuate the production of NF κ B [29] and increase the production of glutathione [30], which in turn is involved in cytokine regulation [31]. Further, choline supplementation in diet supports PC biosynthesis, reduces oxidative stress in terms of lipid peroxidation and promotes conservation of retinol and α -tocopherol [32,33]. Also in this study, choline administration by oral or *i.n.* routes reduced the lipid peroxidation significantly in terms of TBARS levels compared with that of OVA-challenged saline treated mice.

Elevated levels of 8-isoprostanes, the most reliable biomarker to measure oxidative stress, have been observed in respiratory diseases such as cystic fibrosis, COPD and asthma [34,35]. The increase in oxidative stress markers, namely hydroxyoctadecadienoic acid and 8-iso-PGF_{2 α} , induced by CDD has been demonstrated in liver and plasma in mice [15]. Studies suggest that fatty liver induced by CDD is associated with low level of antioxidants [16,17]. In this study, isoprostanes increased significantly in OVA-challenged mice, but choline treatment reduced isoprostanes level significantly by both the routes. Previously, isoprostanes have been reported as potent constrictor of smooth muscle as observed *in vitro* in human and guinea pig airways [36]. In addition, isoprostanes have been shown to elicit AHR in isolated perfused mouse lungs [37], cause airway obstruction and airway plasma exudation in guinea pigs *in vivo* [38]. Our previous study has shown that choline treatment inhibited the development of AHR in mice [39]. The effective dose of choline used in this study is within the dose range used in earlier study [39]. Oral and *i.n.* choline treatment suppressed IgG1 and IgE levels and cysteinyl-leukotriene levels (data not shown).

The development of oxidant/antioxidant imbalance in the lungs of asthmatics may lead to activation of redox-sensitive transcription factor NF κ B [40]. ROS appear to serve as messengers modulating the activation of NF κ B that further regulates

many of the cytokines, such as IL-4, IL-5, IL-9, IL-15 and TNF- α [40]. Administration of choline and α -lipoic acid caused significant reduction of NF κ B activity and Th2 cytokines (IL-4 and IL-5; data not shown). In addition, we sought to determine whether increased NF κ B levels lead to enhanced pro-inflammatory cytokine. Interestingly, TNF- α was increased in OVA-challenged mice over saline control mice, which was suppressed with choline administration. Therefore, these results strongly suggest that choline inhibits TNF- α expression in asthma by inhibiting the activity of NF κ B. It has been reported that ROS are needed for TNF- α -induced NF κ B activation [41]; therefore, 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 defences. Oxidant/antioxidant balance in the airways is important for asthma and improvement in the antioxidant status may improve the inflammatory process. Two strategies are suggested to treat oxidative stress in asthma; firstly, reducing exposure to oxidants and secondly, augmenting antioxidant defences. Limiting exposure to environmental oxidants such as nitrites and ozone might decrease asthmatic exacerbations [42,43], but this seems difficult to implement in daily life.

In conclusion, it may be too early to include choline in the list of recognised antioxidants, but the results of this study demonstrate that choline administration by oral or *i.n.* route limits oxidative stress and attenuates the immunological responses in a mouse model of allergic airway disease. Further studies with choline on mechanistic aspects and clinical trials in asthmatics will be worthwhile to explore its therapeutic potential.

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Address

Allergy and Immunology Section, Institute of Genomics and Integrative Biology, Delhi, India (A. K. Mehta, N. Arora, B. P. Singh); Department of Biotechnology, University of Pune, Ganeshkhind, Pune, India (A. K. Mehta); Department of Pulmonary Medicine, V. P. Chest. Institute, University of Delhi, Delhi, India (S. N. Gaur).

Correspondence to: Bhanu Pratap Singh, Scientist F, Allergy and Immunology Section, Room no. 509, Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road, Delhi 110007, India. Tel.: +91-11-27666157; fax: +91-11-27667471; e-mail: singhbp1951@yahoo.com

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